

## Stable hydrolysis-resistant synthetic polyribosylribitolphosphate derivatives as vaccines against *Haemophilus influenzae type b*

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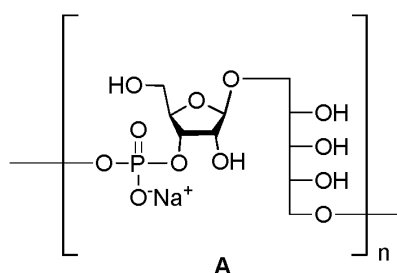
### Field of the invention

The present invention provides a stable synthetic saccharide of Hib polyribosylribitolphosphate (PRP) derivative and conjugate thereof. Said saccharide, said conjugate  
10 and pharmaceutical compositions thereof are hydrolysis-resistant, long-term stable and useful for the prevention and/or treatment of diseases associated with *Haemophilus influenzae*, and more specifically of diseases associated with *Haemophilus influenzae type b*.

### 15 Background of the invention

*Haemophilus influenzae type b* (Hib) is a serious human health problem worldwide, being responsible of a variety of diseases including meningitis, pneumonia, epiglottitis, and other diseases of the respiratory tract especially in children under the  
20 age of 5. 30% of the survivors of the diseases present sequels ranging from auditive problems to mental retardation.

Purified capsular polysaccharide of *Haemophilus influenzae* is able to induce protective immunity in adults. However, the immune response in children is very poor  
25 and practically absent in infants under 2 years old. The capsular polysaccharide isolated from the Hib bacteria presents a ribosylribitolphosphate repeating unit:



30 A variety of vaccines for prevention of Hib infection were developed and are consisting of synthetic polyribosylribitolphosphate (PRP) or mixtures of synthetic PRP of different lengths conjugated to carrier protein (WO 9210936 A1, EP 0276516 A2, EP 0320942 A2, WO 0116146 A1). Several vaccines have recently been licensed in the USA and elsewhere for pediatric use.

The synthesis of Hib oligosaccharides was reported to be a useful alternative to costly and cumbersome isolation of Hib PRP from bacteria (IN 2013/DEL/02989, US 6,765,091, WO2016044164 A1 and EP 0320942A). In *Journal of Carbohydrate Chemistry* **1992**, 11, 265 a solid phase synthesis of Hib oligosaccharides is described. The acetylated and methylated Hib disaccharides were investigated by mass-spectrometry in *Carbohydrate Research* **1979**, 73, 59.

As mentioned above, polyribosylribitolphosphate (PRP) is the active ingredient in a variety of vaccines that have proven successful in preventing disease caused by *Haemophilus influenzae type b* (Hib).

Long-term stability of the Hib vaccine is required to maintain the immunogenicity of said vaccine and this can be a challenge for Hib-conjugate vaccines given the potential susceptibility of the phosphodiester bond in the PRP polymer to hydrolytic cleavage. Usually, metabolism of the capsular polysaccharide of vaccine reduces the immunogenicity of the vaccine. It is known that aluminum oxide used as adjuvant accelerates the hydrolysis of PRP and depolymerization of PRP causes instability of the Hib vaccine and reduced immunogenicity of Hib vaccine (Vaccine 19, 1999, p1169-1178).

With the consequence that commercially available licensed Hib vaccines need to be stored at 2°C – 8°C and are usually stable at these conditions for only 24 – 36 months. Moreover, liquid Hib vaccine formulations are prone to being damaged by freezing which is assumed to be caused by the aluminum salt present in the liquid formulation. Sensitivity towards freeze damaging impedes the storage and transport of liquid Hib vaccines which have to be cooled since the use of ice has to be avoided to prevent freezing.

It is also reported that the purified natural polysaccharide component of the bacterial cell wall contained in commercially available vaccines may be responsible for inhibition of b receptors leading to increased bronchoconstriction. Furthermore, it is well-known that the Hib vaccines increase the risk of seizure symptom in infants under 3 years old. Thus, new Hib vaccine having enhanced immunogenicity by administering a smaller amount of dose and reduced side effects is requested.

It is the objective of the present invention to provide a synthetic saccharide of Hib polyribosylribitolphosphate (PRP) derivative and conjugate thereof should be metabolic stable, hydrolysis-resistant and shelf-stable in liquid formulations. Said saccharide, said conjugate and pharmaceutical compositions thereof have enhanced

immunogenicity and thus should be particularly useful for the prevention and/or treatment of diseases associated with *Haemophilus influenzae*, and more specifically of diseases associated with *Haemophilus influenzae type b*.

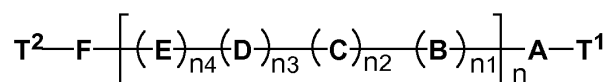
- 5 The objective of the present invention is solved by the teaching of the independent claims. Further advantageous features, aspects and details of the invention are evident from the dependent claims, the description, the figures, and the examples of the present application.

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### Description of the invention

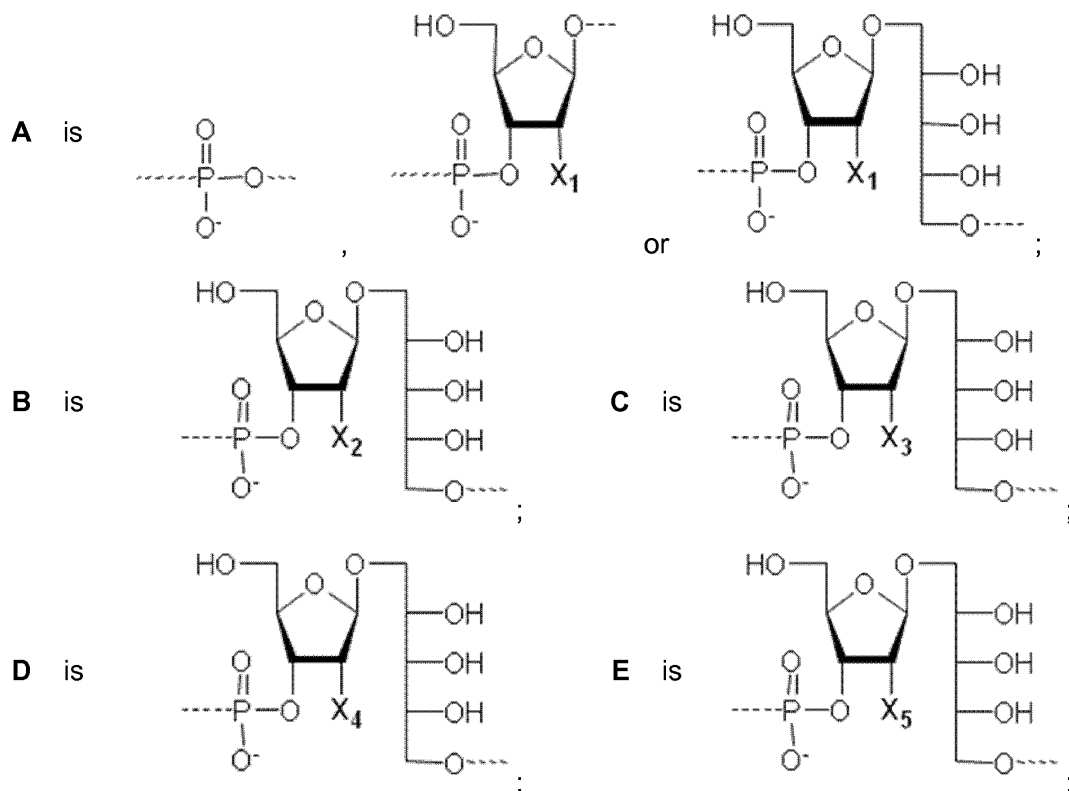
It is the objective of the present invention to provide saccharide of general formula (I)

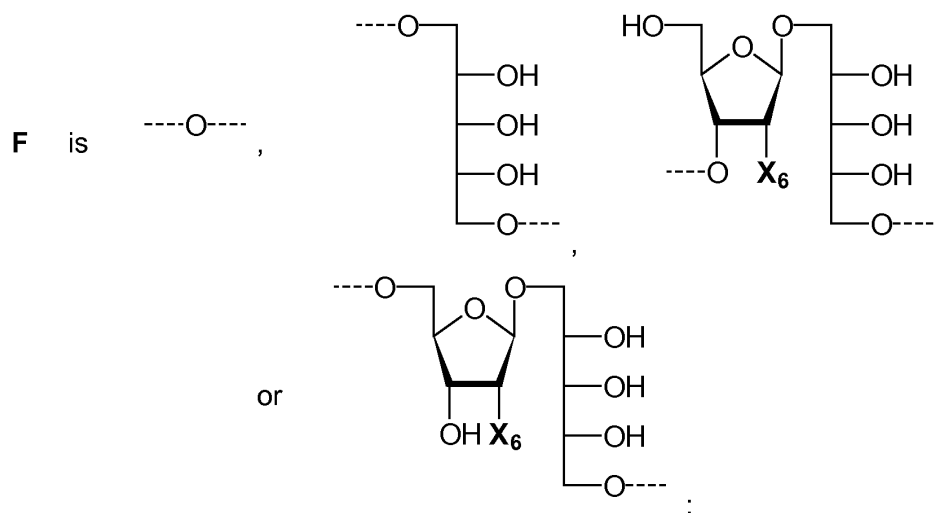
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(I)

wherein





**T**<sup>1</sup> and **T**<sup>2</sup> represent  $-H$ ,  $-L-NH_2$  or  $-L-COOH$ ; wherein if **T**<sup>1</sup> is  $-L-NH_2$  or  $-L-COOH$ , then **T**<sup>2</sup> is  $-H$  and if **T**<sup>1</sup> is  $-H$ , then **T**<sup>2</sup> is  $-L-NH_2$  or  $-L-COOH$ , or wherein one of **T**<sup>1</sup> and **T**<sup>2</sup> represents  $-H$  and the other one of **T**<sup>1</sup> and **T**<sup>2</sup> represents  $-L-NH_2$  or  $-L-COOH$ ;

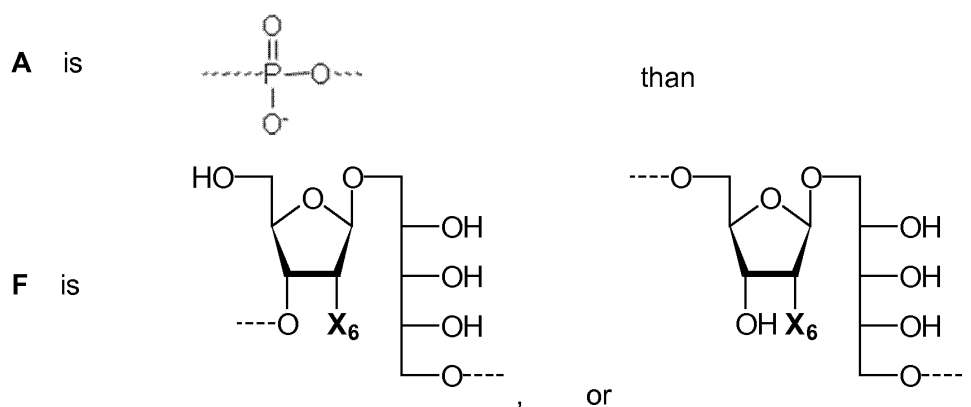
- 5 **X**<sub>1</sub>, **X**<sub>2</sub>, **X**<sub>3</sub>, **X**<sub>4</sub>, **X**<sub>5</sub>, and **X**<sub>6</sub> represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-Cl$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$  and at least one of **X**<sub>1</sub>, **X**<sub>2</sub>, **X**<sub>3</sub>, **X**<sub>4</sub>, **X**<sub>5</sub>, and **X**<sub>6</sub> is not  $-OH$ ; preferred at least 50% of **X**<sub>1</sub>, **X**<sub>2</sub>, **X**<sub>3</sub>, **X**<sub>4</sub>, **X**<sub>5</sub>, and **X**<sub>6</sub> are not  $-OH$ ; more preferred at least two of **X**<sub>1</sub>, **X**<sub>2</sub>, **X**<sub>3</sub>, **X**<sub>4</sub>, **X**<sub>5</sub>, and **X**<sub>6</sub> are not  $-OH$ ;
- 10 most preferred all of **X**<sub>1</sub>, **X**<sub>2</sub>, **X**<sub>3</sub>, **X**<sub>4</sub>, **X**<sub>5</sub>, and **X**<sub>6</sub> are not  $-OH$ ;

**n** is an integer selected from 1, 2, 3, or 4;

**n**<sub>1</sub>, **n**<sub>2</sub>, **n**<sub>3</sub> and **n**<sub>4</sub> are independently of each other selected from 0 and 1; and **n**<sub>1</sub>+**n**<sub>2</sub>+**n**<sub>3</sub>+**n**<sub>4</sub> ≥ 1;

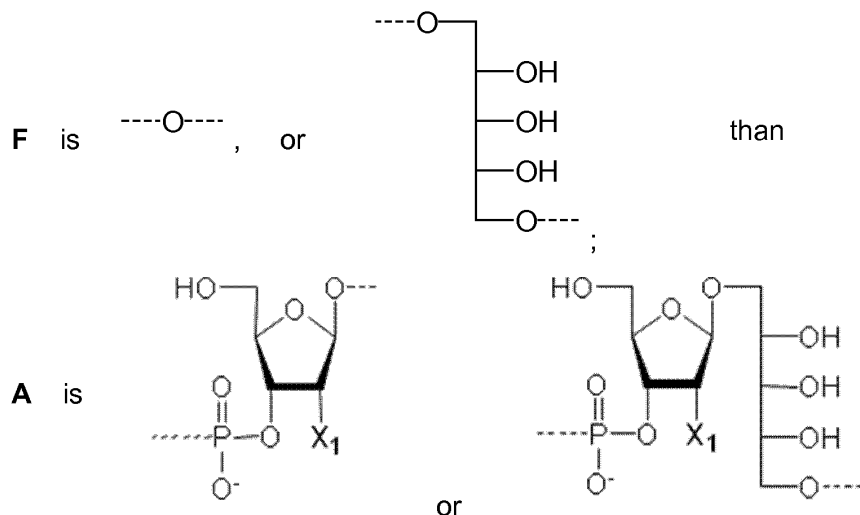
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under the proviso that if **n**<sub>1</sub>+**n**<sub>2</sub>+**n**<sub>3</sub>+**n**<sub>4</sub> = 1 and if **n** = 1 and if



and

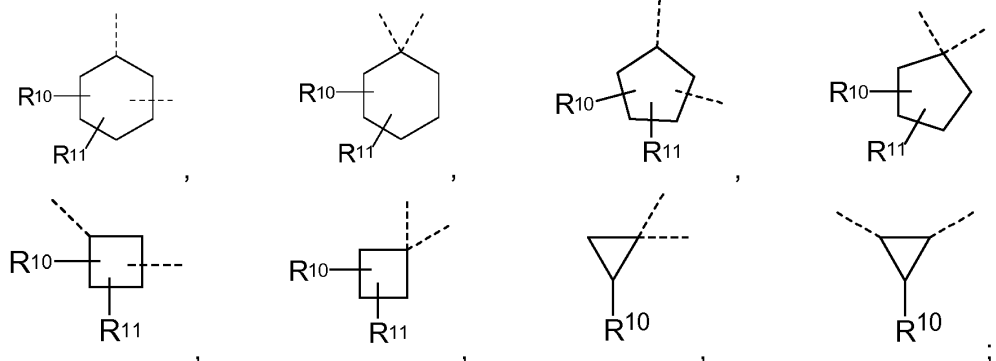
under the proviso that if  $n1+n2+n3+n4 = 1$  and if  $n = 1$  and if



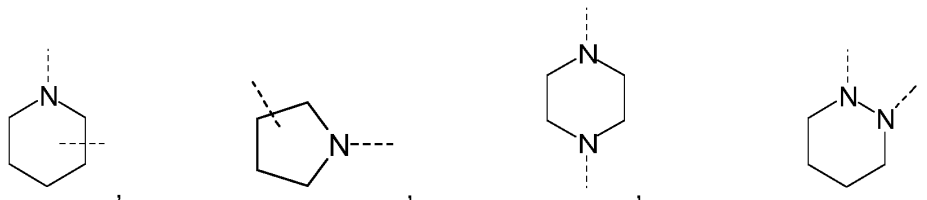
and

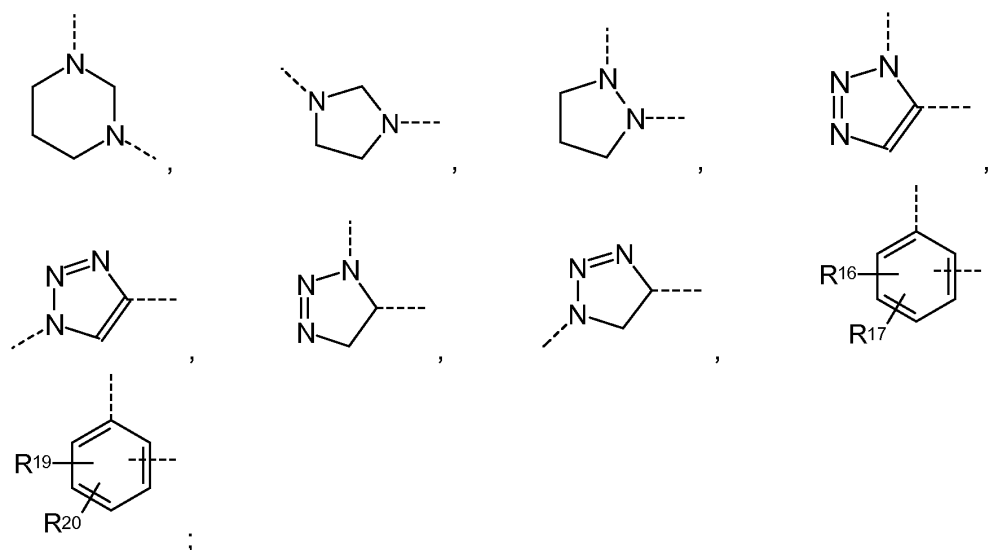
**L** is selected from:  $-L^a-$ ,  $-L^a-L^e-$ ,  $-L^a-L^b-L^e-$ ,  $-L^a-L^b-L^d-L^c-L^e-$ ,  
5  $-L^a-L^d-L^e-$ ,

wherein  $-L^a-$  is selected from:  $-(CH_2)_a-$ ,  $-(CF_2)_a-$ ,  $-(CH_2-CH_2-O)_a-C_2H_4-$ ,  
 $-(CH_2-CH_2-O)_a-CH_2-$ ,  $-(CR^{10}R^{11})_a-$ ,

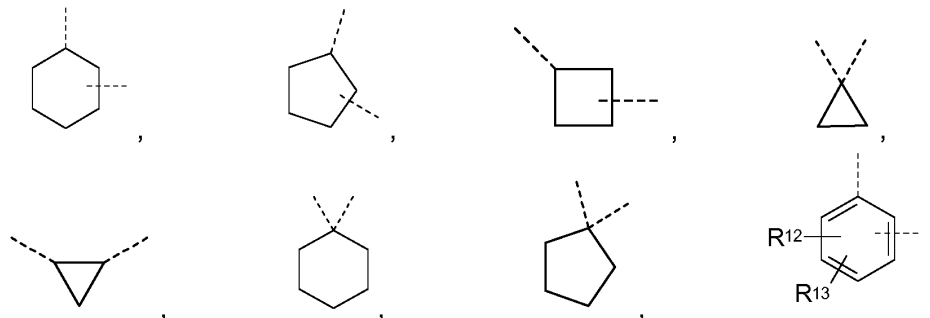


10  $-L^b-$  and  $-L^c-$  are independently of each other selected from:  $-O-$ ,  $-S-$ ,  
 $-NH-C(O)-NH-$ ,  $-NH-C(S)-NH-$ ,  $-NH-C(O)-$ ,  $-C(O)-NH-$ ,  $-S-S-$ ,  
 $-NH-C(O)-O-$ ,  $-NR^9-$ ,  $-NR^{18}-$ ,  $-SO_2-$ ,  $-OP(O)(OH)-O-$ ,

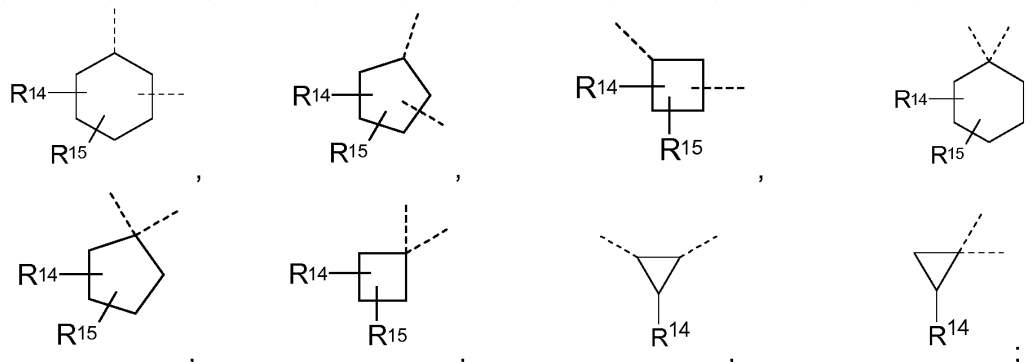




$-L^d-$  represents  $-(CH_2)_d-$ ,  $-(CF_2)_d-$ ,  $-(CR^{12}R^{13})_d-$ ,  
 $-(CH_2-CH_2-O)_d-C_2H_4-$ ,  $-(CH_2-CH_2-O)_d-CH_2-$ ,



5  $-L^e-$  is selected from:  $-(CH_2)_{e1}-$ ,  $-(CF_2)_{e1}-$ ,  $-C_2H_4-(O-CH_2-CH_2)_{e2}-$ ,  
 $-CH_2-(O-CH_2-CH_2)_{e2}-$ ,  $-(CH_2)_{e1}-O-(CH_2)_{e2}-$ ,  $-(CH_2)_{e1}-S-(CH_2)_{e2}-$ ,  
 $-(CR^{14}R^{15})_{e1}-$ ,  $-(CR^{14}R^{15})_{e1}-O-(CR^{21}R^{22})_{e2}-$ ,  $-(CR^{14}R^{15})_{e1}-S-(CR^{21}R^{22})_{e2}-$ ,



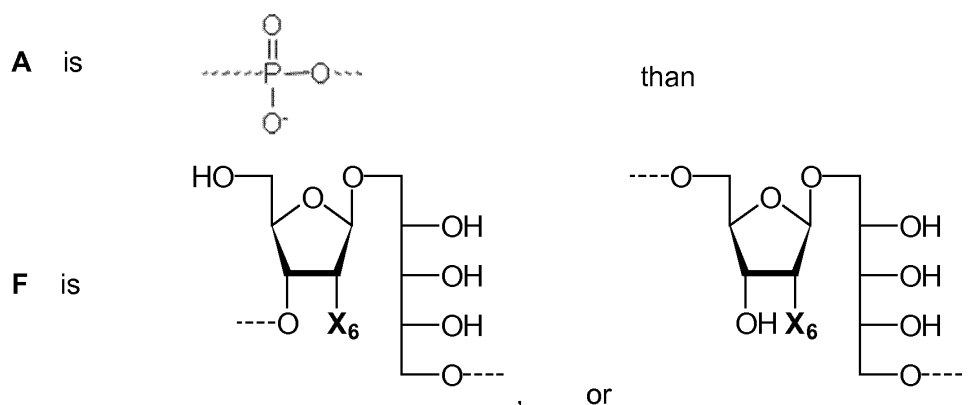
$R^9$  and  $R^{18}$  are independently of each other selected from:  $-CH_3$ ,  $-C_2H_5$ ,  
 10  $-C_3H_7$ , and  $-C(O)CH_3$ ;

$R^{10}$ ,  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$ ,  $R^{19}$ ,  $R^{20}$ ,  $R^{21}$  and  $R^{22}$  are independently of each other selected from:  $-H$ ,  $-F$ ,  $-Cl$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-C_3H_7$ ,  $-C_5H_9$ ,  $-C_6H_{13}$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-CH_2F$ ,  $-CHF_2$ ,  $-CF_3$ ,  $-C(O)-NH_2$ ,  $-SCH_3$ ,  $-SC_2H_5$ ,  $-NHC(O)CH_3$ ,  $-N(CH_3)_2$ , and  $-N(C_2H_5)_2$ ;

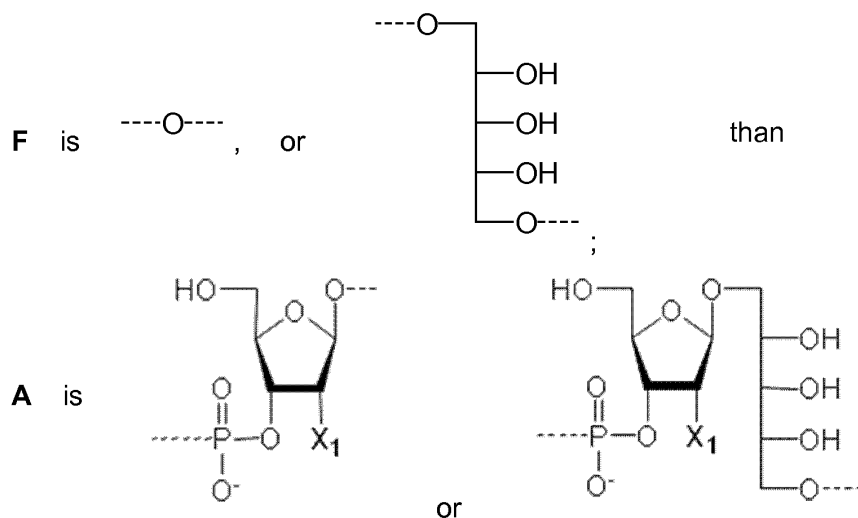
$a$ ,  $d$ ,  $e1$  and  $e2$  are independently of each other an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10;

and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

The smallest saccharides claimed by formula (I) are disaccharides so that the disclaimer under the proviso that if  $n1+n2+n3+n4 = 1$  and if  $n = 1$  and if



and under the proviso that if  $n1+n2+n3+n4 = 1$  and if  $n = 1$  and if

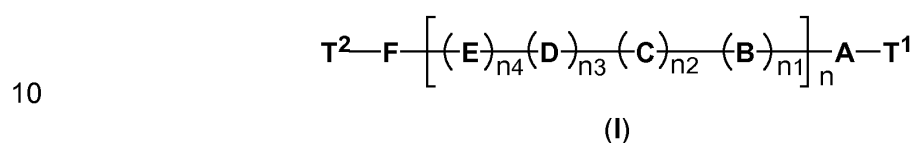


could be replaced by the condition that “saccharide of general formula (I) is at least a disaccharide”.

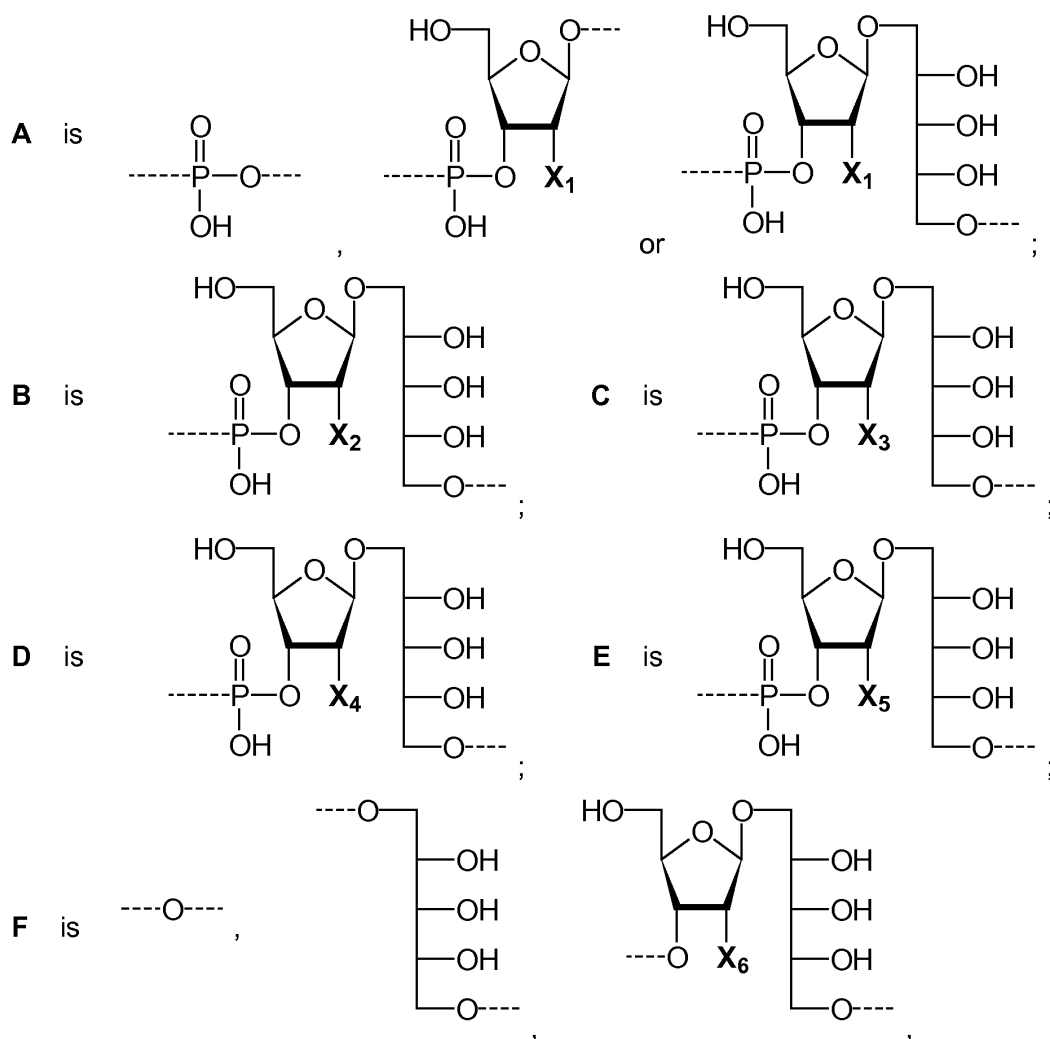
Alternatively the afore-mentioned disclaimer could be replaced by the condition that if

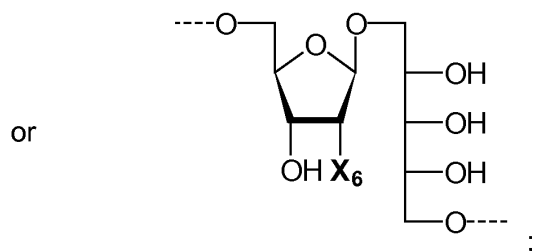
- 5  $n_1+n_2+n_3+n_4 = 1$  than  $n \neq 1$  ( $n$  different from 1) and if  
 $n = 1$  than  $n_1+n_2+n_3+n_4 \neq 1$  ( $n_1+n_2+n_3+n_4$  different from 1).

Thus, alternatively preferred is the saccharide of general formula (I)



wherein





$T^1$  and  $T^2$  represent  $-H$ ,  $-L-NH_2$  or  $-L-COOH$ ; wherein if  $T^1$  is  $-L-NH_2$  or  $-L-COOH$ , then  $T^2$  is  $-H$  and if  $T^1$  is  $-H$ , then  $T^2$  is  $-L-NH_2$  or  $-L-COOH$ , or wherein one of  $T^1$  and  $T^2$  represents  $-H$  and the other one of  $T^1$  and  $T^2$  represents  $-L-NH_2$  or  $-L-COOH$ ;

$X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-Cl$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$  and at least one of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  is not  $-OH$ ; preferred at least 50% of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  are not  $-OH$ ; more preferred at 80% of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  are not  $-OH$ ; most preferred all of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  are not  $-OH$ ;

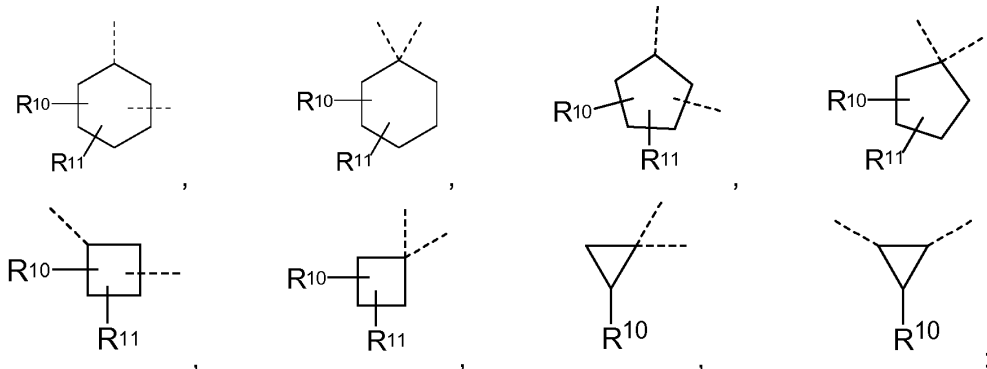
$n$  is an integer selected from 1, 2, 3, or 4;

$n_1$ ,  $n_2$ ,  $n_3$  and  $n_4$  are independently of each other selected from 0 and 1; and

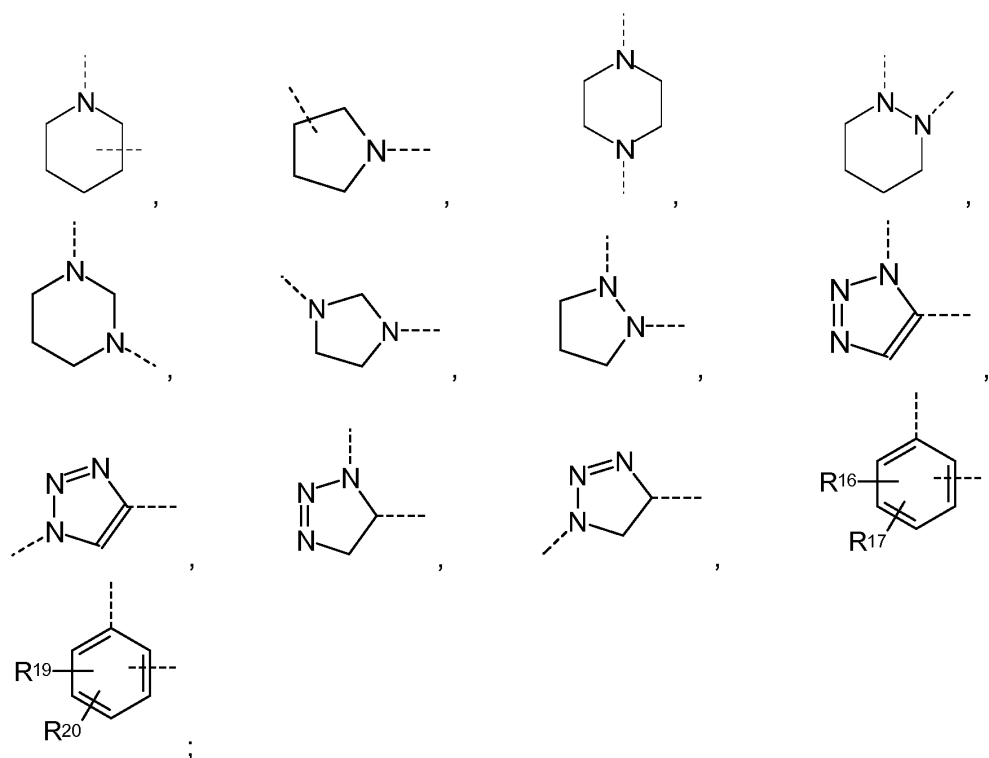
$n_1+n_2+n_3+n_4 \geq 2$ ;

$L$  is selected from:  $-L^a-$ ,  $-L^a-L^e-$ ,  $-L^a-L^b-L^e-$ ,  $-L^a-L^b-L^d-L^c-L^e-$ ,  $-L^a-L^d-L^e-$ ,

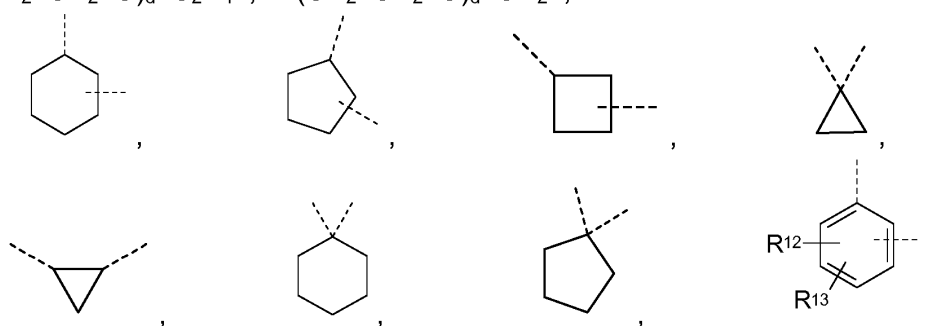
wherein  $-L^a-$  is selected from:  $-(CH_2)_a-$ ,  $-(CF_2)_a-$ ,  $-(CH_2-CH_2-O)_a-C_2H_4-$ ,  $-(CH_2-CH_2-O)_a-CH_2-$ ,  $-(CR^{10}R^{11})_a-$ ,



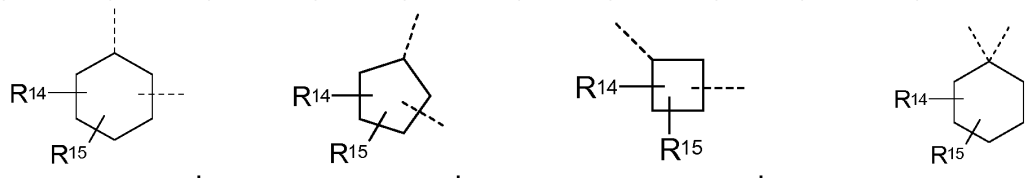
$-L^b-$  and  $-L^c-$  are independently of each other selected from:  $-O-$ ,  $-S-$ ,  $-NH-C(O)-NH-$ ,  $-NH-C(S)-NH-$ ,  $-NH-C(O)-$ ,  $-C(O)-NH-$ ,  $-S-S-$ ,  $-NH-C(O)-O-$ ,  $-NR^9-$ ,  $-NR^{18}-$ ,  $-SO_2-$ ,

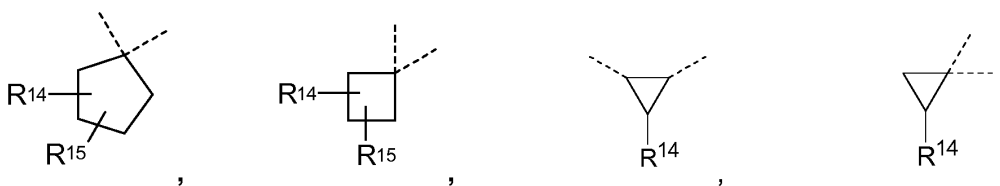


$-L^d-$  represents  $-(CH_2)_d-$ ,  $-(CF_2)_d-$ ,  $-(CR^{12}R^{13})_d-$ ,  
 $-(CH_2-CH_2-O)_d-C_2H_4-$ ,  $-(CH_2-CH_2-O)_d-CH_2-$ ,



5  $-L^e-$  is selected from:  $-(CH_2)_{e1}-$ ,  $-(CF_2)_{e1}-$ ,  $-C_2H_4-(O-CH_2-CH_2)_{e2}-$ ,  
 $-CH_2-(O-CH_2-CH_2)_{e2}-$ ,  $-(CH_2)_{e1}-O-(CH_2)_{e2}-$ ,  $-(CH_2)_{e1}-S-(CH_2)_{e2}-$ ,  
 $-(CR^{14}R^{15})_{e1}-$ ,  $-(CR^{14}R^{15})_{e1}-O-(CR^{21}R^{22})_{e2}-$ ,  $-(CR^{14}R^{15})_{e1}-S-(CR^{21}R^{22})_{e2}-$ ,





$R^9$  and  $R^{18}$  are independently of each other selected from:  $-\text{CH}_3$ ,  $-\text{C}_2\text{H}_5$ ,  $-\text{C}_3\text{H}_7$ , and  $-\text{C}(\text{O})\text{CH}_3$ ;

- 5  $R^{10}$ ,  $R^{11}$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ ,  $R^{16}$ ,  $R^{17}$ ,  $R^{19}$ ,  $R^{20}$ ,  $R^{21}$  and  $R^{22}$  are independently of each other selected from:  $-\text{H}$ ,  $-\text{F}$ ,  $-\text{Cl}$ ,  $-\text{CH}_3$ ,  $-\text{C}_2\text{H}_5$ ,  $-\text{C}_3\text{H}_7$ ,  $-\text{C}_5\text{H}_9$ ,  $-\text{C}_6\text{H}_{13}$ ,  $-\text{OCH}_3$ ,  $-\text{OC}_2\text{H}_5$ ,  $-\text{CH}_2\text{F}$ ,  $-\text{CHF}_2$ ,  $-\text{CF}_3$ ,  $-\text{C}(\text{O})-\text{NH}_2$ ,  $-\text{SCH}_3$ ,  $-\text{SC}_2\text{H}_5$ ,  $-\text{NHC}(\text{O})\text{CH}_3$ ,  $-\text{N}(\text{CH}_3)_2$ , and  $-\text{N}(\text{C}_2\text{H}_5)_2$ ;
- 10 a, d, e1 and e2 are independently of each other an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10;  
and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.
- 15 More preferably  $\text{X}_1$ ,  $\text{X}_2$ ,  $\text{X}_3$ ,  $\text{X}_4$ ,  $\text{X}_5$ , and  $\text{X}_6$  represent independently of each other  $-\text{H}$ ,  $-\text{F}$ ,  $-\text{CH}_3$ ,  $-\text{C}_2\text{H}_5$ ,  $-\text{CN}$ ,  $-\text{OCH}_3$ ,  $-\text{OC}_2\text{H}_5$ ,  $-\text{OCH}(\text{CH}_3)_2$ ,  $-\text{OCH}_2\text{F}$ ,  $-\text{OCF}_3$ ,  $-\text{OCO}-\text{N}(\text{CH}_3)_2$ ,  $-\text{O}-\text{C}_2\text{H}_4-\text{O}-\text{CH}_3$ ,  $-\text{O}-\text{CH}_2-\text{CF}_3$ , and still more preferably  $-\text{H}$ ,  $-\text{F}$ ,  $-\text{CH}_3$ ,  $-\text{CN}$ ,  $-\text{OCH}_3$ ,  $-\text{OC}_2\text{H}_5$ ,  $-\text{OCH}_2\text{F}$ ,  $-\text{OCF}_3$ ,  $-\text{OCO}-\text{N}(\text{CH}_3)_2$ .
- 20 The saccharides of formula (I) are at least disaccharides, while at least trisaccharides are preferred and more preferred is that the saccharide of general formula (I) is at least a tetrasaccharide.
- 25 The saccharides of the present invention bear basic and/or acidic groups and they may form salts with organic or inorganic acids or bases. Examples of suitable acids for such acid addition salt formation are hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, acetic acid, citric acid, oxalic acid, malonic acid, salicylic acid, *p*-aminosalicylic acid, malic acid, fumaric acid, succinic acid, ascorbic acid, maleic acid,
- 30 acid, sulfonic acid, phosphonic acid, perchloric acid, nitric acid, formic acid, propionic acid, gluconic acid, lactic acid, tartaric acid, hydroxymaleic acid, pyruvic acid, phenylacetic acid, benzoic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, methanesulfonic acid, ethanesulfonic acid, nitrous acid, hydroxyethanesulfonic acid, ethylenesulfonic acid, *p*-toluenesulfonic acid, naphthylsulfonic acid, sulfanilic acid,
- 35 camphorsulfonic acid, china acid, mandelic acid, *o*-methylmandelic acid, hydrogen-

benzenesulfonic acid, picric acid, adipic acid, d-o-tolytartaric acid, tartronic acid, (o, m, p)-toluic acid, naphthylamine sulfonic acid, and other mineral or carboxylic acids well known to those skilled in the art. The salts are prepared by contacting the free base form with a sufficient amount of the desired acid to produce a salt in the conventional manner.

Examples for suitable inorganic or organic bases are, for example, NaOH, KOH, NH<sub>4</sub>OH, trialkylamine, triethylamine, tetraalkylammonium hydroxide, lysine or arginine and the like. Salts may be prepared in a conventional manner using methods well known in the art, for example by treatment of a solution of the saccharide of the general formula (I) with a solution of base, selected out of the group mentioned above.

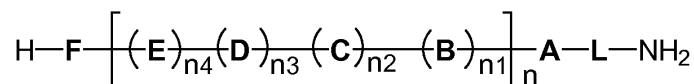
Most preferably, phosphate groups of the saccharide of the general formula (I) build salts or zwitterionic form with sodium or triethylammonium cations.

Preferred are salts with an inorganic cation and more preferred are salts with an inorganic cation which has a single positive charge such as Li<sup>+</sup>, Na<sup>+</sup>, and K<sup>+</sup>. Most preferred are Na<sup>+</sup> salts.

It was surprisingly found that the saccharides of general formula (I) are stable in basic aqueous media as well as suspensions containing aluminum phosphate or aluminum hydroxide, such as the commonly used adjuvant Alhydrogel. While natural Hib PRP hydrolyzes within one day in basic aqueous media or in the presence of aluminum salts, the saccharides of general formula (I) as well as conjugates thereof are stable over several days even at elevated temperatures. The increased stability is particularly advantageous for their use in vaccines against *Haemophilus influenzae*. Thus the saccharides of general formula (I) as well as conjugates thereof are particularly useful for shelf-stable liquid vaccines against *Haemophilus influenzae*, which can be stored at ambient temperature and which are not prone to being damaged by freezing which may occur for instance during transport.

Surprisingly, it was also found that the saccharides of general formula (I) conjugated to an immunogenic carrier are able to provide a protective immune response against *Haemophilus influenzae* bacteria in a human and/or animal host. Additionally, the saccharides of general formula (I) conjugated to an immunogenic carrier are able to elicit substantial IgM and IgG responses in rabbits that are comparable in terms of kinetics and IgG production to the corresponding commercially available Hib PRP conjugate vaccines. Antibodies elicited by the saccharides of general formula (I) conjugated to an immunogenic carrier are cross-reacting with the natural Hib PRP, thus indicating the ability of these antibodies to bind to *Haemophilus influenzae* bacteria and to confer protection against *Haemophilus influenzae* infections.

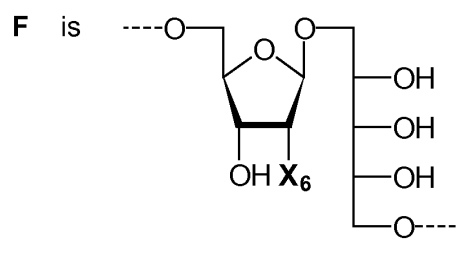
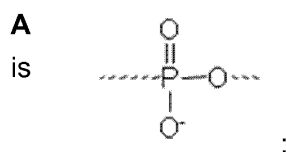
Preferred is a saccharide of the formula (II-1)



5

(II-1)

wherein



**B, C, D, E, L, n1, n2, n3** and **n4** have the meanings as defined above;

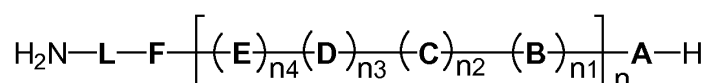
**n** is 1 or 2; and

10

**X<sub>2</sub> - X<sub>6</sub>** are -H, -F, or -OCH<sub>3</sub>;

and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

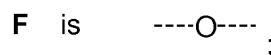
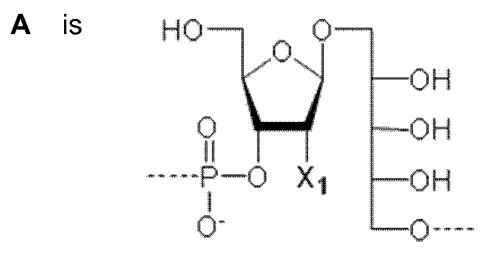
15 Also preferred is a saccharide of the formula (II-2)



(II-2)

20

wherein



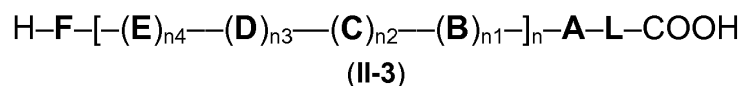
**B, C, D, E, L, n1, n2, n3** and **n4** have the meanings as defined above;

**n** is 1 or 2; and

**X<sub>1</sub> - X<sub>5</sub>** are -H, -F, or -OCH<sub>3</sub>;

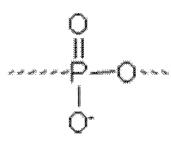
and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

5 Also preferred is a saccharide of the formula (II-3)

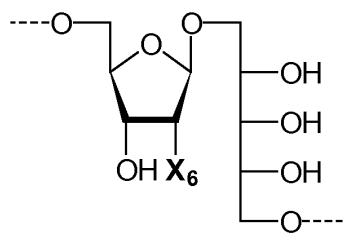


10 wherein

**A** is



**F** is



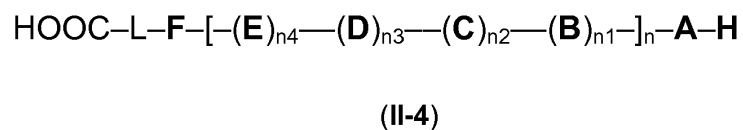
**B, C, D, E, L, n1, n2, n3** and **n4** have the meanings as defined above;

**n** is 1 or 2; and

15 **X<sub>2</sub> - X<sub>6</sub>** are -H, -F, or -OCH<sub>3</sub>;

and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

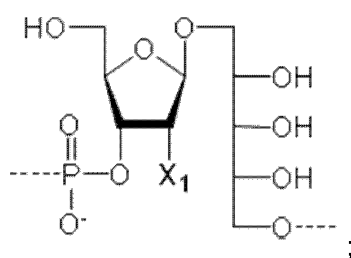
20 Also preferred is a saccharide of the formula (II-4)



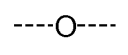
25

wherein

**A** is



**F** is



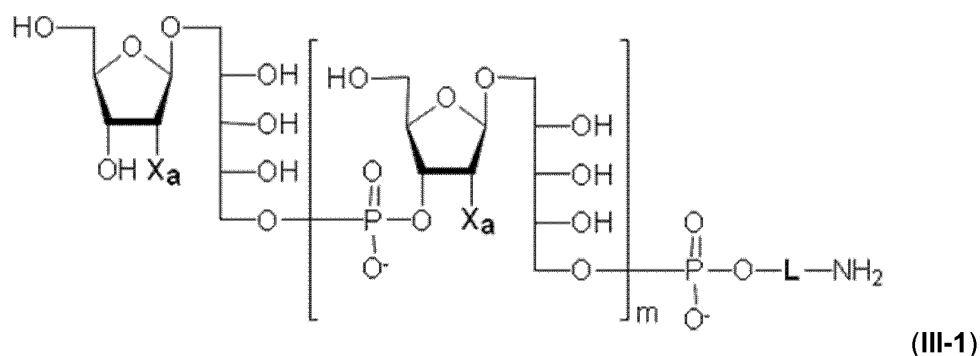
**B, C, D, E, L, n1, n2, n3** and **n4** have the meanings as defined above;

**n** is 1 or 2; and

**X<sub>1</sub> - X<sub>5</sub>** are -H, -F, or -OCH<sub>3</sub>;

and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

Further preferred is saccharide of the formula (III-1)



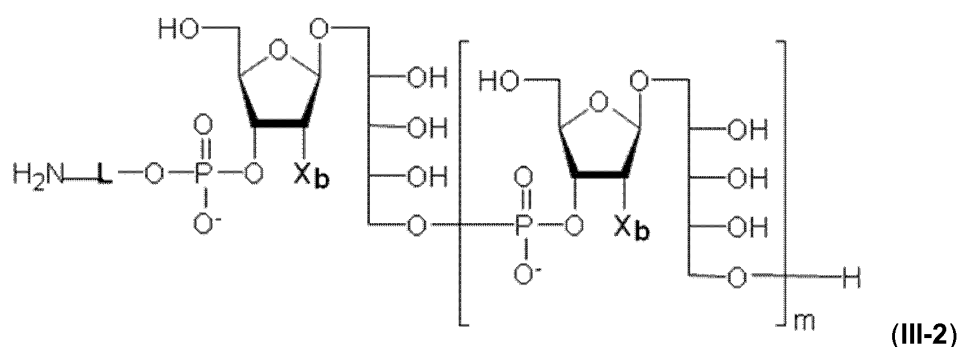
wherein

**L** has the meaning as defined above;

**m** is an integer selected from 1 to 9; and

**X<sub>a</sub>** is -H, -F or -OCH<sub>3</sub>.

Yet further preferred is saccharide of the formula (III-2)



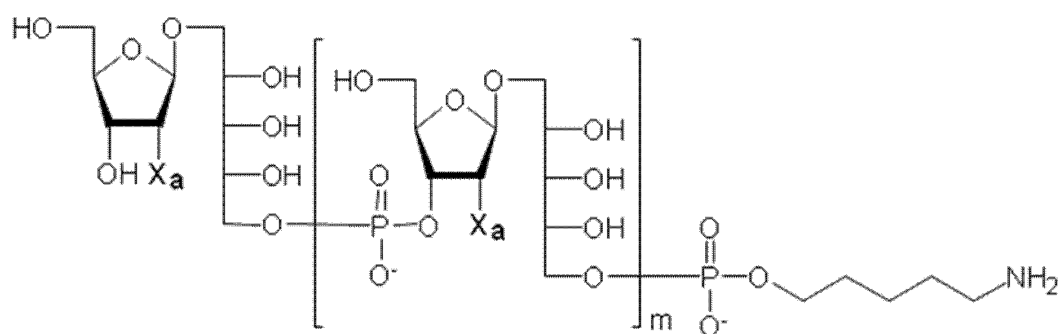
wherein

**L** has the meaning as defined above;

**m** is an integer selected from 1 to 9; and

**X<sub>b</sub>** is -H, -F or -OCH<sub>3</sub>.

More preferred is a saccharide of the formula (IV-1)

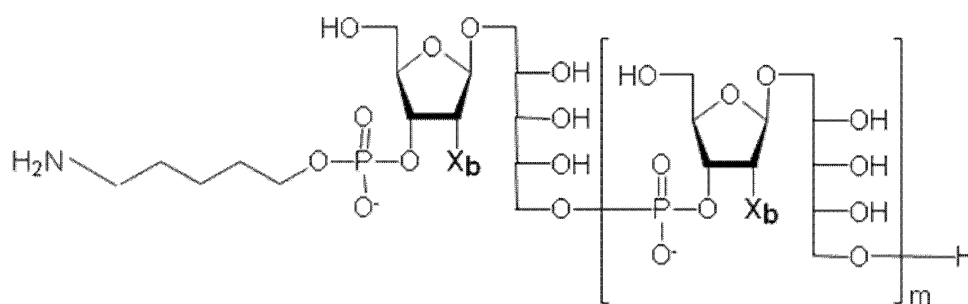


(IV-1)

wherein

- 5     **m** is an integer selected from 1 to 9; and  
       **X<sub>a</sub>** is -H, -F or -OCH<sub>3</sub>.

Still more preferred is a saccharide of the formula (IV-2)



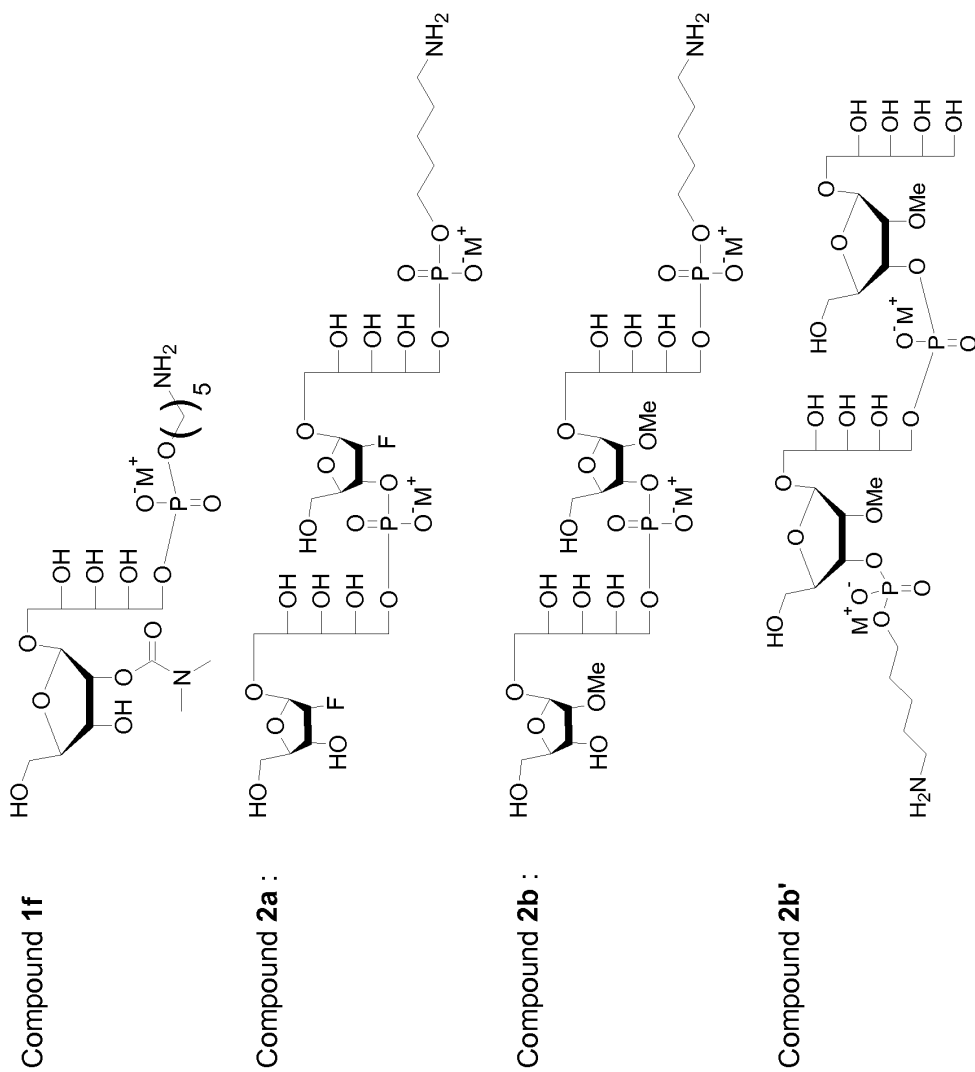
(IV-2)

wherein

- 10     **m** is an integer selected from 1 to 9; and  
       **X<sub>b</sub>** is -H, -F or -OCH<sub>3</sub>.

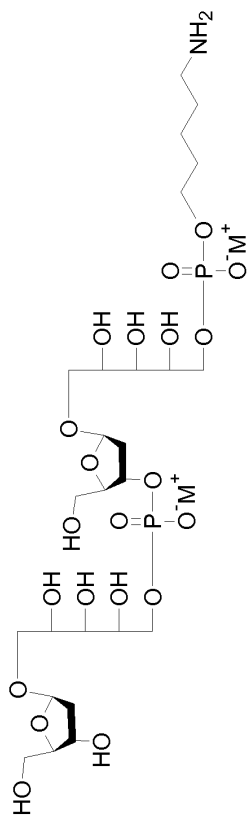
15

Most preferred is a saccharide selected from the group consisting of:

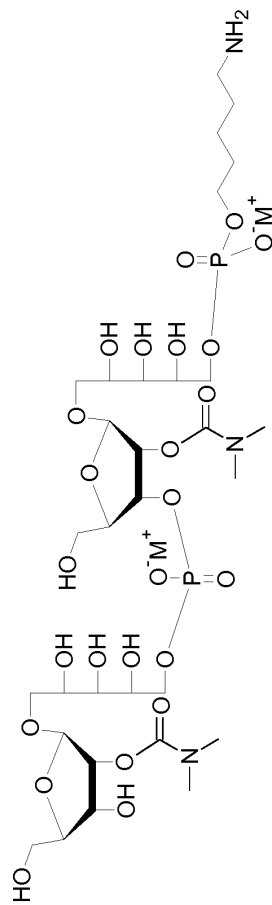


18

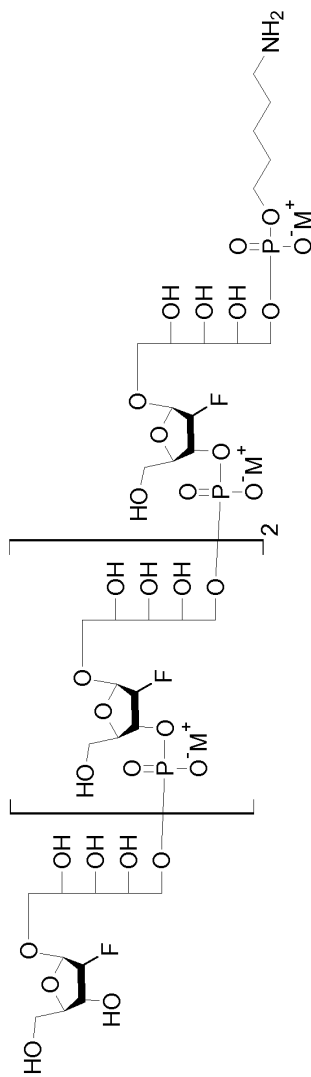
Compound **2c** :



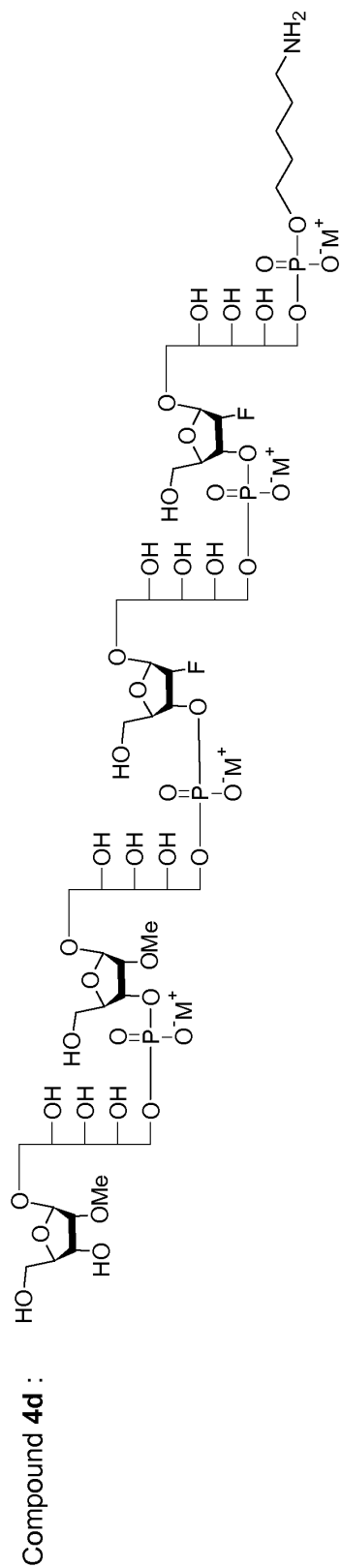
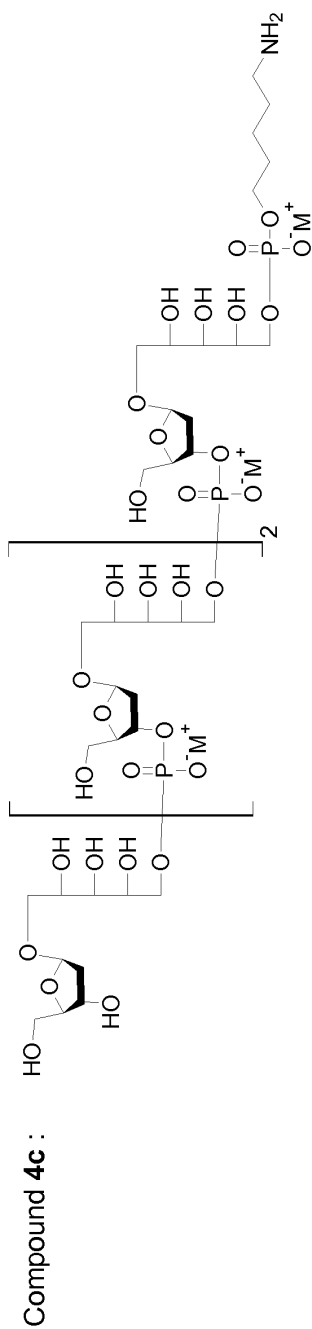
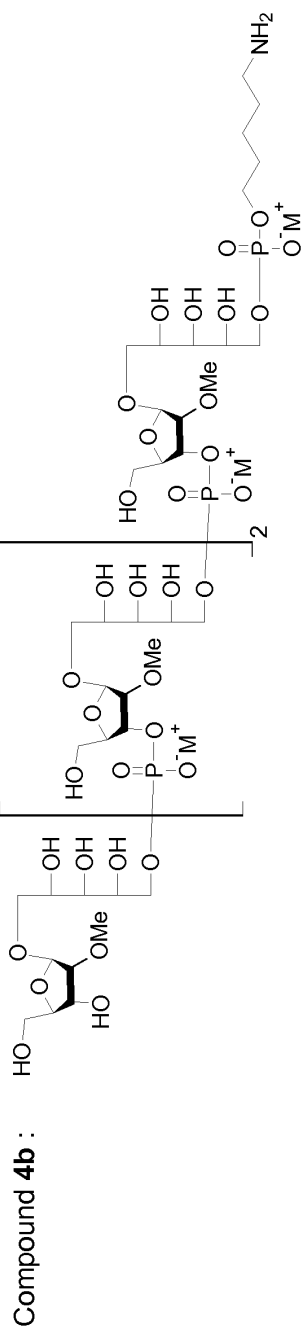
Compound **2f**:



Compound **4a** :

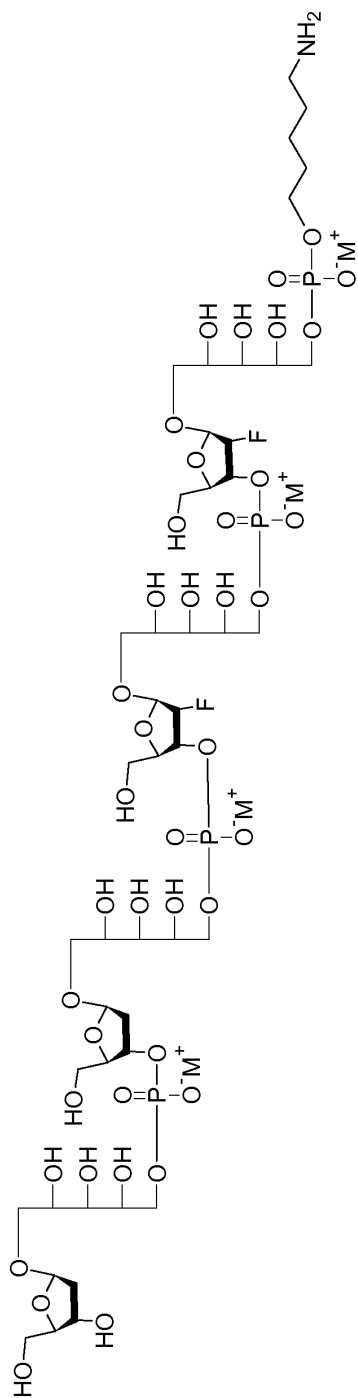


19

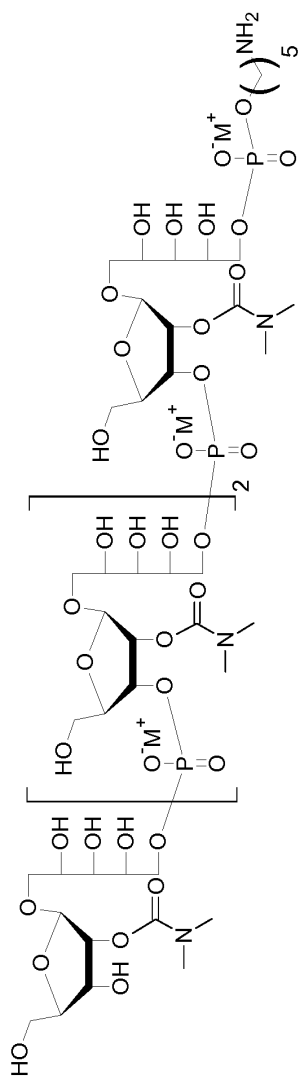


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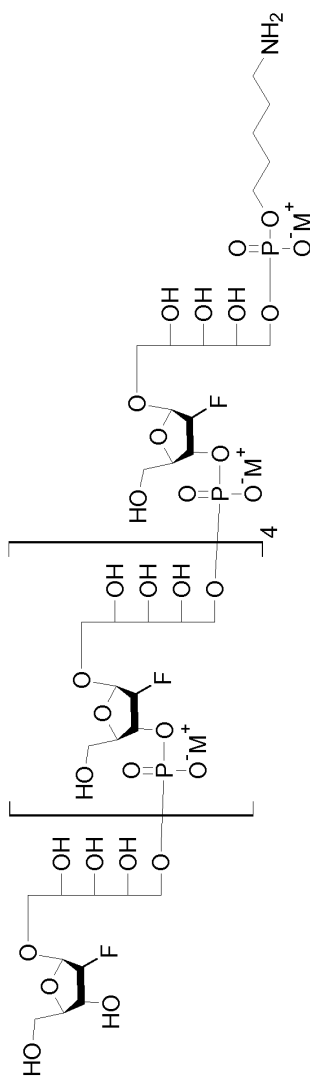
Compound **4e** :



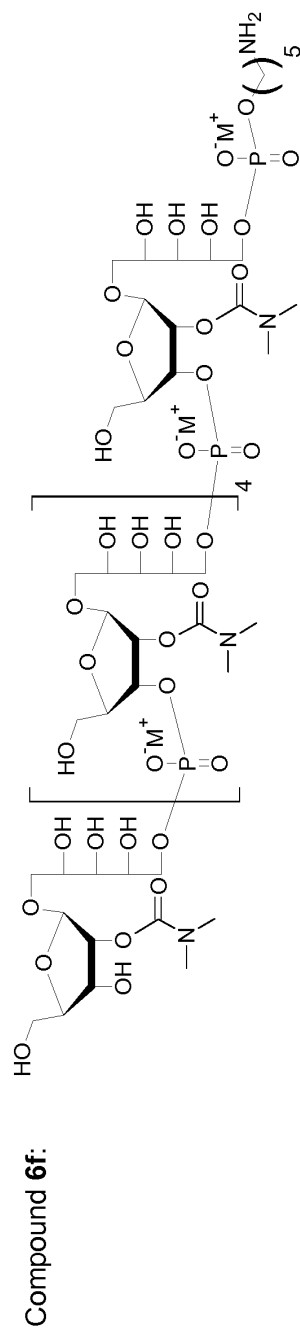
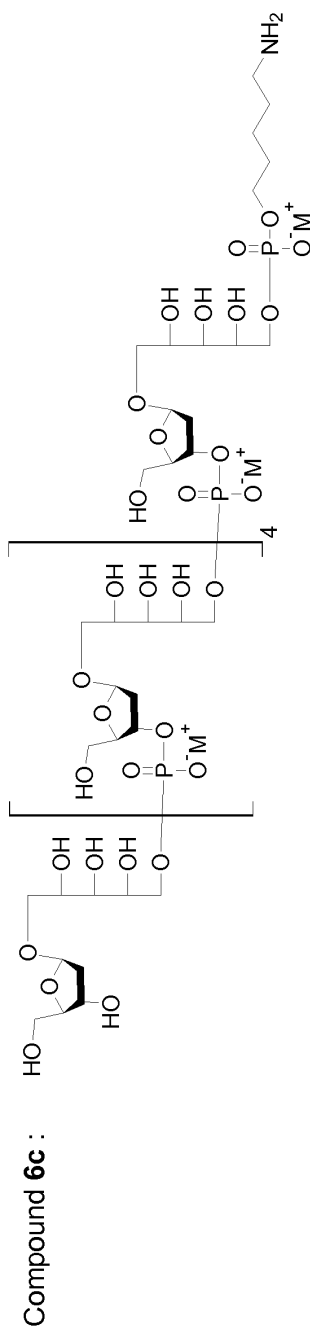
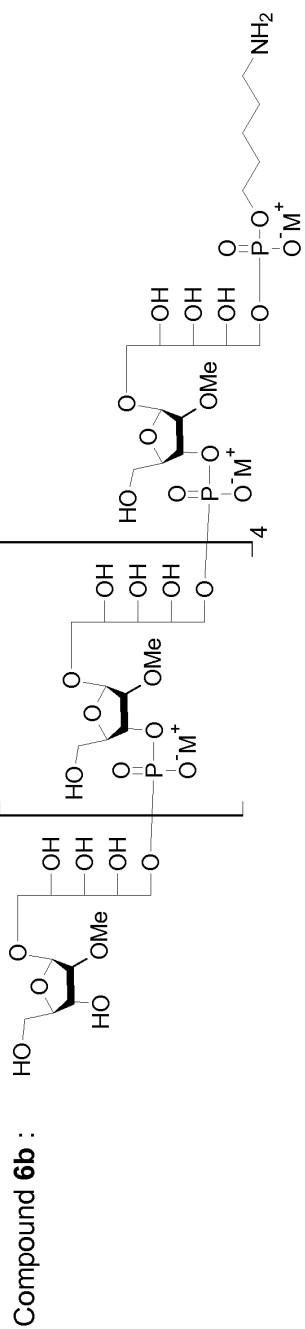
Compound **4f**:

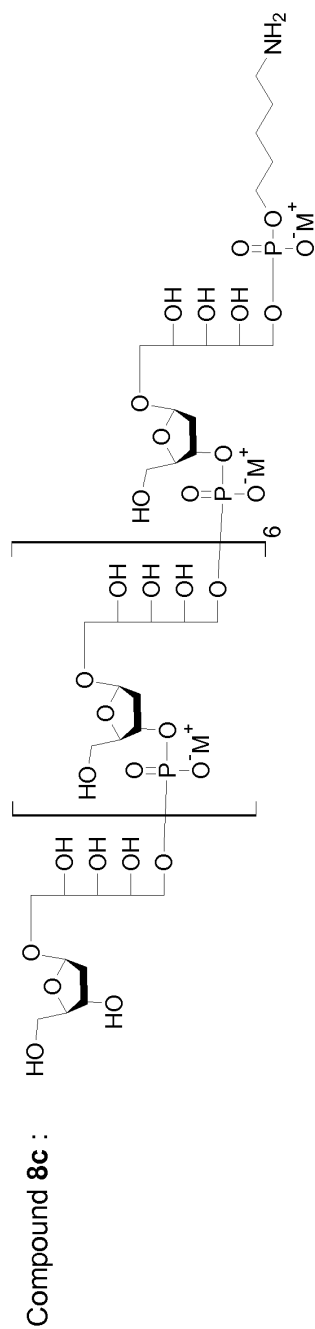
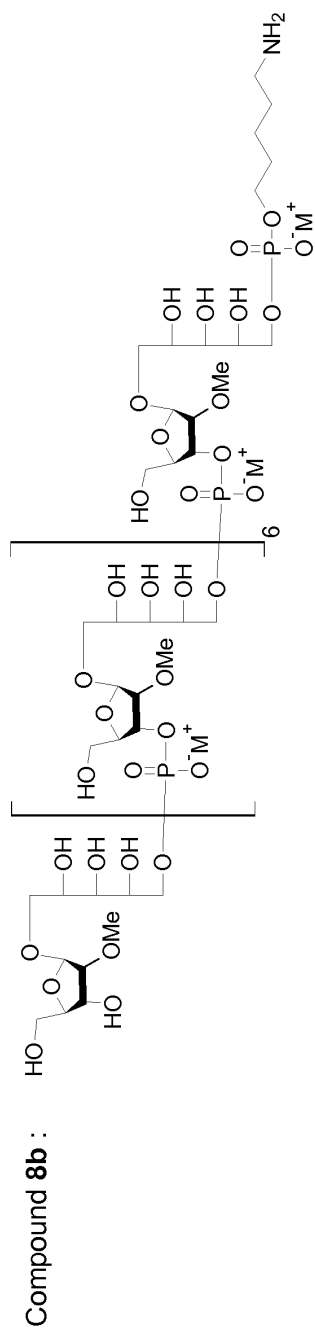
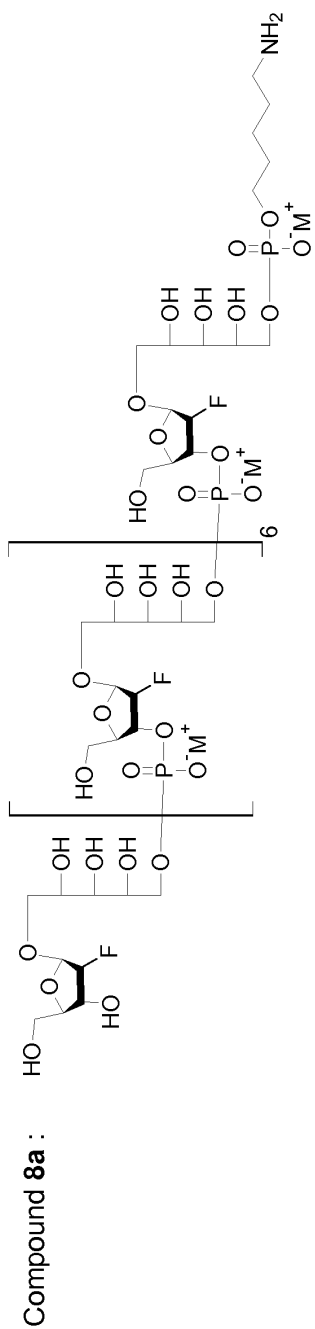


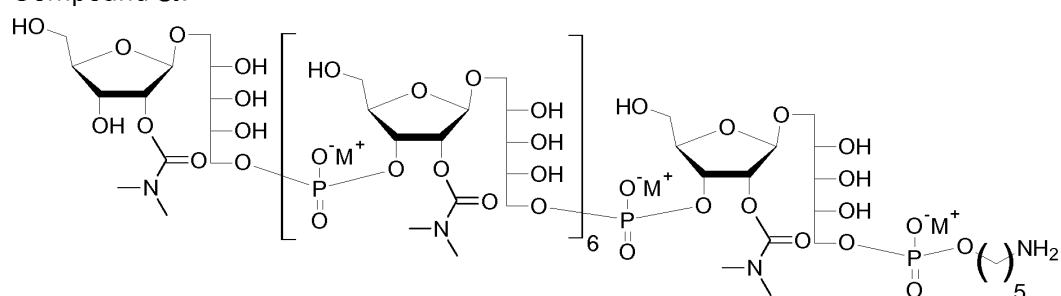
Compound **6a** :



21



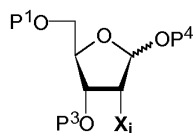
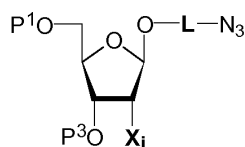
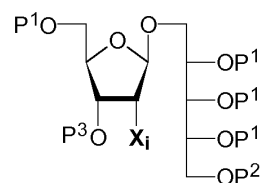
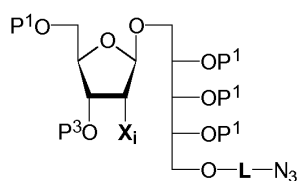
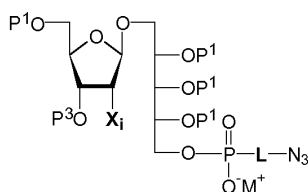
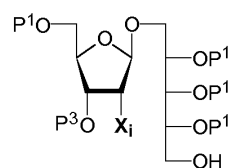
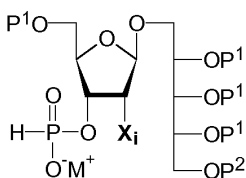
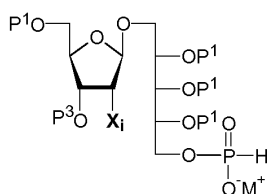
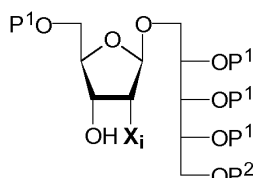


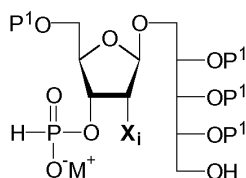
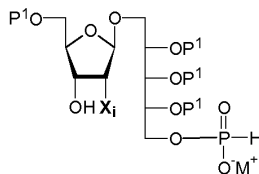
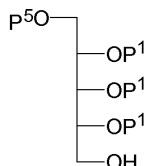
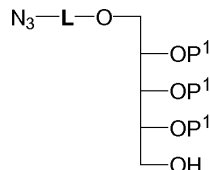
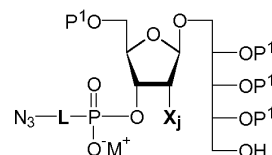
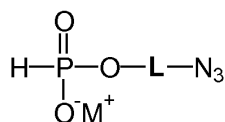
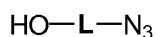
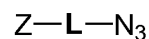
Compound **8f**:

## 5 Synthetic Approach

### 1. Stepwise synthesis of the inventive saccharide.

Disaccharide repeating units having ribosylribitol back bone protected by appropriate protecting groups **A1\*-3** – **A1\*-5**, **B1\*-1** – **B1\*-6**, **C1\*-3**, half repeating units having ribose or ribitol back bone **A1\*-1**, **A1\*-2**, **C1\*-1**, **C1\*-2**, and terminal units comprising a linker **L**, or **T1\*** – **T3\*** can be applied for various synthetic routes of the inventive saccharide. **A1\*-1** – **A1\*-5**, **B1\*-1** – **B1\*-6**, **C1\*-1** – **C1\*-3** and **T1\*** – **T3\*** have the following structures:

**A1\*-1****A1\*-2****A1\*-3****A1\*-4****A1\*-5****B1\*-1****B1\*-2****B1\*-3****B1\*-4**

**B1\*-5****B1\*-6****C1\*-1****C1\*-2****C1\*-3****T1\*****T2\*****T3\***

Z = -Br, -Cl, -I, -OTf,  
-OMs, or -OTs

wherein  $X_i$  and  $X_j$  represent independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;

$P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$  and  $P^5$  represent protecting groups that when bound to -O- cannot be identical to  $X_i$  and/or  $X_j$ ;

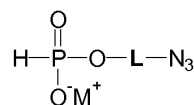
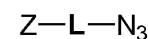
- 5 L has the meaning as defined above; and  
 $M^+$  represents  $Na^+$ ,  $K^+$ ,  $NH_4^+$ ,  $H^+$  or  $Et_3NH^+$ .

- A person skilled in the art may envision, that the protecting groups  $P^1$  to  $P^5$  employed in the synthesis of the inventive saccharides of the general formulae (I), (II-1), (II-2), (III-1), (III-2), (IV-1) and (IV-2) as well as conjugates with immunogenic carriers thereof cannot be identical to  $X_i$  and/or  $X_j$ , when bound to an oxygen atom.

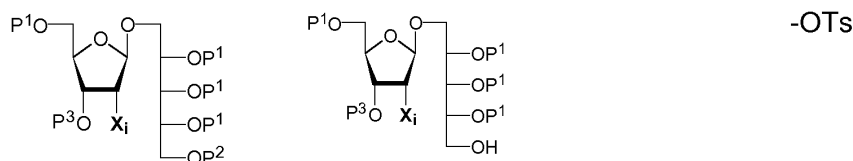
Some of useful synthetic routes are available by the combinations of the above-mentioned building blocks.

15

In an embodiment of the present invention, the following building blocks **A1\*-3**, **B1\*-1** and **T1\*** or **T3\*** may be used for the synthesis of the inventive saccharide (Scheme 1).

**A1\*-3****B1\*-1****T1\*****T3\***

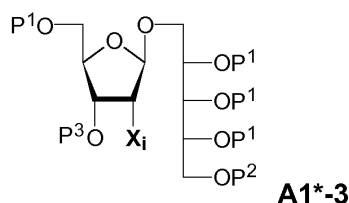
Z = -Br, -Cl, -I,  
-OTf, -OMs, or



As starting building block **A1\*-3** may be used.

The synthetic method **A** comprising the following steps:

- i) Providing a starting building block **A1\*-3**

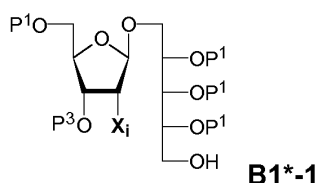


- 5                    wherein  $X_i$  represents  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1, P^2$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

- ii) Removing  $P^3$  group from **A1\*-3**;

- iii) Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii);

- 10                  iv) Coupling an building block **B1\*-1** with a resulting compound after step iii)



- wherein  $X_i$  represents  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

- 15                  v) Optionally repeating the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block **A1\*-3** in the step i), wherein  $t$  is an integer 0 to 20;

- vi) Removing  $P^2$  protecting group from a resulting compound after step v);

- vii) Coupling the compound **T1\*** with a resulting compound after step vi);

- 20                  viii) Removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the above-mentioned synthetic method **A**, the compound **T3\*** also can be used instead of **T1\*** in the step vii). In the step viii), removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

When the half repeating unit **A1\*-1** is used as starting building block instead of the starting building block **A1\*-3** in the abovementioned synthetic method **A**, then after step v), the following steps vi') and vii') may be performed instead of the steps vi) -

5 viii):

vi') Coupling a resulting compound after step v) with **T2\***;

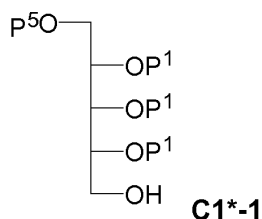
vii') Removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

10 The half repeating unit **C1\*-1** can be used as termination building block after the step v) in the abovementioned synthetic method **A** and then the following steps vi'') - xi'') may be performed instead of the steps vi) - viii):

vi'') Removing  $P^3$  protecting group from a resulting compound after step v);

15 vii'') Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step vi'');

viii'') Coupling a resulting compound after step vii'') with **C1\*-1**;



wherein  $P^1$  and  $P^5$  are protecting groups;

20 ix'') Removing  $P^2$  protecting group from a resulting compound after the step viii'');

x'') Coupling the compound **T1\*** with a resulting compound after step ix'');

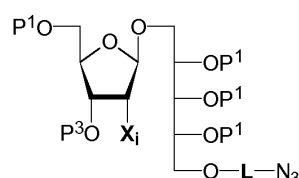
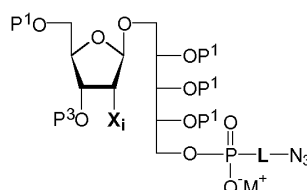
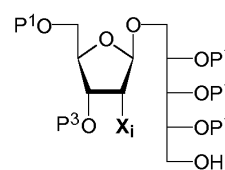
xi'') Removing  $P^1$  and  $P^5$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

25

In step iii) and vii'') of synthetic method **A**, a phosphoramidite group can also be introduced instead of a hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii) or step vi''). **T1\*** is then replaced by bis(diisopropylamino)benzyloxyphosphine and  $HO-L-N_3$ .

30

In another embodiment of the present invention, the following building blocks **A1\*-4** or **A1\*-5**, and **B1\*-1** may be used for the synthesis of the inventive saccharide (Scheme 2).

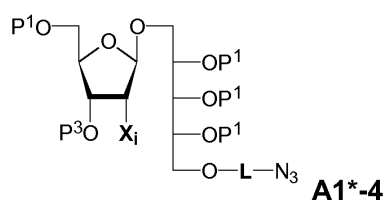
**A1\*-4****A1\*-5****B1\*-1**

As starting building block **A1\*-4** or **A1\*-5** may be used.

The synthetic method **B** comprising the following steps:

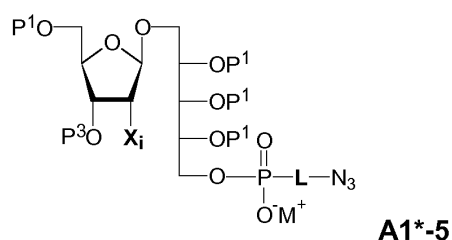
- i) Providing a starting building block **A1\*-4** or **A1\*-5**

5



wherein L is a linker and has the same meaning as defined above;

$X_i$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;



10

wherein L is a linker and has the same meaning as defined above;

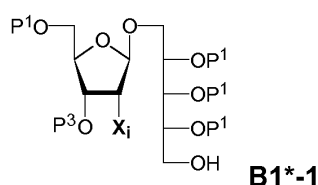
$X_i$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

- ii) Removing  $P^3$  group from **A1\*-4** or **A1\*-5**

15

- iii) Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii);

- iv) Coupling an building block **B1\*-1** with a resulting compound after step iii)



wherein  $X_i$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

20

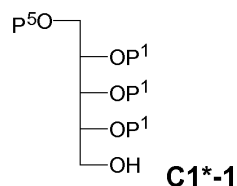
- v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block **A1\*-4** or **A1\*-5** in the step i), wherein  $t$  is an integer 0 to 20;
- vi) Removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vi), removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

10

The half repeating unit **C1\*-1** can be used as termination building block after the step v) in the abovementioned synthetic method **B** and then the following steps vi'') - ix'') may be performed instead of the step vi):

- vi'') Removing  $P^3$  protecting group from a resulting compound after step v);
- vii'') Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step vi'');
- viii'') Coupling a resulting compound after step vii'') with **C1\*-1**;

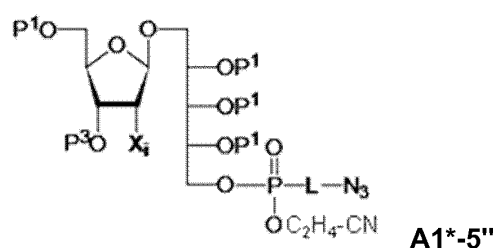
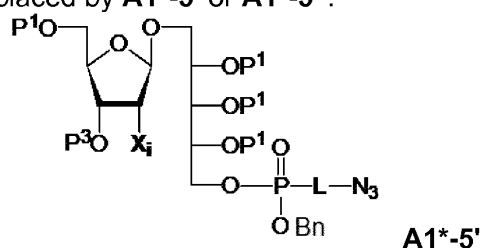


20

wherein  $P^1$  and  $P^5$  are protecting groups;

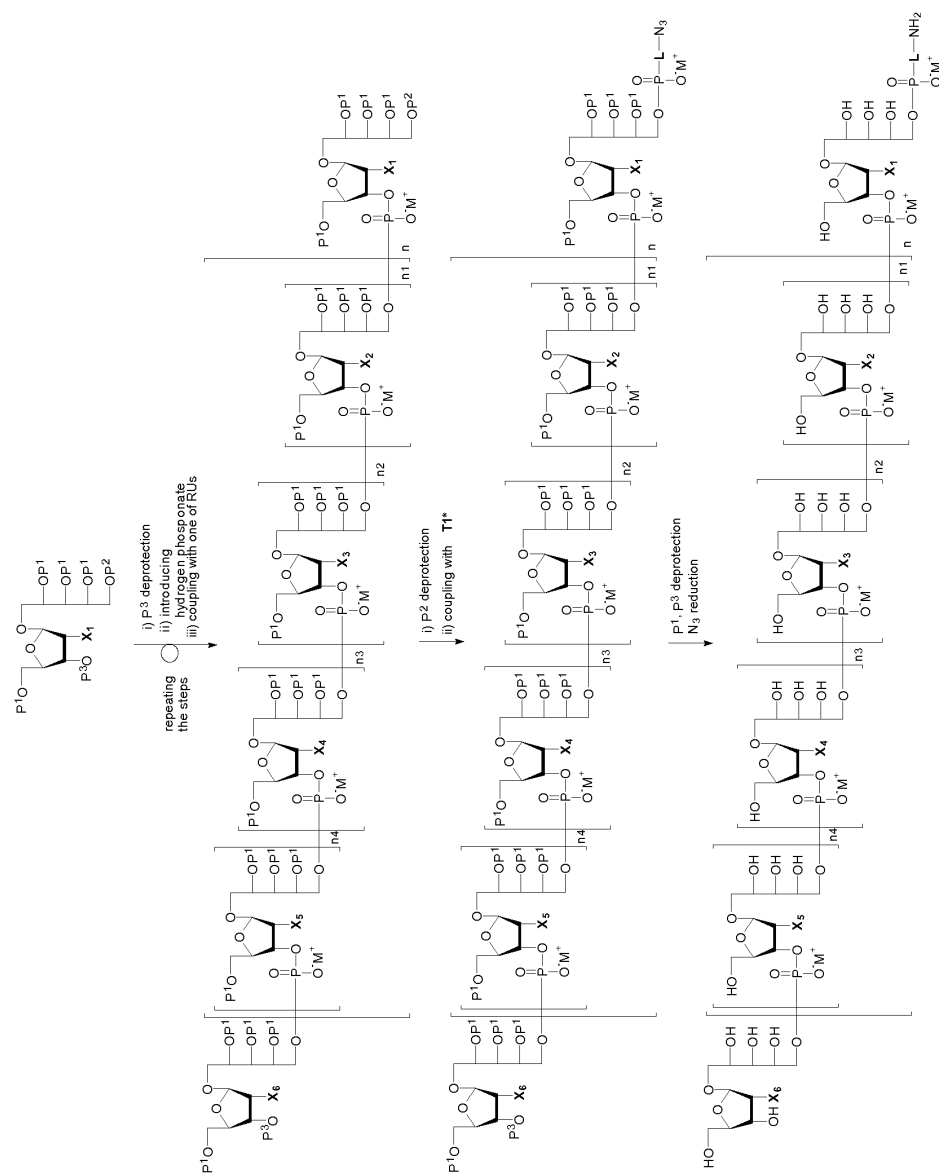
- ix'') Removing  $P^1$  and  $P^5$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In step iii) and vii'') of synthetic method **B**, a phosphoramidite group can also be introduced instead of a hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii) or step vi''). Starting building block **A1\*-5** is then replaced by **A1\*-5'** or **A1\*-5''**.

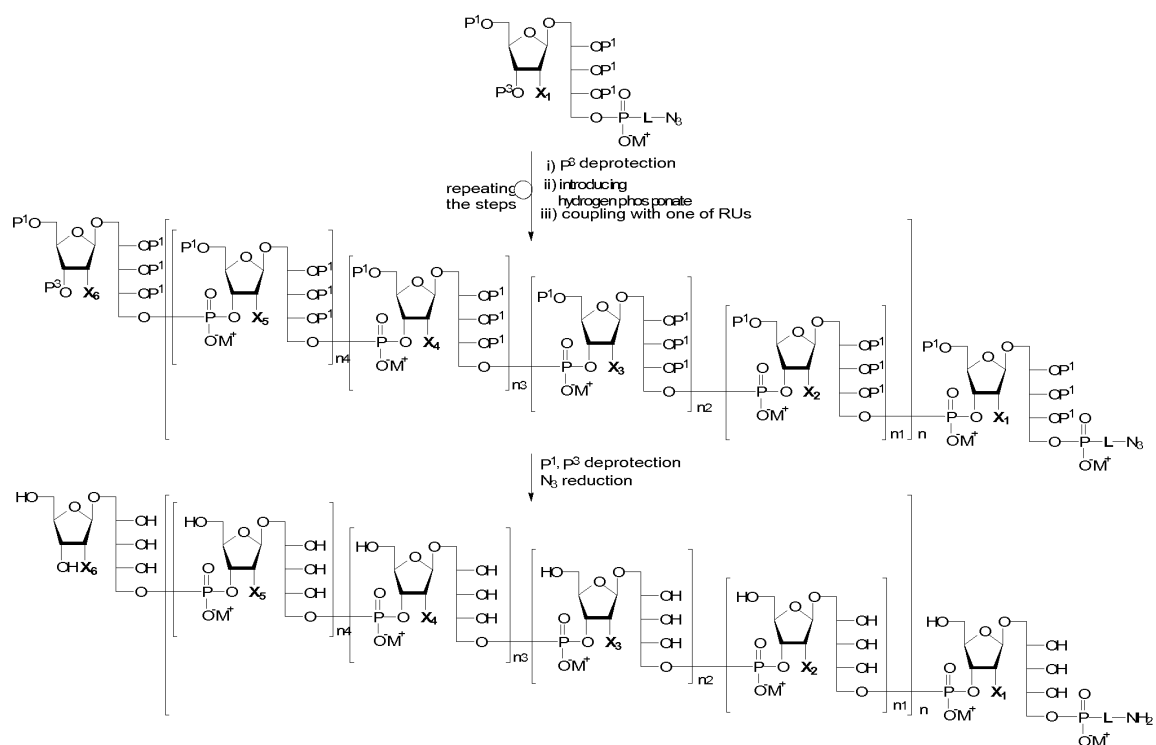


25

**Scheme 1.** Synthesis of the Hib capsular oligosaccharide derivatives by the synthetic method **A**



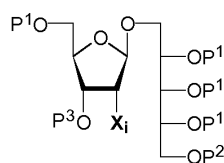
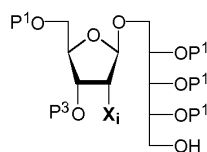
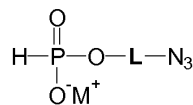
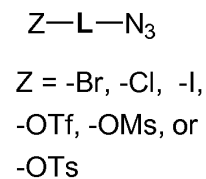
**Scheme 2.** Synthesis of the Hib capsular oligosaccharide derivatives by the synthetic method **B**



5

In an embodiment of the present invention, the following building blocks **A1\*-3**, **B1\*-1** and **T1\*** or **T3\*** may be used for the synthesis of the inventive saccharide (Scheme 1).

10

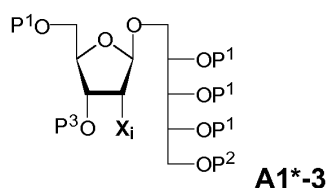
**A1\*-3****B1\*-1****T1\*****T3\***

As starting building block **A1\*-3** may be used.

The synthetic method **C** comprising the following steps:

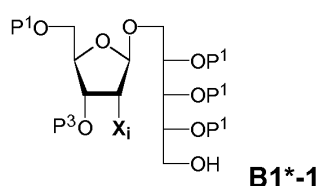
15

i) Providing a starting building block **A1\*-3**



wherein  $X_i$  represents  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1, P^2$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

- ii) Removing  $P^3$  group from **A1\*-3**;
- 5    iii) Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii);
- iv) Coupling an building block **B1\*-1** with a resulting compound after step iii)

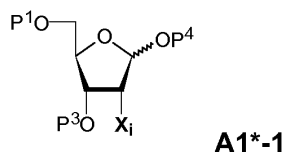


wherein  $X_i$  represents  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

- 10    v) Optionally repeating the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block **A1\*-3** in the step i), wherein  $t$  is an integer 0 to 20;
- vi) Removing  $P^3$  protecting group from a resulting compound after step v);
- 15    vii) Coupling the compound **T1\*** or **T3\*** with a resulting compound after step vi);
- viii) Removing  $P^1$  and  $P^2$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

20    In the step viii), removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

The half repeating unit **A1\*-1** can be used as a starting building block



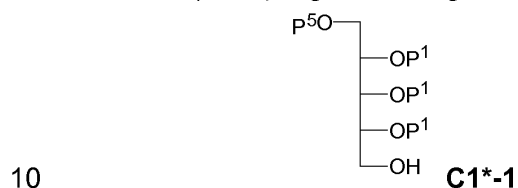
25

instead of **A1\*-3** in the step i) in the abovementioned synthetic method **C** and then a step for removing  $P^4$  protecting group is added into the step viii).

The half repeating unit **C1\*-1** can be used as termination building block after the step v) in the abovementioned synthetic method **C** and then the following steps vi'') - xi'') may be performed instead of the steps vi) - viii):

- 5 vi'') Removing  $P^3$  protecting group from a resulting compound after step v);  
 vii'') Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step vi'');

viii'') Coupling a resulting compound after step vii'') with **C1\*-1**;

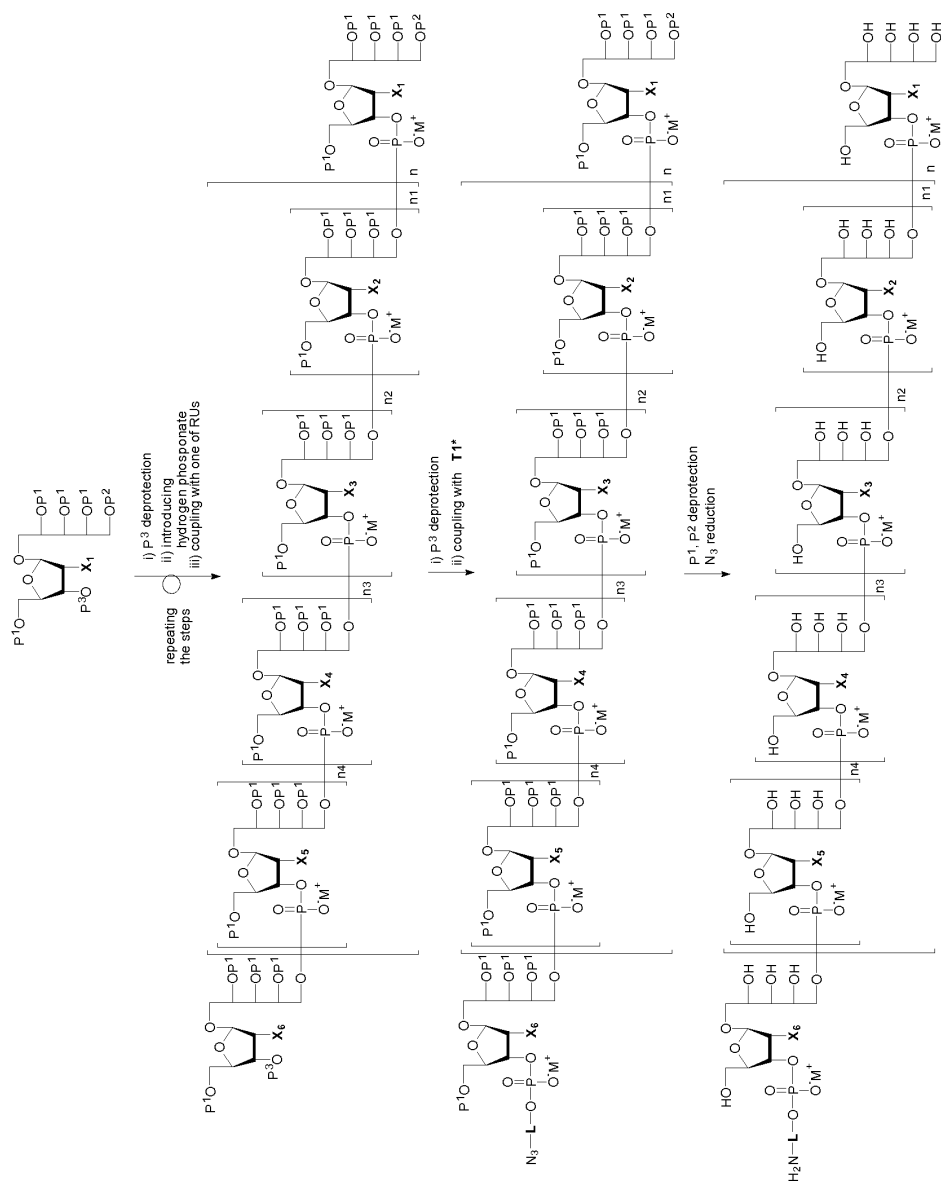


wherein  $P^1$  and  $P^5$  are protecting groups;

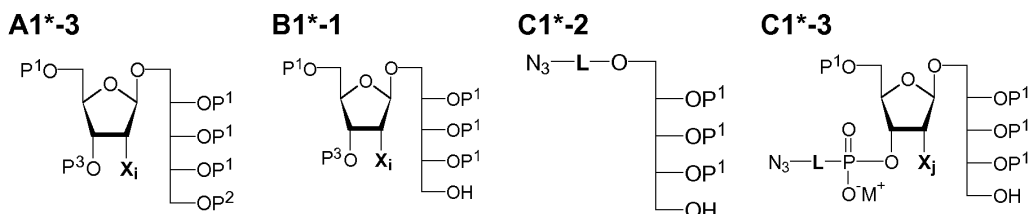
- ix'') Removing  $P^5$  protecting group from a resulting compound after the step viii'');  
 x'') Coupling the compound **T1\*** or **T3\*** with a resulting compound after step ix'');  
 15 xi'') Removing  $P^1$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

20 In step iii) and vii'') of synthetic method **C**, a phosphoramidite group can also be introduced instead of a hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii) or step vi''). **T1\*** is then replaced by bis(diisopropylamino)benzyloxyposphine and  $HO-L-N_3$ .

**Scheme 3.** Synthesis of the Hib capsular oligosaccharide derivatives by synthetic method C



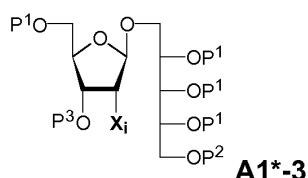
In another embodiment of the present invention, the following building blocks **A1\*-3**, **B1\*-1**, and **C1\*-2** or **C1\*-3** may be used for the synthesis of the inventive saccharide.



5 As starting building block **A1\*-3** may be used.

The synthetic method **D** comprising the following steps:

i) Providing a starting building block **A1\*-3**



10

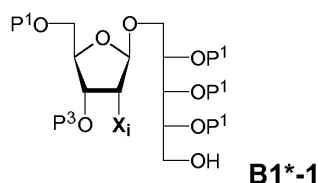
wherein L is a linker and has the same meaning as defined above;

$X_i$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$ ,  $P^2$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

ii) Removing  $P^3$  group from **A1\*-3**;

15 iii) Introducing hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii);

iv) Coupling an building block **B1\*-1** with a resulting compound after step iii)



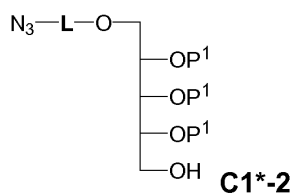
20

wherein  $X_i$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

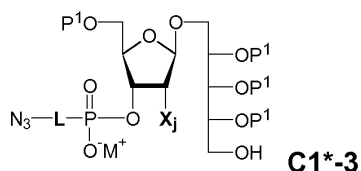
v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block **A1\*-3** in the step i), wherein  $t$  is an integer 0 to 20;

vi) Removing  $P^3$  group from a resulting compound after the step v);

25 vii) Coupling a building block **C1\*-2** or **C1\*-3** with a resulting compound after the step vi)



wherein  $P^1$  is protecting group;



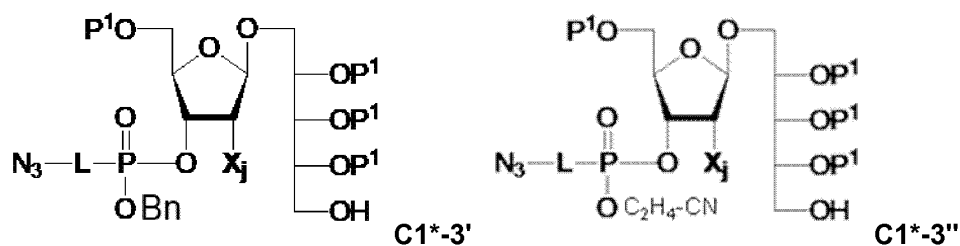
- 5 wherein  $X_j$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  is protecting group that when bound to  $-O-$  is not identical to  $X_j$ ;

viii) Removing  $P^1$  and  $P^2$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

10

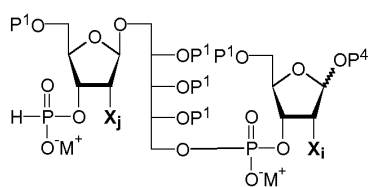
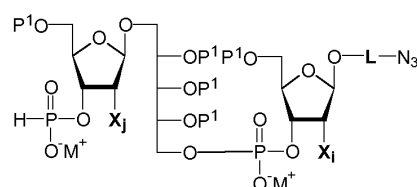
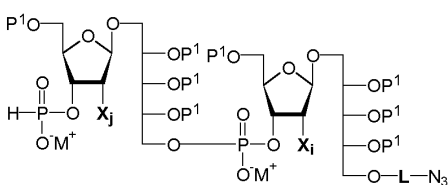
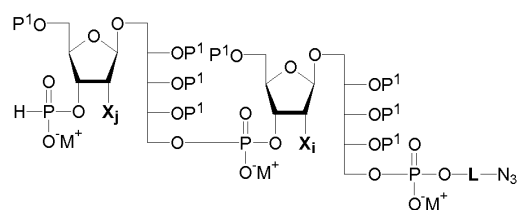
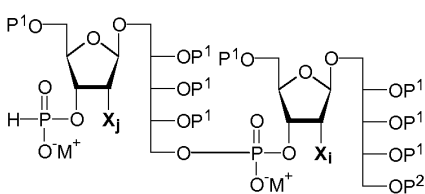
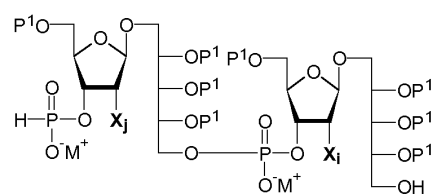
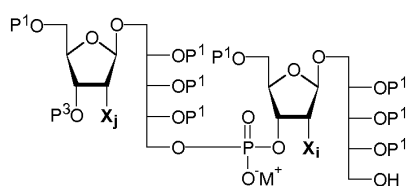
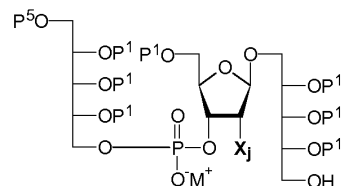
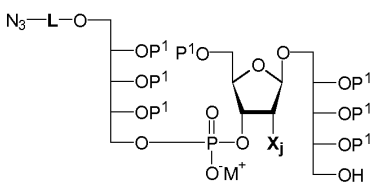
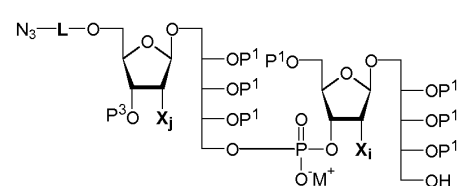
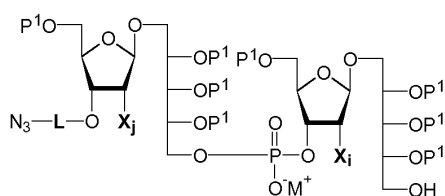
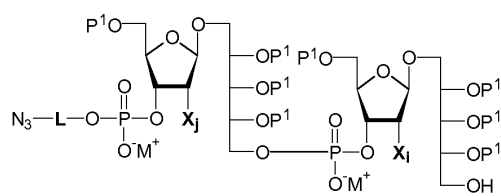
In the step viii), removing  $P^1$  and  $P^2$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

- 15 In step iii), a phosphoramidite group can also be introduced instead of a hydrogen phosphonate group at C-3 position of a ribose of a resulting compound after step ii). Starting building block **C1\*-3** is then replaced by **C1\*-3'** or **C1\*-3''**.



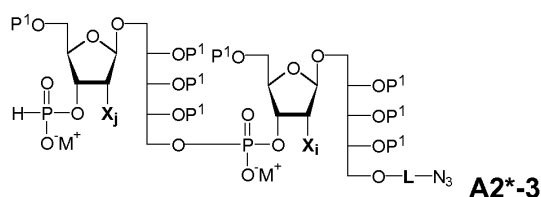
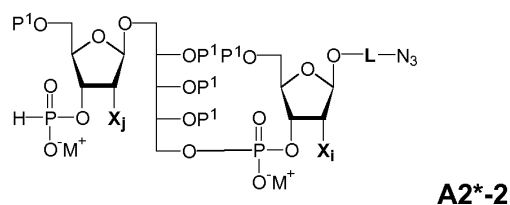
20

In one embodiment of the present invention, the following building blocks consisting of two repeating units are useful for synthesis of the inventive saccharide by [2+2] synthetic approach:

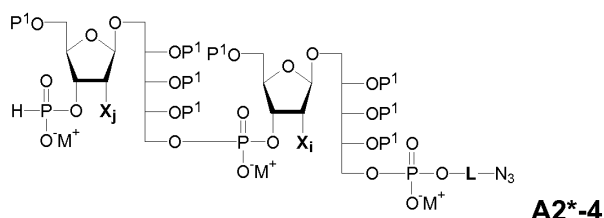
**A2\*-1****A2\*-2****A2\*-3****A2\*-4****B2\*-1****B2\*-2****B2\*-3****B2\*-4****C2\*-1****C2\*-2****C2\*-3****C2\*-4**

A synthetic method **E-1** comprises the following steps (Scheme 4):

- i) Providing a starting building block selected from **A2\*-2**, **A2\*-3**, or **A2\*-4**



5



wherein L is a linker and has the same meaning as defined above;

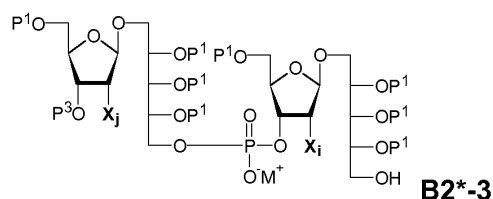
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$X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;

$P^1$  represents protecting group that when bound to  $-O-$  is not identical to  $X_i$  and  $X_j$ ; and

- ii) Coupling a building block **B2\*-3** with the starting building block selected from **A2\*-2**, **A2\*-3**, or **A2\*-4**

15



wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

20

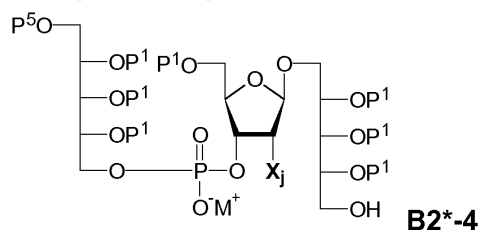
- iii) Removing  $P^3$  group from a resulting compound after the step ii);  
iv) Introducing hydrogen phosphonate at C-3 position of ribose of a resulting compound after the step iii);

- v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;
- vi) Removing  $P^1$  and  $P^3$  protecting groups from a resulting compound after step v) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vi), removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

After the step v) of the synthetic method **E-1**, a further building block **B2\*-4** may be alternatively coupled with the resulting compound after the step v). In this case, the synthetic method **E-1** comprises the following steps instead of the step vi):

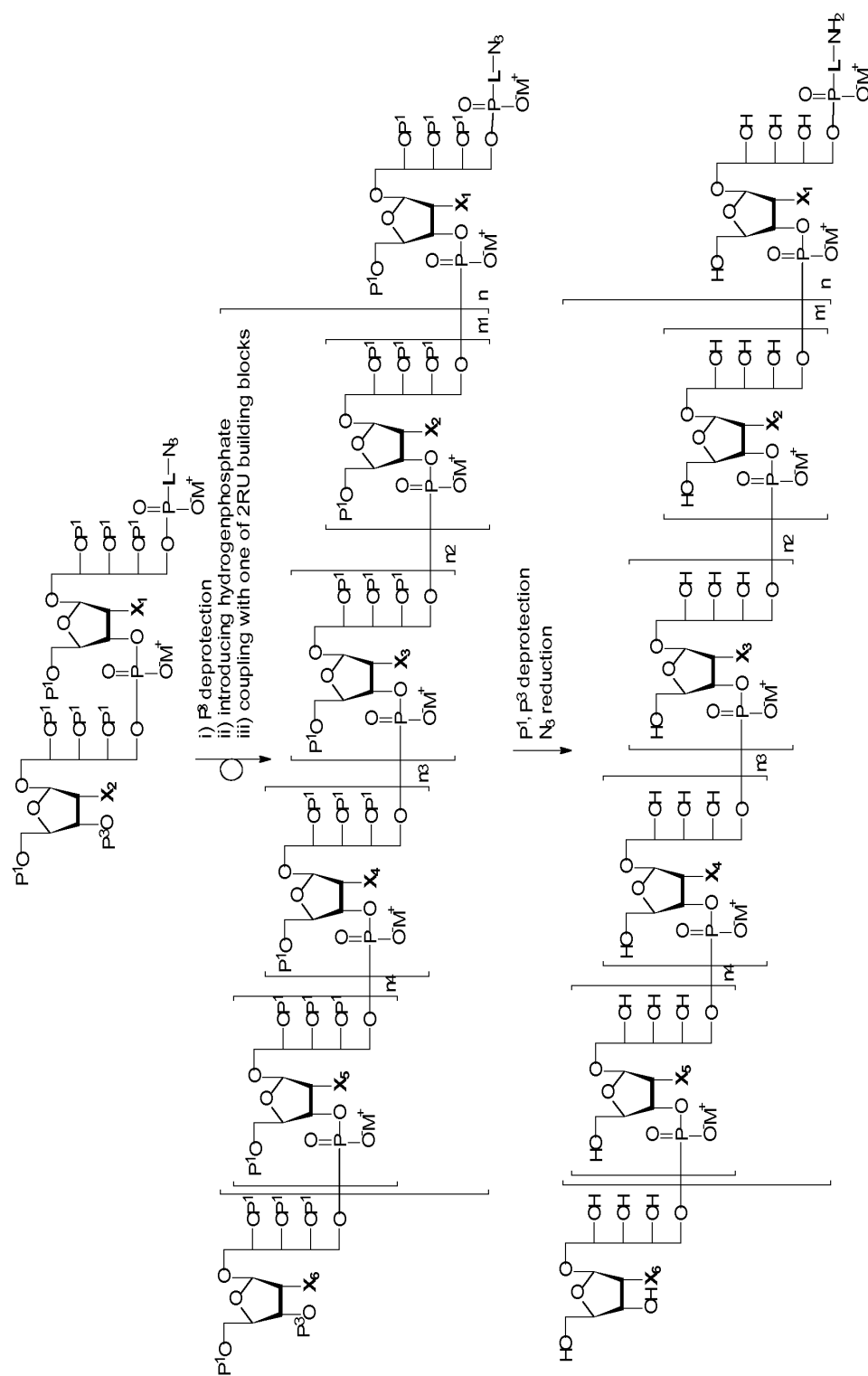
- vi') coupling a building block **B2\*-4** with a resulting compound after the step v);



wherein  $X_j$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^5$  are protecting groups that when bound to  $-O-$  are not identical to  $X_j$ ;

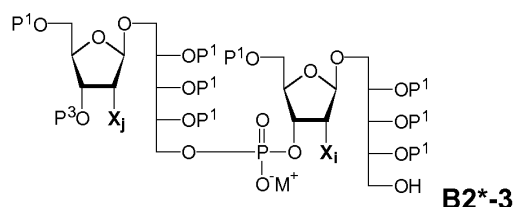
- vii') Removing  $P^1$  and  $P^5$  protecting groups from a resulting compound after the step vi') and transforming an azido group to an amine group for obtaining the compound of the formula (I).

**Scheme 4.** Synthesis of the Hib capsular oligosaccharide derivatives by method E-1

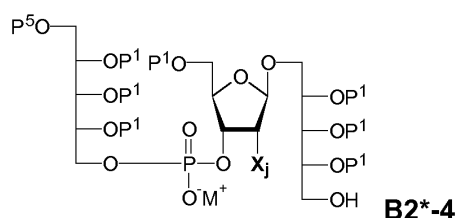


Of course, **B2\*-3** or **B2\*-4** can be used as a starting building block. In this case, an alternative synthetic method **E-2** comprises the following steps:

- i) Providing a starting building block selected from **B2\*-3**, or **B2\*-4**

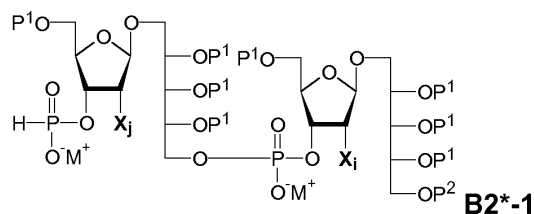


- 5 wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;



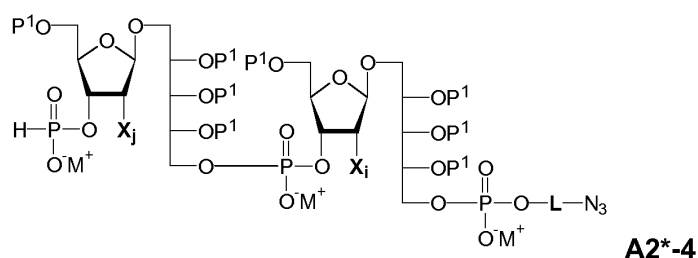
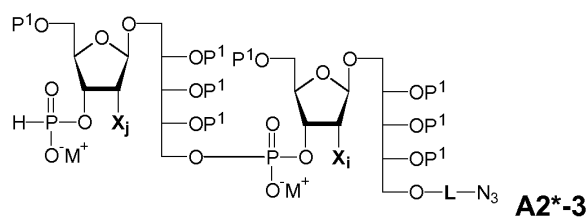
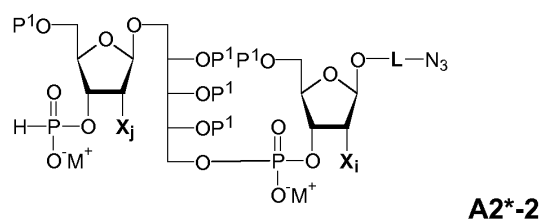
- 10 wherein  $X_j$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^5$  are protecting groups that when bound to  $-O-$  are not identical to  $X_j$ ;

- ii) Coupling a building block **B2\*-1** with the starting building block selected from **B2\*-3**, or **B2\*-4**



- 15 wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^2$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- 20 iii) Removing  $P^2$  protecting group from a resulting compound after the step ii);  
 iv) Optionally repeat the steps i) - iii) for  $t$  times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;  
 v) Coupling a resulting compound after the step iv) with a building block selected from **A2\*-2**, **A2\*-3**, or **A2\*-4**



5

wherein L is a linker and has the same meaning as defined above;

$X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  represents protecting group that when bound to  $-O-$  is not identical to  $X_i$  and/or  $X_j$ ;

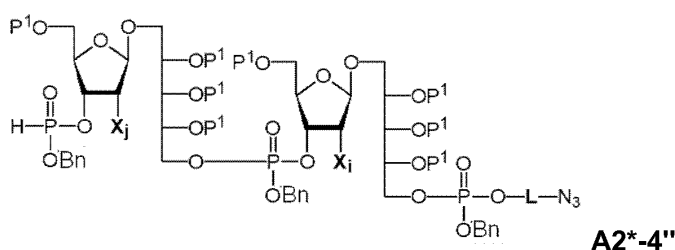
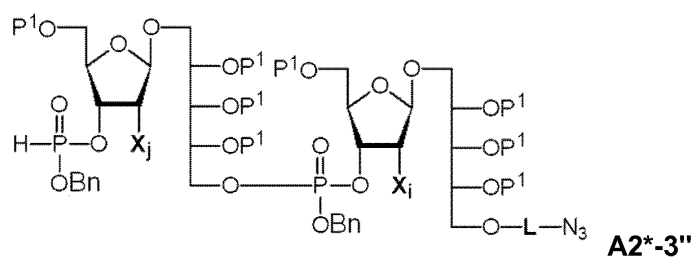
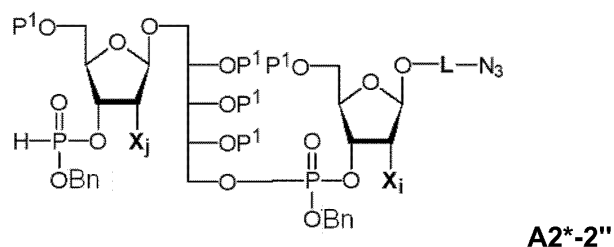
10

vi) Removing  $P^1$ ,  $P^3$  and  $P^5$  protecting groups from a resulting compound after step v) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

15 In the step vi), removing  $P^1$ ,  $P^3$  and  $P^5$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

20 An alternative synthetic method **E-3** differs from synthetic method **E-1** in that phosphoramidite is employed instead of phosphonate chemistry. Thus, synthetic method **E-3** comprises the following steps:

i) Providing a starting building block selected from **A2\*-2''**, **A2\*-3''**, or **A2\*-4''**

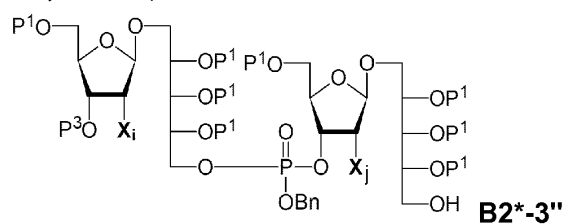


5

wherein L is a linker and has the same meaning as defined above;  
 $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  
 $P^1$  represents protecting group that when bound to  $-O-$  is not identical to  $X_i$  and  $X_j$ ; and

10

- ii) Coupling a building block **B2\*-3''** with the starting building block selected from **A2\*-2''**, **A2\*-3''**, or **A2\*-4''**



15

wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

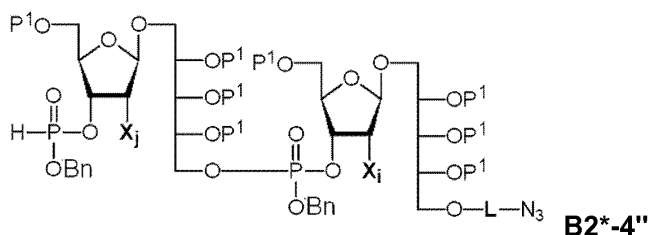
- iii) Removing  $P^3$  group from a resulting compound after the step ii);

- iv) Introducing phosphoramidite group at C-3 position of ribose of a resulting compound after the step iii);
- v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;
- 5 vi) Removing  $P^1$  and  $P^3$  protecting groups from a resulting compound after step v) and transforming an azido group to an amine group for obtaining the compound of the formula (I).
- 10 In the step vi), removing  $P^1$  and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

After the step v) of the synthetic method **E-3**, a further building block **B2\*-4''** may be alternatively coupled with the resulting compound after the step v). In this case, the synthetic method **E-3** comprises the following steps instead of the step vi):

15

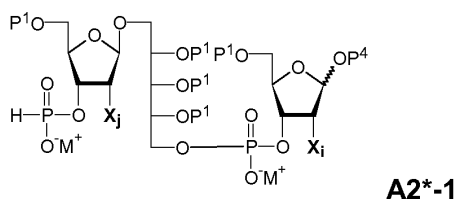
vi') coupling a building block **B2\*-4''** with a resulting compound after the step v);

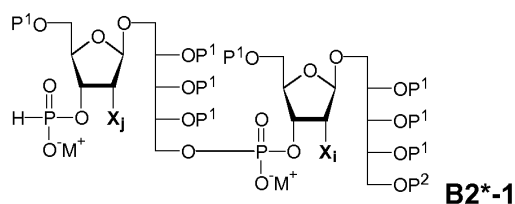


- 20 wherein  $X_j$  represents  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^5$  are protecting groups that when bound to  $-O-$  are not identical to  $X_j$ ;
- vii') Removing  $P^1$  and  $P^5$  protecting groups from a resulting compound after the step vi') and transforming an azido group to an amine group for obtaining the compound of the formula (I).
- 25

A synthetic method **F-1** comprises the following steps (Scheme 5):

- i) Providing a starting building block selected from **A2\*-1** or **B2\*-1**

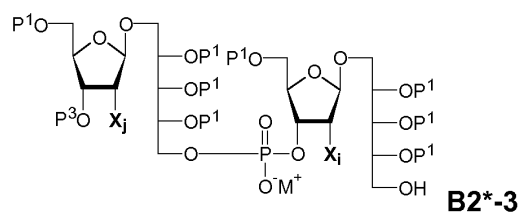




wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1, P^2$  and  $P^4$  represent protecting group that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

5

- ii) Coupling a building block **B2\*-3** with the starting building block selected from **A2\*-1** or **B2\*-1**

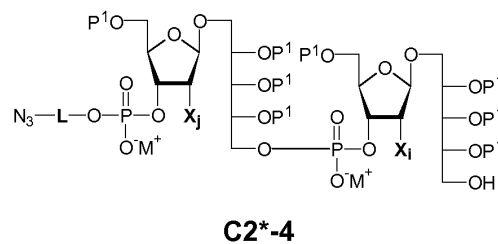
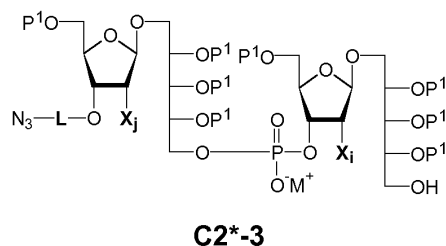
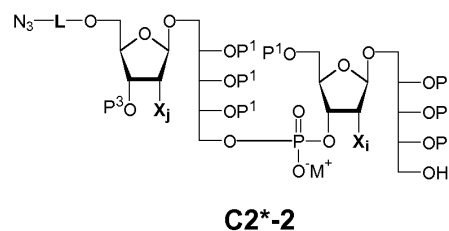
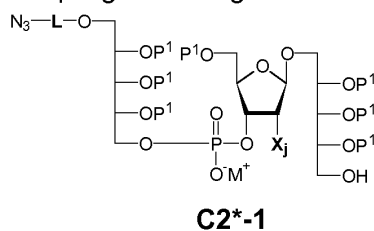


10

wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- iii) Removing  $P^3$  group from a resulting compound after the step ii);  
 iv) Introducing hydrogen phosphonate at C-3 position of ribose of a resulting compound after the step iii);  
 v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;  
 vi) Coupling a building block selected from **C2\*-1**, **C2\*-2**, **C2\*-3** or **C2\*-4**

15



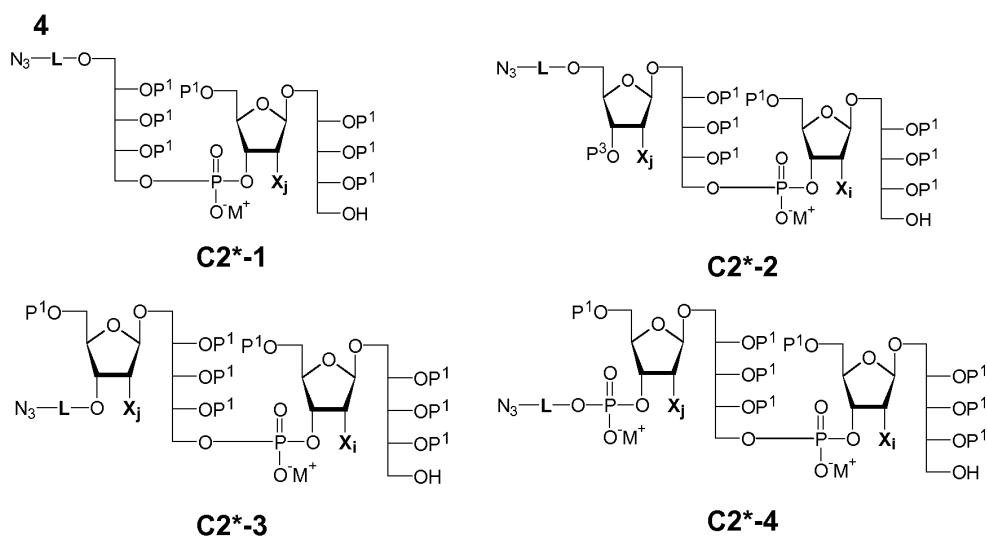
wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- 5       vii) Removing  $P^1, P^2, P^3$  and  $P^4$  protecting groups from a resulting compound after step vi) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

10       In the step vii), removing  $P^1, P^2, P^3$  and  $P^4$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

Of course, **C2\*-1**, **C2\*-2**, **C2\*-3**, or **C2\*-4** can be used as a starting building block. In this case, an alternative synthetic method **F-2** comprises the following steps:

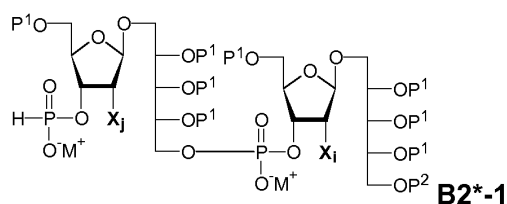
- 15       i) Providing a starting building block selected from **C2\*-1**, **C2\*-2**, **C2\*-3**, or **C2\*-4**



wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

20

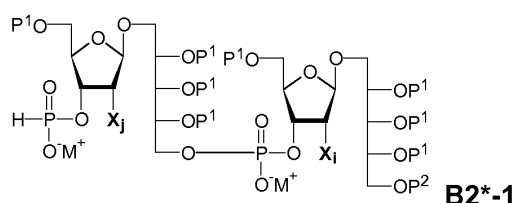
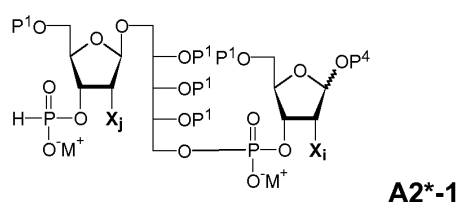
- ii) Coupling a building block **B2\*-1** with the starting building block selected from **C2\*-1**, **C2\*-2**, **C2\*-3**, or **C2\*-4**



wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^2$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- iii) Removing  $P^2$  protecting group from a resulting compound after the step ii);  
 5 iv) Optionally repeat the steps i) - iii) for  $t$  times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;  
 v) Coupling a starting building block selected from **A2\*-1** or **B2\*-1**

10



15

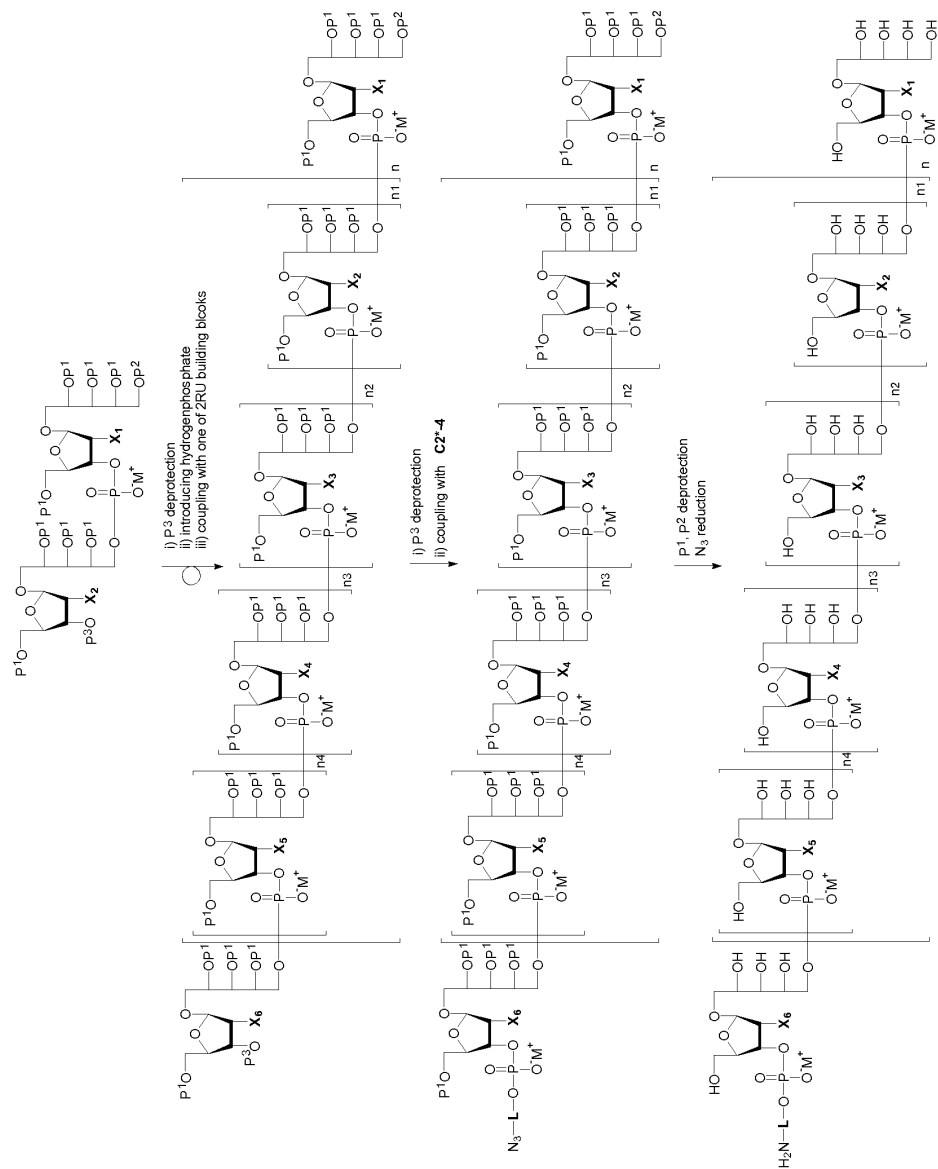
wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$ ,  $P^2$  and  $P^4$  represent protecting group that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

20

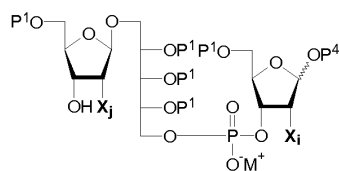
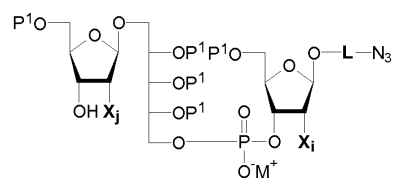
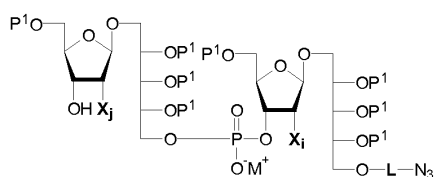
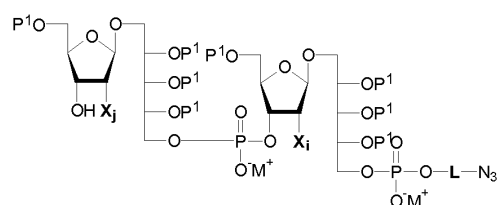
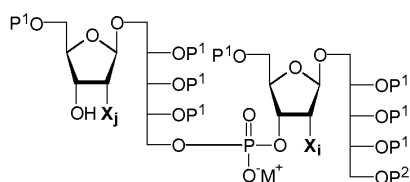
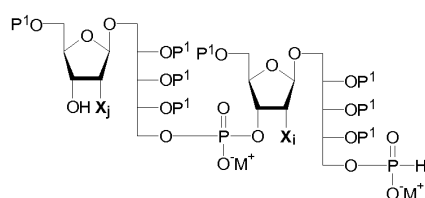
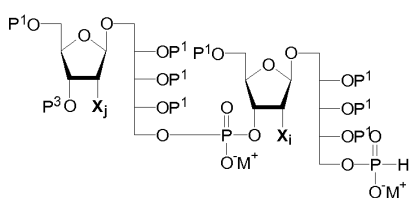
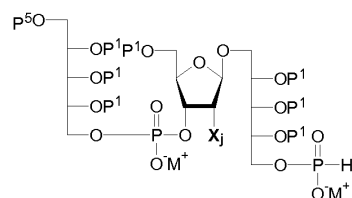
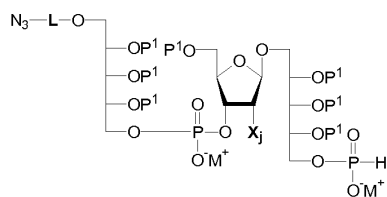
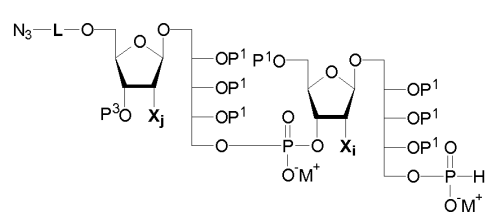
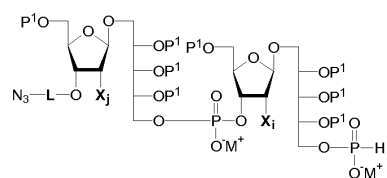
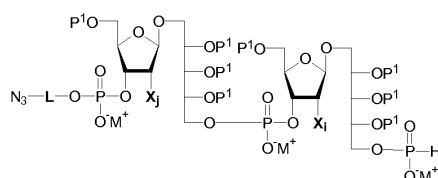
- vi) Removing  $P^1$ ,  $P^2$ ,  $P^3$  and  $P^4$  protecting groups from a resulting compound after step v) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vi), removing  $P^1$ ,  $P^2$ ,  $P^3$  and  $P^4$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

**Scheme 5. Synthesis of the Hib capsular oligosaccharide derivatives by method F-1**

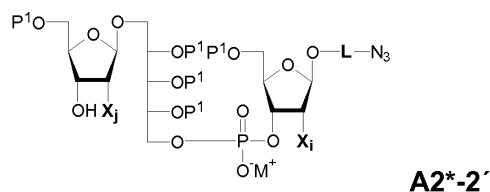


Further, the following building blocks consisting of two repeating units are useful for synthesis of the inventive saccharide by [2+2] synthetic approach:

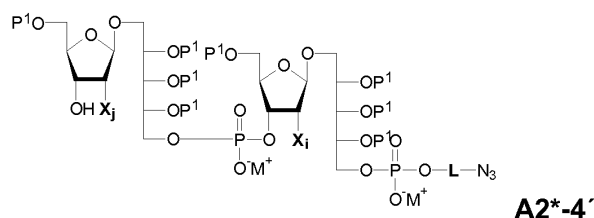
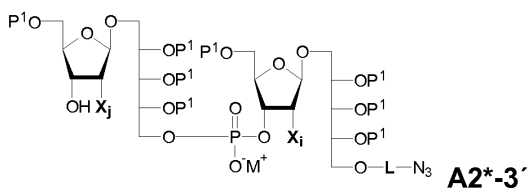
**A2\*-1'****A2\*-2'****A2\*-3'****A2\*-4'****B2\*-1'****B2\*-2'****B2\*-3'****B2\*-4'****C2\*-1'****C2\*-2'****C2\*-3'****C2\*-4'**

A synthetic method **G-1** comprises the following steps:

- i) Providing a starting building block selected from **A2\*-2'**, **A2\*-3'**, or **A2\*-4'**



5



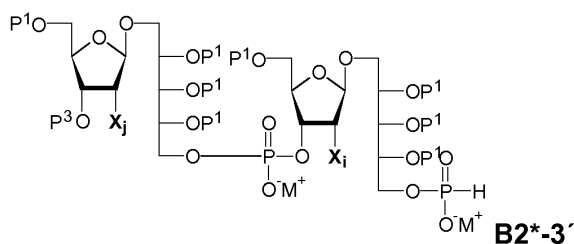
10

wherein L is a linker and has the same meaning as defined above;

$X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ; P<sup>1</sup> represents protecting group that when bound to —O— is not identical to  $X_i$  and  $X_j$ ;

15

- ii) Coupling a building block **B2\*-3'** with the starting building block selected from **A2\*-2'**, **A2\*-3'**, or **A2\*-4'**



wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ; P<sup>1</sup> and P<sup>3</sup> are protecting groups that when bound to —O— are not identical to  $X_i$  and  $X_j$ ;

20

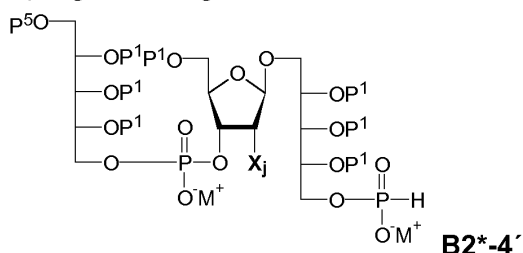
- iii) Removing P<sup>3</sup> group from a resulting compound after the step ii);

- iv) Optionally repeat the steps i) - iii) for **t** times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein **t** is an integer 0 to 10;
- v) Removing P<sup>1</sup> and P<sup>3</sup> protecting groups from a resulting compound after step iv) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step v), removing P<sup>1</sup> and P<sup>3</sup> protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

After the step iv) of the synthetic method **G-1**, a further building block **B2\*-4'** may be alternatively coupled with the resulting compound after the step v). In this case, the synthetic method **G-1** comprises the following steps instead of the step vi):

- 15 v') Coupling a building block **B2\*-4'** with a resulting compound after the step vi);

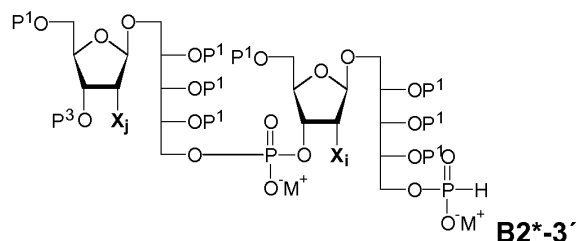


wherein  $X_j$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^5$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

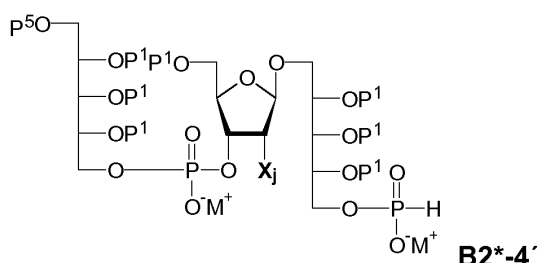
- vi') Removing P<sup>1</sup> and P<sup>5</sup> protecting groups from a resulting compound after the step  
20 v') and transforming an azido group to an amine group for obtaining the  
compound of the formula (I).

Of course, **B2\*-3'** or **B2\*-4'** can be used as a starting building block. In this case, an alternative synthetic method **G-2** comprises the following steps:

- 25 i) Providing a starting building block selected from **B2\*-3'**, or **B2\*-4'**

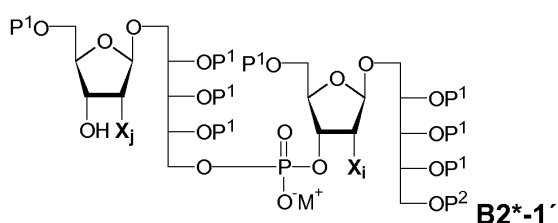


wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;



wherein  $X_j$  represents  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^5$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$ ;

ii) Coupling a building block **B2\*-1'** with the starting building block selected from **B2\*-3.** or **B2\*-4**



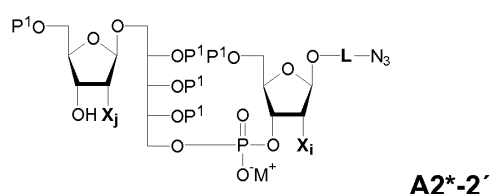
wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^2$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

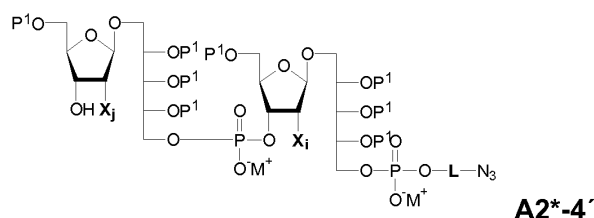
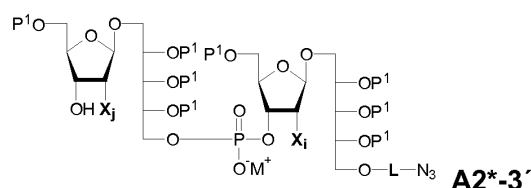
iii) Removing P<sup>2</sup> protecting group from a resulting compound after the step ii);

iv) Introducing hydrogen phosphonate at C-5 position of ribitol of a resulting compound after the step iii);

v) Optionally repeat the steps i) - iv) for **t** times by using a resulting compound after the step iv) instead of the starting building block in the step i), wherein **t** is an integer 0 to 10;

vi) Coupling a resulting compound after the step v) with a building block selected from **A2\*-2'**, **A2\*-3'**, or **A2\*-4'**



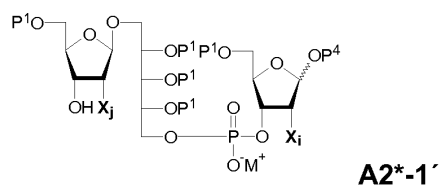


- 5 wherein  $L$  is a linker and has the same meaning as defined above;  
 $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$   
represents protecting group that when bound to  $-O-$  is not identical to  $X_i$   
and  $X_j$ ;  
vii) Removing  $P^1$ ,  $P^3$  and  $P^5$  protecting groups from a resulting compound after  
10 step vi) and transforming an azido group to an amine group for obtaining the  
compound of the formula (I).

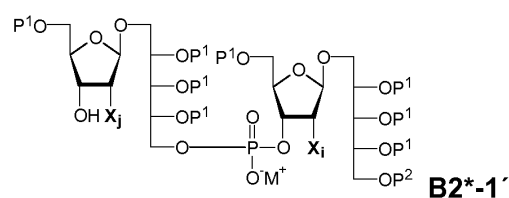
In the step vii), removing  $P^1$ ,  $P^3$  and  $P^5$  protecting groups and transforming an azido  
group to an amine group can be performed stepwise, but preferred at the same time  
15 under the same reaction condition.

A synthetic method **H-1** comprises the following steps:

- i) Providing a starting building block selected from **A2\*-1'** or **B2\*-1'**

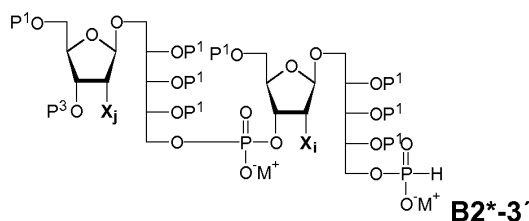


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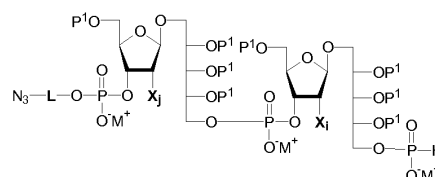
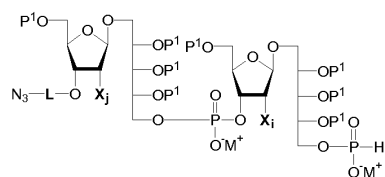
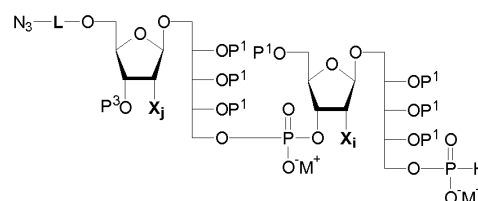
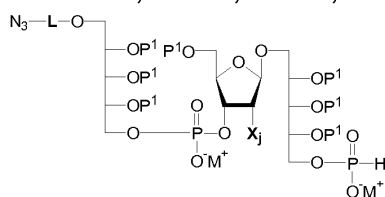
wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1, P^2$  and  $P^4$  represent protecting group;

- 5 ii) Coupling a building block **B2\*-3'** with the starting building block selected from **A2\*-1'** or **B2\*-1'**



wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups;

- 10 iii) Removing  $P^3$  group from a resulting compound after the step ii);  
 iv) Optionally repeat the steps i) - iii) for  $t$  times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;  
 v) Coupling a resulting compound after the step iv) with a building block selected from **C2\*-1'**, **C2\*-2'**, **C2\*-3'**, or **C2\*-4'**



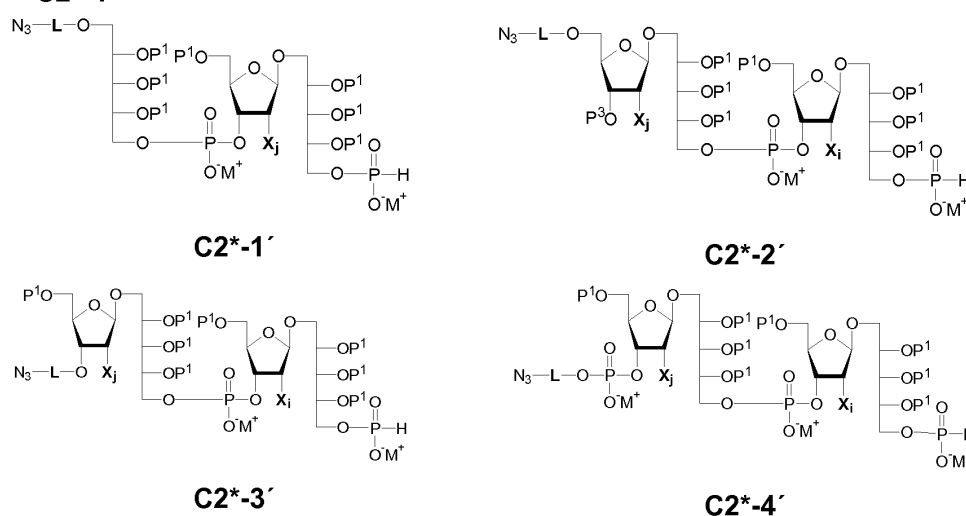
- 15 wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups;

- 20 vi) Removing  $P^1, P^2, P^3$  and  $P^4$  protecting groups from a resulting compound after step v) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vi), removing  $P^1$ ,  $P^2$ ,  $P^3$  and  $P^4$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

- 5 Of course, **C2\*-1'**, **C2\*-2'**, **C2\*-3'**, or **C2\*-4'** can be used as a starting building block. In this case, an alternative synthetic method **H-2** comprises the following steps:

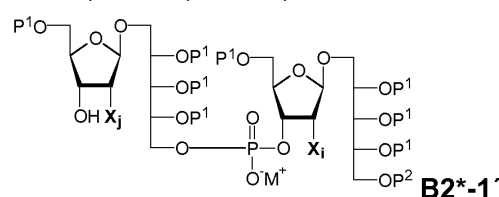
- i) Providing a starting building block selected from **C2\*-1'**, **C2\*-2'**, **C2\*-3'**, or **C2\*-4'**



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wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- ii) Coupling a building block **B2\*-1'** with the starting building block selected from **C2\*-1'**, **C2\*-2'**, **C2\*-3'**, or **C2\*-4'**



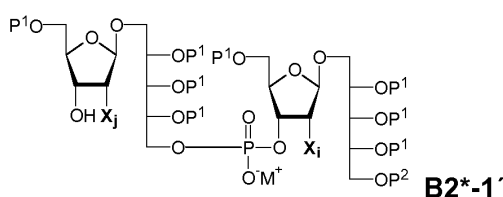
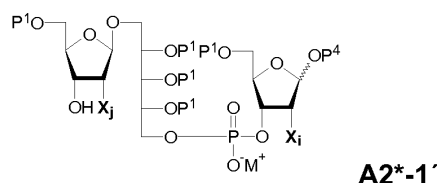
15

wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^2$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- iii) Removing  $P^2$  protecting group from a resulting compound after the step ii);
- 20 iv) Introducing hydrogen phosphonate at C-5 position of ribitol of a resulting compound after the step iii);

- v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iii) instead of the starting building block in the step i), wherein  $t$  is an integer 0 to 10;
- vi) Coupling a resulting compound after the step v) with a building block selected from **A2\*-1'** or **B2\*-1'**

5



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wherein  $X_i$  and  $X_j$  represents independently of each other  $X_1, X_2, X_3, X_4, X_5$  or  $X_6$ ;  $P^1, P^2$  and  $P^4$  represent protecting group that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

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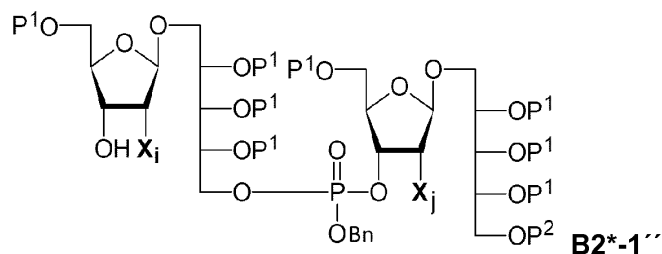
- vii) Removing  $P^1, P^2, P^3$  and  $P^4$  protecting groups from a resulting compound after step vi) and transforming an azido group to an amine group for obtaining the compound of the formula (I).

20

In the step vii), removing  $P^1, P^2, P^3$  and  $P^4$  protecting groups and transforming an azido group to an amine group can be performed stepwise, but preferred at the same time under the same reaction condition.

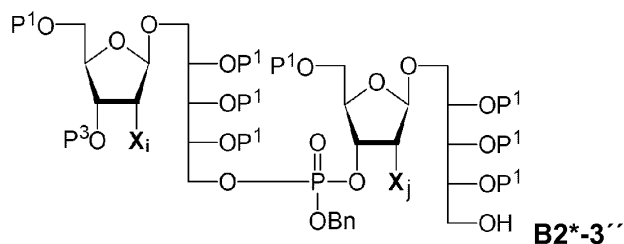
A synthetic method **J-1** comprises the following steps:

- i) Providing starting building block **B2\*-1''**



wherein  $X_i$  and  $X_j$  represent independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$ ,  $P^2$  and  $P^3$  represent protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- ii) Introducing phosphoramidite group at C-3 position of a ribose of starting block **B2\*-1''**;
- iii) Coupling building block **B2\*-3''** with the resulting compound after step ii);



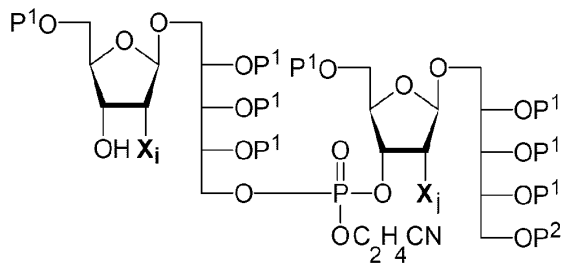
wherein  $X_i$  and  $X_j$  represent independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- iv) Removing  $P^3$  group from a resulting compound after the step iii);
- v) Optionally repeat the steps i) - iv) for  $t$  times by using a resulting compound after the step iv) instead of the building block **B2\*-3''** in the step iii), wherein  $t$  is an integer 0 to 10;
- vi) Coupling a compound  $HO-L-N_3$  with a resulting compound after step v);
- vii) Removing  $P^1$ ,  $P^2$ , and  $P^3$  protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vii), removing  $P^1$ ,  $P^2$ , and  $P^3$  protecting groups and transforming an azido group to an amine group can be performed stepwise, or at the same time under the same reaction condition.

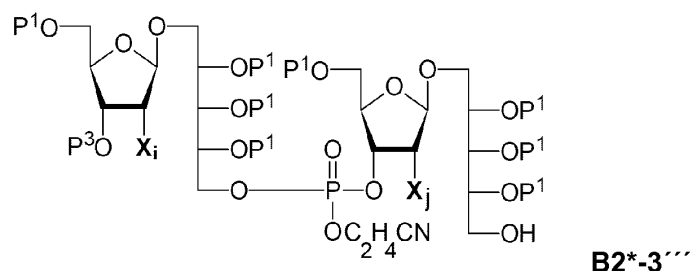
A synthetic method **J-2** comprises the following steps:

- i) Providing starting building block **B2\*-1'''**



wherein  $X_i$  and  $X_j$  represent independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$ ,  $P^2$  and  $P^3$  represent protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- ii) Introducing phosphoramidite group at C-3 position of a ribose of starting block **B2\*-1'''**;
- iii) Coupling building block **B2\*-3'''** with the resulting compound after step ii);

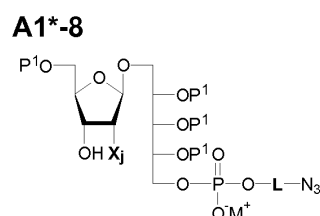
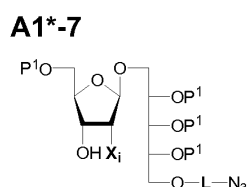
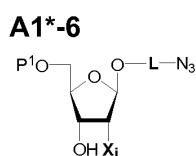


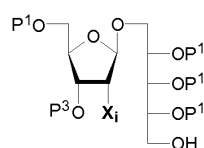
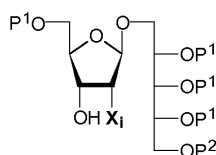
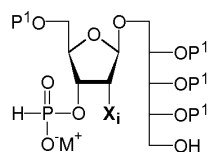
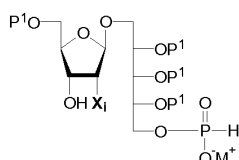
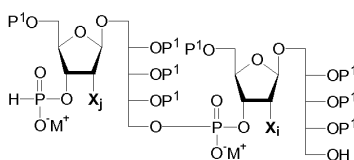
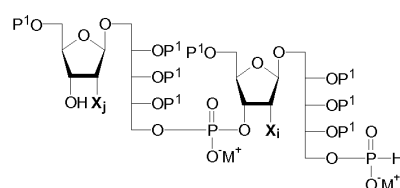
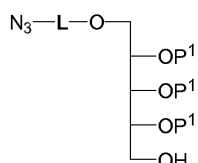
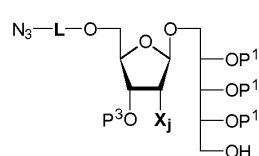
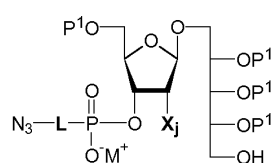
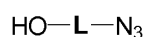
wherein  $X_i$  and  $X_j$  represent independently of each other  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$  or  $X_6$ ;  $P^1$  and  $P^3$  are protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

- iv) Removing P<sup>3</sup> group from a resulting compound after the step iii);
- v) Optionally repeat the steps i) - iv) for **t** times by using a resulting compound after the step iv) instead of the building block **B2\*-3'''** in the step iii), wherein **t** is an integer 0 to 10;
- 15 vi) Coupling a compound HO-L-N<sub>3</sub> with a resulting compound after step v);
- vii) Removing P<sup>1</sup>, P<sup>2</sup>, and P<sup>3</sup> protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I).

In the step vii), removing P<sup>1</sup> P<sup>2</sup>, and P<sup>3</sup> protecting groups and transforming an azido group to an amine group can be performed stepwise, or at the same time under the same reaction condition.

Furthermore, Hib capsular oligosaccharide derivatives of the present invention can  
25 be synthesized by polycondensation method I. The following building blocks are  
suitable for the polycondensation method I :



**B1\*-1****B1\*-4****B1\*-5****B1\*-6****B2\*-2****B2\*-2'****C1\*-1****C1\*-2****C1\*-3****T2\***

wherein  $X_i$ ,  $X_j$ ,  $P^1$ ,  $P^2$ ,  $P^3$ , and  $L$  have the same meanings as defined above.

A repeating unit having two functional groups, i.e. hydrogen phosphonate or phosphoramidite and hydroxy group is suitable for the polycondensation reaction. For quenching the polycondensation reaction, a quenching building block having a hydroxyl group may be used. As a repeating unit, disaccharides **B1\*-5**, **B1\*-6** and tetrasaccharides **B2\*-2**, **B2\*-3'** are suitable for the polycondensation. As quenching building block, **T2\***, **A1\*-6**, **A1\*-7**, **A1\*-8**, **B1\*-1**, **B1\*-4**, **C1\*-1**, **C1\*-2**, and **C1\*-3** may be used.

When **B1\*-5** or **B2\*-2** is used as repeating unit, **T2\***, **C1\*-1**, **C1\*-2**, and **C1\*-3** can be used as the quenching building block. When **B1\*-6** or **B2\*-2'** is used as repeating unit, **T2\***, **A1\*-6**, **A1\*-7** and **A1\*-8** can be used as the quenching building block.

Synthetic method I by polycondensation comprising the following steps:

- i) Providing at least one repeating unit suitable for polycondensation reaction;
- ii) Performing the polycondensation reaction of the at least one repeating unit;
- iii) Quenching the polycondensation reaction of the at least one repeating unit by a quenching building block;

iv) Removing P protecting groups and transforming an azido group to an amine group for obtaining the compound of the formula (I) or a mixture of the compounds of the formula (I);

wherein when **B1\*-5** or **B2\*-2** is used as the repeating unit, **T2\***, **C1\*-1**, **C1\*-2** or **C1\*-3** is used as the quenching building block; and

when **B1\*-6** or **B2\*-2'** is used as repeating unit, **T2\***, **A1\*-6**, **A1\*-7** or **A1\*-8** is used as the quenching building block.

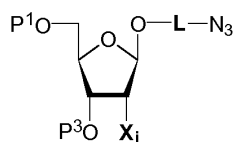
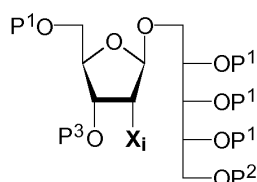
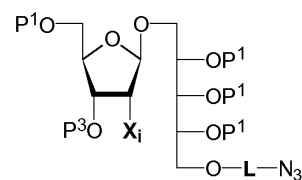
Alternatively, at least one repeating unit and a quenching building block may be provided at the same time and polycondensation reaction of the at least one repeating unit and quenching of the polycondensation reaction are performed automatically in an one-pot system.

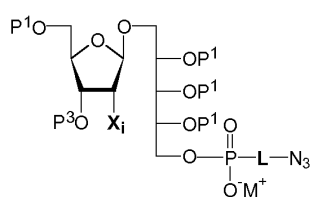
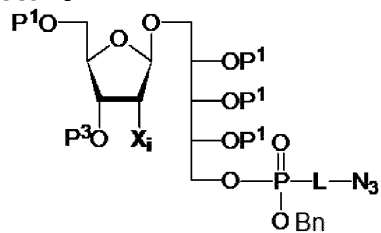
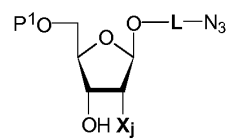
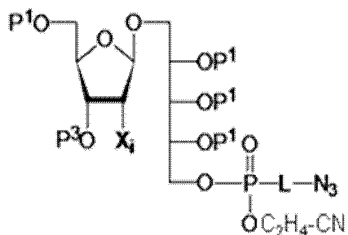
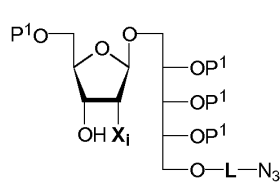
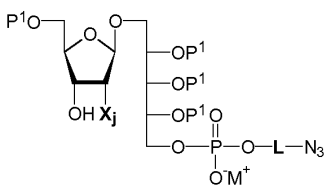
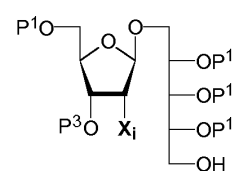
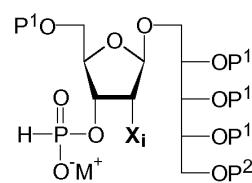
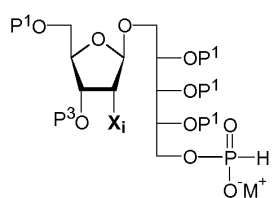
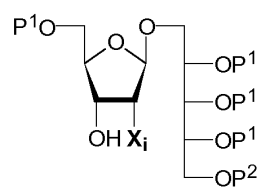
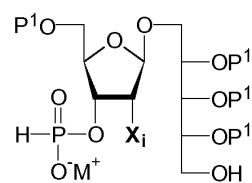
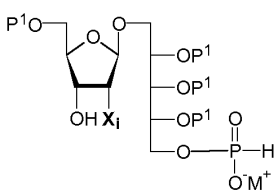
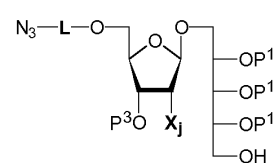
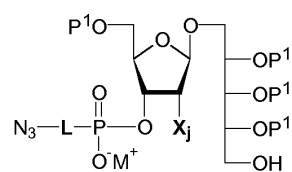
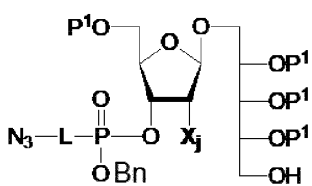
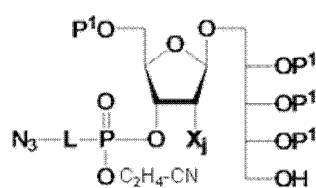
A molar ratio between sum of at least one repeating unit and a quenching building block plays a critical role for length and diversity of oligosaccharide(s).

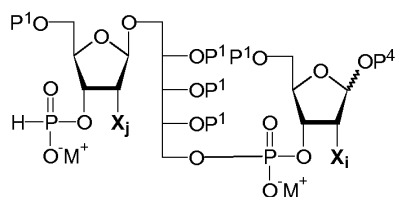
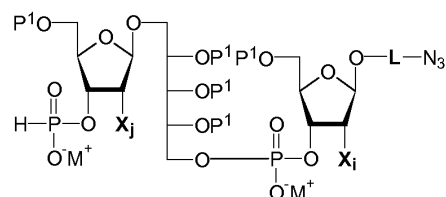
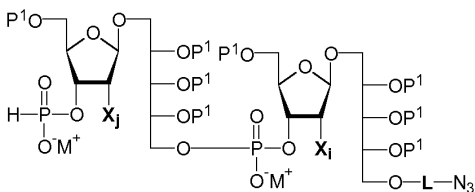
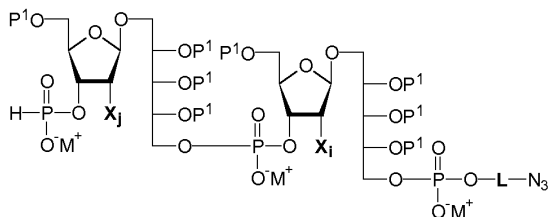
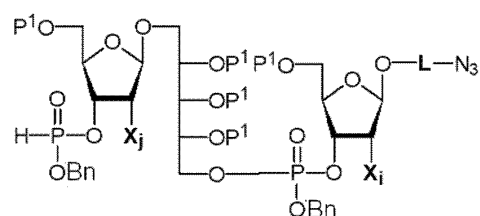
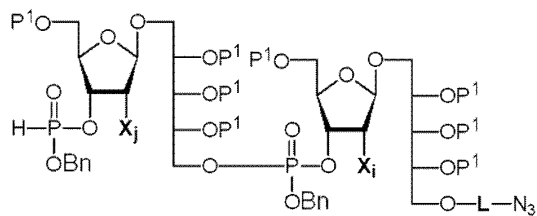
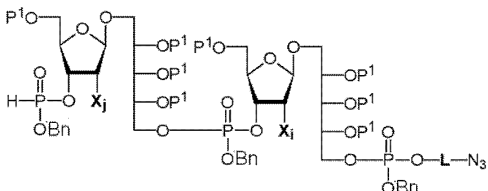
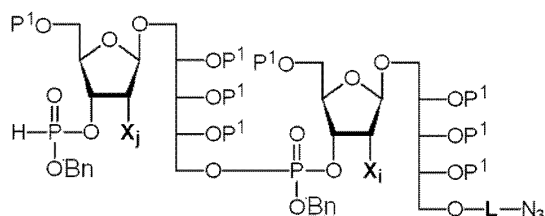
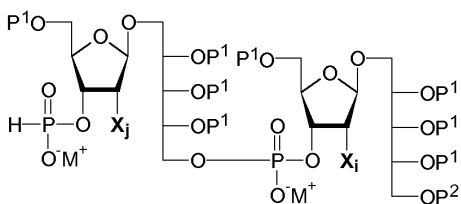
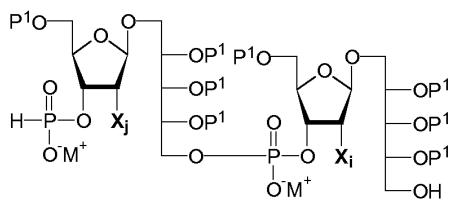
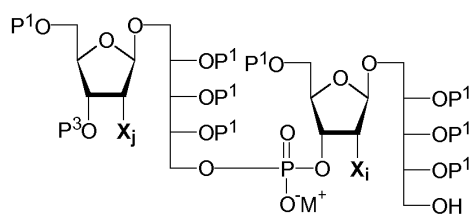
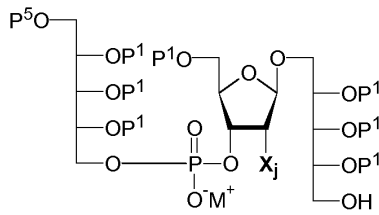
At least one repeating unit and a quenching building are provided in the range of a molar ratio between the repeating unit and quenching building block of 20:1 to 1:1, preferred 20:1 to 3:1, more preferred 15:1 to 4:1, most preferred 10:1 to 5:1.

Thus, an embodiment of the present invention is intermediate compound for synthesis of the saccharide of the formula (I) is selected from the group consisting of:

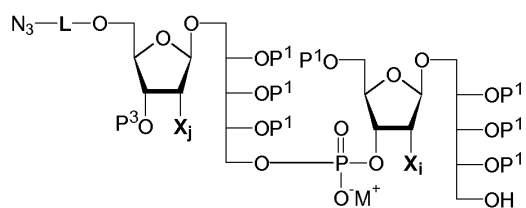
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**A1\*-2****A1\*-3****A1\*-4**

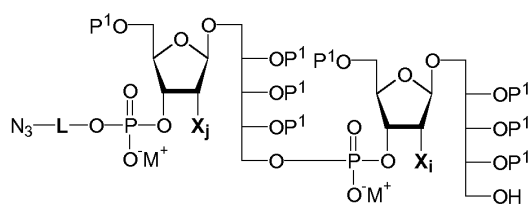
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**A2\*-1****A2\*-2****A2\*-3****A2\*-4****A2\*-2''****A2\*-3''****A2\*-4''****B2\*-4''****B2\*-1****B2\*-2****B2\*-3****B2\*-4**

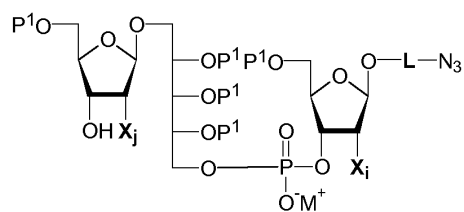
**C2\*-2**



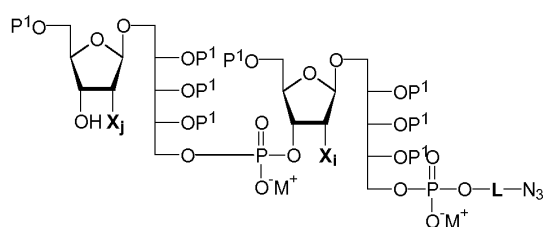
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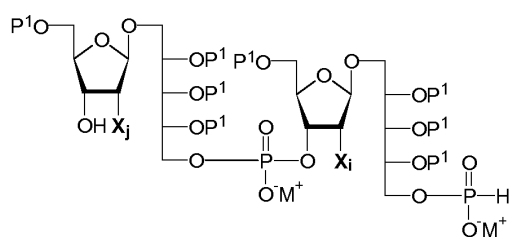
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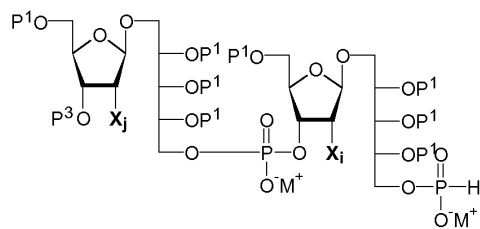
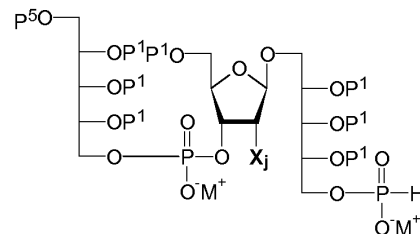
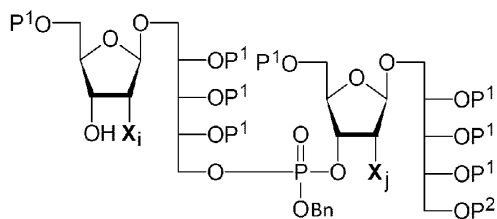
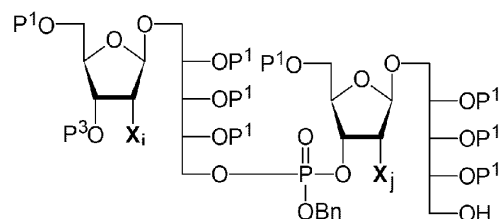
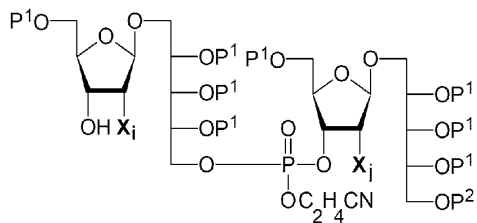
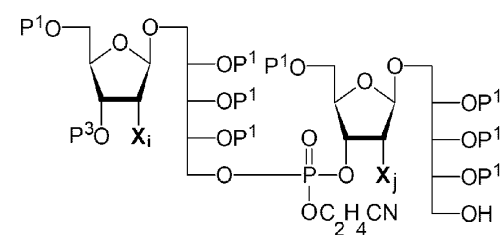
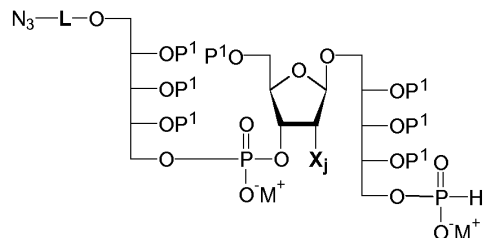
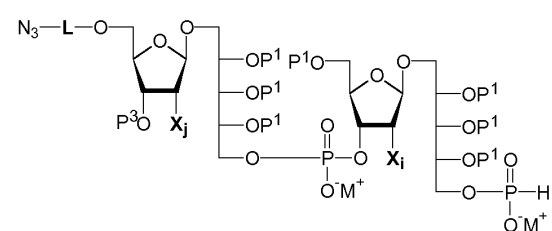
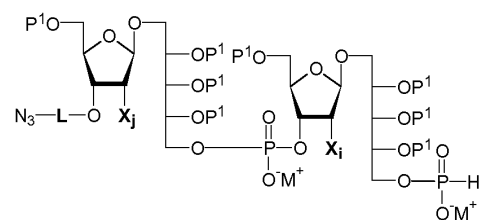
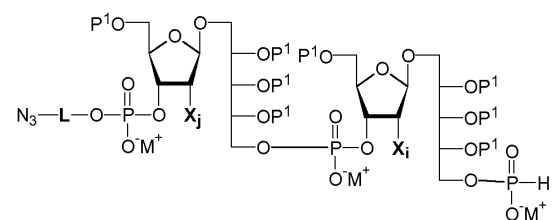


**A2\*-4'**



**B2\*-2'**



**B2\*-3'****B2\*-4'****B2\*-1''****B2\*-3''****B2\*-1'''****B2\*-3'''****C2\*-1'****C2\*-2'****C2\*-3'****C2\*-4'**

wherein  $P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$ , and  $P^5$  represent protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

$X_i$  and  $X_j$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$ , wherein

if only one group  $X_i$  or  $X_j$  is present,  $X_i$  or  $X_j$  cannot be  $-OH$ ;

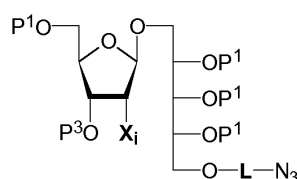
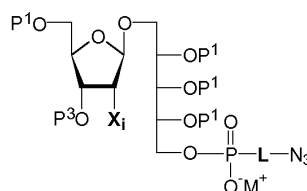
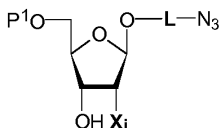
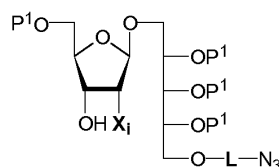
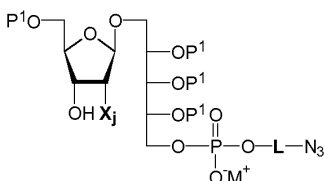
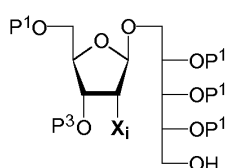
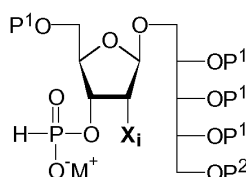
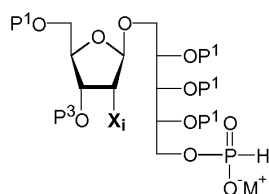
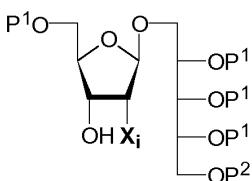
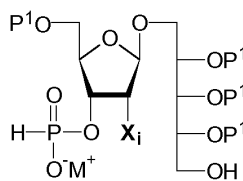
- 5 if both groups  $X_i$  and  $X_j$  are present,  $X_i$  and  $X_j$  cannot be simultaneously  $-OH$ ,

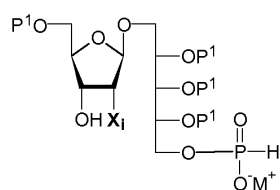
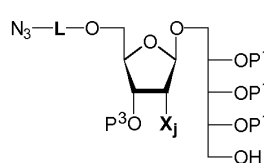
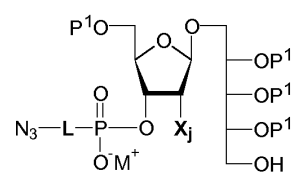
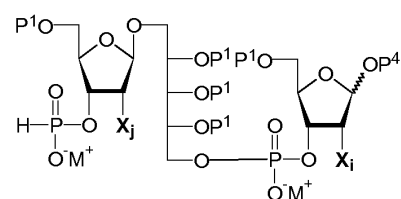
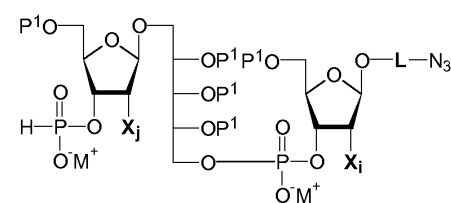
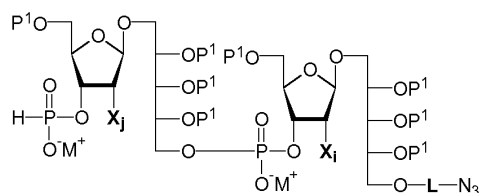
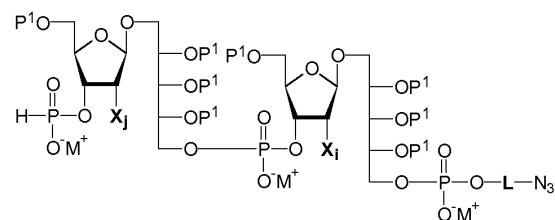
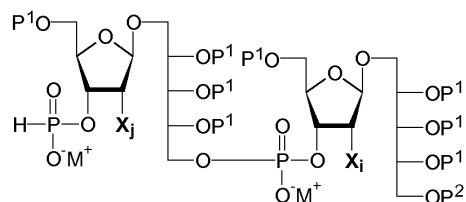
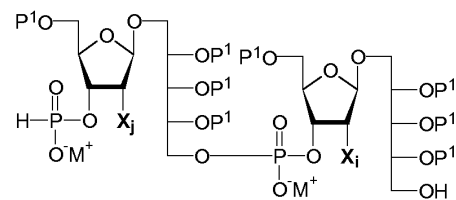
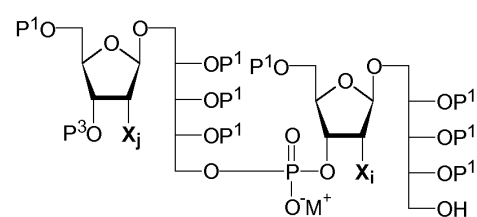
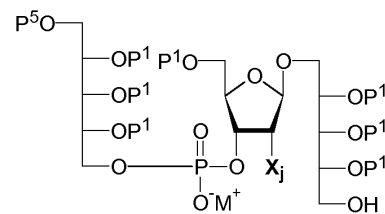
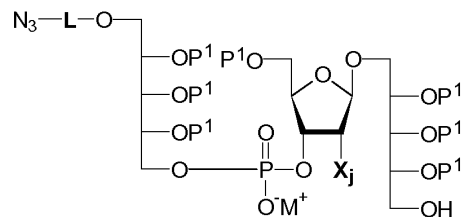
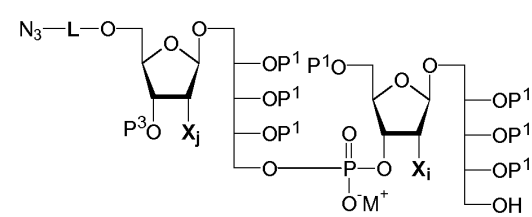
L has the meaning defined above; and

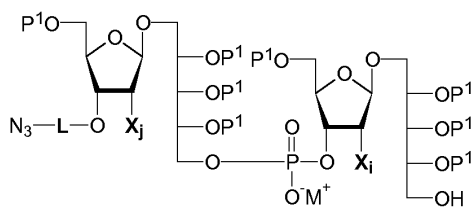
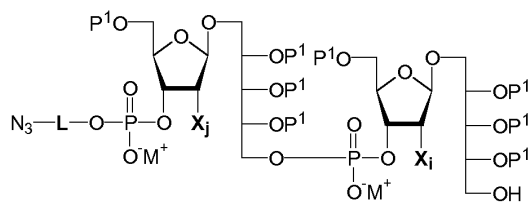
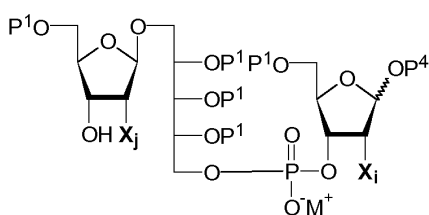
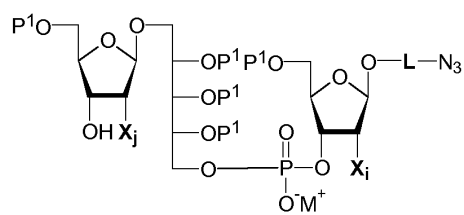
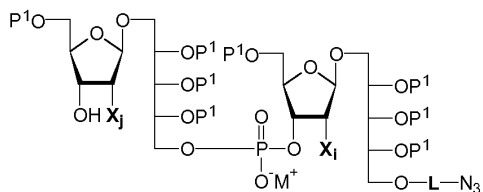
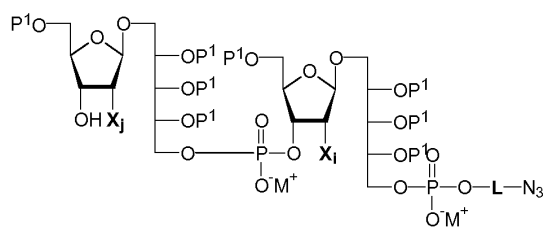
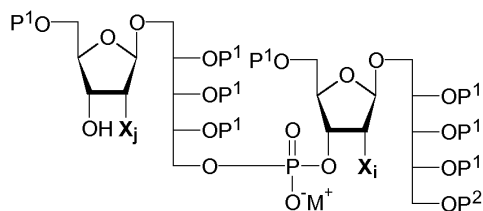
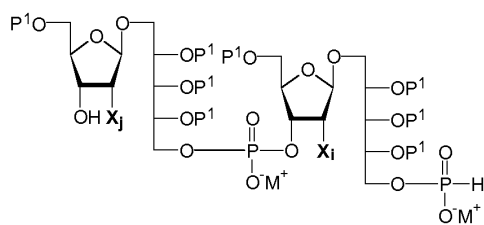
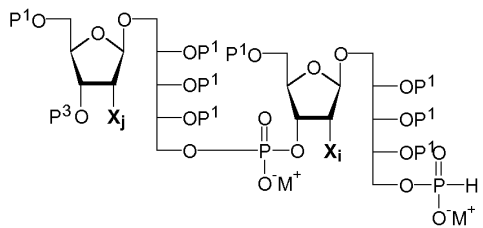
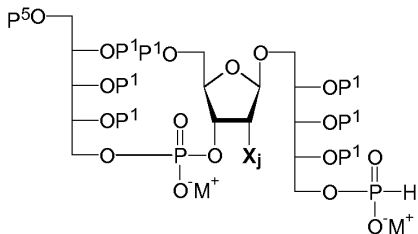
$M^+$  represents  $Na^+$ ,  $K^+$ ,  $NH_4^+$ , or  $HNEt_3^+$ :

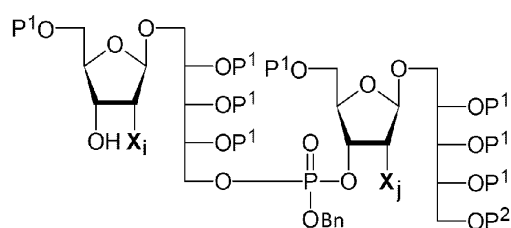
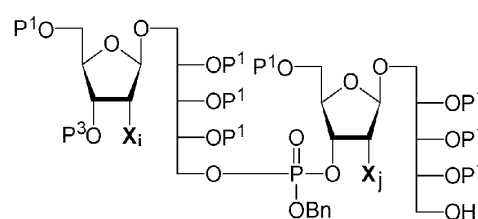
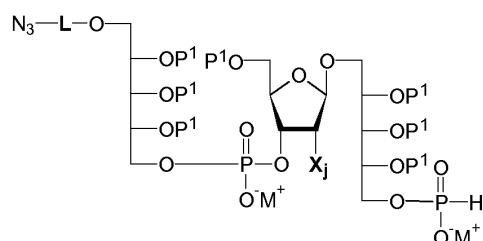
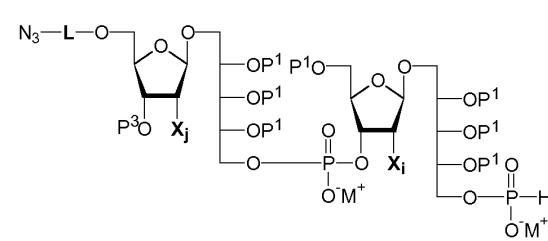
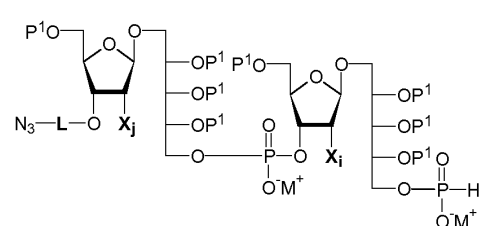
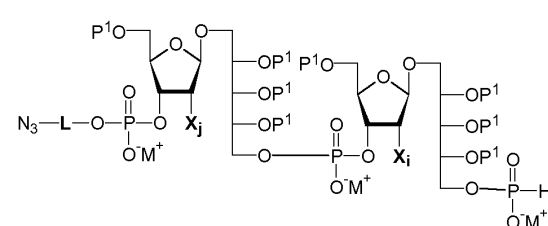
Alternatively, an embodiment of the present invention is intermediate compound for synthesis of the saccharide of the formula (I) is selected from the group consisting of:

**A1\*-4, A1\*-5, A1\*-6, A1\*-7, A1\*-8, B1\*-1, B1\*-2, B1\*-3, B1\*-4, B1\*-5, B1\*-6, C1\*-2, C1\*-3, A2\*-1, A2\*-2, A2\*-3, A2\*-4, B2\*-1, B2\*-2, B2\*-3, B2\*-4, C2\*-1, C2\*-2, C2\*-3, C2\*-4, A2\*-1', A2\*-2', A2\*-3', A2\*-4', B2\*-1', B2\*-2', B2\*-3', B2\*-4', B2\*-1'', B2\*-3'', C2\*-1', C2\*-2', C2\*-3' and C2\*-4'**:

**A1\*-4****A1\*-5****A1\*-6****A1\*-7****A1\*-8****B1\*-1****B1\*-2****B1\*-3****B1\*-4****B1\*-5**

**B1\*-6****C1\*-2****C1\*-3****A2\*-1****A2\*-2****A2\*-3****A2\*-4****B2\*-1****B2\*-2****B2\*-3****B2\*-4****C2\*-1****C2\*-2**

**C2\*-3****C2\*-4****A2\*-1'****A2\*-2'****A2\*-3'****A2\*-4'****B2\*-1'****B2\*-2'****B2\*-3'****B2\*-4'**

**B2\*-1''****B2\*-3''****C2\*-1'****C2\*-2'****C2\*-3'****C2\*-4'**

wherein  $P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$ , and  $P^5$  represent protecting groups;

$X_i$  and  $X_j$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,

5  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$ , wherein

if only one group  $X_i$  or  $X_j$  is present,  $X_i$  or  $X_j$  cannot be  $-OH$ ;

if both groups  $X_i$  and  $X_j$  are present,  $X_i$  and  $X_j$  cannot be simultaneously  $-OH$ ,

L has the meaning defined above; and

$M^+$  represents  $Na^+$ ,  $K^+$ ,  $NH_4^+$ , or  $HNEt_3^+$ :

10

The term "protecting groups" as used herein refers to commonly used protection groups in organic synthesis, preferably for hydroxyl groups.

15 More specifically,  $P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$ ,  $P^5$  and  $P^6$  preferably are suitable protecting groups for hydroxyl groups, more preferably different suitable protecting groups for hydroxyl groups capable of being removed subsequently one after another by a suitable sequence of deprotection reactions. Therefore protecting groups  $P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$ ,  $P^5$  and  $P^6$  for hydroxyl groups may be selected from the group consisting of or

comprising: acetyl, benzyl, benzoyl, p-methoxybenzyl, p-methoxyphenyl, para-bromobenzyl, o-nitrophenyl, p-nitrophenyl, allyl, methyl, isopropyl, levulinyl, dimethoxytrityl(DMTr), trityl, 2-naphthylmethyl (Nap), pivaloyl, triisopropylsilyl, *tert*-butyldimethylsilyl, *tert*-butyldiphenylsilyl, *tert*-butylmethoxyphenylsilyl, triethylsilyl, trimethylsilyl, 2-trimethylsilylethoxymethyl.

More specifically, in a preferred embodiment of the present invention P<sup>1</sup>, P<sup>5</sup> and P<sup>6</sup> may be benzyl or p-methoxybenzyl, P<sup>2</sup> may be allyl, dimethoxytrityl (DMTr), 2-naphthylmethyl (Nap) or trityl, P<sup>3</sup> may be levulinyl (Lev) or 2-naphthylmethyl (Nap), P<sup>4</sup> may be methyl or acetyl.

In a particularly preferred embodiment, P<sup>1</sup> may be benzyl, P<sup>2</sup> may be 2-naphthylmethyl and P<sup>3</sup> may be levulinyl.

Removing a DMTr group as P<sup>2</sup> protecting group by treatment with trichloroacetic acid leads to a primary alcohol at C-5 position of ribitol, which is coupled with a hydrogen phosphonate or phosphoramidite at C-3 position of ribose of a partner building block. Cleavage of a levulinyl group as P<sup>3</sup> protecting group by treatment with hydrazine acetate leads to a secondary alcohol at the C-3 position of ribose, which also reacts with hydrogen phosphonate or phosphoramidite at C-5 position of a ribitol of partner building block.

Deprotection of a benzyl group as P<sup>1</sup> protecting group is carried out preferred by Pd-catalyzed hydrogenation reaction. The same reaction condition may apply to the transformation of an azido group to an amine group.

For introducing a hydrogen phosphonate group at C-3 position of ribose or at C-5 position of ribitol of a building block or a resulting compound after deprotection of P<sup>3</sup> or P<sup>2</sup> protecting group, a hydroxyl group at C-3 position of ribose or at C-5 position of ribitol is preferred reacted with trichlorophosphine and triethylamine in the presence of imidazole.

For introducing a phosphoramidite group at C-3 position of ribose or at C-5 position of ribitol of a building block or a resulting compound after deprotection of P<sup>3</sup> or P<sup>2</sup> protecting group, a hydroxyl group at C-3 position of ribose or at C-5 position of ribitol is preferred reacted with bis(diisopropylamino)-benzyloxyphosphine in the presence of diisopropylammonium tetrazolide.

Pivaloyl chloride is used as a coupling or condensation reagent for coupling a pair of building block. As described above, for the coupling reaction, one coupling partner

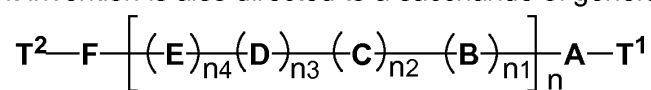
has a hydroxyl group and the other coupling partner has hydrogen phosphonate group.

- For the polycondensation reaction, a building block has two functional groups, i.e.  
5 hydroxyl group and hydrogen phosphonate group.

### Linker

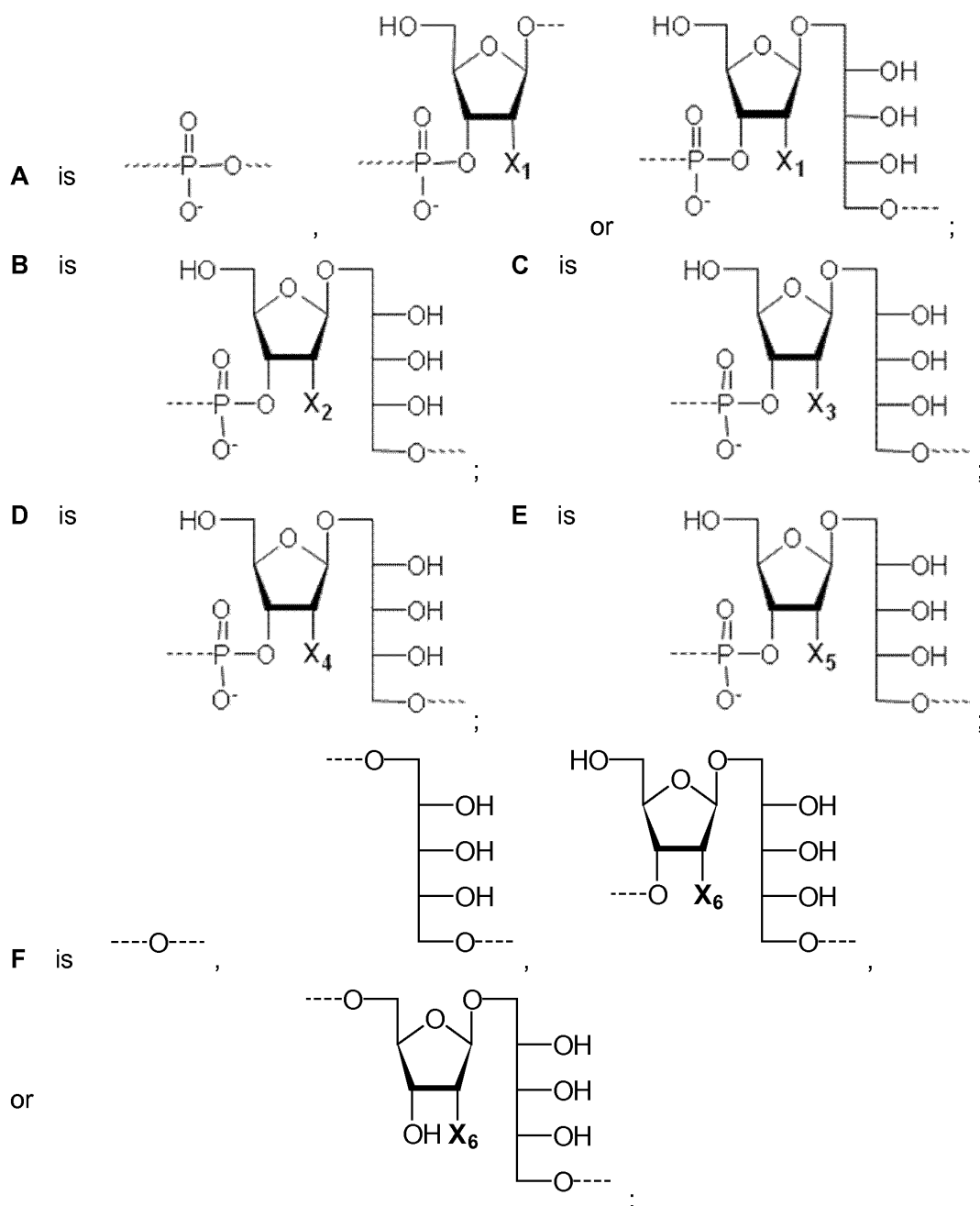
- Therefore the present invention is related to a saccharide of any of the general  
10 formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) containing a  $-O-L-NH_2$  group which is suitable for conjugation with an immunogenic carrier through the nitrogen atom of the  $-O-L-NH_2$  group. The present invention is also related to a saccharide of any of the general formulae containing a  $-O-L-COOH$  group which is suitable for conjugation with an immunogenic carrier through the carboxylic acid of the  
15 the  $-O-L-COOH$  group. The linker L may be any non-immunogenic moiety as defined above. Thus the linker L although being part of the saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) as well as of any conjugate of a saccharide of any of the general formulae (I), (II-1), (II-2), (III-1), (III-2), (IV-1) and (IV-2) with an immunogenic carrier does not affect the  
20 immunogenic properties of the saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) as well as of any conjugate of the saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) with an immunogenic carrier.
- 25 Any bifunctional linker of the formula  $HO-L-NH_2$  or  $HO-L-COOH$  can be used in the inventive saccharides of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1) and (III-2) as well as their conjugates with an immunogenic carrier. In one embodiment of the present invention the saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), and (III-2) the Linker  $-L-$  is selected from:  $-L^a-$ ,  
30  $-L^a-L^e-$ ,  $-L^a-L^b-L^e-$ ,  $-L^a-L^d-L^e-$ ; wherein  
 $-L^a-$  is selected from:  $-(CH_2)_o-$ ,  $-(CH_2-CH_2-O)_o-C_2H_4-$ ,  $-(CH_2-CH_2-O)_o-CH_2-$ ;  
 $-L^b-$  represents  $-O-$ , or  $-O-P(O)(OH)-O-$ ;  
 $-L^d-$  is selected from  $-(CH_2)_q-$ ,  $-(CF_2)_q-$ ,  $-(CH_2-CH_2-O)_q-C_2H_4-$ , and  $-(CH_2-CH_2-O)_q-CH_2-$ ;  
35  $-L^e-$  is selected from:  $-(CH_2)_{p1}-$ ,  $-(CF_2)_{p1}-$ ,  $-C_2H_4-(O-CH_2-CH_2)_{p1}-$ ,  $-CH_2-(O-CH_2-CH_2)_{p1}-$  and  $-(CH_2)_{p1}-O-(CH_2)_{p2}-$ ;  
and o, q, p1 and p2 are independently of each other an integer selected from 1, 2, 3, 4, 5, and 6.

Thus, the present invention is also directed to a saccharide of general formula (I)



(I)

wherein



$T^1$  and  $T^2$  represent  $-H$ ,  $-L-NH_2$ ; wherein if  $T^1$  is  $-L-NH_2$ , then  $T^2$  is  $-H$  and if  $T^1$  is  $-H$ , then  $T^2$  is  $-L-NH_2$  or  $-L-COOH$ ;

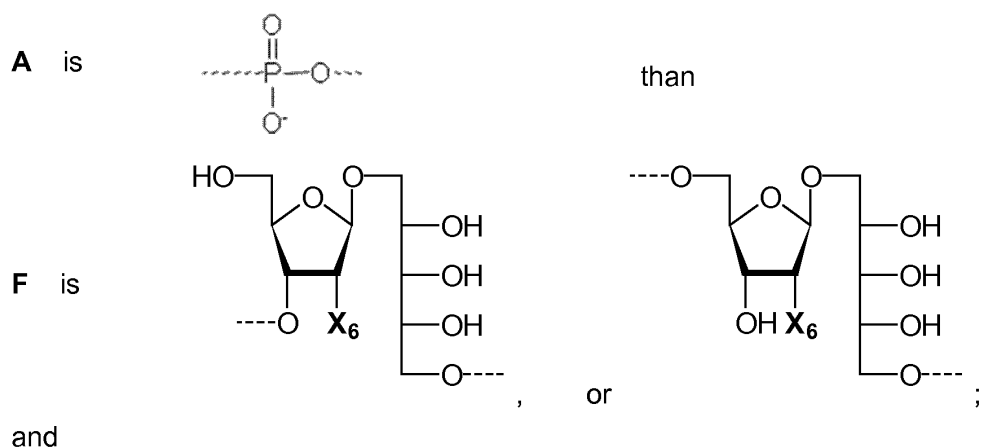
$X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  
 5  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$ , and at least two of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  are not  $-OH$ ;

$n$  is an integer selected from 1, 2, 3, or 4;

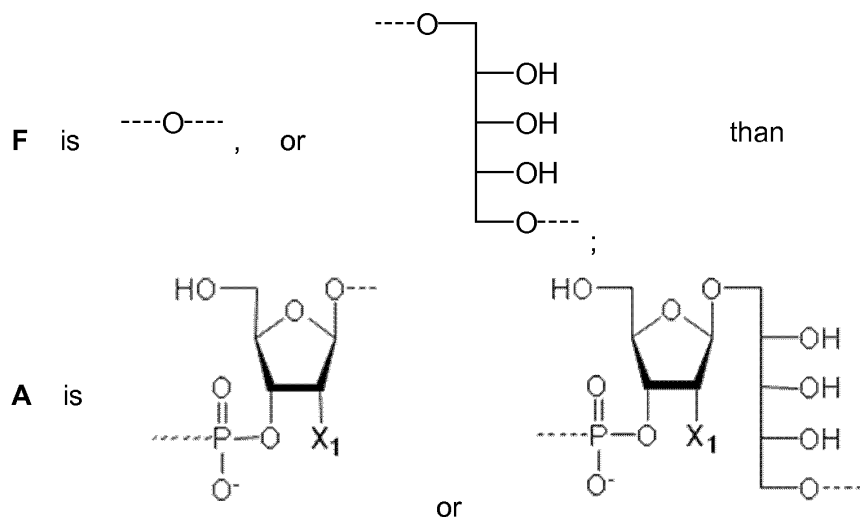
$n_1$ ,  $n_2$ ,  $n_3$  and  $n_4$  are independently of each other selected from 0 and 1

10 and  $n_1+n_2+n_3+n_4 \geq 1$ ;

under the proviso that if  $n_1+n_2+n_3+n_4 = 1$  and if  $n = 1$  and if



15 under the proviso that if  $n_1+n_2+n_3+n_4 = 1$  and if  $n = 1$  and if



- L** is a linker and **L** is selected from:  $-\text{L}^{\text{a}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{e}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{b}}-\text{L}^{\text{e}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{d}}-\text{L}^{\text{e}}-$ ;  
 wherein  $-\text{L}^{\text{a}}-$  is selected from:  $-(\text{CH}_2)_o-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{C}_2\text{H}_4-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{CH}_2-$ ;  
 5  $-\text{L}^{\text{b}}-$  represents  $-\text{O}-$ , or  $-\text{O}-\text{P}(\text{O})(\text{OH})-\text{O}-$ ;  
 $-\text{L}^{\text{d}}-$  is selected from  $-(\text{CH}_2)_q-$ ,  $-(\text{CF}_2)_q-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_q-\text{C}_2\text{H}_4-$ , and  $-(\text{CH}_2-\text{CH}_2-\text{O})_q-\text{CH}_2-$ ;  
 $-\text{L}^{\text{e}}-$  is selected from:  $-(\text{CH}_2)_{p1}-$ ,  $-(\text{CF}_2)_{p1}-$ ,  $-\text{C}_2\text{H}_4-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$ ,  $-\text{CH}_2-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$  and  $-(\text{CH}_2)_{p1}-\text{O}-(\text{CH}_2)_{p2}-$ ;  
 10 and o, q, p1 and p2 are independently of each other an integer selected from 1, 2, 3, 4, 5, and 6;

- and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

- It is also preferred that the inventive saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), and (III-2) has a linker  $-\text{L}-$  which is  $-\text{L}^{\text{a}}-\text{L}^{\text{e}}-$ ; and wherein  
 20  $-\text{L}^{\text{a}}-$  is selected from:  $-(\text{CH}_2)_o-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{C}_2\text{H}_4-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{CH}_2-$ ;  
 $-\text{L}^{\text{e}}-$  is selected from:  $-\text{C}_2\text{H}_4-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$ ,  $-\text{CH}_2-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$ ,  $-(\text{CH}_2)_{p1}-$ , and  $-(\text{CH}_2)_{p1}-\text{O}-(\text{CH}_2)_{p2}-$ ; and  
 o, p1, p2, are independently of each other an integer selected from 1, 2, 3, 4, 5, and 6.

- 25 In one embodiment the saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), and (III-2) the Linker  $-\text{L}-$  is  $-\text{L}^{\text{a}}-$ , wherein  
 $-\text{L}^{\text{a}}-$  represents:  $-(\text{CH}_2)_{o1}-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_{o2}-\text{C}_2\text{H}_4-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_{o2}-\text{CH}_2-$ ;  
 o1 is an integer selected from 2, 3, 4, 5 and 6;  
 30 o2 is an integer selected from 1, 2, 3, 4, 5 and 6.

### Glycoconjugates

- Another aspect of the present invention refers to a saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) conjugated with  
 35 an immunogenic carrier through the nitrogen atom of the  $-\text{O}-\text{L}-\text{NH}_2$  group. Said saccharide is also defined as a glycoconjugate obtained by reacting a saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) with an immunogenic carrier. Thus said saccharide of any of the general formulae

(I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) conjugated with an immunogenic carrier through the nitrogen atom of the  $-O-L-NH_2$  group and said glycoconjugate obtained by reacting a saccharide of any of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) with an immunogenic carrier have the same meaning.

Said glycoconjugate proved to be efficient as a vaccine for immunization against diseases associated with bacteria.

Saccharides are known by the person skilled in the art as generally TI-2 (T cell independent-2) antigens and poor immunogens. TI-2 antigens are antigens, which are recognized only by mature B cells through the cross linking of surface exposed immunoglobulin receptors. Without T cell help, no immunological memory is generated and neither isotype switching from IgM to other IgG subclasses, nor B cells affinity maturation occurs. Moreover, saccharides are known poor immunogens in humans due to the structural homology to human glycolipids and glycoproteins. Due to their poor immunogenic properties, saccharides manifest poor ability to produce both antibody production by B cells, as well as the formation of memory cells, features which are essential for the production of potent vaccines.

Therefore, to produce a potent saccharide-based vaccine, the saccharides of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) are conjugated to an immunogenic carrier to provide glycoconjugates, which present increased immunogenicity in comparison with the saccharides.

Saccharide-protein conjugate consisting of at least one synthetic saccharide of the general formula (I) and an immunogenic carrier to which the at least one saccharide (I) is covalently bound.

In this context the term "immunogenic carrier" is defined as a structure, which is conjugated to the saccharide to form a glycoconjugate that presents an increased immunity in comparison with the saccharide *per se*. Thus, the conjugation of the saccharides of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) to the immunogenic carrier has as effect the stimulation of the immune response against the saccharide of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) without inducing an immune response against the said immunogenic carrier.

Preferred immunogenic carriers are carrier proteins. For the person skilled in the art, a carrier protein is a protein selected from the group comprising or consisting of: a diphtheria toxoid, a mutated diphtheria toxoid, a modified diphtheria toxoid, a mutated and modified diphtheria toxoid, a tetanus toxoid, a modified tetanus toxoid, a mutated tetanus toxoid, outer membrane protein (OMP), bovine serum albumin (BSA), keyhole limpet hemocyanine (KLH) or cholera toxoid (CT). The term "toxoid" as used herein refers to a bacterial toxin (usually an exotoxin), whose toxicity has been inactivated or suppressed either by chemical (formalin) or heat treatment, while other properties, typically immunogenicity, are maintained. A mutated toxoid as used herein is a recombinant bacterial toxin, which has been amended to be less toxic or even non-toxic by amending the wild-type amino acid sequence. Such a mutation could be a substitution of one or more amino acids. Such a mutated toxoid presents on its surface a functionality that can react with the functional group Y of the interconnecting molecule to provide a modified toxoid.

15 An embodiment of the present invention is saccharide (I) covalently linked to an immunogenic carrier through the nitrogen atom of the -O-L-NH<sub>2</sub>-group. -L- of the -O-L-NH<sub>2</sub>-group is the linkage to the immunogenic carrier in form of a covalent bond (oxygen atom of O-L-NH<sub>2</sub>-group directly or indirectly linked to the immunogenic carrier) or in form of the -L-NH-, wherein the nitrogen atom is linked directly or indirectly to the carrier protein

Directly linked means to a functional group or side chain of the immunogenic carrier and indirectly linked refers to a linkage through an additional functional group with which the immunogenic carrier and/or the nitrogen of the -O-L-NH<sub>2</sub>-group.

If a carrier protein is used as immunogenic carrier, for the coupling of saccharide to a carrier protein, chemical activation of the saccharide and sometimes of the protein is necessary. The choice of the conjugation method to saccharide to proteins is restricted due to the pH and temperature sensitivity of the proteins, and their limited solubility in most organic solvents. The procedure has to be performed under mild conditions in order to prevent denaturation of the protein and degradation of the saccharide. As such, conjugation reactions are carried out in buffers at or near neutral pH. To date, numerous protocols have been reported for the covalent attachment of carbohydrates to protein. The following linking methods can be used for linking synthetic saccharide (I) to the carrier protein:

Reductive amination is the one of the most popular glycoconjugation methods for the binding of free oligosaccharides via the reducing-end to the ε-amino group of the

lysine residues in proteins. In the present invention the amino group of synthetic saccharide (I) can be further functionalized with an aldehyde function, e.g. using glutaraldehyde, and glycoconjugation between synthetic saccharide functionalized with aldehyde and a carrier protein having lysine residues can be formed by reductive amination.

Maleimide-Thiol conjugation as the irreversible reaction of maleimide with thiols to afford stable linkages has also been employed frequently for the preparation of glycoconjugates. For example, maleimide can be prepared from a synthetic saccharide (I) bearing an amine linker at the reducing-end. The saccharide-maleimide construct can then react with thiol side-chains in cysteines of a carrier protein, resulting in the stable glycoconjugate. Inversely, a carrier protein can be activated with maleimide and a synthetic saccharide (I) can be further functionalized with a thiol function.

A glycoconjugation method using squarate ester can be applied. At first, a squaric acid amide ester of synthetic saccharide (I) can be prepared by the coupling of amine group with squarate ester. The squaric acid amide ester of synthetic saccharide (I) can be further coupled with the  $\epsilon$ -amino group of the lysine residues of a carrier protein and squaric acid diamides are formed.

Activated esters including di(*N*-succinimidyl) adipate, di(*N*-succinimidyl) glutarate (DSG), *N*-( $\gamma$ -maleimidobutyryloxy) sulfosuccinimide ester (sulfo-GMBS), succinimidyl (4-iodoacetyl) aminobenzoate (sulfo-SIAB), succinimidyl-3-(bromoacetamido)propionate (SBAP), 2-pyridyldithiol-tetraoxatetradecane-*N*-hydroxysuccinimide (PEG-4-SPDP) can be applied. (see **Figure 1**). Preferred, di(*N*-succinimidyl) adipate can be used for the preparation of glycoconjugate.

Zero-length cross linking method with carbodiimides, most of all EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) can be used. *N*-substituted carbodiimides can react with carboxylic acids of a carrier protein to form highly reactive, *O*-acylisourea derivatives. This active species then can react with a primary amine of synthetic saccharide (I) to form an amide bond.

As further conjugation method click chemistry can be used. Click chemistry is the [3+2] cycloaddition of alkyne and azide. Preferably, strain-promoted azide-alkyne cycloaddition (SPAAC) can be applied for the glycoconjugation. Strained alkynes such as cyclooctyne are advantageous for a bioconjugation due to the non-toxic reaction condition. A carrier protein can be simply labelled with an azide-containing

amino acid and conjugated with synthetic saccharide (I) functionalized with an alkyne. Inversely, synthetic saccharide (I) functionalized with an azide can be conjugated with a carrier protein labeled with an alkyne.

5 Native chemical ligation (NCL) can be used for glycoconjugation. To apply this method to glycoconjugation, synthetic saccharide (I) should be further functionalized with a thioester, for example, benzylthioester, ethylthioester, or phenylthioester. In the native chemical ligation the thiolate group of an *N*-terminal cysteine residue of a carrier protein attacks the thioester of functionalized synthetic saccharide (I). This  
10 reversible transthioesterification step is chemoselective and regioselective and leads to form a thioester intermediate and this intermediate rearranges by an intramolecular *S,N*-acyl shift that results in the formation of a native amide bond at the ligation site. Consequently the synthetic saccharide (I) and a carrier protein are conjugated with an amide bond.

15 Traceless Staudinger ligation can be also used for glycoconjugation. In this reaction, the phosphine bears a thioester electrophile; after reaction with an azide, the products are an amide and the leaving phosphine oxide. The Staudinger ligation has been demonstrated to be ideal for labeling azide-bearing proteins both in  
20 vitro, on living cells, and in living animals, due to the demonstrated non-toxicity of both azides and phosphines at relevant concentrations. For glycoconjugating applications, synthetic saccharide (I) is functionalized with thioester bearing phosphine and azide group is introduced into a carrier protein of interest in several ways for subsequent phosphine probe introduction. Usually, azidohomoalanine can  
25 be incorporated in place of methionine of a protein. A carrier protein modified with azidohomoalanine can be effectively and specifically conjugated with synthetic saccharide (I) by the Staudinger ligation.

Photo-cross-linking is an important chemical method in the field of molecular biology.  
30 Recently, much attention has been devoted to the application of this method to the identification of ligand-binding regions. Photo-cross-linking methods can be applied for glycoconjugation. Photoaffinity labeling requires functional groups that can be activated photochemically to generate highly reactive intermediates, usually nitrenes or carbenes. As functional groups, benzophenone, arylazide, aryl-, or alkyl diazirine  
35 are applied. Recently, alkyl- or aryl diazirine is preferred used for photoaffinity labeling (M. R. Bond *et al.* *Nature Protocols* 2009, 4(7), 1044-1063; M. R. Bond *et al.* *Bioconjugate Chemistry* 2011, 22 (9), 1811-1823). For conjugation of synthetic saccharide (I) with a carrier protein, synthetic saccharide (I) can be functionalized with an alkyl- or aryl diazirine, for example, 3-trifluoromethyl-3-phenyldiazirine. After

UV-irradiation a reactive carbene is produced from synthetic saccharide (I) functionalized with aryldiazirine and this carbene coupled with a carrier protein by a C-C covalent bond.

- 5 It is especially preferred that the saccharide of the general formula (I) and/or preferably saccharides **2a**, **2b**, **2c**, **4a**, **4b**, **4c**, **4d**, **4e**, **6a**, **6b**, **6c**, **8a**, **8b** and **8c** are conjugated to the non-toxic mutated diphtheria toxin CRM<sub>197</sub> presenting as a functionality a primary amine functionality of a lysine residue.
- 10 CRM<sub>197</sub> like wild-type diphtheria toxin is a single polypeptide chain of 535 amino acids (58 kD) consisting of two subunits linked by disulfide bridges having a single amino acid substitution of glutamic acid for glycine. It is utilized as a carrier protein in a number of approved conjugate vaccines (e.g. Prevnar) for diseases such as pneumococcal bacterial infections.
- 15 Preferably, di(*N*-succinimidyl) adipate is first attached to a synthetic saccharide (I) having a primary amino group. Activated saccharide (I) is subsequently condensed with a carrier protein such as CRM<sub>197</sub>, to afford the glycoconjugate. Also, the use of the related disuccinimidyl glutarate can be applied (**Figure 2 (A)**).
- 20 Said saccharide conjugated with a carrier protein has preferably a purity of  $\geq 95\%$ , preferably  $\geq 96\%$ , more preferably  $\geq 97\%$ , still more preferably  $\geq 98\%$ , and most preferably  $\geq 99\%$ .
- 25 Another aspect of the present invention relates to the use of the inventive saccharides and their glycoconjugates as drugs, i.e. as pharmaceutically active agents applicable in medicine.

- 30 It was found that the saccharides of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) and said glycoconjugate obtained by reacting the saccharides of general formulae (I), (II-1), (II-2), (III-1), (III-2), (IV-1) and (IV-2) with an immunogenic carrier are suitable for pharmaceutically active agent in medicine. Said saccharides and said glycoconjugates are suitable to elicit an immune response in an animal, and therefore are useful raising a protective immune response in a
- 35 human and/or animal host.

Preferred, said saccharides and said glycoconjugates are useful for prevention and/or treatment of diseases associated with *Haemophilus influenzae type b*, preferably diseases selected from meningitis, pneumonia, and epiglottitis.

Surprisingly, it was found that the novel conjugates of the present invention are also suitable to raise an immune response in human and/or animal host and therefore, are suitable for protection against diseases associated with *Haemophilus influenzae* type b. Thus, the inventive conjugates disclosed herein are useful for prevention or treatment of diseases associated with *Haemophilus influenzae* type b. Such diseases include, but are not restricted to meningitis, pneumonia, epiglottitis, and other diseases of the respiratory tract. Moreover, it was found that the treatment of an animal with the novel conjugate of the current invention lead to the formation of immunoglobuline IgG-isotypes, which prove the development of memory B-cells in the living organism. The presence of memory B-cells demonstrates immunological memory. Thus, it has been shown that the conjugates of the current invention are capable to induce a long term protection in an animal against *Haemophilus influenzae* type b.

Therefore, conjugates according to the present invention are suitable for the use as a pharmaceutically active agent applicable in medicine, especially for use in vaccination against diseases caused or associated with *Haemophilus influenzae* type b.

## **Vaccine composition**

One aspect of the present invention relates to pharmaceutical compositions, especially vaccine compositions containing at least one saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2) (IV-1), and (IV-2), pharmaceutically acceptable salts thereof, glycoconjugate thereof, together with at least one pharmaceutically acceptable adjuvant, carrier, cryoprotectant, lyoprotectant, excipient and/or diluent.

Thus the inventive syntheses for the synthetic saccharides of the general formula (I) may further comprise step B)

B) preparing a salt of a saccharide of the general formula (I) or preparing a lyophilisate of a saccharide of the general formula (I) or of the salt of a synthetic saccharide of the general formula (I).

In a preferred embodiment, the inventive syntheses for the saccharide of the formula (I) may further comprise step B)

B) preparing a salt of a saccharide of the general formula (I) or preparing a lyophilisate of a saccharide of the formula (I) or of the salt of a saccharide of the formula (I).

Pharmaceutical compositions according to the present invention comprise the at least one saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) pharmaceutically acceptable salts thereof. Especially the saccharides **2a, 2b, 2c, 4a, 4b, 4c, 4d, 4e, 6a, 6b, 6c, 8a, 8b** and **8c** obtained according to the total chemical synthesis disclosed herein are used in these vaccines. The saccharides of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) as well as the saccharides **2a, 2b, 2c, 4a, 4b, 4c, 4d, 4e, 6a, 6b, 6c, 8a, 8b** and **8c** obtained by the total chemical synthesis disclosed herein have a purity of at least 95%, more preferably of at least 96%, still more preferably of at least 97%, still more preferably of at least 98%, and most preferably of at least 99%.

The saccharides of the present invention as well as the pharmaceutical compositions containing at least one saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) and especially the vaccines containing at least one inventive saccharide, especially saccharides **2a, 2b, 2c, 4a, 4b, 4c, 4d, 4e, 6a, 6b, 6c, 8a, 8b** and **8c** are highly useful for use as a vaccine in immunization against diseases caused or associated with by *Haemophilus influenzae type b*, preferably meningitis, pneumonia, and epiglottitis.

According to the present invention saccharides **2a, 2b, 2c, 4a, 4b, 4c, 4d, 4e, 6a, 6b, 6c, 8a, 8b** and **8c** as well as any other inventive saccharide disclosed herein is useful for the manufacture of said pharmaceutical composition for use as vaccine for immunization against above-mentioned diseases.

The vaccine may be prepared in the form of a suspension or may be lyophilized. The suspension form may be stored frozen. In the lyophilized form, it is preferable to add one or more stabilizers. Vaccination can be performed at any age. The vaccine may be administered subcutaneously, by spray, by injection, orally, intraocularly, intratracheally or nasally. The amount of vaccine of the invention to be administered a human or animal and the regime of administration can be determined in accordance with standard techniques well known to those of ordinary skill in the pharmaceutical and veterinary arts taking into consideration such factors as the particular antigen, the adjuvant (if present), the age, sex, weight, species and condition of the particular animal or patient, and the route of administration.

A method of inducing immune response against *Haemophilus influenzae type b* in a subject comprises administering of the saccharide of the present invention, a mixture thereof, the conjugation thereof or pharmaceutical composition thereof. A method of

treating or preventing diseases caused or associated with by *Haemophilus influenzae* type *b*, preferably meningitis, pneumonia, and epiglottitis in a subject comprises administering of at least one synthetic saccharide of the present invention or a mixture thereof, the conjugation thereof or the composition or vaccine thereof.

5

In the present invention, the amount of the saccharide of any one of the formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) to provide an efficacious dose for vaccination against *Haemophilus influenzae* type *b* can be from between 0.02 µg to about 10 µg per kg body weight. The saccharide of any one of the formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) optionally conjugated to an immunogenic carrier, preferred carrier protein such as CRM<sub>197</sub> can be administered as a single dose or in a series (i.e., with a "booster" or "boosters"). For example, a child could receive a single dose early in life, then be administered a booster dose up to ten years later, as is currently recommended for other vaccines to prevent childhood diseases.

15

Intravenous and parenteral administration is preferred. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions or the saccharides of any one of the formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) according to the present invention optionally conjugated to an immunogenic carrier can also be lyophilized.

20

Lyophilized saccharides of the invention will ultimately be reconstituted with a liquid component to give material suitable for administration to a patient. The reconstitution will typically take place at the point of use. Thus a saccharide of the invention and an oil-in-water emulsion adjuvant or a buffer solution of an adjuvant may be kept separately in a packaged or distributed vaccine kit, ready for final formulation at the time of use. In a kit containing two containers, one will include liquid for reconstitution and the second container includes lyophilized material. For stability reasons, the lyophilized component of the invention may include a stabilizer such as lactose, sucrose and/or mannitol, as well as mixtures thereof. Using a sucrose/mannitol mixture can speed up the drying process. A lyophilized component may also include sodium chloride. Soluble components in the lyophilized material will be retained in the composition after reconstitution, and so final liquid vaccines may thus contain lactose and/or sucrose.

30

35

Formulation of the vaccines of the present invention can be accomplished using methods known by the art. Obviously, the choice of suitable carriers and other

additives will depend on the exact route of administration and the nature of the particular dosage form.

The vaccine compositions of the present invention may contain one or more  
5 adjuvants. As defined herein, an "adjuvant" is a substance that serves to enhance the immunogenicity of a saccharide of any one of the formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) according to the present invention optionally conjugated to an immunogenic carrier. An immune adjuvant may enhance an  
10 immune response to an antigen that is weakly immunogenic when administered alone, e.g., inducing no or weak antibody titers or cell-mediated immune response. Further an adjuvant may increase antibody titers to the antigen, and/or lowers the dose of the antigen effective to achieve an immune response in the individual. Thus, adjuvants are often given to boost the immune response and are well known to the skilled person. Suitable adjuvants to enhance effectiveness include, by way of  
15 example and not limitation, aluminum adjuvants (e.g., aluminum salts such as aluminum hydroxide, aluminum phosphate, aluminum sulfate or combinations thereof), Freund's Adjuvant (Complete or Incomplete), BAY R1005 (N-(2-Deoxy-2-L-leucylamino- $\beta$ -D-glucopyranosyl)-N-octadecyldodecanoylamide hydroacetate), DC-chol (dimethylaminoethane)-carbamoyl cholesterol, PCPP  
20 (poly[di(carboxylatophenoxy) phosphazene]), monophosphoryl lipid A, CpG oligonucleotides, QS-21 (Quillaja saponaria saponin immunologic adjuvant), cholera toxin and formyl methionyl peptide.

Alternative adjuvants include oil-in-water emulsion formulations for example MF59 as  
25 described in PCT Publ. No. WO 90/14837, SAF-1 (Syntex Adjuvant Formulation threonyl-MDP (0.05-1%) in an emulsion vehicle [5% squalane, 2.5% Pluronic® L121, 0.2% Polysorbate 80 and phosphate buffered saline (pH 7.4)], and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), saponin adjuvants,  
30 such as Stimulon™ (Cambridge Bioscience, Worcester, Mass.) may be used or particles generated thereof. Various oil-in-water emulsion adjuvants are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. Squalane, the saturated analog to squalene, is preferred oil. Other preferred oils are the  
35 tocopherols. Mixtures of oils can be used. Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens); copolymers of ethylene oxide

(EO), propylene oxide (PO), and/or butylene oxide (BO); octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, such as octoxynol-9 (Triton X-100, or toctylphenoxypolyethoxyethanol); phospholipids such as phosphatidylcholine (lecithin); polyoxyethylene fatty ethers derived from lauryl, cetyl, 5 stearyl and oleyl alcohols (known as Brij surfactants). Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Further adjuvants may be cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon), macrophage colony 10 stimulating factor (M-CSF), tumor necrosis factor (TNF).

The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and 15 the preparation desired. Compositions of the invention may include an antimicrobial, particularly when packaged in multiple dose format. Antimicrobials such as thiomersal and 2-phenoxyethanol are commonly found in vaccines, but it is preferred to use either a mercury-free preservative or no preservative at all.

A composition may include a temperature protective agent. Examples include 20 glycerin, propylene glycol, and/or polyethylene glycol (PEG).

The vaccine compositions of the present invention may further comprises at least one of diphtheria antigen, tetanus antigen, pertussis antigen, hepatitis B antigen, inactivated polio vaccine and inactivated rotavirus vaccine.

25 The term "diphtheria antigen" as used herein is preferably a diphtheria toxoid. The diphtheria toxoid is the formaldehyde-inactivated toxin of Corynebacterium diphtheriae, used as an active immunizing agent against diphtheria. The preparation of diphtheria toxoids is well documented (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 12). Any suitable diphtheria toxoid may be used. The concentration 30 of diphtheria toxoid is generally between 5 and 100 Lf(Limit of Flocculation)/mL. A preferred concentration is between 10 and 50 Lf/mL. A more preferred concentration is between 20 and 40 Lf/mL. Most preferably, the concentration is about 30 Lf/mL.

The term "tetanus antigen" as used herein is preferably a tetanus toxoid. The 35 tetanus toxoid is well-known for a person skilled in the art (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 33). Any suitable tetanus toxoid may be used. The concentration of tetanus toxoid is generally between 1 and 50 Lf/mL. A preferred concentration is between 2 and 9 Lf/mL. A more preferred concentration is between 5 and 8 Lf/mL. Most preferably, the concentration is about 6.5 Lf/mL.

The term "pertussis antigen" as used herein may be cellular (e.g. whole cell) or acellular. The preparation of both types of antigen is well documented (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 23). For cellular pertussis antigens, the concentration of pertussis antigens is generally between 5 and 50 OU/mL. A preferred concentration is between 10 and 40 OU/mL. A more preferred concentration is between 25 and 35 OU/mL. Most preferably, the concentration is about 30 OU/mL. Where acellular antigens are used, it is preferred to use pertussis holotoxin (PT) and filamentous haemagglutinin (FHA), more preferably combined with pertactin (also known as PRN or 69kDa antigen) and optionally, agglutinogens (also known as fimbriae) 2 and 3. PT is a toxic protein and, when present in the pertussis antigen, it is preferably detoxified. Detoxification may be by chemical and/or genetic means. A preferred detoxified mutant is the 9K/129G double mutant.

The term "hepatitis B antigen" as used herein may be hepatitis B surface antigen. The preparation of hepatitis B surface antigen is well documented (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 15).

The term "inactivated polio vaccine (IPV)" as used herein, preferably consists of inactivated (killed) poliovirus strains of all three poliovirus types 1-3. In the present invention virus inactivation refers to elimination of the infectious ability of a virus. In the two methods of inactivation (chemical and physical), most commonly used chemicals are formaldehyde and betapropiolactone. The preparation of diphtheria toxoids is well documented (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 27).

The term "inactivated rotavirus vaccine" as used herein, preferably may be the bovine(UK strain)/human reassortant vaccine, the human neonatal RV3 strain or the bovine/human neonatal 116E strain. In the present invention virus inactivation refers to elimination of the infectious ability of a virus. In the two methods of inactivation (chemical and physical), most commonly used chemicals are formaldehyde and betapropiolactone. The preparation of inactivated rotavirus vaccine is well documented (e.g. Plotkin et al, Vaccines, 6<sup>th</sup> edition, 2012, Chapter 30).

Compositions of the invention are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions that may be buffered to a selected pH. Pharmaceutically acceptable carriers for liquid formulations may be aqueous or nonaqueous solutions, suspensions, emulsions or oils. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, and

injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic solutions, emulsions or suspensions, including saline and buffered media. The pH of a composition after reconstitution is preferably between 6 and 8, and more preferably between 6.5 and 7.5 (e.g. about 7). Compositions of the invention may be maintained by the use of a buffer e.g. a Tris buffer, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate and triethanolamine buffer. Thus compositions of the invention will preferably include a buffer. The isotonic agent may be an ionic isotonic agent such as a salt or a non-ionic isotonic agent such as a carbohydrate. Examples of ionic isotonic agents include but are not limited to NaCl, CaCl<sub>2</sub>, KCl and MgCl<sub>2</sub>. Examples of non-ionic isotonic agents include but are not limited to sorbitol and glycerol.

In a preferred embodiment of the invention, the vaccine composition is formulated as a sterile liquid, pyrogenfree, phosphate-buffered physiological saline, with or without a preservative.

A pharmaceutically acceptable preservative can be employed to increase the shelf life of the compositions. Benzyl alcohol may be suitable, although a variety of preservatives including, for example, parabens, thimerosal, chlorobutanol, or benzalkonium chloride may also be employed. A suitable concentration of the preservative will be from 0.02% to 2% based on the total weight although there may be appreciable variation depending upon the agent selected.

The composition of the invention can be formulated as single dose vials, multidose vials or as pre-filled syringes.

Another aspect of the present invention relates to pharmaceutical formulations and pharmaceutical compositions containing at least one saccharide of any one of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) optionally conjugated with an immunogenic carrier or the vaccine as an active ingredient, together with at least one pharmaceutically acceptable adjuvant, carrier, excipient, solvent and/or diluents.

Further preferred, the pharmaceutical composition is formulated in the form of a lyophilisate or liquid buffer solution.

The vaccine or pharmaceutical composition can also be administered in form of its pharmaceutically active salt optionally using substantially nontoxic pharmaceutically acceptable carrier, excipients, adjuvants or diluents. The vaccine or pharmaceutical

composition of the present invention is prepared in a conventional solid or liquid carrier or diluents and a conventional pharmaceutically-made adjuvant at suitable dosage level in a known way. The preferred preparations and formulations are in administrable form which is suitable for oral application. These administrable forms, for example, include pills, tablets, film tablets, coated tablets, capsules, powders and deposits. Other than oral administrable forms are also possible. The inventive vaccine or pharmaceutical composition may be administered by any appropriate means, including but not limited to inhalation, injection (intravenous, intraperitoneal, intramuscular, subcutaneous) by absorption through epithelial or mucocutaneous linings (oral mucosa, rectal and vaginal epithelial linings, nasopharyngeal mucosa, intestinal mucosa); orally, rectally, transdermally, topically, intradermally, intragastrically, intracutaneously, intravaginally, intravasally, intranasally, intrabuccally, percutaneously, sublingually, or any other means available within the pharmaceutical arts.

The vaccine or pharmaceutical composition of the present invention, containing at least one saccharide of any of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2), preferred the saccharides **2a**, **2b**, **2c**, **4a**, **4b**, **4c**, **4d**, **4e**, **6a**, **6b**, **6c**, **8a**, **8b** and **8c** or pharmaceutically acceptable salt thereof as an active ingredient will typically be administered in admixture with suitable carrier materials suitably selected with respect to the intended form of administration, i.e. oral tablets, capsules (either solid-filled, semi-solid filled or liquid filled), powders for constitution, oral gels, elixirs, dispersible granules, syrups, suspensions, and the like, and consistent with conventional pharmaceutical practices. For example, for oral administration in the form of tablets or capsules, the active ingredient may be combined with any oral nontoxic pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable binders, lubricants, disintegrating agents and coloring agents may also be incorporated in the mixture. Powders and tablets may be comprised of from about 5 to about 95 percent of the tetrasaccharide.

Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethyl-cellulose, polyethylene glycol and waxes. Among the lubricants that may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose, guar gum and the like. Sweetening and flavoring agents and preservatives may also be included

where appropriate. Some of the terms noted above, namely disintegrants, diluents, lubricants, binders and the like, are discussed in more detail below.

5 Additionally, the vaccine or pharmaceutical composition of the present invention may be formulated in sustained release form to provide the rate controlled release of any one or more of the components or active ingredients to optimize the therapeutic effects. Suitable dosage forms for sustained release include layered tablets containing layers of varying disintegration rates or controlled release polymeric matrices impregnated with the active components and shaped in tablet form or  
10 capsules containing such impregnated or encapsulated porous polymeric matrices.

Liquid form preparations include solutions, suspensions and emulsions. As an example may be mentioned water or water-propylene glycol solutions for parenteral injections or addition of sweeteners and opacifiers for oral solutions, suspensions  
15 and emulsions. Liquid form preparations may also include solutions for intranasal administration.

Aerosol preparations suitable for inhalation may include solutions and solids in powder form, which may be in combination with a pharmaceutically acceptable  
20 carrier such as inert compressed gas, e.g. nitrogen.

For preparing suppositories, a low melting wax such as a mixture of fatty acid glycerides such as cocoa butter is first melted, and the active ingredient is dispersed homogeneously therein by stirring or similar mixing. The molten homogeneous  
25 mixture is then poured into convenient sized molds, allowed to cool and thereby solidifies.

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for either oral or parenteral administration.  
30 Such liquid forms include solutions, suspensions and emulsions.

The inventive vaccine or pharmaceutical composition containing at least one saccharide of any of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2), preferably the saccharides **1f**, **2a**, **2b**, **2b'**, **2c**, **2f**, **4a**, **4b**, **4c**, **4d**,  
35 **4e**, **4f**, **6a**, **6b**, **6c**, **6f**, **8a**, **8b**, **8c** and **8f** may also be deliverable transdermally. The transdermal compositions may take the form of creams, lotions, aerosols and/or emulsions and can be included in a transdermal patch of the matrix or reservoir type as are conventional in the art for this purpose.

The term capsule refers to a special container or enclosure made of methyl cellulose, polyvinyl alcohols, or denatured gelatins or starch for holding or containing compositions comprising the active ingredients. Hard shell capsules are typically made of blends of relatively high gel strength bone and pork skin gelatins. The capsule itself may contain small amounts of dyes, opaquing agents, plasticizers and preservatives.

Tablet means compressed or molded solid dosage form containing the active ingredients with suitable diluents. The tablet can be prepared by compression of mixtures or granulations obtained by wet granulation, dry granulation or by compaction well known to a person skilled in the art.

Oral gels refer to the active ingredients dispersed or solubilized in a hydrophilic semi-solid matrix.

Powders for constitution refer to powder blends containing the active ingredients and suitable diluents which can be suspended in water or juices.

Suitable diluents are substances that usually make up the major portion of the composition or dosage form. Suitable diluents include sugars such as lactose, sucrose, mannitol and sorbitol, starches derived from wheat, corn rice and potato, and celluloses such as microcrystalline cellulose. The amount of diluents in the composition can range from about 5 to about 95% by weight of the total composition, preferably from about 25 to about 75%, more preferably from about 30 to about 60% by weight, and most preferably from about 40 to 50% by weight.

The term disintegrants refers to materials added to the composition to help it break apart (disintegrate) and release the medicaments. Suitable disintegrants include starches, "cold water soluble" modified starches such as sodium carboxymethyl starch, natural and synthetic gums such as locust bean, karaya, guar, tragacanth and agar, cellulose derivatives such as methylcellulose and sodium carboxymethylcellulose, microcrystalline celluloses and cross-linked microcrystalline celluloses such as sodium croscarmellose, alginates such as alginic acid and sodium alginate, clays such as bentonites, and effervescent mixtures. The amount of disintegrant in the composition can range from about 1 to about 40% by weight of the composition, preferably 2 to about 30% by weight of the composition, more preferably from about 3 to 20% by weight of the composition, and most preferably from about 5 to about 10% by weight.

Binders characterize substances that bind or "glue" powders together and make them cohesive by forming granules, thus serving as the "adhesive" in the formulation. Binders add cohesive strength already available in the diluents or bulking agent. Suitable binders include sugars such as sucrose, starches derived from wheat, corn  
5 rice and potato; natural gums such as acacia, gelatin and tragacanth; derivatives of seaweed such as alginic acid, sodium alginate and ammonium calcium alginate; cellulosic materials such as methylcellulose and sodium carboxymethylcellulose and hydroxypropyl-methylcellulose; polyvinylpyrrolidone; and inorganics such as magnesium aluminum silicate. The amount of binder in the composition can range  
10 from about 1 to 30% by weight of the composition, preferably from about 2 to about 20% by weight of the composition, more preferably from about 3 to about 10% by weight, even more preferably from about 3 to about 6% by weight.

Lubricant refers to a substance added to the dosage form to enable the tablet,  
15 granules, etc. after it has been compressed, to release from the mold or die by reducing friction or wear. Suitable lubricants include metallic stearates such as magnesium stearate, calcium stearate or potassium stearate; stearic acid; high melting point waxes; and water soluble lubricants such as sodium chloride, sodium benzoate, sodium acetate, sodium oleate, polyethylene glycols and d'l-leucine.  
20 Lubricants are usually added at the very last step before compression, since they must be present on the surfaces of the granules and in between them and the parts of the tablet press. The amount of lubricant in the composition can range from about 0.05 to about 15% by weight of the composition, preferably 0.2 to about 5% by weight of the composition, more preferably from about 0.3 to about 3%, and most  
25 preferably from about 0.3 to about 1.5% by weight of the composition.

Glidants are materials that prevent caking and improve the flow characteristics of granulations, so that flow is smooth and uniform. Suitable glidants include silicon dioxide and talc. The amount of glident in the composition can range from about  
30 0.01 to 10% by weight of the composition, preferably 0.1% to about 7% by weight of the total composition, more preferably from about 0.2 to 5% by weight, and most preferably from about 0.5 to about 2% by weight.

Coloring agents are excipients that provide coloration to the composition or the  
35 dosage form. Such excipients can include food grade dyes and food grade dyes adsorbed onto a suitable adsorbent such as clay or aluminum oxide. The amount of the coloring agent can vary from about 0.01 to 10% by weight of the composition, preferably from about 0.05 to 6% by weight, more preferably from about 0.1 to about 4% by weight of the composition, and most preferably from about 0.1 to about 1%.

Techniques for the formulation and administration of the vaccine of the present invention may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton PA. A suitable vaccine composition comprising at least one saccharide of any one of the general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2), preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** and/or pharmaceutically acceptable salts thereof may be a solution of such saccharide(s) in a suitable liquid pharmaceutical carrier or any other formulation such as tablets, pills, film tablets, coated tablets, dragees, capsules, powders and deposits, gels, syrups, slurries, suspensions, emulsions, and the like.

A therapeutically effective dosage of the saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2), preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** refers to that amount of the compound that results in an at least a partial immunization against a disease. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical, pharmacological, and toxicological procedures in cell cultures or experimental animals. The dose ratio between toxic and therapeutic effect is the therapeutic index. The actual amount of the composition administered will be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgement of the prescribing physician.

The mentioned vaccine formulation displays an extraordinary stability at room temperature due to the modular constitution of the compounds of the present invention, wherein said vaccine formulation may be maintained at a temperature of at least 25 °C for a period of at least 3 months prior to reconstitution. The temperature-stability of the herein described vaccine formulations constitutes a particular advantage of the present invention over the vaccines directed against *Haemophilus influenzae type b*, which were described up to present.

In a preferred embodiment of the invention the said period is comprises 6 months or at least 12 months.

35

### **Antibody**

The present invention refers also to an antibody against at least one synthetic saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2). The antibody is produced by the monoclonal hybridoma. The antibody is for

diagnostics, prophylaxis, and treatment of pneumonia, meningitis, otitis media, bacteremia and acute exacerbation of chronic bronchitis, sinusitis, arthritis and conjunctivitis. Another embodiment of the present invention is use of the antibody for manufacture of medicaments or devices for diagnosis, prophylaxis, and treatment  
5 of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

The term "antibody" as used herein encompasses polyclonal and monoclonal antibody preparations, as well as preparations including hybrid antibodies, F(ab')<sub>2</sub> fragments, F(ab) molecules, single domain antibodies and functional fragments  
10 thereof which exhibit immunological binding properties of the parent antibody molecule. The antibody according to the invention may be polyclonal or monoclonal.

The saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2), preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** or the antibody thereof can be used for preparing a  
15 pharmaceutical composition especially vaccines for the treatment or prevention of diseases caused by *Haemophilus influenzae type b*. The saccharide of the present invention or the antibody thereof can be used for the treatment or prevention of a disease caused by *Haemophilus influenzae type b*.

20 Further the present invention refers to the use of at least one saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2) preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** according to the invention or at least one antibody against at least one saccharide of  
25 the present invention in immunological assays for diagnostics of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

Such assays comprise, for instance, microarray and ELISA useful for diagnosis of diseases caused by *Haemophilus influenzae type b*. Therefore another aspect of  
30 the present invention refers to the use of any one of saccharides of formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2) preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** for diagnosis of diseases caused by *Haemophilus influenzae type b*.

35 Any one of saccharides of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2) preferably the saccharides **1f, 2a, 2b, 2b', 2c, 2f, 4a, 4b, 4c, 4d, 4e, 4f, 6a, 6b, 6c, 6f, 8a, 8b, 8c** and **8f** or a mixture of such saccharides could be immobilized on a microarray surface or any other surface and used for an *in vitro* method of detecting *Haemophilus influenzae type b*. A method of identifying

*Haemophilus influenzae type b* comprises the use of at least one saccharide of the present invention. Furthermore, the synthetic saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1), and (IV-2) preferably the saccharides **1f**, **2a**, **2b**, **2b'**, **2c**, **2f**, **4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **6a**, **6b**, **6c**, **6f**, **8a**, **8b**, **8c** and **8f** or a mixture of such saccharides can be used as an analytical standard for immuno-assays.

### Diagnostic devices

Another aspect of the present invention is related to a solid support comprising at least one saccharide of general formula (I), preferred saccharides of the formulae (II-1), (II-2), (II-3), (II-4), more preferred saccharide of the formulae (III-1), (III-2), (IV-1), and (IV-2), preferably the saccharides **1f**, **2a**, **2b**, **2b'**, **2c**, **2f**, **4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **6a**, **6b**, **6c**, **6f**, **8a**, **8b**, **8c** and **8f**.

This solid support is preferable a part of a diagnostic device. The solid support and the diagnostic device are used for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*, wherein the at least one saccharide of general formula (I) is immobilized on said solid support by preferably covalent bonding.

The other embodiment of the present invention is the diagnostic device wherein the solid support is selected from the group comprising a glass slide, glass plate, a microtitre plate, microspheres, or beads.

The even other embodiment of the present invention is the diagnostic device wherein the at least one saccharide of general formula (I) is covalently bound using a linking molecule.

Moreover, the present invention shows that the saccharide according to formula (I) can be used in immunological assays for meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

Such assays comprise, for instance, microarray and ELISA useful for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

Therefore another aspect of the present invention refers to the use of a saccharide of formula (I) for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

Saccharide (I) according to the invention is immobilized on a solid support by covalent bonding. At least one synthetic saccharide (I) unbound or immobilized on a solid support is used for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

- 5 Use of saccharide (I) for the diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b* is based on the detection of the presence of antibodies specific for at least one saccharide of general formula (I). Thus, a diagnostic device comprising at least one saccharide of general formula (I) is used for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus*  
10 *influenzae type b*.

- It is further preferred that a saccharide of formula (I) used for meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b* is substantially pure, having a purity of  $\geq 95\%$ , preferably  $\geq 96\%$ , more preferably  $\geq 97\%$ , still more preferably  $\geq$   
15  $98\%$ , and most preferably  $\geq 99\%$ .

- There are different possibilities for the choice of an assay system in which a saccharide of formula (I) is used for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*. An assay conducted for  
20 diagnostic purposes according to the invention may be an immune assay like a solid-phase enzyme immunoassay (EIA), an enzyme linked immunosorbent assay (ELISA), especially an "indirect" ELISA or a radioimmune assay (RIA). For the use of a saccharide of formula (I) in such assays it could be necessary to immobilize the saccharide of formula (I) on a solid support.

- 25 Thus other embodiment of the present invention is solid support on which at least one saccharide (I) is immobilized by covalent attachment through a linker or spacer. Even other embodiment of the invention is saccharide immobilized by covalent bonding on the solid support directly or indirectly through the nitrogen atom of the -O-  
30 L-NH<sub>2</sub> group (**Figure 2 (B)**).

- Therefore a saccharide of formula (I) may be immobilized on a solid support, particularly for diagnostic applications. One preferred embodiment of the present invention is a saccharide of general formula (I) immobilized on a solid support by  
35 covalent bonding. One particularly preferred embodiment of the present invention is a saccharide of general formula (I) immobilized on a solid support by direct or indirect covalent bonding. Thereby direct covalent bonding is especially preferred.

Further preferred the solid support is selected from the group comprising or consisting of: a glass slide, a microtitre plate, test tubes, microspheres, nanoparticle or beads.

- 5 It is particularly preferred that the solid support is a glass slide or a microtitre plate. A microtitre plate or microplate or microwell plate is a flat plate with multiple "wells" used as small test tubes. Typically a microtitre plate having 6, 24, 96, 384 or even 1536 sample wells can be used. Microplates are produced from many different materials, like polycarbonate for microtitre plate used for PCR. The most common is  
10 polystyrene as used for most optical detection microplates. It can be colored white by the addition of titanium dioxide for optical absorbance or luminescence detection or black by the addition of carbon for fluorescent biological assays.

- "Direct covalent bonding" as used herein refers to immobilization of a compound of  
15 general formula (I) by reacting a functional group of the saccharide of general formula (I) with a functional group of the material the solid support is made from. It is preferred that the functional group of the saccharide of general formula (I) is amine as defined above in -O-L-NH<sub>2</sub>. Possible reactive, functional groups of the solid support may be: phenyl, amino groups, hydroxyl groups, thiols, carbonyls, carboxyls,  
20 vinyls, halides such as fluorides, chlorides, bromides and iodides, maleimides; succinimide esters.

- "Indirect covalent bonding" as used herein refers to immobilization of a saccharide of general formula (I) on a solid support wherein the saccharide of general formula (I) is  
25 covalently linked to a second compound which mediates the immobilization to the solid support. It is preferred that this second compound is a protein which does not cause an immune reaction. It is important that the second compound itself is most probably not bound by any antibody present in the blood or serum of a patient to avoid false positive results. Further the second compound should be able to be  
30 immobilized on the solid carrier, by covalent or non-covalent bonding. It is preferred that this second compound is selected from the group comprising or consisting of bovine serum albumin (BSA), human serum albumin (HAS), gelatin or casein. The immobilization using indirect covalent bonding therefore refers preferably to covalent bonding of a saccharide of general formula (I) to a protein as a second compound  
35 (eg using the free amino groups of a protein) and subsequently binding of the protein to the solid support by covalent bonding or non covalent interaction between the solid support and the protein. Possible non-covalent interactions are: hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions. Many polymers, such as polystyrene and polypropylene are hydrophobic in nature. Nevertheless

there are also manufacturers which supply solid support having specialized surfaces optimized for different adhesion conditions.

However, immobilization, especially using indirect covalent bonding, may also occur by strong adhesion. Thus, an effective immobilization according to the present invention may be realized not only by chemical bonding, but also unbound by immobilization related to physisorption. As key feature for physisorption acts the phenomenon that the force for adhesion is caused by van der Waals force. The term "unbonded" refers to a bonding other than covalent bonding.

10

Chemisorption as immobilization form according to the present invention uses chemical bonds between solid support and a saccharide of formula (I). Such bond may be covalent, but may also be ionic.

15 In a preferred embodiment of the present invention immobilization of a saccharide of the formula (I) on a solid support is realized by direct covalent bonding namely a chemical reaction between these two reactants, preferably by a substitution reaction. In a more preferred embodiment of the present invention the solid support is modified with a functional group which is capable of leaving the solid support upon reaction  
20 with the compound of the present invention. Such functional group may be bound directly to a composing molecule of the solid support or may be bound to a linker which is directly bound to the composing molecule of the solid support. Thus, in a more preferred embodiment of the present invention the solid support is modified to bearing a suitable leaving group. Suitable leaving groups may be halides such as  
25 chlorides, bromides and iodides; hydroxides, oximes, maleimides, succinimide, alcohols and esters. Such leaving group may be or may be incorporated in maleimide;  $\alpha$ -iodoacetyl;  $\alpha$ -bromoacetyl; *N*-hydroxysuccinimide ester (NHS) and 2-pyridyldithiols. In yet more preferred embodiment the leaving group on the solid support is capable of reacting with amines, alcohols and thiols, preferably upon  
30 proton exchange. In most preferred embodiment of the present invention the solid support is functionalized with a succinimidyl hydroxide functional group, more preferably *N*-succinimidyl hydroxide, which will leave the solid support upon reaction with a compound of the present invention as *N*-hydroxysuccinimide.

35 Modification of the solid support by introduction of a suitable leaving group is preferably carried out by reaction of an unmodified carrier with a reactive bifunctional molecule, preferably a bifunctional molecule with a molecular bridge or spacer arm between the two functional groups. In a preferred embodiment of the present

invention functional groups willingly reacting with the solid support comprise sulfosuccinimide esters and succinimides.

One further preferred aspect of the bifunctional linking molecules is the ability of providing the functional group meant to bind with a saccharide of the formula (I) is an appropriate distance to the solid carrier. Such an appropriate distance is provided by a molecular bridge or spacer arm of suitable length. Such a molecular bridge or spacer arm may have a length preferably from 3 Å ( $10^{-10}$  m) to 10 nm, more preferably from 5 Å to 50 Å, and most preferably from 6 Å to 30 Å.

Suitable reactive bifunctional molecules for modification of the solid support comprise N-(γ-maleimidobutyryloxy) sulfosuccinimide ester (sulfo-GMBO), succinimidyl (4-iodoacetyl) aminobenzoate (sulfo-SIAN), succinimidyl-3-(bromoacetamido)propionate (SBAP), disuccinimidyl glutarat (DSG), 2-pyridyldithiol-tetraoxatetradecane-N-hydroxysuccinimide (PEG-4-SPDP).

There are also solid supports commercially available made from polymers with reactive functional introduced for covalent bonding. One example are microplates named Covalink™ NH by Thermo scientific which allow covalent binding through a secondary amine group.

Another aspect of the present invention is the use of the diagnostic device comprising at least one synthetic saccharide of general formula (I) immobilized on a solid support by covalent bonding for diagnosis of meningitis, pneumonia, and epiglottitis caused by *Haemophilus influenzae type b*.

### Kit

One embodiment of the present invention relates to a kit comprising at least one saccharide of general formula (I) immobilized on a solid support by covalent bonding or the saccharide of general formula (I) for immobilization on a solid support. The at least one saccharide of general formula (I) may be used as a marker in said assays. Another embodiment of the present invention relates to a kit comprising at least one antibody against a saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2).

A kit in molecular biology or in medical diagnostics is a package which includes all necessary ingredients for performing a certain method or singular step. Standard chemicals as present in any standard molecular biology or medical laboratory are

normally not included. Nevertheless some of these standard chemicals may be indispensable to carry out the diagnosis or the immobilization properly. It is understood that all ingredients are provided in quantities that allow for a proper execution of the desired reactions for the majority of scientific, diagnostic and industrial applications.

Often, but not always, these ingredients are provided in already prepared solutions ready- or close to ready-for-use. There may be also combinations of different ingredients already added together. A further advantage is that such kits use to be verified. Therefore the operator doesn't have to prove again the viability of the diagnostic method and can save on at least some control experiments. Therefore kits are a very popular tool in laboratories in research, diagnostics and industry.

Such a kit according to the invention shall include at least the following components:

- A) solid support on which at least one saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) is immobilized
- B) at least one antibody, like detection antibody
- C) a standard solution

or a kit according to the invention shall include at least the following components:

- A') solid support on which at least one antibody against a saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) is immobilized
- B) at least one further antibody, like detection antibody
- C) a standard solution

The following components may also be included in such kits:

- D) blocking solution
- E) wash solution
- F) sample buffer

An antibody in the kit may be a specific antibody which can be used as a capture antibody. But preferably it is at least an enzyme-linked secondary antibody used as detection antibody that binds specifically to antibody's Fc region. For quantitative determinations, the optical density (OD) or fluorescence of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule (a standard solution). A blocking solution may be a solution of a non-reacting protein, such as bovine serum albumin or casein, which is added to block any plastic surface in the well that remains uncoated by the antigen. Washing solutions are used to remove unbound components. A sample buffer may

be used to dilute the sample of the patient (blood, serum, urine) so that the concentration of the target molecule is in the range which can normally be detected by the test system used.

- 5 If the kit shall be allowed for the immobilization of a saccharide of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2) on a solid support, the kit should include at least:
- 10 A) At least one saccharide of any one of general formulae (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2), or an antibody against at least one synthetic saccharide of general formula (I), (II-1), (II-2), (II-3), (II-4), (III-1), (III-2), (IV-1) and (IV-2)
- B) A solid support, like a microtiter plate or microarray slide

15 Thereby the solid support may be modified, for example the solid support may be functionalized with a linker molecule as described above.

The following components may also be included in such kits:

- 20 C) blocking solution  
D) wash solution  
E) reaction buffer

### Description of Figures

25

**Figure 1:** Commercially available interconnecting molecules according to the present invention.

30

**Figure 2:** (A) saccharide conjugated with a carrier protein; (B) saccharide conjugated on a solid support.

**Figure 3:** Synthetic scheme of 2'-methoxyribosylribitolphosphate tetramer **29** conjugated to CRM<sub>197</sub> and cysteine.

35

**Figure 4:** <sup>1</sup>H NMR spectra of (A) fragmented Hib CPS after treatment with 0.1 M sodium hydroxide solution for 20 hours and (B) untreated Hib CPS as reference. The comparison shows that Hib CPS hydrolyzes completely within 20 hours under basic conditions.

- Figure 5:** (A) HPLC chromatogram and (B) ESI mass spectrum of fragmented Hib CPS after treatment with 0.1 M sodium hydroxide solution for 20 hours.
- 5 **Figure 6:** HPLC chromatogram of Hib CPS after treatment with Alhydrogel® (A) for 7 days and (B) for 2 days at 37 °C as well as (D) for 7 days and (E) for 2 days at room temperature. HPLC chromatogram of untreated Hib CPS is shown in (C).
- 10 **Figure 7:** HPLC chromatogram of compound **16** after treatment with (A) Alhydrogel®, (B) with aluminum phosphate, (C) with water for 7 days at 37 °C. HPLC chromatograms of untreated compounds **16** and **8** are shown in (D) and (E).
- 15 **Figure 8:** HPLC chromatogram of compound **16** after treatment with (A) Alhydrogel®, (B) with aluminum phosphate, (C) with water for 7 days at 2–8 °C. HPLC chromatograms of untreated compounds **16** and **8** are shown in (D) and (E).
- 20 **Figure 9:** HPLC chromatogram of compound **16** after treatment with (A) Alhydrogel®, (B) with aluminum phosphate, (C) with water for 7 days at room temperature. HPLC chromatograms of untreated compounds **16** and **8** are shown in (D) and (E).
- 25 **Figure 10:** HPLC chromatogram of compound **16** after treatment with (A) Alhydrogel®, (B) with aluminum phosphate, (C) with water for 7 days at 70 °C. HPLC chromatograms of untreated compounds **16** and **8** are shown in (D) and (E).
- 30 **Figure 11:** HPLC chromatogram of compound **30** after treatment with (A) Alhydrogel®, (B) with phosphate buffer, (C) with water for 14 days at room temperature. HPLC chromatograms of untreated compounds **30**, **16** and **8** are shown in (D), (E) and (F).
- 35 **Figure 12:** HPLC chromatogram of compound **30** after treatment with (A) Alhydrogel®, (B) with phosphate buffer, (C) with water for 14 days at 2–8 °C. HPLC chromatograms of untreated compounds **30**, **16** and **8** are shown in (D), (E) and (F).

- 5 **Figure 13:** HPLC chromatogram of (A) compound **30** after treatment with after treatment with 0.1 M sodium hydroxide solution for 3 days; (C) compound **16** after treatment with after treatment with 0.1 M sodium hydroxide solution for 3 days, HPLC chromatograms of untreated compounds **30**, **16** and **8** are shown in (B), (D) and (E).
- 10 **Figure 14:** MALDI mass spectra of (A) compound **30** conjugated to CRM<sub>197</sub> and (B) compound **94**.
- Figure 15:** HPLC-SEC chromatograms of compound **94** in presence of phosphate buffer.
- Figure 16:** shows the structure of conjugate **94**.
- 15 **Figure 17:** Glycan array analysis: (A) Binding of compounds **19**, **24**, **29**, **53** and **62** to antibodies (Hib human reference serum) in the presence of Hib PRP inhibitor (grey bars) or no inhibitor (black bars) after 21 days; (B) Binding of compounds **19**, **24**, **29**, **53** and **62** to antibodies (rabbit typing serum) in the presence of Hib PRP inhibitor (grey bars) or no inhibitor (black bars) after 21 days; Binding of compounds **19**, **24**, **29**, **53**, **62** and natural Hib PRP as reference to antibodies raised by immunization with compound **94** (C) and compound **94** with Alhydrogel (D) after 35 days. Error bars represent the standard deviation of the quadruplicate samples.
- 20
- 25 **Figure 18:** ELISA study: Binding of IgG antibodies from rabbits (n = 4) immunized with unadjuvanted conjugate **94** (■), with conjugate **94** adjuvanted with Alhydrogel (▲), with PBS/Alhydrogel (●) and with HiberiX® (▼) to plates coated with native Hib PRP antigen after 0 days (A – negative control), 14 days (B), 21 days (C) and 35 days (D). Sera were diluted 5-fold with 1% BSA-PBS. Diluted sera (100 µL) were added per well of the microtiter plate which was coated with 1 µg of Hib-PRP polysaccharide, detected with a HRP conjugated goat anti-rabbit secondary antibody diluted to 1 : 10000 and developed using TMB. Absorbance was measured at 450 nm and the data were plotted using the graphpad prism software.
- 30
- 35 **Figure 19:** ELISA study: Binding of IgG antibodies from rabbits (n = 4) immunized with unadjuvanted conjugate **94** (■), with conjugate **94** adjuvanted with

Alhydrogel (▲), with PBS/Alhydrogel (●) and with HiberiX® (▼) to commercial ADi plates precoated with native Hib PRP antigen after 0 days (A – negative control), 14 days (B), 21 days (C) and 35 days (D). Sera were diluted 5-fold with 1% BSA-PBS. Diluted sera (100 µL) were added per well of the microtiter plate which was coated with 1 µg of Hib-PRP polysaccharide, detected with a HRP conjugated goat anti-rabbit secondary antibody diluted to 1 : 10000 and developed using TMB. Absorbance was measured at 450 nm and the data were plotted using the graphpad prism software.

10

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those skilled in the art that the techniques disclosed in the examples, which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those skilled in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments, which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

20

Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as examples of embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the spirit and scope of the invention as described in the following claims.

30

## Examples

### Chemical synthesis

#### 5 Abbreviations:

TLC: thin layer chromatography, EtOAc: ethyl acetate, DCM: dichloromethane, RBF: round bottom flask, ACN: acetonitrile, AcOH: acetic acid, TBAF: tetrabutylammonium fluoride, BnBr: benzyl bromide, DMAP: dimethylaminopyridine, PTFE: polytetrafluoroethylene, AIBN: azobisisobutyronitrile, THF: tetrahydrofuran, NAP: 2-naphthylmethyl, Lev: levulinyll.

#### General information for chemical synthesis

Commercial reagents were used without further purification except where noted.

15 Solvents were dried and redistilled prior to use in the usual way. All reactions were performed in oven-dried glassware under an inert atmosphere unless noted otherwise. Analytical thin layer chromatography (TLC) was performed on Kieselgel 60 F254 aluminium plates precoated with a 0.25 mm thickness of silica gel. The TLC plates were visualized with UV light and by staining with Hanessian solution

20 (ceric sulfate and ammonium molybdate in aqueous sulfuric acid) or sulfuric acid-ethanol solution. Column chromatography was performed on Fluka Kieselgel 60 (230-400 mesh). Optical rotations (OR) were measured with a Schmidt & Haensch UniPol L1000 polarimeter at a concentration (c) expressed in g/100 mL.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were measured with a Varian 400-MR or Varian 600 spectrometer with

25  $\text{Me}_4\text{Si}$  as the internal standard. NMR chemical shifts ( $\delta$ ) were recorded in ppm and coupling constants ( $J$ ) were reported in Hz. High-resolution mass spectra (HRMS) were recorded with an Agilent 6210 ESI-TOF mass spectrometer.

#### General procedure A) Disilyloxy deprotection

30 TBAF (0.21 mL, 0.21 mmol) and AcOH (7  $\mu\text{L}$ , 0.11 mmol) were added to a stirred solution of starting material (0.073 mmol) in THF (1.5 mL) at room temperature in a 10 mL RBF (oven dried) under argon atmosphere. Reaction mixture was stirred at room temperature for 4 h. Reaction was monitored by TLC. After complete consumption of starting material reaction mixture was diluted with DCM (10 mL) and

35 concentrated under vacuum to obtain the crude product. The crude product was by automated flash chromatography using EtOAc in *n*-hexane (20-60%) as the eluent. Concentration of solvent from test tubes containing product (based on TLC) in vacuum resulted in a colourless oil.

**General procedure B) Benzylolation using Bu<sub>2</sub>SnO**

Ag<sub>2</sub>O (0.116 g, 0.5 mmol) and BnBr (8  $\mu$ L, 0.07 mmol) were added to a stirred solution of starting material (0.063 mmol) in DCM (1 mL) at room temperature in a 10 mL RBF (oven dried) under argon atmosphere. Reaction mixture was kept for stirring at room temperature for 6 h. Reaction was monitored by TLC. After complete consumption of starting material reaction mixture was diluted with DCM (30 mL) and filtered through celite pad and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-50%) as the eluent. Concentration of solvent from test tubes containing the product (based on TLC) in vacuum resulted in the colourless oil.

Bu<sub>2</sub>SnO (2.42 g, 9.73 mmol) was added to a clear solution of the diol starting material (4.6 g, 6.48 mmol) in toluene (50 mL) at room temperature under argon atmosphere equipped with a stir bar and stirring of 1400 rpm. Then the reaction mixture was kept under reflux at 130 °C for 6 h. After 6 h, solvent were removed under vacuum and the reaction was azeotropic dried with toluene (5 X 10 mL dry toluene). After complete removal of solvents acetal was dried under vacuum for 20 min. Acetal was removed from vacuum in presence of argon and dissolved in DMF (50 mL). To this solution BnBr (1.16 mL, 9.73 mmol) and TBAI (4.78 g, 12.96 mmol) were added and the reaction mixture was kept for stirring at 100 °C for 20 h. Reaction was monitored by TLC (40% EtOAc in *n*-hexane). After 20 h, reaction mixture was diluted with EtOAc (50 mL) and water (20 mL). The aqueous layer was separated and washed with EtOAc (2 x 30 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> (~2 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by column chromatography on silica gel using EtOAc in *n*-hexane (gradient, 0 to 30%) as the eluent. Concentration of solvent from test tubes resulted in the colourless oil (3.8 g, 73%).

**General procedure C) Protection with Lev**

To a solution of the hydroxyl compound (0.195 mmol) in DCM (3 mL) in a 25 mL RBF under argon atmosphere was added levulinic acid (0.3 mmol, 30  $\mu$ L), *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (0.3 mmol, 58 mg) and DMAP (0.2 mmol, 24 mg). The resulting reaction mixture was stirred at room temperature for 2 h. The reaction was monitored by TLC. After complete consumption of the starting material, the reaction mixture was diluted with DCM (10 mL) and washed with brine (5 mL). The aqueous layer was extracted with DCM (2 x 5 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> (0.2 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-50%) as the

eluent. Concentration of solvent from test tubes containing the products (based on TLC) in vacuum resulted in a white oil.

#### General procedure D) NAP deprotection

- 5 To a solution of the NAP protected compound (0.048 mmol) in dichloromethane:H<sub>2</sub>O (1.8 : 0.2 mL) in a 10 mL RBF (oven dried) under argon atmosphere was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (14 mg, 0.058 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. Reaction was monitored by TLC. Reaction was diluted with DCM (10 mL) and extracted with NaHCO<sub>3</sub> aq. sat. solution  
10 (5 mL) and brine (5 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> (0.2 g), filtered, and the filtrate was concentrated under vacuum for 15 min to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-80%) as the eluent. Concentration of solvent from test tubes containing the products (based on TLC) in vacuum resulted in a white oil.

15

#### General procedure E) Phosphate coupling: phosphoramidite method

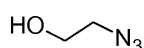
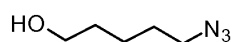
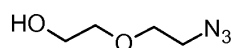
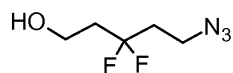
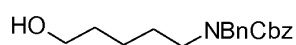
- To a solution of 3-OH compound (0.054 mmol) in DCM (1.2 mL) in a 25 mL RBF (oven dried) under argon atmosphere was added bis(diisopropylamino)-benzyloxyphosphine (0.108 mmol) and diisopropylammonium tetrazolide (0.081  
20 mmol) and the solution stirred at room temperature for 1.5 h. Reaction was monitored by TLC. Reaction mixture was diluted with DCM (10 mL) and quenched with NaHCO<sub>3</sub> aq. sat. solution (5 mL). The aqueous layer was extracted with DCM (2 x 5 mL). The combined organic layer was washed with brine (5 mL), dried over Na<sub>2</sub>SO<sub>4</sub> (0.5 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product.
- 25 The crude product was purified on silica gel column chromatography (column 2 X 10 cm) using EtOAc in *n*-hexane (0-30%) and 2% triethylamine as the eluent. Concentration of solvent from test tubes containing product (based on TLC) in vacuum resulted in a yellow oil. The product was transferred to a smaller RBF (10 mL) using toluene (5 mL) and evaporation of the solvent under vacuum for 20 min  
30 resulted in a white oil. The product was left in high vacuum for 10 min flushed with argon and used immediately for the next step. To a solution of the phosphoramidite (0.054 mmol) in DCM (1.2 mL) in a 10 mL RBF (oven dried) under argon atmosphere was added 5-OH compound (0.036 mmol) and tetrazole 0.45 M solution in ACN (0.24 mL, 0.108 mmol) and the solution stirred at room temperature for 2 h. Then, *t*-butyl peroxide 5.0 - 6.0 M solution in decane (0.015 mL, 0.072 mmol) was added at room  
35 temperature and the reaction mixture stirred for 1 h. Reaction mixture was diluted with DCM (10 mL) and quenched with NaHCO<sub>3</sub> aq. sat. solution (5 mL). The aqueous layer was extracted with DCM (2 x 5 mL). The combined organic layer was washed with brine (5 mL), dried over Na<sub>2</sub>SO<sub>4</sub> (0.3 g), filtered, and the filtrate was

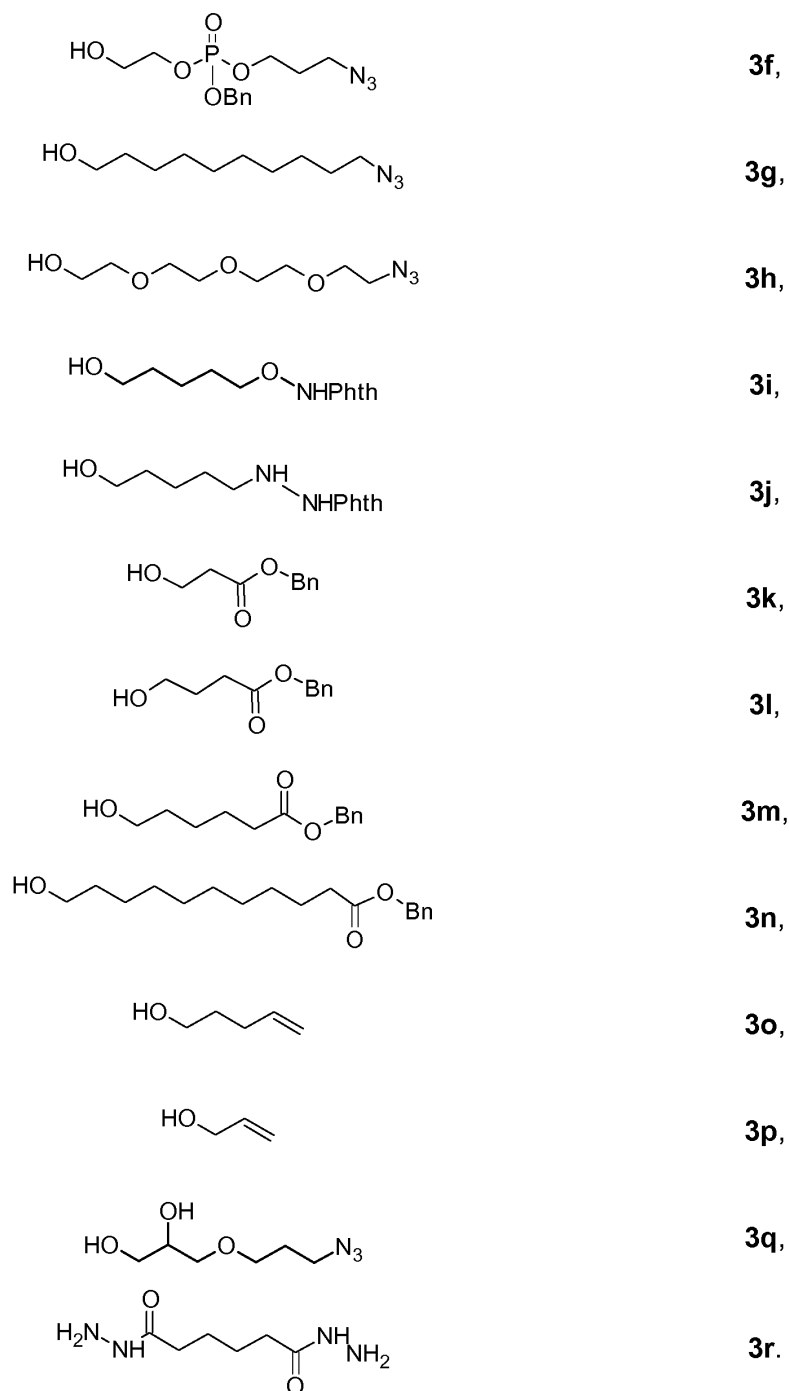
concentrated under vacuum at 35 °C bath temperature of rotary evaporator for 15 min to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-80%). Concentration of solvent from test tubes containing the product (based on TLC) in vacuum resulted in a colorless oil.

**General procedure F) Phosphate coupling with linker: phosphoramidite method (one pot procedure)**

To a solution of the hydroxyl compound (0.016 mmol) in DCM (1.0 mL) in a 10 mL RBF (oven dried) under argon atmosphere was added bis(diisopropylamino)-benzyloxyphosphine (0.032 mmol) and diisopropylammonium tetrazolide (0.024 mmol) and the solution stirred at room temperature for 1.5 h. Then, linker (0.097 mmol) and tetrazole 0.45 M solution in ACN (0.11 mL, 0.049 mmol) and the solution stirred at room temperature for 2 h. Reaction was monitored by TLC. Then, *t*-butyl peroxide 5.0 - 6.0 M solution in decane (0.006 mL, 0.032 mmol) was added at room temperature and the reaction mixture stirred for 1 h. Reaction was monitored by TLC. Reaction mixture was diluted with DCM (5 mL) and quenched with NaHCO<sub>3</sub> aq. sat. solution (3 mL). The aqueous layer was extracted with DCM (2 x 3 mL). The combined organic layer was washed with brine (3 mL), dried over Na<sub>2</sub>SO<sub>4</sub> (0.2 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-80%). Concentration of solvent from test tubes containing the product (based on TLC) in vacuum resulted in a colorless oil.

The following linkers were employed in the synthesis:

**3a,****3b,****3c,****3d,****3e,**

**General procedure G) Lev deprotection**

To a solution of the Lev protected compound (0.060 mmol) in DCM (2 mL), a solution of hydrazine hydrate (0.267 mmol, 13  $\mu$ L) dissolved in acetic acid (0.08 mL) and pyridine (0.12 mL) was added. The resulting reaction mixture was stirred at room

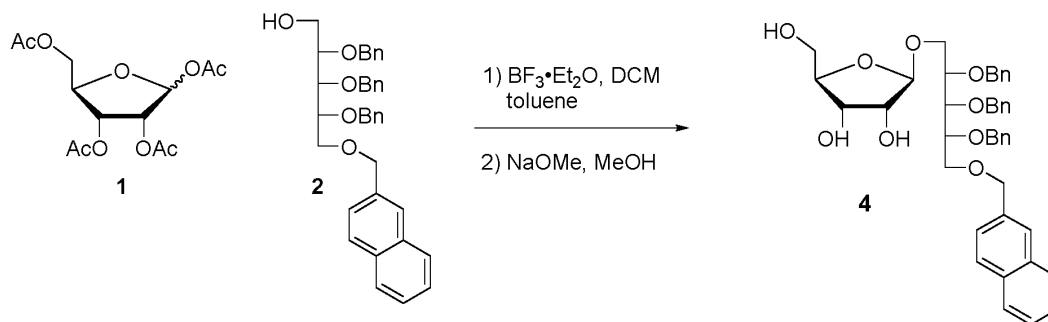
temperature for 2 h. The reaction was quenched by the addition of acetone (0.5 mL) and the solvent removed under vacuum to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-80%) as the eluent. Concentration of solvent from test tubes containing the products (based on TLC) in vacuum resulted in a white oil.

### General procedure H) Hydrogenation

To a solution of the protected compound (20 mg) in a EtOAc:MeOH:H<sub>2</sub>O:AcOH (1.0:0.5:0.25:0.125, 1.825 mL) in a 10 mL round bottom flask equipped with a stir bar was added palladium on carbon (20 mg). Using a balloon, the solution was flushed with hydrogen for 2 minutes and stirred at room temperature under hydrogen pressure for 40 h. Reaction was filtered through PTFE filter (0.45 µm) and the flask washed using H<sub>2</sub>O:MeOH solution (1:1, 5 mL). The filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified on SepPak C18 ec (small) using 100% H<sub>2</sub>O, H<sub>2</sub>O:MeOH (1:1) and 100% MeOH as the eluent. Concentration of solvent from the vial containing the main product in lyophilizer overnight resulted in a white solid.

## 20 Example A. Glycosylation

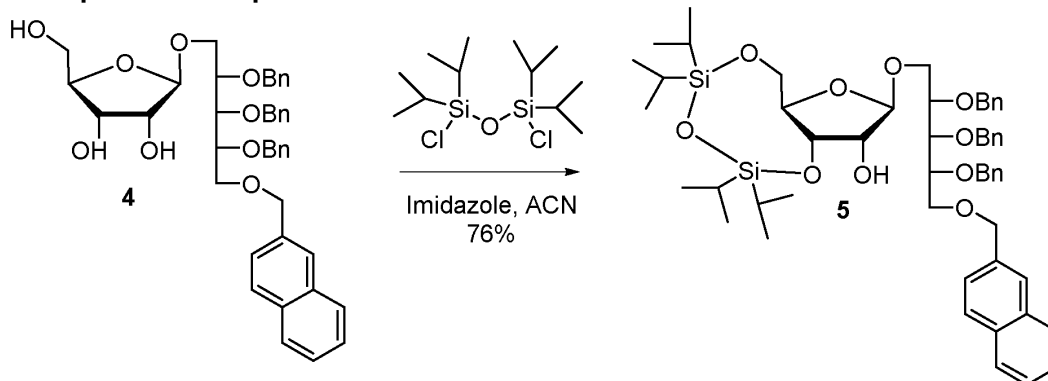
### Example A-1. Compound 4



25 2,3,4-Tri-O-benzyl-5-O-(2-naphthalenylmethyl)-1-O-D-ribitol **2** (40 g, 71.08 mmol) was coevaporated with anhydrous toluene and dried in vacuum. A solution of **2** in anhydrous DCM (320 mL) under argon atmosphere and stirring was cooled to 0 °C and BF<sub>3</sub>·Et<sub>2</sub>O (19.3 mL, 156.38 mmol) added dropwise. After 5 min, a solution of 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose **1** (15.08 g, 47.38 mmol) in toluene (480 mL) was added over 45 minutes. The reaction mixture was warmed to room temperature and stirred for 2 h. Reaction was monitored by TLC. Reaction mixture was quenched by addition of triethylamine (50 mL) and diluted with DCM (200 mL). The reaction

mixture was washed with  $\text{NaHCO}_3$  aq. sat. solution (200 mL) and the aqueous layer extracted with DCM (2 x 150 mL). The combined organic layers were washed with NaCl aq. sat. solution (100 mL) and dried over  $\text{Na}_2\text{SO}_4$  (~20 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product **4** was purified on silica gel column chromatography using EtOAc in *n*-hexane (20-30%) as the eluent. Concentration of solvent from test tubes containing product in vacuum resulted in the yellow oil. The product (31.05 g, 37.82 mmol) was then redissolved in methanol (70 mL) in a 250 mL RBF (oven dried) under argon atmosphere and sodium methoxide 25% wt solution in methanol (4.09 mL, 18.91 mmol) was added. The solution was stirred at room temperature for 4 h. Reaction was monitored by TLC. Reaction mixture was neutralized (pH 7) by addition of Amberlite IR-120H (500 mg), filtered and the solvent evaporated under vacuum to obtain the crude product. The crude product was purified on silica gel column chromatography using EtOAc in *n*-hexane (50-100%) as the eluent. Concentration of solvent from test tubes containing product **4** in vacuum resulted in the yellow oil (11.46 g, 35% over 2 steps). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{42}\text{H}_{46}\text{O}_9\text{Na}^+$   $[\text{M}+\text{Na}]^+$  717.3040, found 717.3057.

## 20 Example A-2. Compound 5



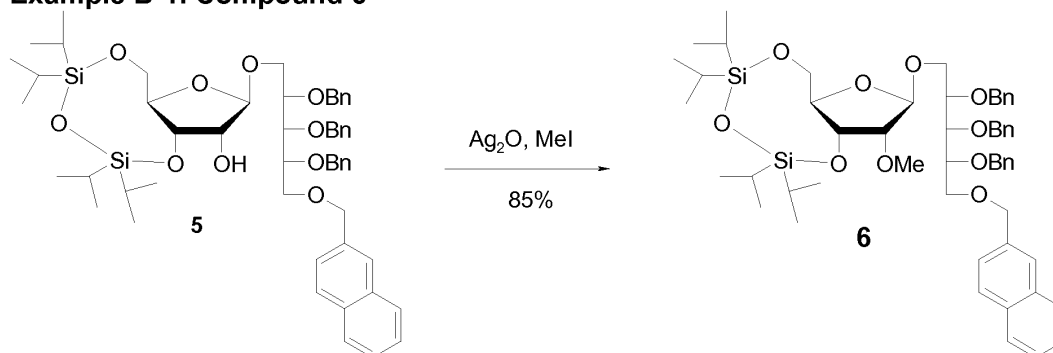
1,3-Dichloro-1,1,3,3-tetraisopropylsiloxane (8.2 g, 11.79 mmol) was added dropwise to a solution of triol **4** (3.53 g, 11.2 mmol) and imidazole (3.05 mg, 44.8 mmol) in acetonitrile (70 mL) in a 100 mL RBF (oven dried) under argon atmosphere. Reaction was monitored by TLC. After stirring at room temperature for 10 min, reaction mixture was quenched by addition of water (10 mL) and diluted with DCM (50 mL) and water (20 mL). The aqueous layer was separated and washed with DCM (2 x 30 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$  (~2 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The

crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-30%) as the eluent. Concentration of solvent from test tubes containing product **5** in vacuum resulted in a colourless oil (8.4 g, 76%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>54</sub>H<sub>72</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 959.4562, found 959.4590.

5

**Example B. 2'-Methoxypolyribosylribitolphosphate**

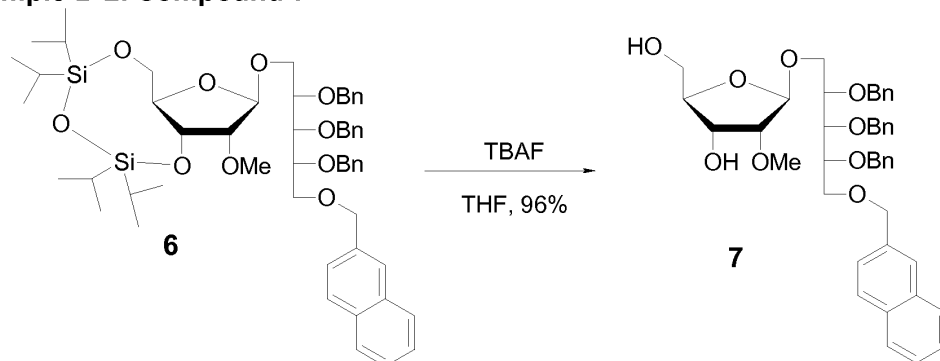
**Example B-1. Compound 6**



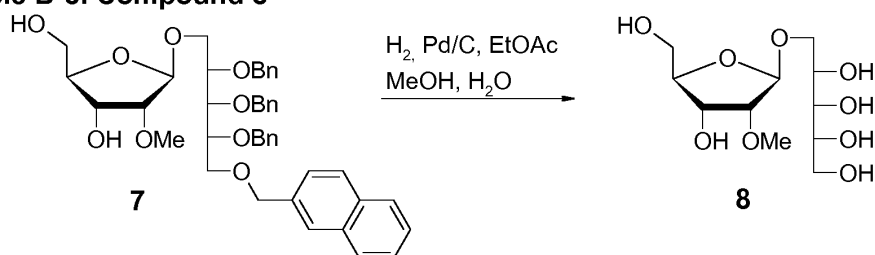
10

Ag<sub>2</sub>O (6 g, 26.0 mmol) was added to a stirred solution of the alcohol **5** (3.5 g, 3.73 mmol) in MeI (6 mL) at room temperature in a 25 mL RBF (oven dried) under argon atmosphere. Reaction mixture was stirred for 2.5 days. Reaction was monitored by TLC. After complete consumption of starting material reaction mixture was diluted with DCM (50 mL) and filtered through Celite® pad and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified on silica gel column chromatography using EtOAc in *n*-hexane (0-20%) as the eluent. Concentration of solvent from test tubes containing product in vacuum resulted in the colourless oil **6** (3.0 g, 85%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>55</sub>H<sub>74</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 973.4718, found 973.4738.

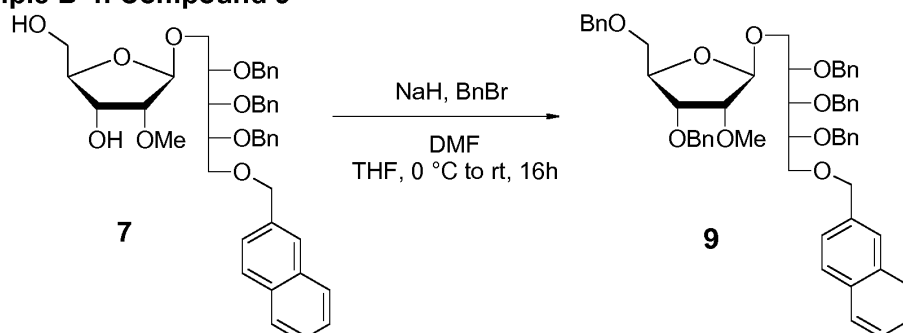
20

**Example B-2. Compound 7**

According to general procedure A) the silylated compound **6** was converted to diol product **7** (4.6 g, 96%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>43</sub>H<sub>48</sub>O<sub>9</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 731.3196, found 731.3217.

**Example B-3. Compound 8**

10 According to general procedure H), the monomer **7** was converted to the deprotected monomer **8** (5 mg, 67%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>22</sub>O<sub>9</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 321.1162, found 321.1187.

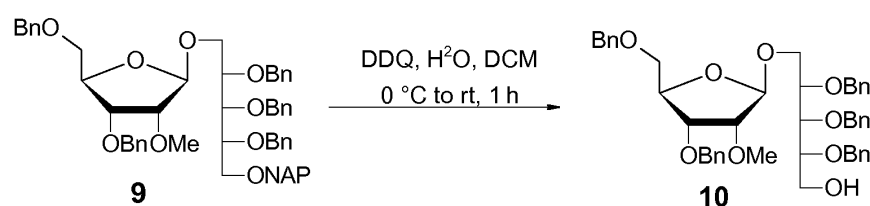
**Example B-4. Compound 9**

15

Benzyl bromide (77 mg, 0.45 mmol) and sodium hydride (16 mg, 0.672 mmol) were added to a stirred solution of the diol **7** (80 mg, 0.112 mmol) in THF:DMF (1.5:0.2, 1.7 mL) solution under argon atmosphere at 0 °C. The reaction was warmed to room

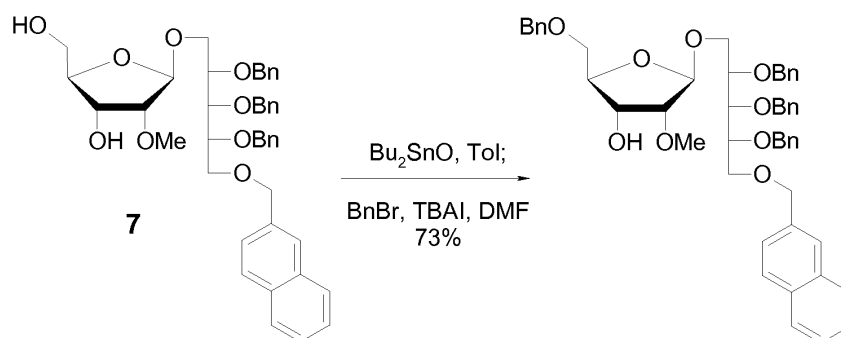
temperature and stirred overnight. The reaction mixture was quenched with ice cold water (1 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> (~0.5 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by flash chromatography using EtOAc in *n*-hexane (0-50%) as the eluent. Concentration of solvent from test tubes containing product **9** in vacuum resulted in a colourless oil (75 mg, 75%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>57</sub>H<sub>60</sub>O<sub>9</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 911.4135, found 911.4162.

#### 10 Example B-5. Compound 10

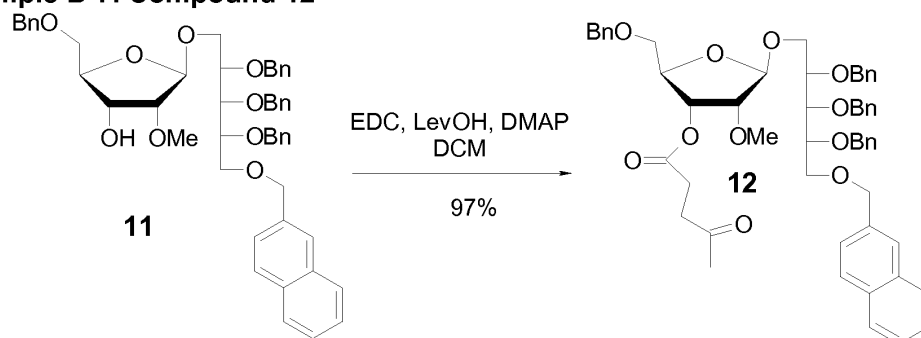


According to general procedure D), the NAP protected intermediate **9** was converted to the 5-hydroxyl compound **10** (189 mg, 89%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>46</sub>H<sub>52</sub>O<sub>9</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 771.3509, found 771.3521.

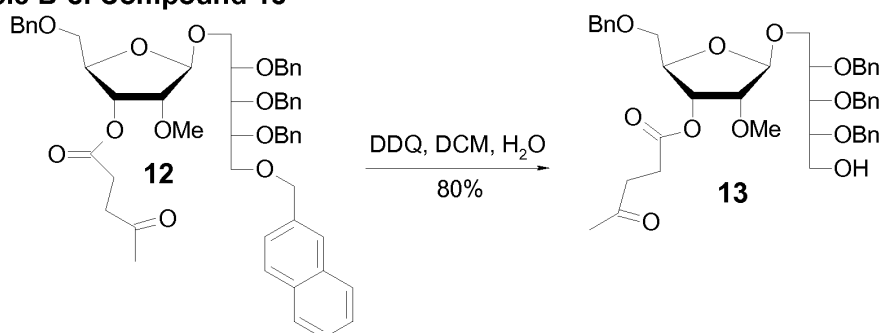
#### Example B-6. Compound 11



According to general procedure B) the diol **7** was converted to the benzylated product **11** (3.8 g, 73%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>50</sub>H<sub>54</sub>O<sub>9</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 821.3666, found 821.3672.

**Example B-7. Compound 12**

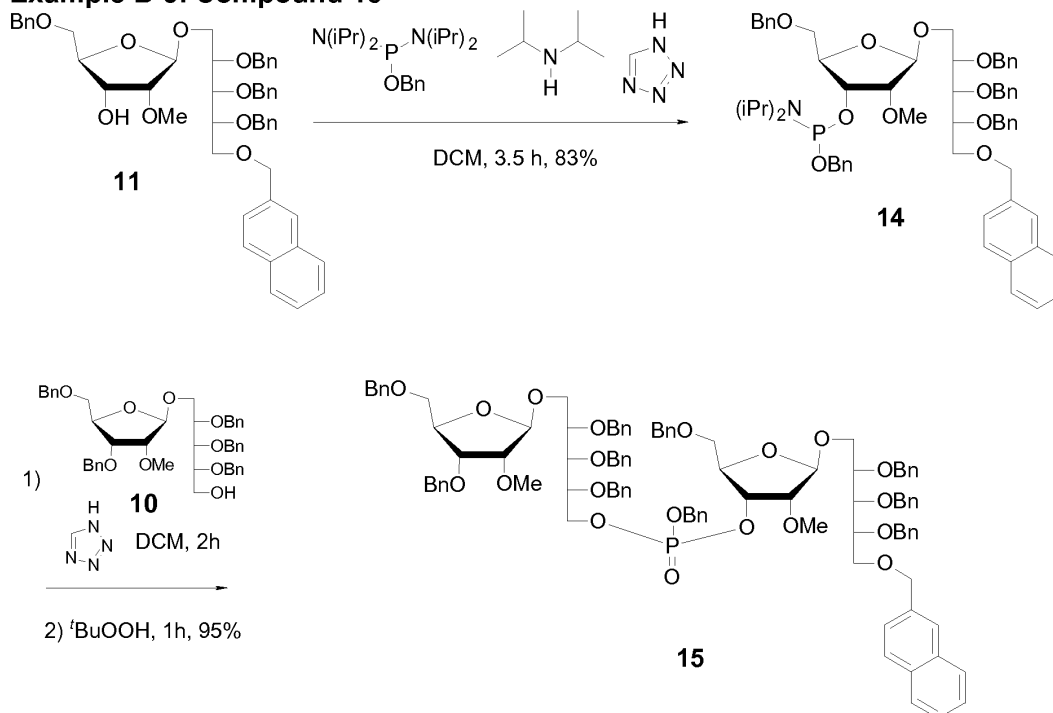
According to general procedure C), the 3-hydroxyl intermediate **11** was converted to the Lev protected compound **12** (1.75 g, 97%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>55</sub>H<sub>60</sub>O<sub>11</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 919.4033, found 919.4062.

**Example B-8. Compound 13**

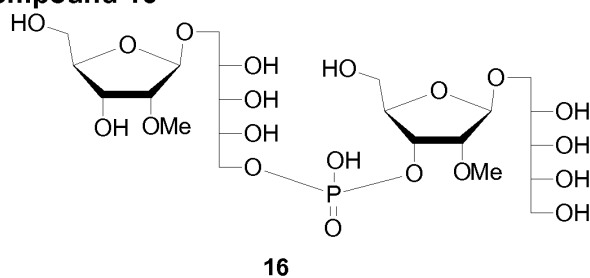
10

According to general procedure D), the NAP protected intermediate **12** was converted to the 5-hydroxyl compound **13** (1.23 g, 80%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>44</sub>H<sub>52</sub>O<sub>11</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 779.3407, found 779.3431.

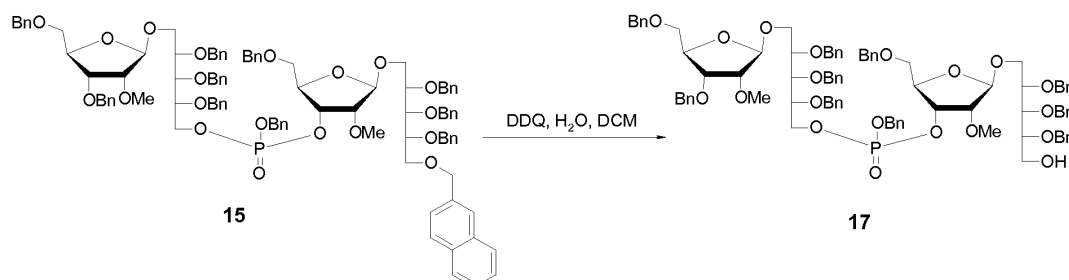
15

**Example B-9. Compound 15**

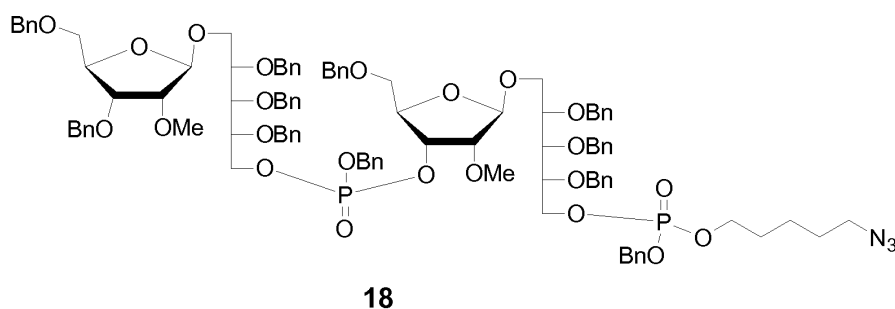
According to general procedure E), the 3-hydroxyl compound **11** and the 5-hydroxyl compound **10** were converted to dimer **15** (60 mg, 95%). **HRMS** (ESI<sup>+</sup>) Calcd for  $C_{103}H_{111}O_{20}PNa^+$  [M+Na]<sup>+</sup> 1721.7304, found 1721.7393.

**Example B-10. Compound 16**

According to general procedure H), the dimer **15** was converted to the deprotected dimer **16** (5.6 mg, 77%). **HRMS** (ESI<sup>+</sup>) Calcd for  $C_{11}H_{22}O_9Na^+$  [M+Na]<sup>+</sup> 681.1983, found 681.2000.

**Example B-11. Compound 17**

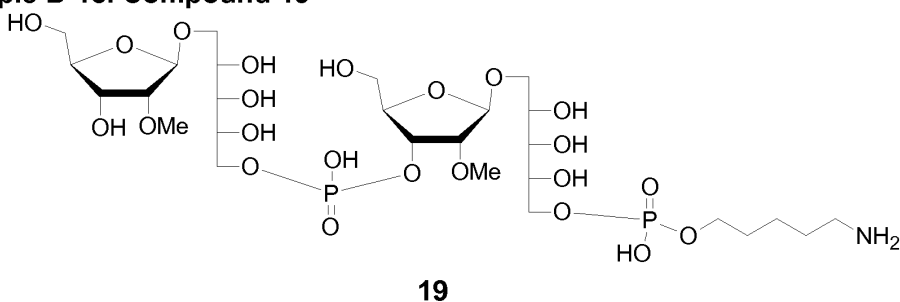
According to general procedure D), the NAP protected dimer **15** was converted to the 5-hydroxyl compound **17** (30 mg, 60%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>92</sub>H<sub>103</sub>O<sub>20</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 1581.6678, found 1581.6734.

**Example B-12. Compound 18**

10

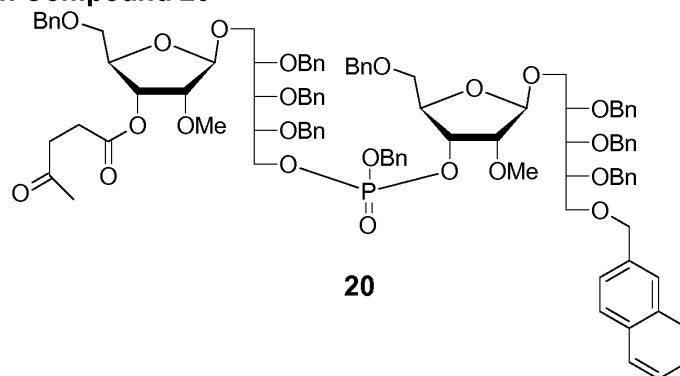
According to general procedure E), the dimer **17** was coupled to the linker 5-azido-pentanol to give dimer **18** (6 mg, 42%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>104</sub>H<sub>119</sub>N<sub>3</sub>O<sub>23</sub>P<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 1863.7641, found 1863.7709.

15

**Example B-13. Compound 19**

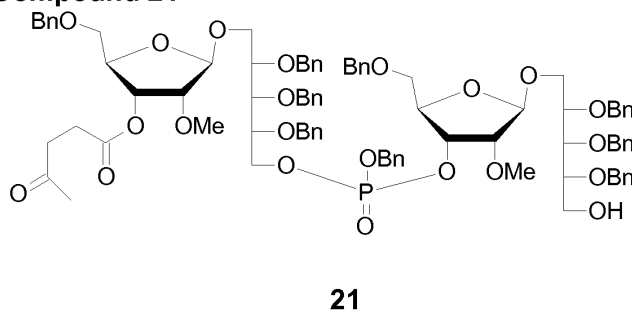
According to general procedure H), the dimer **18** was converted to the deprotected dimer **19** (1.2 mg, 55%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>27</sub>H<sub>5</sub>O<sub>23</sub>P<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 846.2538, found 846.2540.

5 **Example B-14. Compound 20**



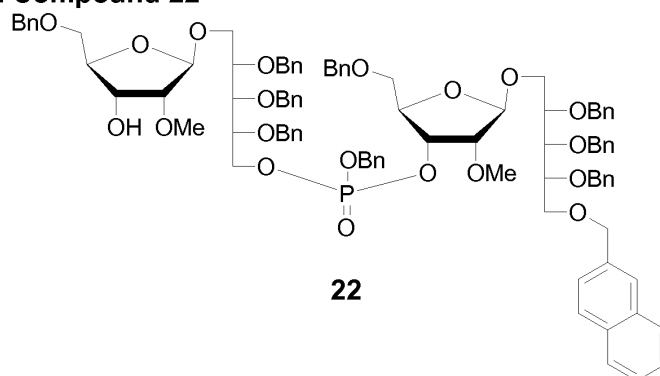
According to general procedure E), the 3-hydroxyl compound **11** and the 5-hydroxyl compound **13** were converted to dimer **20** (1.5 g, 76%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>101</sub>H<sub>111</sub>O<sub>22</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1729.7202, found 1729.7240.

10 **Example B-15. Compound 21**

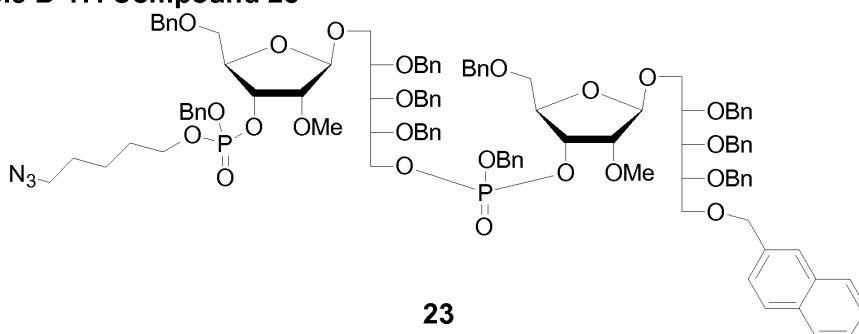


15 According to general procedure D), the NAP protected intermediate **20** was converted to the 5-hydroxyl compound **21** (386 mg, 63%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>90</sub>H<sub>103</sub>O<sub>22</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1589.6576, found 1589.6613.

115

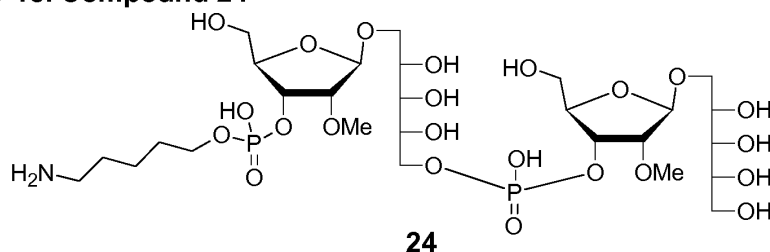
**Example B-16. Compound 22****22**

According to general procedure G), the dimer **20** was converted to 3-hydroxyl dimer **22** (473 mg, 74%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>96</sub>H<sub>105</sub>O<sub>20</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1631.6835, found 1631.6873.

**Example B-17. Compound 23****23**

10

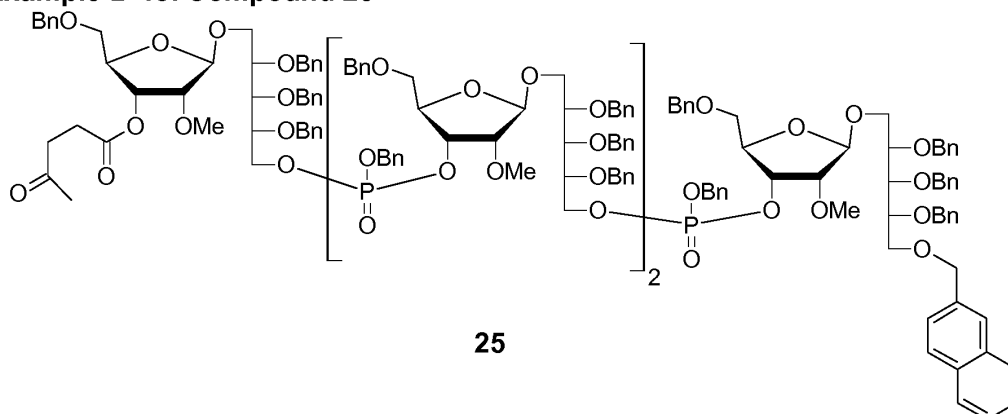
According to general procedure E), the 3-hydroxyl dimer **22** was coupled to the linker 5-azido-pentanol to give dimer **23** (3 mg, 30%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>108</sub>H<sub>121</sub>N<sub>3</sub>O<sub>23</sub>P<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 1912.7764, found 1912.7781.

**Example B-18. Compound 24****24**

15

According to general procedure H), the dimer **23** was converted to the deprotected dimer **24** (1.1 mg, 76%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>27</sub>H<sub>5</sub>O<sub>23</sub>P<sub>2</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 846.2538, found 846.2540.

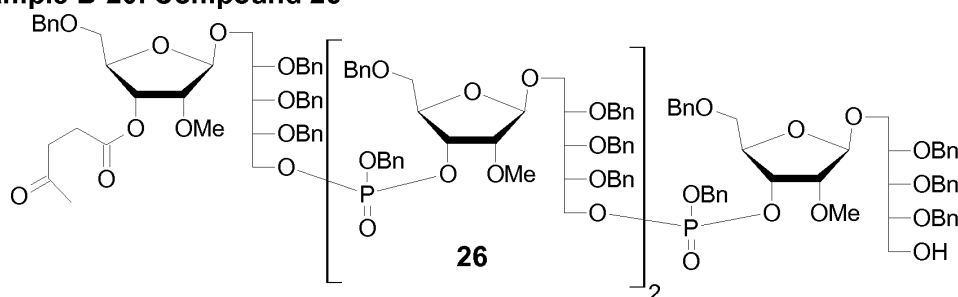
5 **Example B-19. Compound 25**



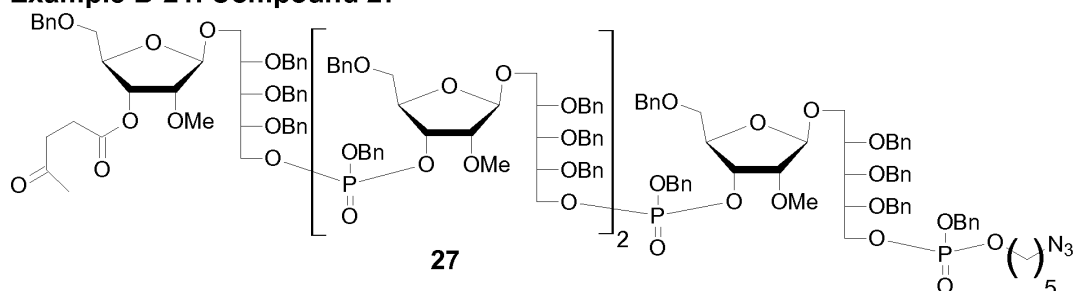
According to general procedure E), the 3-hydroxyl dimer **22** and the 5-hydroxyl dimer **21** were converted to tetramer **25** (367 mg, 86%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>193</sub>H<sub>213</sub>O<sub>44</sub>P<sub>3</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3350.3540, found 3350.3531.

10

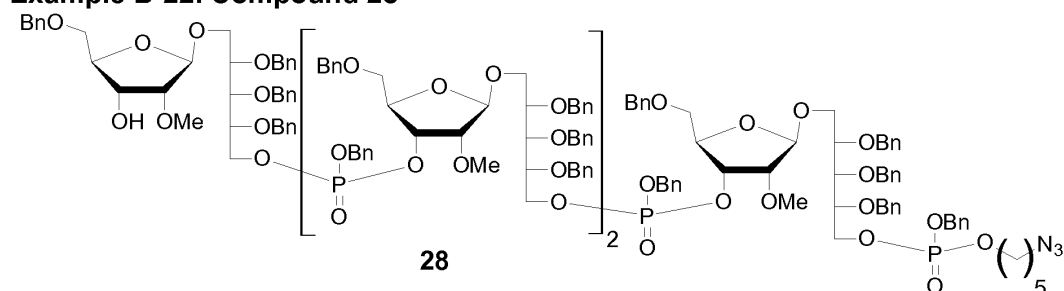
**Example B-20. Compound 26**



15 According to general procedure D), the NAP protected tetramer **25** was converted to the 5-hydroxyl tetramer **26** (125 mg, 75%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>182</sub>H<sub>205</sub>O<sub>44</sub>P<sub>3</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3210.2914, found 3210.2911.

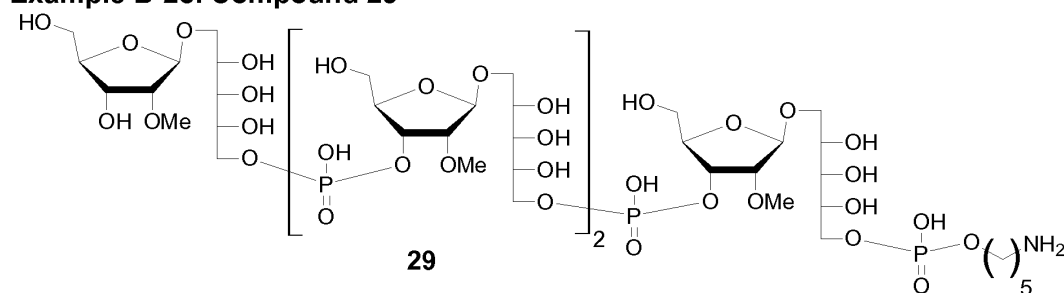
**Example B-21. Compound 27**

According to general procedure E), the tetramer **26** was coupled to the linker 5-azido-pentanol to give tetramer **27** (35 mg, 77%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>194</sub>H<sub>221</sub>N<sub>3</sub>O<sub>47</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3491.3844, found 3492.3787.

**Example B-22. Compound 28**

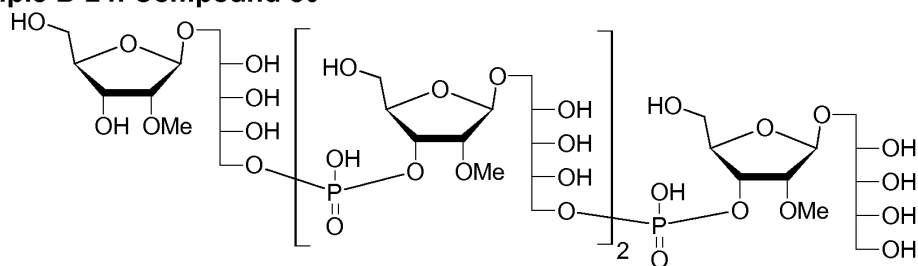
10

According to general procedure G), the tetramer **27** was converted to 3-hydroxyl tetramer **28** (30 mg, 90%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>189</sub>H<sub>215</sub>N<sub>3</sub>O<sub>45</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3393.3476, found 3393.3543.

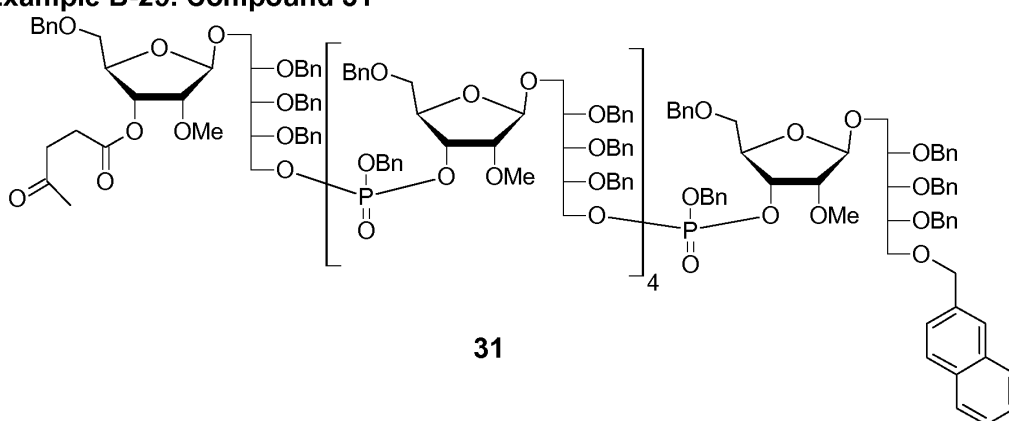
**Example B-23. Compound 29**

20

According to general procedure H), the tetramer **28** was converted to the deprotected tetramer **29** (10 mg, 73%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>49</sub>H<sub>97</sub>NO<sub>45</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 1566.4181, found 1566.4174.

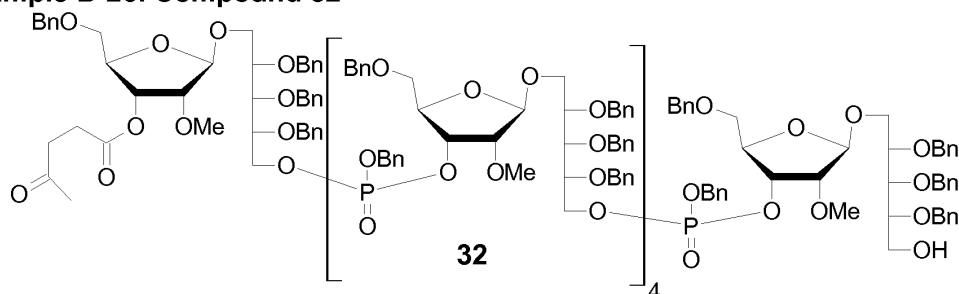
**Example B-24. Compound 30****30**

- 5 Tetramer **30** is prepared in two steps from tetramer **26** according to general procedure G) and general procedure H).

**Example B-25. Compound 31****31**

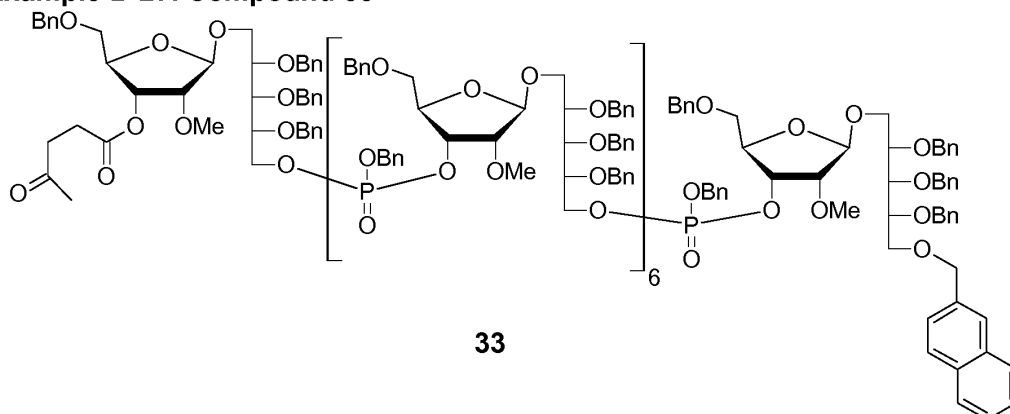
10

According to general procedure E), the 3-hydroxyl dimer **22** and the 5-hydroxyl tetramer **26** were converted to hexamer **31** (85 mg, 85%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>285</sub>H<sub>315</sub>O<sub>66</sub>P<sub>5</sub>Na<sup>+</sup> [M/2+Na]<sup>+</sup> 2496.9888, found 2496.0271.

**15 Example B-26. Compound 32****32**

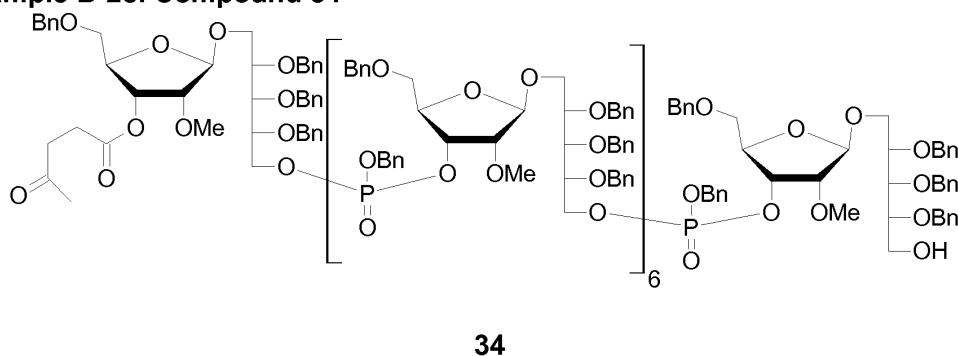
According to general procedure D), the NAP protected hexamer **31** was converted to the 5-hydroxyl hexamer **32** (50 mg, 65%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>274</sub>H<sub>307</sub>O<sub>66</sub>P<sub>5</sub>Na<sup>+</sup> [M/2+Na]<sup>+</sup> 2426.9677, found 2426.9977.

5 **Example B-27. Compound 33**

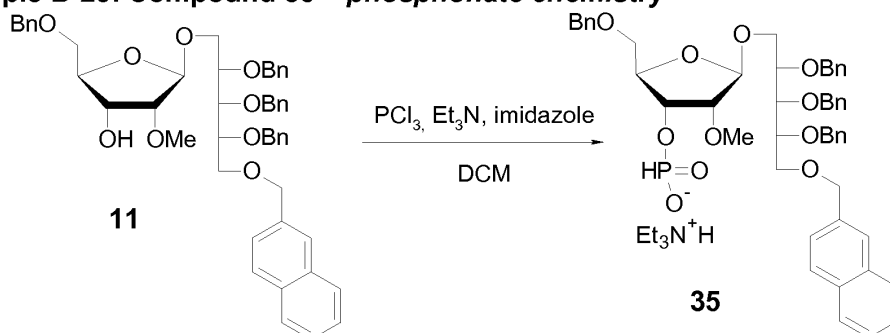


According to general procedure E), the 3-hydroxyl dimer **22** and the 5-hydroxyl hexamer **32** were converted to octamer **33** (50 mg, 77%). **<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*)  $\delta$  7.86 – 7.62 (m, 4H), 7.51 – 7.35 (m, 3H), 7.37 – 7.06 (m, 195H), 5.10 (t, *J* = 5.5 Hz, 1H), 5.03 (dt, *J* = 7.6, 4.2 Hz, 7H), 4.97 – 4.77 (m, 22H), 4.69 – 4.37 (m, 60H), 4.36 – 4.14 (m, 23H), 3.98 – 3.55 (m, 47H), 3.53 – 3.36 (m, 13H), 3.34 – 3.21 (m, 24H), 2.72 (t, *J* = 6.5 Hz, 2H), 2.65 – 2.54 (m, 2H), 2.16 (s, 3H).

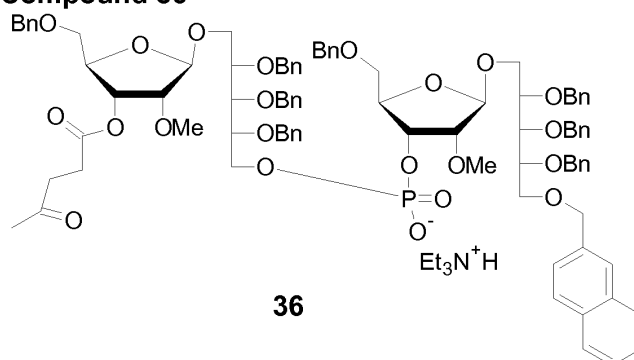
15 **Example B-28. Compound 34**



According to general procedure D), the NAP protected octamer **33** was converted to the 5-hydroxyl octamer **34** (35 mg, 73%). **<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*)  $\delta$  7.43 – 7.07 (m, 195H), 5.10 (t, *J* = 5.4 Hz, 1H), 5.08 – 4.78 (m, 29H), 4.70 – 4.45 (m, 51H), 4.45 – 4.15 (m, 38H), 3.95 – 3.54 (m, 50H), 3.54 – 3.36 (m, 16H), 3.36 – 3.25 (m, 24H), 2.72 (t, *J* = 6.5 Hz, 2H), 2.65 – 2.56 (m, 2H), 2.16 (s, 3H).

**Example B-29. Compound 35 – phosphonate chemistry**

- 5 To a solution of 3-hydroxyl compound **11** (10 mg) and imidazole (5.96 mg, 0.087 mmol) in DCM (2 mL) in a 10 mL RBF (oven dried) under argon atmosphere was added  $\text{PCl}_3$  (4.37  $\mu\text{L}$ , 0.050 mmol) and triethylamine (12.28  $\mu\text{L}$ , 0.087 mmol) at 0 °C. After 5 min, the reaction mixture was warmed to room temperature and stirred for 1 h. Reaction was monitored by TLC. The reaction was diluted with DCM (5 mL),
- 10 quenched by the addition of  $\text{NaHCO}_3$  aq. sat. solution (5 mL) and triethylammonium buffer solution (5 mL). The aqueous layer was extracted with DCM (2 x 10 mL), the combined organic layers were washed with brine (5 mL). The organic layer was dried over  $\text{Na}_2\text{SO}_4$  (0.25 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified on silica gel column chromatography using 10% MeOH in DCM as the eluent. Concentration of solvent
- 15 from test tubes containing product **35** (based on TLC) in vacuum resulted in the yellow oil (10 mg, 83%). **HRMS** (ESI<sup>+</sup>) Calcd for  $\text{C}_{56}\text{H}_{71}\text{NO}_{11}\text{SP}^+$   $[\text{M}+\text{H}]^+$  964.4765, found 964.4759.

**20 Example B-30. Compound 36**

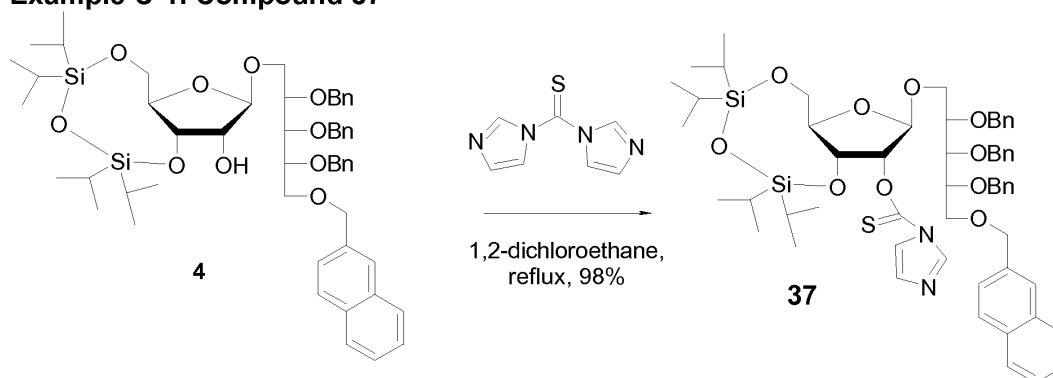
H-phosphonate **35** (10 mg, 0.010 mmol) and compound **13** (7.85 mg, 0.010 mmol) were dissolved in in pyridine (1 mL). Trimethylacetyl chloride (3.83  $\mu\text{L}$ , 0.031 mmol)

was then slowly added. The reaction mixture was stirred for 1 h at room temperature. A solution of iodine (2.6 mg, 0.010 mmol) in pyridine-water (96:4, v/v; 67  $\mu$ L) was added and the reaction was further stirred for 30 min at room temperature. The reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  (5 mL), washed with aq. sat.  $\text{Na}_2\text{S}_2\text{O}_3$  (5 mL) and then with 1.0 M TEAB solution (5 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$  filtered, and concentrated in vacuum. The residue was purified by flash chromatography (DCM/MeOH, 10:1, v/v) to yield **36** (5.5 g, 29%) as colorless oil. **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{94}\text{H}_{104}\text{O}_{22}\text{P}^+ [\text{M}]^-$  1615.6762, found 1615.7056.

10

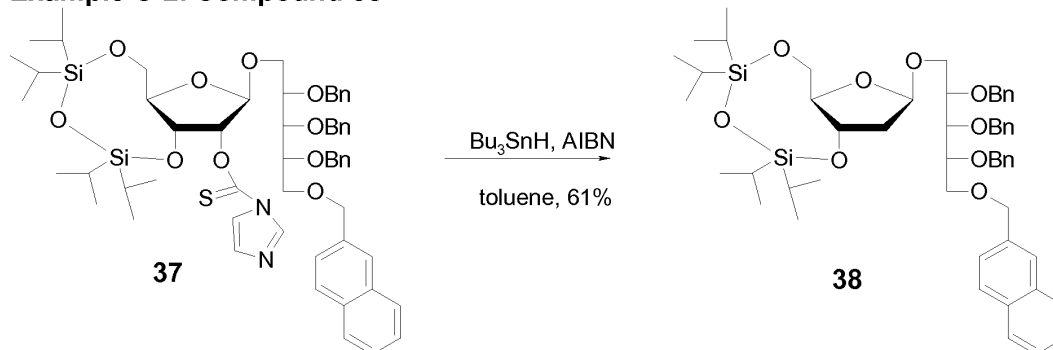
### Example C. 2'-Deoxypolyribosylribitolphosphate

#### Example C-1. Compound 37

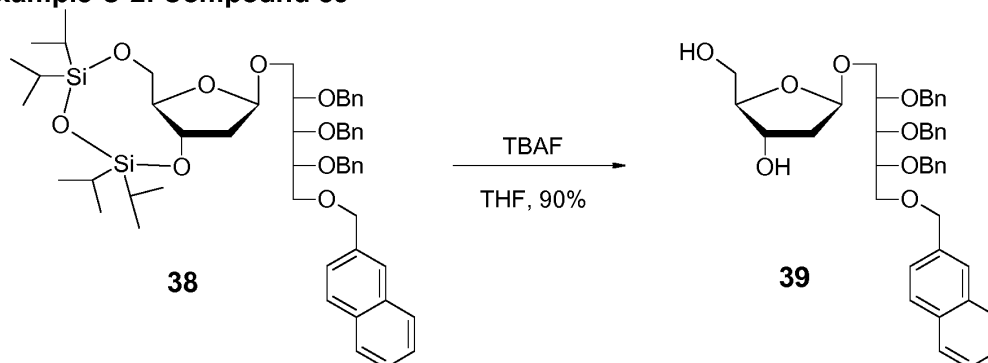


To a solution of the alcohol **4** (860 mg, 0.92 mmol) in 1,2-dichloroethane (5 mL) in a 25 mL RBF (oven dried) under argon atmosphere was added thiocarbonyldiimidazole (327 mg, 1.83 mmol). The solution was stirred at 60  $^{\circ}\text{C}$  for 64 h. Reaction was monitored by TLC. The solvent was evaporated under vacuum to obtain the crude product. The crude product was purified by flash column chromatography using EtOAc in *n*-hexane (0-30%) as the eluent. Concentration of solvent from test tubes containing the product **37** (based on TLC) in vacuum resulted in a white oil (950 mg, 98%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{58}\text{H}_{74}\text{N}_2\text{O}_{10}\text{SSi}_2\text{Na}^+ [\text{M}+\text{Na}]^+$  1069.4500, found 1069.4525.

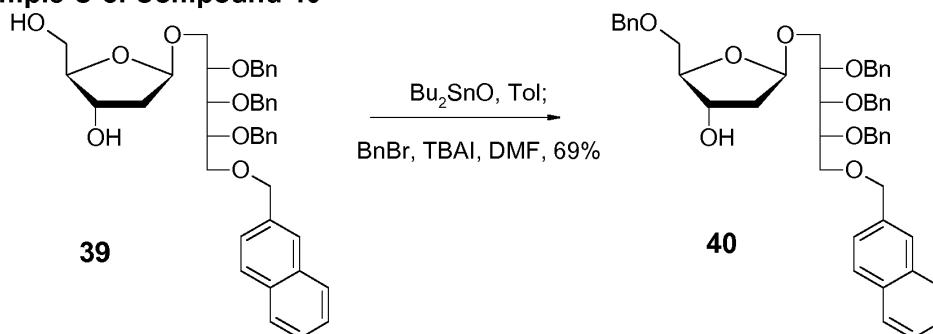
25

**Example C-2. Compound 38**

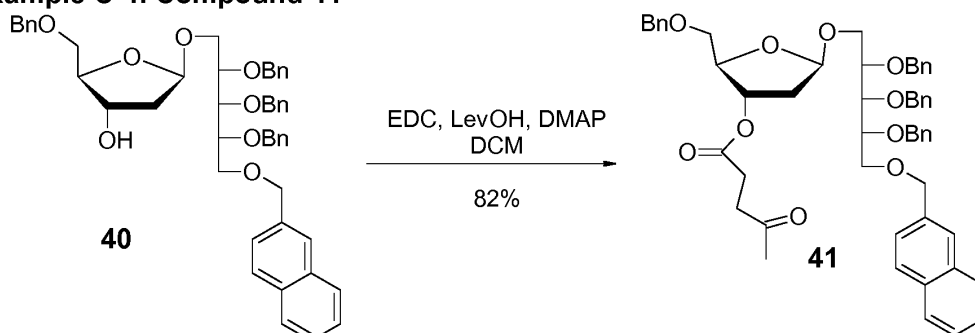
To a solution of  $\text{Bu}_3\text{SnH}$  (0.49 mL, 1.814 mmol) and AIBN (0.2 M in toluene, 0.46 mL, 0.091 mmol) in toluene (20 mL) in a 100 mL RBF (oven dried) under argon atmosphere at 60 °C was added dropwise a solution of the thiocarbamate **37** (950 mg, 0.907 mmol) in toluene (20 mL). The reaction mixture was stirred for 2 h. Reaction was monitored by TLC. Reaction mixture was diluted was quenched with  $\text{NaHCO}_3$  aq. sat. solution (10 mL). The aqueous layer was extracted with ethyl acetate (2 x 20 mL). The combined organic layer was washed with brine (10 mL), dried over  $\text{Na}_2\text{SO}_4$  (0.5 g), filtered, and the filtrate was concentrated under vacuum to obtain the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-50%). Concentration of solvent from test tubes containing the product **38** (based on TLC) in vacuum resulted in a colorless oil (510 mg, 61%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{58}\text{H}_{72}\text{O}_9\text{Si}_2\text{Na}^+$   $[\text{M}+\text{Na}]^+$  943.4613, found 943.4640

**Example C-2. Compound 39**

According to general procedure A) the silylated compound **38** was converted to diol product **39** (330 mg, 90%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{42}\text{H}_{46}\text{O}_8\text{Na}^+$   $[\text{M}+\text{Na}]^+$  701.3090, found 701.3117.

**Example C-3. Compound 40**

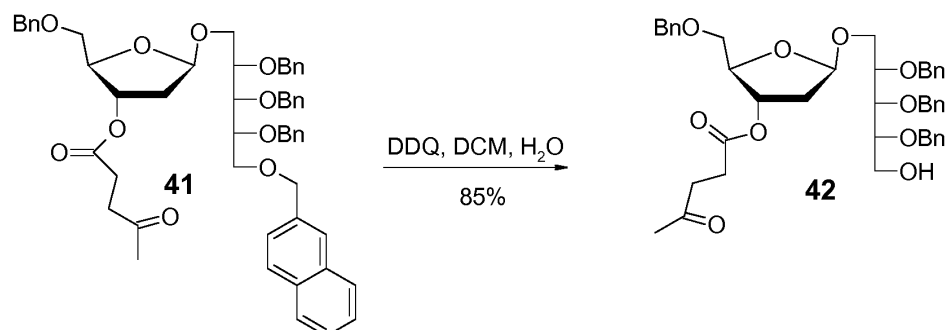
According to general procedure B) the diol **39** was converted to the benzylated product **40** (260 mg, 69%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{49}\text{H}_{52}\text{O}_8\text{Na}^+$   $[\text{M}+\text{Na}]^+$  791.3560, found 791.3565.

**Example C-4. Compound 41**

- 10 According to general procedure C), the 3-hydroxyl intermediate **40** was converted to the Lev protected compound **41** (135 mg, 82%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{54}\text{H}_{58}\text{O}_{10}\text{Na}^+$   $[\text{M}+\text{Na}]^+$  889.3928, found 889.3928.

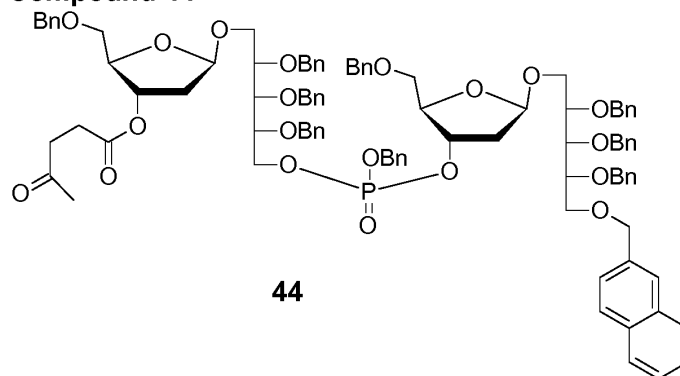
**Example C-5. Compound 42**

15



According to general procedure D), the NAP protected intermediate **41** was converted to the 5-hydroxyl compound **42** (95 mg, 85%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>43</sub>H<sub>50</sub>O<sub>10</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 749.3302, found 749.3325.

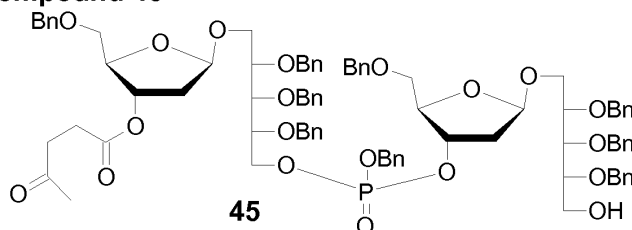
5 **Example C-6. Compound 44**



According to general procedure E), the 3-hydroxyl compound **40** and the 5-hydroxyl compound **42** were converted to dimer **44** (160 mg, 78%). **HRMS** (ESI<sup>+</sup>) Calcd for

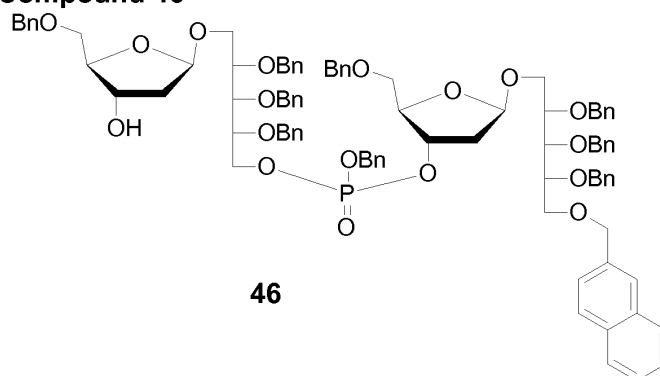
10 C<sub>99</sub>H<sub>107</sub>O<sub>20</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1669.6991, found 1669.6981.

**Example C-7. Compound 45**

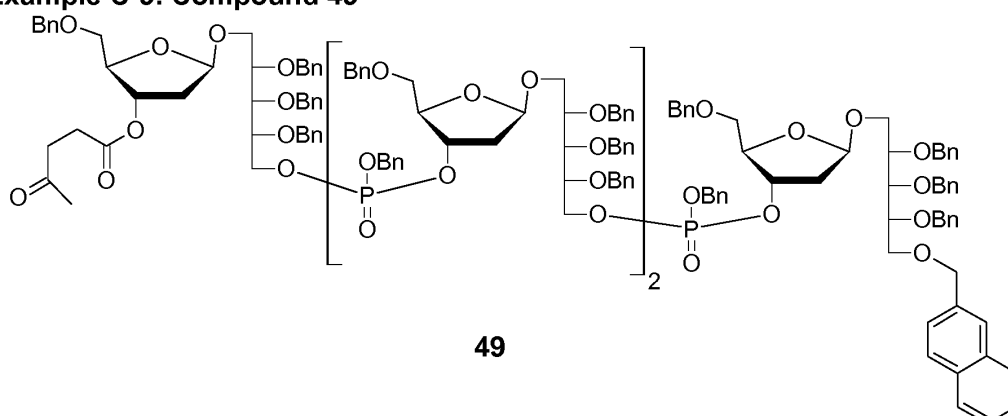


15 According to general procedure D), the dimer **44** was converted to 5-hydroxyl dimer **45** (53 mg, 73%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>99</sub>H<sub>107</sub>O<sub>20</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1529.6365, found 1529.6397.

125

**Example C-8. Compound 46**

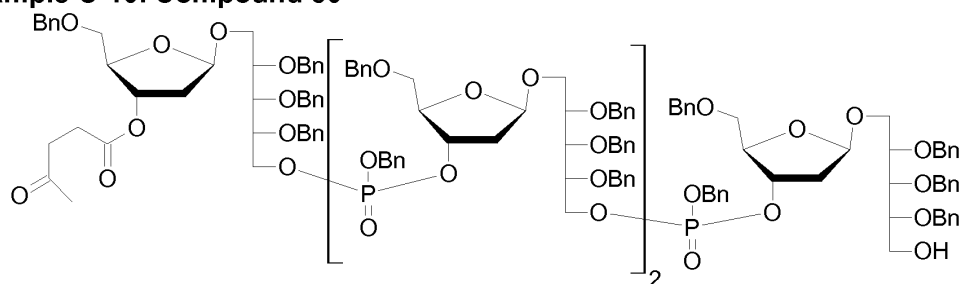
According to general procedure G), the dimer **44** was converted to 3-hydroxyl dimer **46** (85 mg, 91%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>94</sub>H<sub>101</sub>O<sub>18</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1571.6623, found 1571.6656.

**Example C-9. Compound 49**

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According to general procedure E), the 3-hydroxyl dimer **46** and the 5-hydroxyl dimer **45** were converted to tetramer **49** (96 mg, 83%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>189</sub>H<sub>205</sub>O<sub>40</sub>P<sub>3</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3230.3118, found 3230.3111.

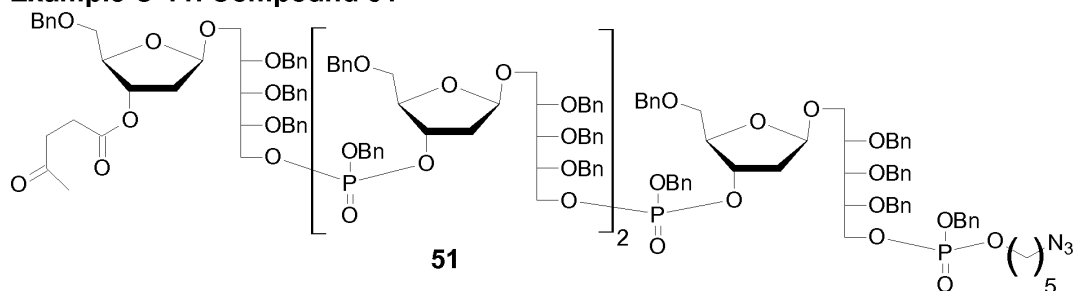
15



50

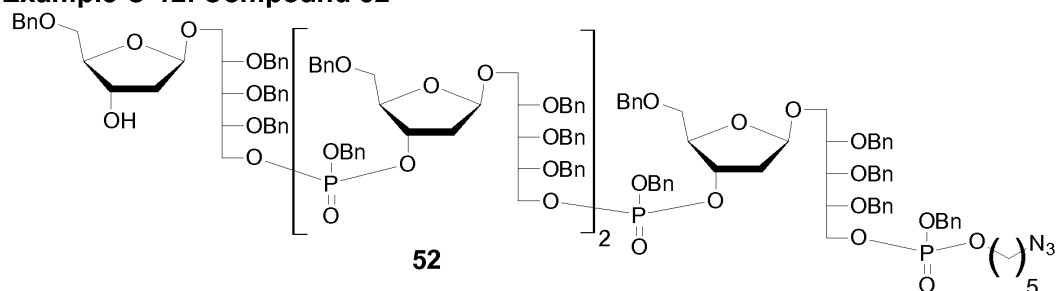
According to general procedure D), the NAP protected tetramer **49** was converted to  
5 the 5-hydroxyl tetramer **50** (50 mg, 56%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>178</sub>H<sub>197</sub>O<sub>40</sub>P<sub>3</sub>Na<sup>+</sup>  
[M+Na]<sup>+</sup> 3090.2492, found 3090.2405.

### Example C-11. Compound 51



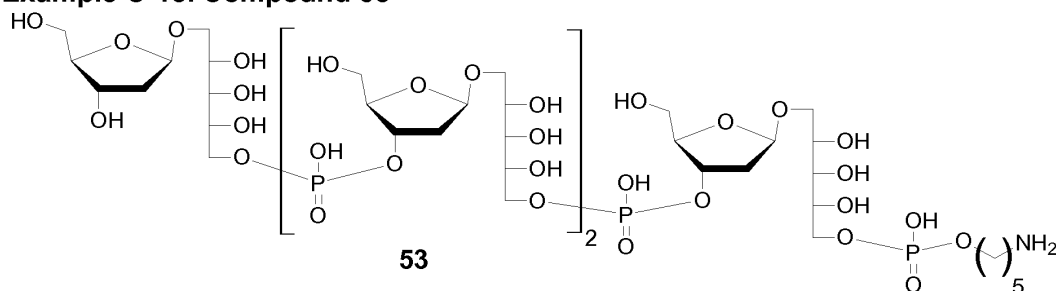
10 According to general procedure E), the tetramer **50** was coupled to the linker 5-azido-  
pentanol to give tetramer **51** (41 mg, 76%). **HRMS** (ESI<sup>+</sup>) Calcd for  
C<sub>190</sub>H<sub>213</sub>N<sub>3</sub>O<sub>43</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3371.3421, found 3371.3321.

### Example C-12. Compound 52



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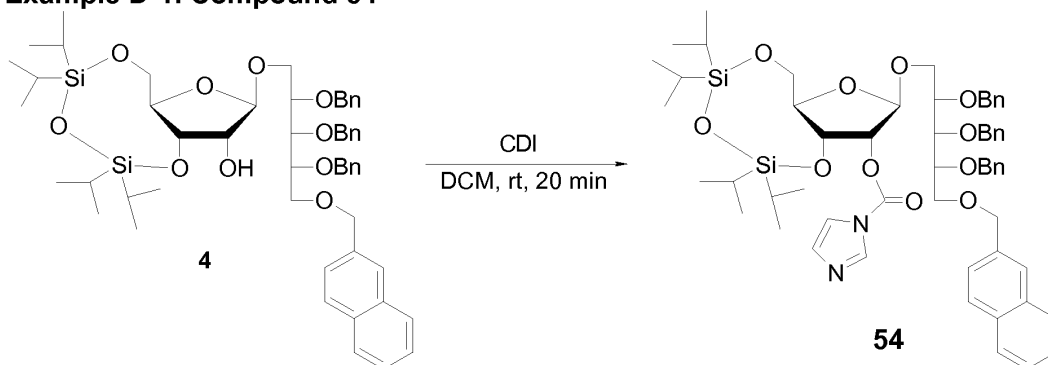
According to general procedure G), the tetramer **51** was converted to 3-hydroxyl tetramer **52** (36 mg, 92%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>185</sub>H<sub>207</sub>N<sub>3</sub>O<sub>41</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 3273.3053, found 3273.3079.

**Example C-13. Compound 53**

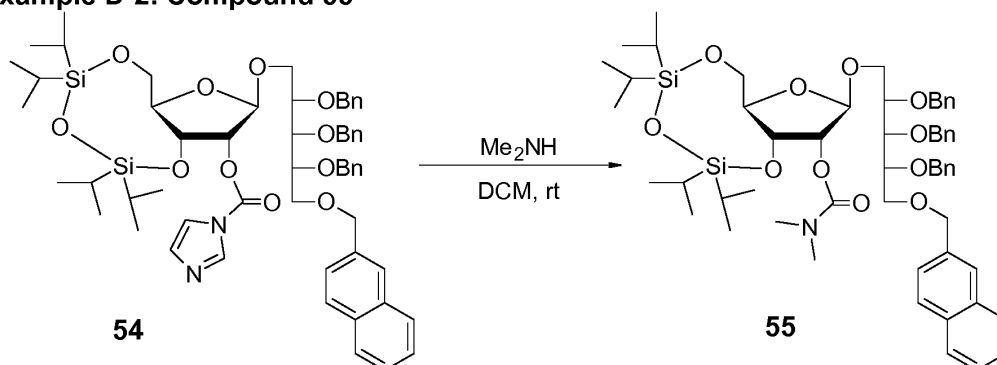
According to general procedure H), the tetramer **52** was converted to the deprotected tetramer **53** (6 mg, 70%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>45</sub>H<sub>89</sub>NO<sub>41</sub>P<sub>4</sub>Na<sup>+</sup> [M+Na/2]<sup>+</sup> 723.1879, found 723.1996.

**Example D. 2'-N,N,-Dimethylaminocarbonylpolyribosylribitolphosphate**

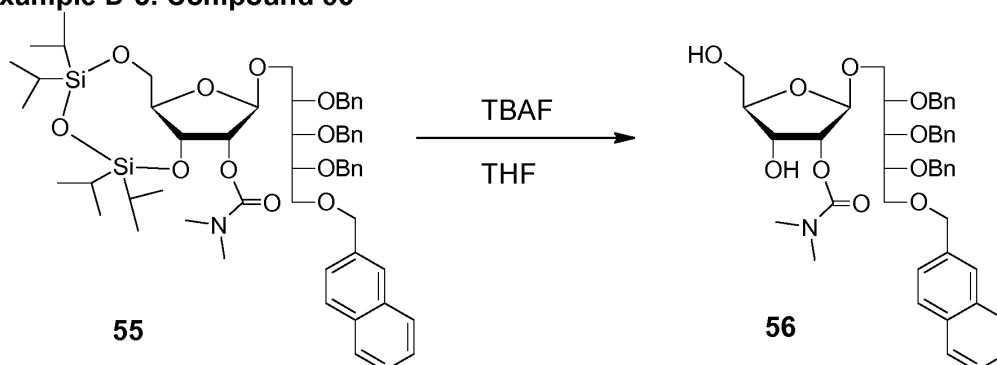
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**Example D-1. Compound 54**

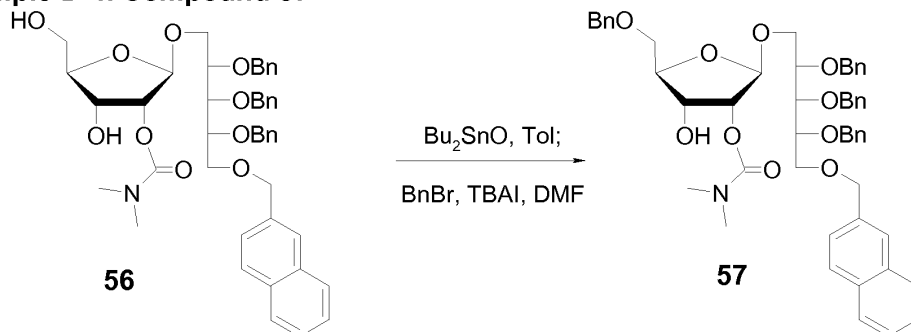
To a solution of alcohol **4** (2.153 g, 2.299 mmol) in DCM (10 mL) at room temperature under argon atmosphere was added 1,1'-carbonyldiimidazole (145 mg, 4.598 mmol). The reaction mixture was stirred for 20 min (when CDI was completely dissolved) and monitored by TLC until complete consumption of the starting material. The solvent was evaporated in vacuum to give the crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-100%) as the eluent. Concentration of solvent from test tubes containing the product **54** (based on TLC) in vacuum resulted in the colourless oil (2.35 g, 99%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>58</sub>H<sub>75</sub>N<sub>2</sub>O<sub>11</sub>Si<sub>2</sub><sup>+</sup> [M+H]<sup>+</sup> 1031.4909, found 1031.4936.

**Example D-2. Compound 55**

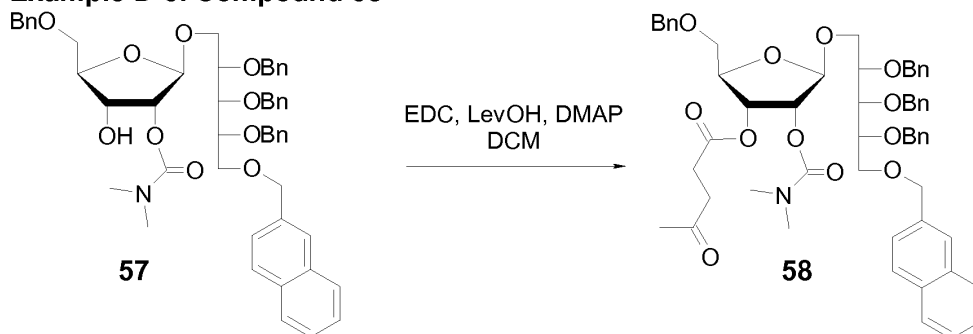
Monomer **54** (2.396 g, 2.32 mmol) was dissolved in ACN (20 mL) at room temperature under argon atmosphere and  $\text{Me}_2\text{NH}$  was added (1.89 g, 23.2 mmol). The reaction mixture was stirring at room temperature for 65 h and monitored by TLC. The solution was filtered off through a pad of celite and washed with DCM (50 mL). The solvent was evaporated in vacuum. The resulting solid was solubilized in DCM (100 mL) and the organic solution was washed with brine (100 mL) and dried with  $\text{Na}_2\text{SO}_4$  (~2 g). The organic solvent was evaporated in vacuum to give to crude product. The crude product was purified by automated flash chromatography using EtOAc in *n*-hexane (0-100%) as the eluent. Concentration of solvent from test tubes containing the product **55** (based on TLC) in vacuum resulted in the colourless oil (2 g, 85%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{57}\text{H}_{77}\text{NO}_{11}\text{Si}_2\text{Na}^+$   $[\text{M}+\text{Na}]^+$  1030.4933, found 1030.4958.

**Example D-3. Compound 56**

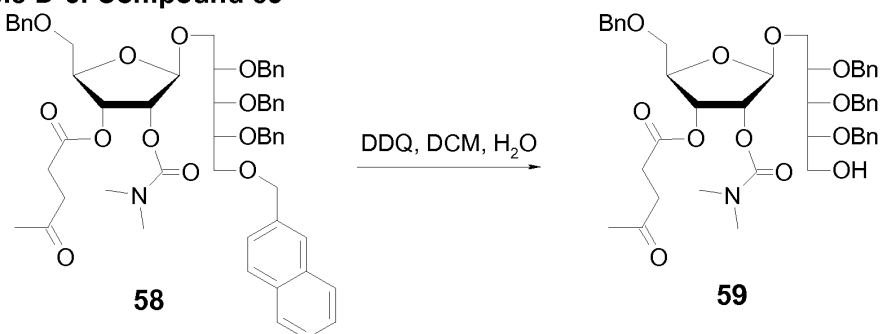
According to general procedure A) the silylated compound **55** was converted to diol product **56** (1.1 g, 73%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{45}\text{H}_{51}\text{NO}_{10}\text{Na}^+$   $[\text{M}+\text{Na}]^+$  788.3411, found 788.3431.

**Example D-4. Compound 57**

According to general procedure B) the diol **56** was converted to the benzylated product **57** (91 mg, 16%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{52}\text{H}_{57}\text{NO}_{10}\text{Na}^+$   $[\text{M}+\text{Na}]^+$  878.3880, found 878.3930.

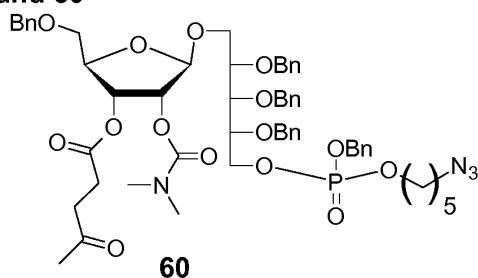
**Example D-5. Compound 58**

According to general procedure C), the 3-hydroxyl intermediate **57** was converted to the Lev protected compound **58** (46 mg, 92%). **HRMS** ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{57}\text{H}_{63}\text{NO}_{12}\text{Na}^+$   $[\text{M}+\text{Na}]^+$  976.4248, found 976.4318.

**Example D-6. Compound 59**

According to general procedure D), the monomer **58** was converted to 5-hydroxyl monomer **59** (23 mg, 60%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>46</sub>H<sub>55</sub>NO<sub>12</sub>Na<sup>+</sup> [M+Na]<sup>+</sup> 836.3622, found 836.3682.

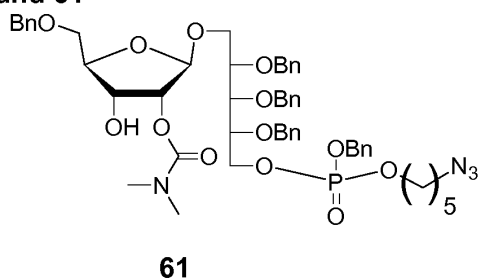
5 **Example D-7. Compound 60**



According to general procedure E), the monomer **59** was coupled to the linker 5-azido-pentanol to give monomer **60** (19 mg, 70%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>58</sub>H<sub>71</sub>N<sub>4</sub>O<sub>15</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1117.4551, found 1117.4628.

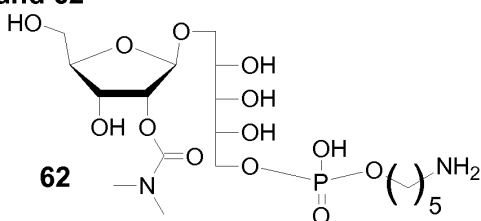
10

**Example D-8. Compound 61**



15 According to general procedure G), the monomer **60** was converted to 3-hydroxyl monomer **61** (12 mg, 71%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>53</sub>H<sub>65</sub>N<sub>4</sub>O<sub>13</sub>PNa<sup>+</sup> [M+Na]<sup>+</sup> 1019.4183, found 1019.4246.

**Example D-9. Compound 62**



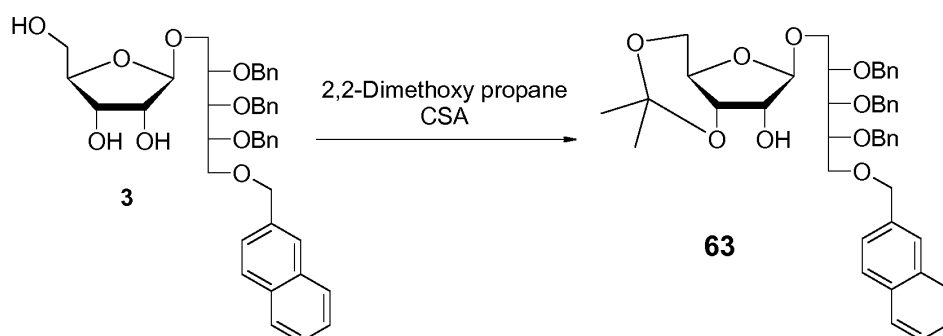
20

According to general procedure H), the monomer **61** was converted to the deprotected monomer **62** (3 mg, 60%). HRMS (ESI<sup>+</sup>) Calcd for C<sub>18</sub>H<sub>38</sub>N<sub>2</sub>O<sub>13</sub>PNa<sup>+</sup> [M+H]<sup>+</sup> 521.2112, found 521.2125.

5

### **Example E. 2'-Fluoropolyribosylribitolphosphate**

#### **Example E-1. Compound 63**

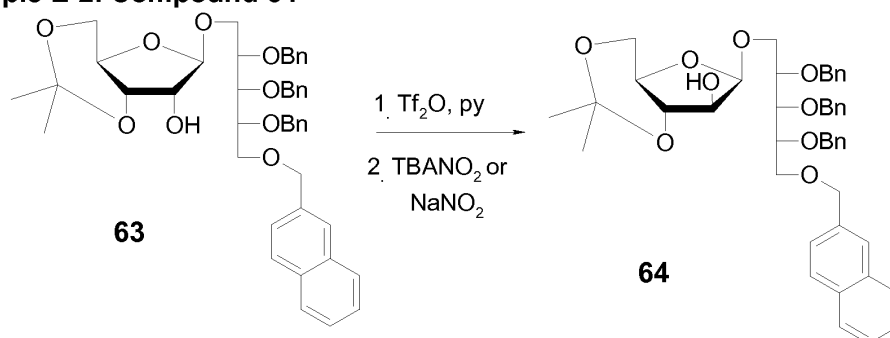


10

To compound **4** in acetone was added 2,2-dimethoxypropane (1.2 equiv.) followed by camphorsulfonic acid (0.5 equiv). After 6 h of stirring, the reaction mixture was concentrated under reduced pressure which on column chromatographic purification gave the pure compound **63**.

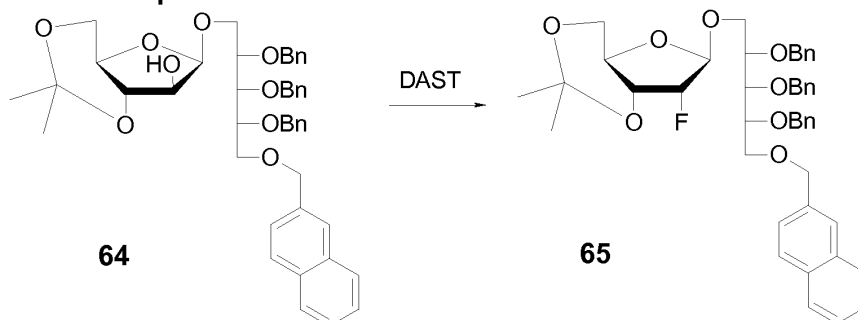
15

#### **Example E-2. Compound 64**



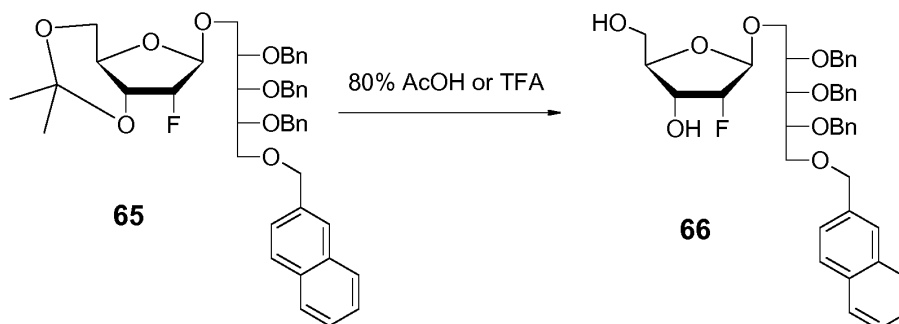
20

Compound **63** was treated at 0 °C with triflic anhydride and pyridine in DCM. After two hours, tetrabutylammonium nitrite and sodium nitrite were added and the mixture was stirred at r.t. for additional 5 hours. The product was extracted into hexane (200 mL). The extract was washed with aqueous sodium hydroxide (2N), brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure to give a brown oil. Purification by flash chromatography (eluting with hexane:ether) gave **64**.

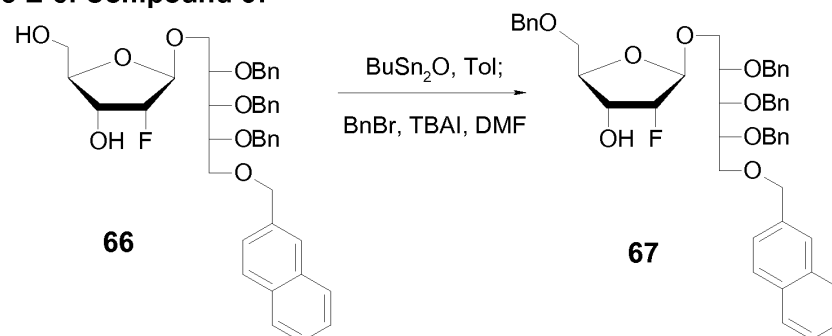
**Example E-3. Compound 65**

5

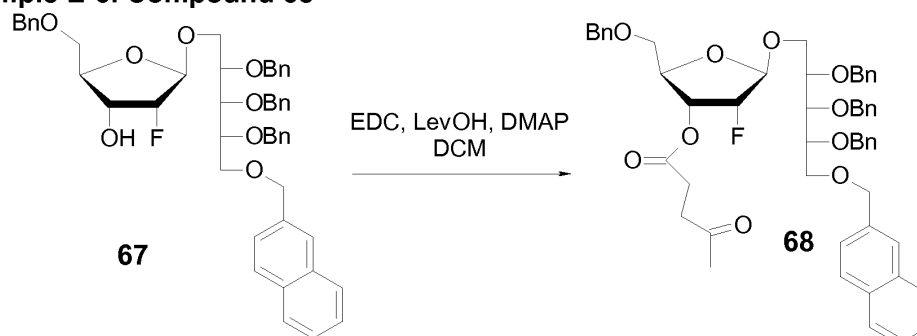
DAST (diethylamino sulfur trifluoride) (1.2 equiv.) was added dropwise to a stirred solution of **64** in dichloromethane at -78°C. The mixture was allowed to warm to room temperature overnight with the cooling bath in place. After 20 h, the solution was poured slowly into a vigorously stirred mixture of ice and excess saturated aqueous sodium bicarbonate. When effervescence had ceased, the product was extracted into hexane (200 mL). The extract was washed with aqueous sodium hydroxide (2N), brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure to give a brown oil. Purification by flash chromatography (eluting with hexane:ether) gave **65**.

**Example E-4. Compound 66**

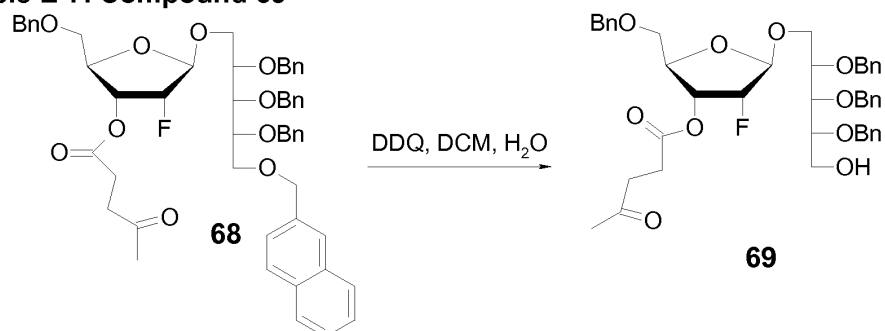
Compound **65** was taken in 80% acetic acid and stirred for 2 h at 80 °C. After completion of reaction (TLC), the acidic solution was concentrated under reduced pressure to give a crude product which was purified by column chromatography to yield compound **66**.

**Example E-5. Compound 67**

5 According to general procedure B) the diol **66** was converted to the benzylated product **67**.

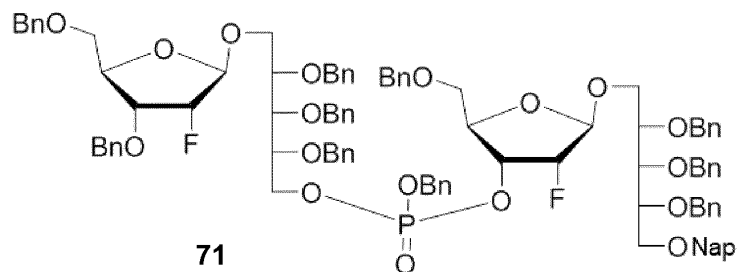
**Example E-6. Compound 68**

10 According to general procedure C), the 3-hydroxyl intermediate **67** was converted to the Lev protected compound **68**.

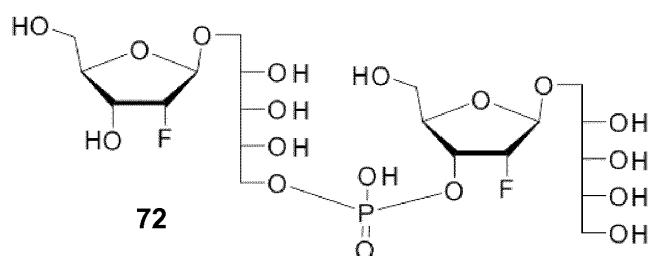
**Example E-7. Compound 69**

15

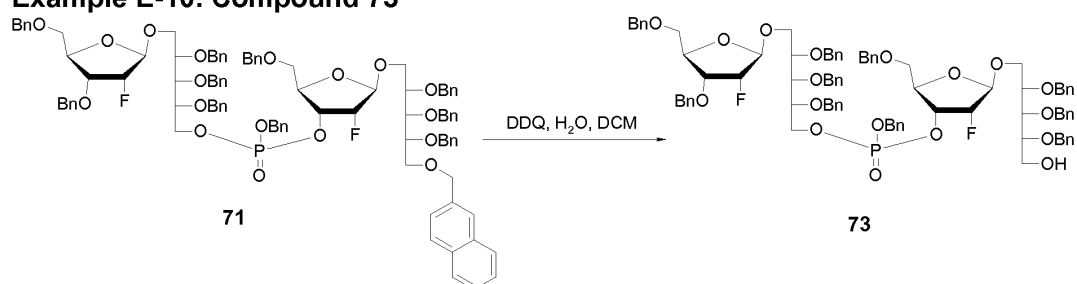
According to general procedure D), the NAP protected intermediate **68** was converted to the 5-hydroxyl compound **69**.

**Example E-8. Compound 71**

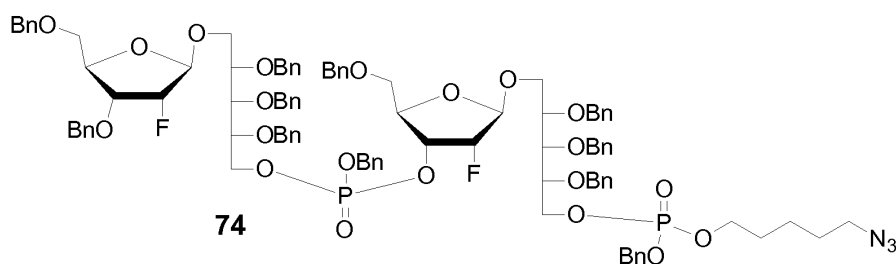
- 5 According to general procedure E), the 3-hydroxyl compound **67** and the 5-hydroxyl compound **69** were converted to dimer **71**.

**Example E-9. Compound 72**

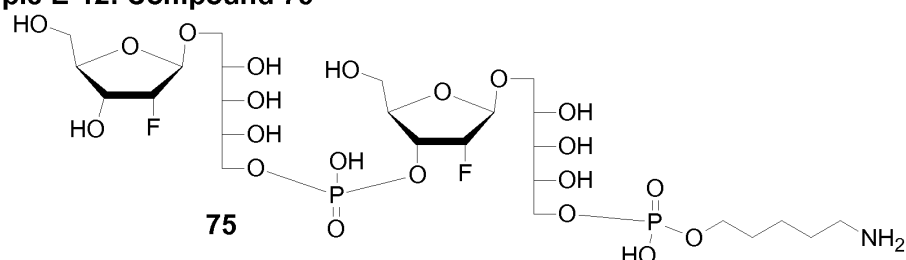
- 10 According to general procedure H), the dimer **71** was converted to the deprotected dimer **72**.

**Example E-10. Compound 73**

- 15 According to general procedure D), the NAP protected dimer **71** was converted to the 5-hydroxyl compound **73**.

**Example E-11. Compound 74**

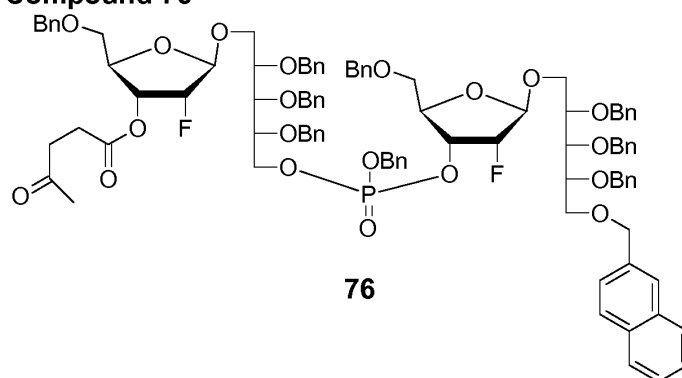
- 5 According to general procedure E), the dimer **73** was coupled to the linker 5-azido-pentanol to give dimer **74**.

**Example E-12. Compound 75**

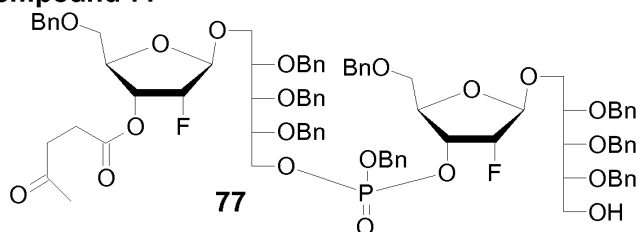
- 10 According to general procedure H), the dimer **74** was converted to the deprotected dimer **75**.

**Example F. 2'-substituted polyribosylribitolphosphates**

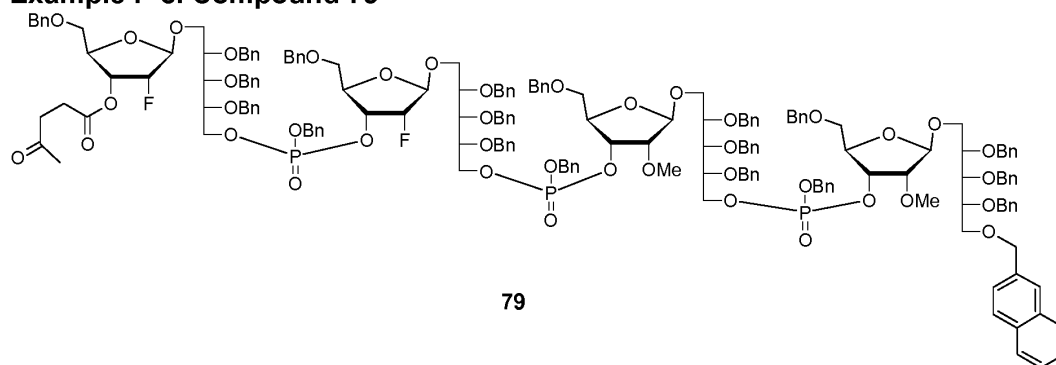
15

**Example F-1. Compound 76**

- 20 According to general procedure E), the 3-hydroxyl compound **67** and the 5-hydroxyl compound **69** were converted to dimer **76**.

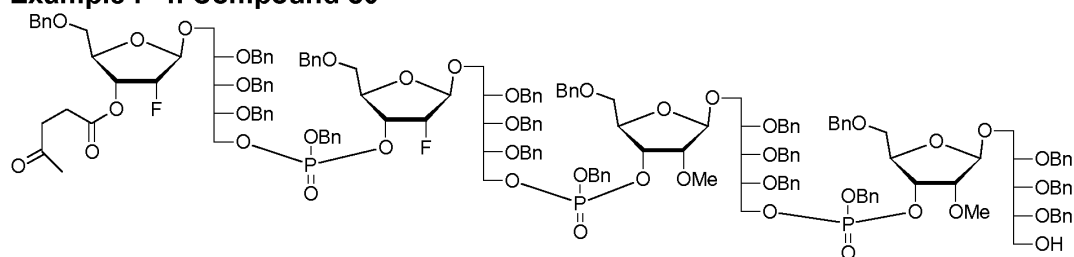
**Example F-2. Compound 77**

- 5 According to general procedure D), the NAP protected intermediate **76** was converted to the 5-hydroxyl compound **77**.

**Example F-3. Compound 79**

10

- According to general procedure E), the 3-hydroxyl dimer **22** and the 5-hydroxyl dimer **77** were converted to tetramer **79**.

**Example F-4. Compound 80**

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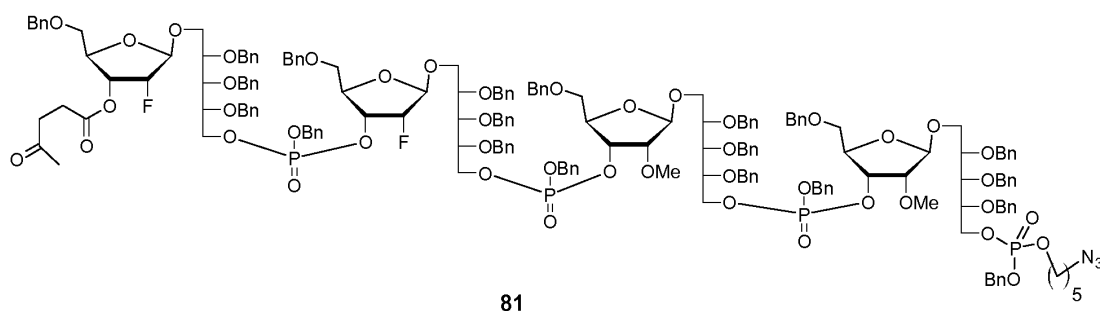
**80**

- According to general procedure D), the NAP protected tetramer **79** was converted to the 5-hydroxyl tetramer **80**.

**Example F-5. Compound 81**

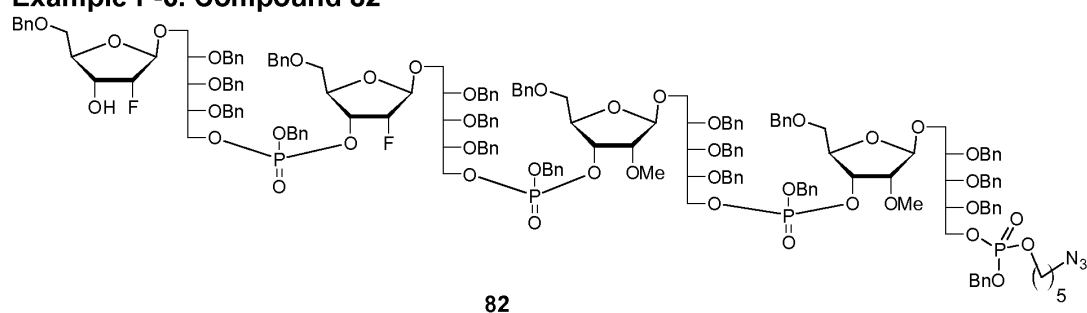
20

137



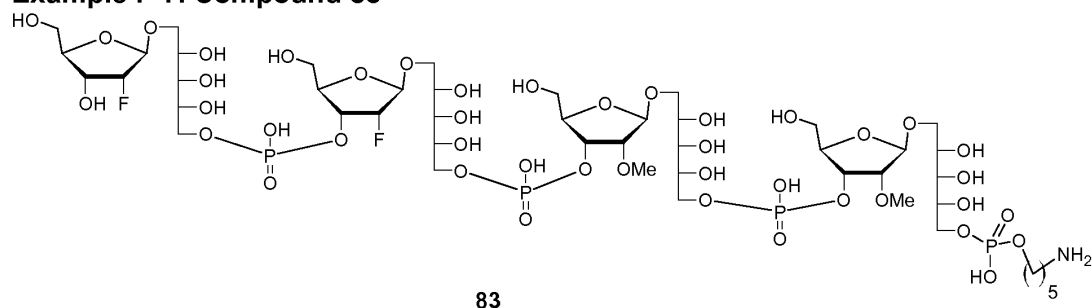
According to general procedure E), the tetramer **80** was coupled to the linker 5-azido-pentanol to give tetramer **81**.

5

**Example F-6. Compound 82**

According to general procedure G), the tetramer **81** was converted to 3-hydroxyl tetramer **82**.

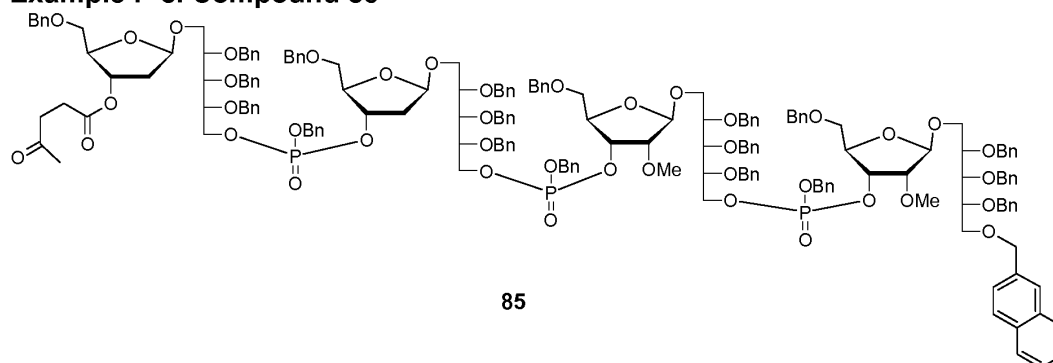
10

**Example F-7. Compound 83**

According to general procedure H), the tetramer **82** was converted to the deprotected tetramer **83**.

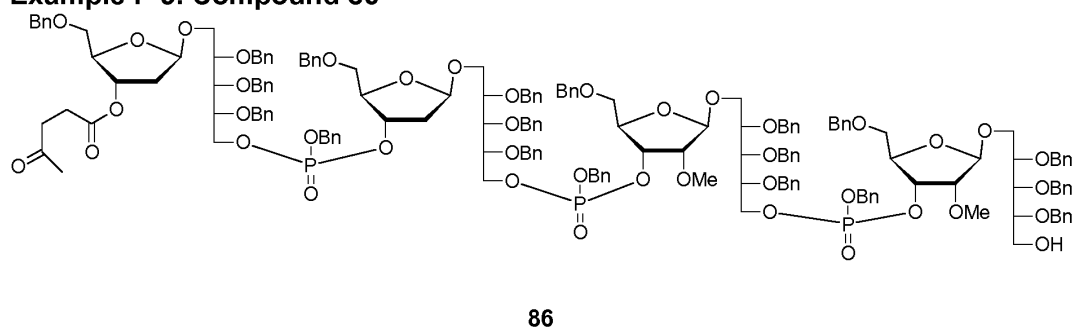
15

### Example F-8. Compound 85



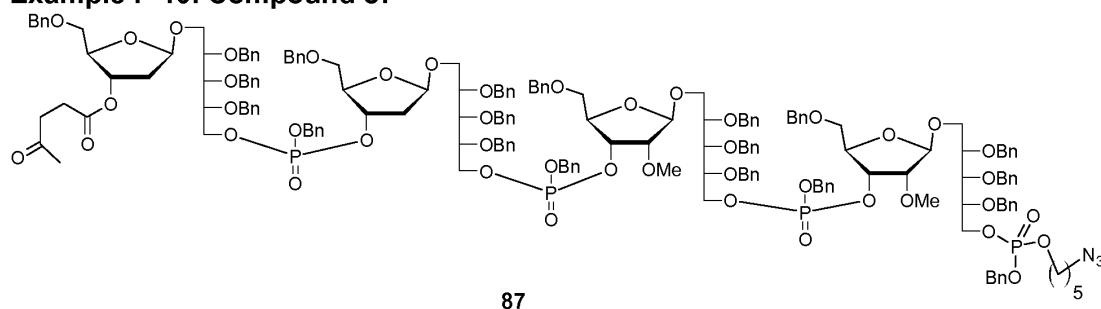
According to general procedure E), the 3-hydroxyl dimer **22** and the 5-hydroxyl dimer **45** were converted to tetramer **85**.

### Example F-9. Compound 86

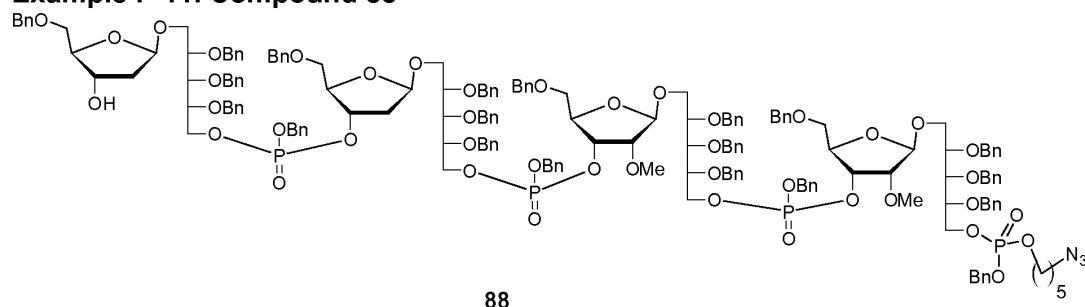


10 According to general procedure D), the NAP protected tetramer **85** was converted to the 5-hydroxyl tetramer **86**.

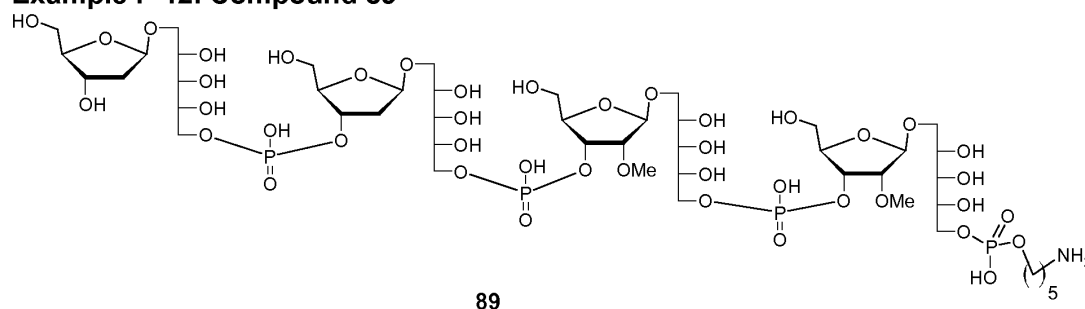
### Example F-10. Compound 87



15 According to general procedure E), the tetramer **86** was coupled to the linker 5-azido-  
pentanol to give tetramer **87**.

**Example F-11. Compound 88**

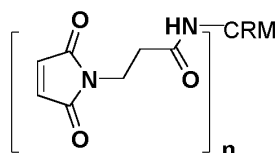
According to general procedure G), the tetramer **87** was converted to 3-hydroxyl  
 5 tetramer **88**.

**Example F-12. Compound 89**

10 According to general procedure H), the tetramer **88** was converted to the deprotected  
 tetramer **89**.

**Example G. Synthesis of a stabilized saccharide-CRM197-cysteine conjugate**

15

**Example G-1. Compound 90**

20 **Making CRM<sub>197</sub> ready for the conjugation:** CRM<sub>197</sub> (3.0 mg, 0.0517 μmol) from the  
 stock solution was transferred to the Amicon filter (0.5 mL, MWCO 30KDa) and  
 centrifuged for 3 min at 10000RPM. 300 μL of the 1X PBS was added to the  
 remaining protein in Amicon filter and centrifuged again for 3 min at 10000 RPM.  
 Then 400 μL of 1X PBS buffer (pH 7.4) was added to Amicon filter and centrifuged

for 3 min at 10000 RPM. Then the filter containing protein was reversed inside of a new Amicon micro centrifuge tube (1.5 mL) and centrifuged for 1 min at 1000 RPM to collect the protein in micro centrifuge tubes (volume~100-120  $\mu$ L).

- Conjugation procedure:** CRM<sub>197</sub> in 1X PBS buffer (~100  $\mu$ L) was diluted with 1.3 mL of 1X PBS buffer in the reaction vial at r.t. equipped with a stir bar. 3-(Maleimido)propionic acid *N*-hydroxysuccinimide ester (0.688 mg, 2.58  $\mu$ mol) was transferred to a Type I glass-2 mL reaction vial, dissolved with 20  $\mu$ L of DMSO, added it to the reaction vial containing CRM197 in 1X PBS buffer. Stirred the reaction at rt for 4 h. RM was clear colorless solution.
- Washing steps:** The reaction mixture was transferred to the Amicon filter (0.5 mL, MWCO 30KDa) and centrifuged for 3 min at 10000 RPM and repeated the process till whole reaction mixture was transferred and centrifuged. Then added 400  $\mu$ L of 1X PBS buffer to the reaction vial and rinsed and transferred it to the Amicon filter. This washing was repeated four more times with 400  $\mu$ L of the PBS buffer solution.
- Finally, Amicon filter containing CRM197-maleimide was reversed inside of a new Amicon micro centrifuge tube (1.5 mL) and centrifuged for 1 min at 1000 RPM to collect ~130  $\mu$ L CRM197-maleimide in micro centrifuge tubes. Added 950  $\mu$ L of PBS solution (pH 7.4) to it and stored the vial at 2-8 °C (total volume = ~1.08 mL). The CRM197-Maleimide conjugate was analyzed by MALDI, SDS-page, Western blot, SEC-HPLC and protein estimation by BCA. The CRM197-Maleimide conjugate was analyzed by MALDI and found between 21-24. BCA estimation also showed the good recovery of the protein. SDS page showed the CRM-maleimide conjugate **90** was higher molecular weight than CRM<sub>197</sub> and stable. Western blot shows that CRM anti-goat antibodies are recognizing the CRM-conjugate **90** whereas the Hib Anti-rabbit antibodies did not recognize the CRM-maleimide conjugate **90** which was without sugar. Sample was stored at 2-8 °C till further use.

#### **Example G-2. Compound 94 saccharide–CRM197–cysteine conjugate**

The synthetic route for compound **94** is outlined in **Figure 3**.

- DTT method:** Compound **29** (5 mg, 0.003 mmol) was dissolved in pH 7.4 PBS buffer (5 mL) and DSP (dithiobis(succinimidyl propionate)) (20 mg, 0.049 mmol) in DMSO (2.5 mL) was added at room temperature overnight. After that, dithiothreitol (DTT) (4 mg, 0.025 mmol) was added and the solution stirred at 40 °C for further 2 h. Compound **91** was purified through Sephadex G-25 column chromatography (4.5 mg, 88%). **HRMS** (ESI<sup>+</sup>) Calcd for C<sub>51</sub>H<sub>99</sub>NO<sub>46</sub>P<sub>4</sub>S [M+2Na+2K-4H]<sup>+</sup> 1736.2793, found 1736.279.

**Making saccharide-thiol ready for the conjugation: TCEP method:** 100  $\mu$ L of TCEP suspension was taken in an 1.5 ml vial and added 300  $\mu$ L of 1X PBS solution (pH 7.4) was added. Centrifuged, and the supernatant solution was removed. And

the washing with PBS repeated once more. And finally the residue was taken in 100  $\mu$ L of PBS solution and added to the Sugar-disulfide **91** (1.38 mg, 0.292  $\mu$ mol) in PBS buffer (0.15 mL) in a 2 mL type I glass vial. Shaken gently using orbital shaker for 1 h at rt. The RM was transferred to vial, and glass reaction vial was rinsed with 200  $\mu$ L of PBS solution and transferred to the vial. Centrifuged, collected the supernatant (~325  $\mu$ L) of PBS solution in a 2 mL type I glass vial. Added 200  $\mu$ L of PBS solution to the residue and mixed well, centrifuged, the supernatant was collected and transferred to the 2 mL type I glass vial again. So, total volume of the reduced thiol **78** solution was ~525  $\mu$ L. ~25  $\mu$ L were kept for analysis.

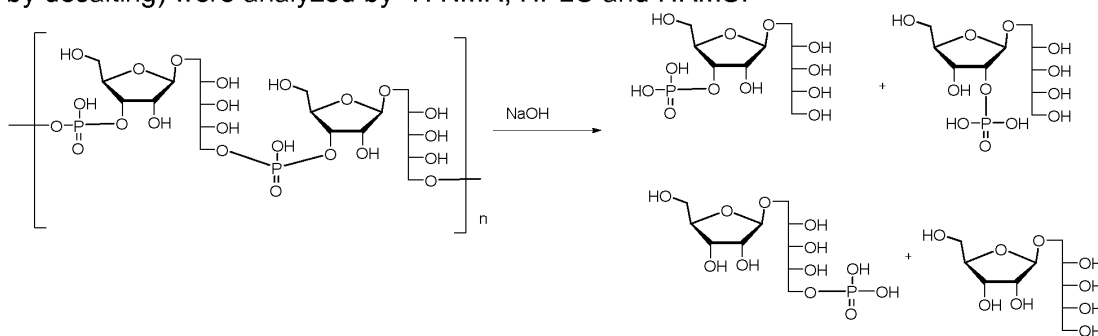
- 10 **Conjugation procedure:** CRM<sub>197</sub>-maleimide **90** in 1X PBS buffer (~850  $\mu$ L) was taken in a reaction vial at rt equipped with a stir bar. The saccharide-thiol **92** in PBS solution (~500  $\mu$ L) in 1X PBS buffer was added to the CRM<sub>197</sub>-maleimide **90** solution dropwise. The vial was rinsed with 50  $\mu$ L PBS solution and the solution was transferred to the reaction mixture at rt. RM was clear solution and stirred at r.t. for 20 h. [An aliquot of ~40  $\mu$ L of the reaction mixture was taken out for analysis purposes]. Then cysteine (0.14 mg) in phosphate buffer (pH 7.4, 30  $\mu$ L) was added to the RM containing **93** and stirred for an hour at rt.

- Washing steps:** The reaction mixture containing **94** was transferred to the Amicon filter (0.5 mL, MWCO 30KDa) and centrifuged for 3 min at 10000 RPM and repeated the process till whole reaction mixture was transferred and centrifuged. Then 400  $\mu$ L of 1X PBS buffer were added to the reaction vial and rinsed and the solution was transferred to the Amicon filter. This washing was repeated four more times with 400  $\mu$ L of the PBS buffer solution. Finally, the filter containing CRM<sub>197</sub>-maleimide-saccharide-cysteine **94** was reversed inside of a new Amicon micro centrifuge tube (1.5 mL) and centrifuged for 1 min at 1000 RPM to collect ~150  $\mu$ L CRM<sub>197</sub>-maleimide-suagr-Cysteine in micro centrifuge tube. Added 200  $\mu$ L of PBS solution (pH 7.4) to the Amicon filter and rinsed well and transferred the solution to the micro centrifuge tube and diluted the solution with 750  $\mu$ L of PBS solution (pH 7.4) and stored the vial at 2-8 °C (total volume = ~1100  $\mu$ L). An aliquot of 40  $\mu$ L of the conjugate **94** was washed with milliQ water using Amicon filter and analyzed using MALDI (sinapinic acid matrix) and loading between 3.4 - 4.2 was obtained. The conjugate **94** was further successfully analyzed using SDS-Page, western blot and SEC-HPLC. Protein estimation using BCA method showed that good recovery of the protein in the conjugate.

### Example H. Stability tests

#### Example H-1. Stability of Hib capsular polysaccharide in basic medium

Stability study of the inventive saccharides is critical for the development of stable liquid Hib glycoconjugate vaccines. As a control we examined first the stability and cleavage of Hib polyribosylribitolphosphate capsular polysaccharide (CPS). Accordingly, Hib CPS was fragmented using standard procedures as described in *Vaccine*, **2000**, 18, 1982-1993: 1 mg Hib CPS in 0.43 mL of 0.1 M NaOH, 20 h. Fragments obtained after purification (filtered through 30 kDa Amicon filters followed by desalting) were analyzed by  $^1\text{H}$  NMR, HPLC and HRMS.



**Figure 4A** shows the  $^1\text{H}$  NMR spectrum of fragmented Hib CPS and **Figure 4B** shows the  $^1\text{H}$  NMR spectrum of untreated Hib CPS. **Figure 5A** shows the HPLC chromatogram of fragmented Hib CPS and **Figure 5B** shows the ESI mass spectrum of treated Hib CPS. This set of initial experiments demonstrated that the Hib CPS was cleaved to its smallest 2 and 3-O phosphate fragments in basic medium.

#### Example H-2. Stability of Hib capsular polysaccharide in aluminum hydroxide suspension

Having established the stability data of the control Hib CPS, then the stability of Hib CPS in aluminum hydroxide was investigated, where it is known to be unstable in a vaccine formulation {0.5 mg Hib CPS, 3 mL Alhydrogel® (Brentag) and 22 mL milli-Q water} at various temperatures {37 °C (A1) and rt (A3)}. At 37 °C cleavage to smallest fragments were observed after 2 days and at rt this pattern was observed after day 7 (**Figure 6**). From this experiment it was confirmed that, Hib CPS decomposes to 2 and 3-O phosphate fragments in 2 to 7 days depending on temperature in Aluminum hydroxide the most common adjuvant used in commercial vaccines.

#### Example H-3. Stability of compound 16

Stability studies of synthesized 2'-methoxyribosylribitolphosphate dimer **16** were conducted in presence of various adjuvants. As a first step stability of **16** was studied

using  $\text{Al}(\text{OH})_3$ ,  $\text{AlPO}_4$  and water at various temperatures {37 °C (A1), 2-8 °C (A2), rt (A3) and 70 °C (A4)}.

5 The amount of  $\text{Al}(\text{OH})_3$  and  $\text{AlPO}_4$  used for this study was similar to the amount present in commercial Hib vaccines. Analysis of samples at different temperatures using HPLC after a week is shown in **Figures 7-10**. Compounds **16** and **8** were used as reference since decomposition of dimer **16** should lead to monomer **8**. Every 24 h 40  $\mu\text{L}$  of solution was aliquoted from each vial and centrifuged for 4 min at 5000 rpm. Supernatant (25  $\mu\text{L}$ ) was taken for HPLC analysis. Even after 7 days, no  
10 decomposition was observed. From this study it was clear that 2'-methoxy-riboseylribitol dimer **16** was stable at 37 °C (A1), 2-8 °C (A2), r.t. (A3) and at 70 °C.

The stability of dimer **16** was further investigated under aqueous basic conditions as applied in **Example H-1**. From the HPLC chromatogram in **Figure 13D** it was  
15 concluded that dimer **16** got cleaved completely after 4 days, while the natural Hib CPS got already cleaved completely within 20 hours. This demonstrates that the 2'-methoxyriboseylribitolphosphate oligomers are very stable even under extreme basic conditions compared to natural Hib CPS.

#### 20 **Example H-4. Stability of compound 29**

Stability studies of synthesized 2'-methoxyriboseylribitolphosphate tetramer bearing a linker **29** were conducted in presence of various adjuvants. As a first step stability of **29** was studied using  $\text{Al}(\text{OH})_3$ , phosphate buffer (PBS) and water at various  
25 temperatures { 2-8 °C (A2), and r.t. (A3)}.

The amount of  $\text{Al}(\text{OH})_3$  used for this study was similar to the amount present in commercial Hib vaccines. Analysis of samples at different temperatures using HPLC after a week is shown in **Figures 11 and 12**. Compounds **29**, **16** and **8** were used as reference since decomposition of tetramer **29** should lead to dimer **16** and monomer  
30 **8**. Every 24 h 40  $\mu\text{L}$  of solution was aliquoted from each vial and centrifuged for 4 min at 5000 rpm. Supernatant (25  $\mu\text{L}$ ) was taken for HPLC analysis. Even after 7 days, no decomposition was observed. From this study it was clear that 2'-methoxy-riboseylribitolphosphate tetramer **29** is stable in presence of  $\text{Al}(\text{OH})_3$ , in phosphate buffer (PBS) and in water at 2-8 °C (A2) and r.t. (A3).

35 The stability of tetramer **29** was further investigated under aqueous basic conditions as applied in **Example H-1**. From the HPLC chromatogram in **Figure 13A** it was concluded that tetramer **29** got cleaved completely after 4 days, while the natural Hib CPS got already cleaved completely within 20 hours. This demonstrates that the 2'-

methoxyribosylribitolphosphate oligomers are very stable even under extreme basic conditions compared to natural Hib CPS.

**Example H-5. Stability of conjugate 94 in presence of phosphate buffer**

- 5 ~5 µg of compound **94** in two vials were each diluted to 500 µL of PBS pH 7.4; one vial was stored at 25 °C and another at 2-8 °C. The samples were analyzed by bicinchoninic acid assay (BCA) and HPLC-SEC (column: TSKgel G2000SWxl, buffer: 50 mM Tris, 20 mM NaCl, pH 7.2) after one day and after 15 days. The data showed that still after 15 days the conjugate **94** was present and stable at both  
10 temperature conditions 25 °C and 2 – 8 °C.

**Example H-6. Stability of compound 94 in presence of aluminum hydroxide**

- Two vials containing ~5 µg of compound **94** and Alhydrogel® (0.125 mg/dose) were each filled up to 500 µL of PBS pH 7.4; one vial was stored at 25 °C and another at  
15 2-8 °C. The samples were analyzed by bicinchoninic acid assay (BCA), HPLC-SEC column: TSKgel G2000SWxl, buffer: 50 mM Tris, 20 mM NaCl, pH 7.2), SDS-page and western blot after one day and after one week. The data showed that ~75% of the conjugate **94** were adsorbed onto Alhydrogel® and the adsorbed conjugate was stable at both temperature conditions. No degradation products were observed  
20 during this course of time.

**Table 1:** BCA assay of conjugate **94** in presence of phosphate buffer and aluminum hydroxide.

|                         | protein concentration<br>at t = 0 in µg / mL | temperature<br>in °C | final protein concentration<br>in µg / mL |
|-------------------------|----------------------------------------------|----------------------|-------------------------------------------|
| <b>94</b> + PBS         | 155                                          | 2 – 8                | 119 after 15 d                            |
| <b>94</b> + PBS         | 155                                          | 25                   | 107 after 15 d                            |
| <b>94</b> + Alhydrogel® | 113                                          | 2 – 8                | 32 after 1 d                              |
| <b>94</b> + Alhydrogel® | 113                                          | 25                   | 36 after 1 d                              |
| <b>94</b> + Alhydrogel® | 113                                          | 2 – 8                | 34 after 7 d                              |
| <b>94</b> + Alhydrogel® | 113                                          | 25                   | 39 after 7 d                              |

## **Example I. Glycan array analysis**

### **Example I-1. Immunization in Rabbits**

Immunization experiments were performed on rabbits (Chong et al. Infect. Immun.,  
5 **1997**, p. 4918–4925; Fernández-Santana et al. Science, **2004**, 305 (5683), p. 522-  
525.). Rabbits were housed and handled according to international animal  
regulations (EU Directive 2010/63/EU) and sanctioned by governmental authorities  
(Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-  
Vorpommern).  
10 Four groups with four rabbits per each group were immunized in a prime-boost  
regime with unadjuvanted conjugate **94** containing 5 µg saccharide **29** or conjugate  
**94** (containing 5 µg Hib saccharide **29**) adjuvanted with Alhydrogel. The negative  
control group received PBS/ Alhydrogel only. The positive control group received the  
approved vaccine ActHIB® (5 µg PRP, corresponding to half the human dose), a  
15 conjugate of native PRP to tetanus toxoid (TT). Preimmune serum and antiserum of  
bleeding day 21 (following two immunizations) were taken and analyzed on a glycan  
array. The ActHIB-specific serum was obtained after three administrations in  
contrast to two immunisations of the HIB analogues of the present invention.

### **Example I-2. Glycan array analysis**

Saccharides in PBS were printed on N-Hydroxy succinimide activated glass slides  
(CodeLink slides, Surmodics) using an S3 microarray spotter (Scienion). Slides were  
incubated overnight in a humidity saturated chamber, quenched for 2 h with 100 mM  
ethanolamine, 50 mM sodium phosphate, pH 7.5, washed with water and dried.

25 For incubation, slides were blocked for 1 h with 3% (w/v) BSA-PBS, washed with  
PBS and water, and dried by centrifugation. A 64 well incubation grid was attached.  
Sera were diluted in 3% BSA-PBS, 0.1% (v/v) Tween-20, incubated at 37 °C for 15  
min, and centrifuged for 2 min at 3220 rpm. Serum dilutions were applied to the slide  
30 and incubated for 1 h at room temperature. Wells were washed three times with PBS  
+ 0.1% Tween-20 (PBS-T). Secondary antibodies (anti-rabbit IgG FITC, anti-rabbit  
IgM AlexaFluor 647, anti-human IgG-Fc AlexaFluor 488 and IgM AlexaFluor 594)  
were incubated on the slides for 30 min at r.t. Wells were washed twice with PBS-T,  
the incubation grid was removed, and the slide washed with PBS and water. After  
35 drying by centrifugation, the slide was scanned using a GenePix 4300A (Molecular  
Devices) microarray reader.

The primary immune response was assessed by glycan array screening of serum  
samples retrieved at day 0, day 21 and day 35. 2'-Methoxyribosylribitolphosphate

dimer **19**, 2'-methoxyribosylribitolphosphate dimer **24**, 2'-Methoxyribosylribitolphosphate tetramer **29**, 2'-deoxyribosylribitolphosphate tetramer **53** and 2'-dimethylaminocarbonylribosylribitolphosphate **62** as well as natural Hib PRP were printed on NHS ester-activated microarray slides. After immunization with conjugate **94** 5 adjuvanted with Alhydrogel and without adjuvant, antibodies of the IgG and IgM subtypes against the Hib PRP derivatives **19**, **24**, **29**, **53** and **62** as well as against the natural Hib PRP polysaccharide in all immunized rabbits were detected.

10 The glycan array screening also showed that anti-Hib antibodies present in human reference serum (**Figure 17A**) and in rabbit typing serum (**Figure 17B**) bind to the Hib PRP derivatives **19**, **24**, **29**, **53** and **62**. The presence of natural Hib PRP inhibits the binding of the Hib PRP derivatives **19**, **24**, **29** and **62**, but not of the 2'-deoxyribosylribitolphosphate **53**.

15 Serum IgG antibodies were detectable 21 days after the first immunization with the conjugate **94** adjuvanted with Alhydrogel and 35 days after the first immunization with undajuvanted conjugate **94**. Antibodies cross-reacting with the natural Hib PRP polysaccharide indicates the potential of these antibodies to bind to *Haemophilus influenzae* bacteria and to confer protection against *Haemophilus influenzae* 20 infection.

### **Example J. ELISA studies**

- 25 • ELISA plates (high-binding, EIA/RIA Plate, 96 well, flat bottom with low evaporation lid, company: costar® 3361)
- commercial rabbit anti-Hib-PRP IgG ELISA assembly kit (Alpha Diagnostic Intl. Inc, # 980-130-PRG; Lot: 170531K5, Expiry: 06/2018)
- antigen: Phosphoribosylphosphate (PRP) capsular polysaccharide from 30 *Haemophilus influenza b* (NIBSC, Code:12/306)
- test sera: supplied by BioGenes GmbH.
- detection antibody: goat anti rabbit IgG peroxidase conjugate (Sigma, #A4914 ).
- Phosphate Buffered Saline (PBS): Made in-house from stock (Biochrom GmbH, Cat: L182-10)
- 35 • blocking solution: 1 % FCS (v/v) in PBS.
- antibody diluent: PBS+1% BSA (w/v).
- wash buffer: PBS+0.1% Tween 20 (PBS-T)

- developing solution: 1 step<sup>TM</sup> Ultra TMB-ELISA developer. (ThermoScientific, Cat #: 34028)
  - stop solution- 2M Sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) (Made in-house)
  - plate reader: Anthos ht 2.
- 5 software: WinRead 2.36 for absorbance measurements and GraphPad Prism 7 for data plotting and analysis.

### Example J-1. Immunization schedule and sera collection

10 All immunization experiments were performed at BioGenes GmbH Berlin. The experimental groups included i) PBS with Alhydrogel (negative control), ii) compound **94** without Alhydrogel, iii) compound **94** with Alhydrogel, and iv) the commercial vaccine HiberiX® (positive control). Briefly, rabbits (n=4) were immunized in a prime boost regime with the constructs for the respective experimental groups as mentioned in Table 1. The mice were immunized following a prime-boost regime and  
15 sera were collected on day 0 (pre-immune), day-14, day-21, and day-35.

Table 2. Immunization schedule and antigen dose information of rabbits (n=4).

| Group                     | Rabbits | amount antigen [µg/dose] | amount Alhydrogel [mg/dose] | Excipient     | Immunization schedule [days] | Sera collection [days] |
|---------------------------|---------|--------------------------|-----------------------------|---------------|------------------------------|------------------------|
| <b>94</b>                 | 4       | 0.5                      | 0                           | 1X PBS pH 7.4 | d0, d14, d28                 | d0, d14, d21, d28, d35 |
| <b>94</b> with Alhydrogel | 4       | 0.5                      | 0.32                        | 1X PBS pH 7.4 | d0, d14, d28                 | d0, d14, d21, d28, d35 |
| PBS with Alhydrogel       | 4       | 0                        | 0.32                        | 1X PBS pH 7.4 | d0, d14, d28                 | d0, d14, d21, d28, d35 |
| HiberiX®                  | 4       | 5                        | –                           |               | d0, d14, d28                 | d0, d14, d21, d28, d35 |

### Sera Collection and Handling:

20 The sera from different experimental groups and respective time points were stored at -20 °C. Sera from the individual rabbits (20 µl) of specific experimental groups were pooled and stored at -20 °C. Individual rabbit sera (20 µl) were aliquoted as a separate stock and stored at -20 °C till further use.

**Example J-2. Enzyme linked immunosorbent assay (ELISA) of sera using in-house antigen Coated plates**

5

**Coating of plates with antigen:**

The antigen, Hib PRP capsular polysaccharide (stock concentration 1 mg/mL) was diluted to a working antigen concentration of 10 µg/mL in PBS pH 7.4. 100 µL was added in each well (1 µg antigen/ well) and incubated overnight at 4 °C.

10

**Washing:**

After overnight adsorption of the antigen, the plates were washed 3X with PBS-T (200 µL/well) and the excess fluid per well was removed by inverting the plate and tapping on a clean dry tissue towel.

15

**Blocking:**

The plates were blocked using 300 µL of blocking solution (PBS+ 1% FCS) for 2 h at RT.

20

**Washing:**

After blocking, the plates were washed 3X with PBS-T (200 µL/well) and the excess fluid per well was removed by inverting the plate and by tapping on a clean dry tissue towel.

25

**Dilution of Sera and Incubations:**

Pooled (n=4 rabbits/group) pre-immune and test sera of the different experimental groups and individual rabbit test sera (n=4) were diluted to their respective dilutions, in the antibody diluent (PBS+1% BSA). 100 µL of the diluted sera samples of the different experimental groups (100 were added in duplicates to the corresponding wells and incubated on a shaker set at 250 rpm for 1 h at RT. 100 µL/well of the antibody diluent (PBS+1 % BSA) formed the experimental blank. After incubation with sera, the plates were washed 5X with PBS-T (200 µL/well) and the excess fluid per well was removed by inverting the plate and by tapping on a clean dry tissue towel.

30  
35

**Incubation (detection antibody):**

The detection antibody, goat anti-rabbit IgG peroxidase was diluted 1: 10,000 in the antibody diluent (PBS+1 % BSA) and 100 µL was added to the well and incubated on a shaker at 250 rpm for 1 h at RT. After the incubation with detection antibody, the plates were washed 5X with PBS-T (200 µL/well) and the excess fluid per well was removed by inverting the plate and by tapping on a clean dry tissue towel.

**Developing:**

To each well, 100 µL of the ready to use TMB substrate (normalized to RT form 4 °C) was added and incubated in dark for 15 min. The blue color of the enzymatic reaction was stopped by adding 100 µL/well of 2M H<sub>2</sub>SO<sub>4</sub> solution resulting in a yellow colored solution. The absorption of the yellow colored solution was measured at 450 nm with a correction wavelength of 630 nm using a plate reader. The absorption values were analyzed by plotting a graph using the Graphpad Prism software.

**Results:**

As seen in **Figure 18A**, the pre-immune sera of all the experimental groups across all the dilutions did not show any significant response and the readings were equal to 0. In the post-immune/test sera, the day-14 HiberiX group (inverted triangles) showed an immune response considerably higher than the PBS control group (filled circles) that increased up to titers of 1:2500 in the day-21 and day-35 groups (**Figures 18B – D**). In the experimental groups immunized with adjuvanted compound **94** (triangles), the antibody titers were lower than that of the PBS group on day-14 which substantially increased to that of the HiberiX group at a titer of 1:25000 but 1-fold lower. Although the experimental group immunized with the unadjuvanted compound **94** (squares) showed immune titers comparable to the HiberiX group at day-14, no boosting response was observed at the day-21 time point where the adjuvanted group showed a boosting response. However, after the second boost, the unadjuvanted compound **94** showed an immune titer comparable to the adjuvanted group, but lower than that of the Hiberix group.

**Example J-3. Enzyme linked immunosorbent assay (ELISA) of sera using commercial ADi plates**

The rabbit anti-Hib-PRP IgG ELISA kit (ADi) detects Hib -PRP specific IgG antibodies in the sera of vaccinated animals. The kit includes a 96 well microtiter plate pre-coated with the antigen Hib-PRP polysaccharide, supplied with the wash buffers and

the peroxide conjugated detection antibody. The kit also includes a set of calibrators (0.5, 1.0, 2.5, and 5.0 U/mL) and a positive control/reference sera. The calibrators provided a control of the internal assay parameters and differentiates false positives in a sera dilution of 1:50 and above. The 0.5 U/mL value becomes the lowest detectable titer below which values of tests would be a false positive. The assay was performed in a normal ELISA format by following the instructions from the manufacturer. Briefly, the sera to be tested was diluted to an appropriate dilution range of 1:100 and above in the diluent provided in the kit. 100 µL of the diluted sera along with the calibrator, positive, and negative control was added to pre-determined wells and incubated for 1 h at room temperature. The wells were then washed 4 times with the kit wash buffer and 100 µL of the detection antibody is added and further incubated for 30 min followed by 5 washes. The wells were developed by adding 100 µL of the TMB substrate and incubating for 15 min followed by stopping the reaction using 100 µL stop solution resulting in a yellow colored solution. The absorption of the yellow colored solution was measured at 450 nm with a correction wavelength of 630 nm using a microplate reader. The absorption values were analyzed by plotting a graph using the Graphpad Prism software.

#### Results:

The Hib-PRP polysaccharide (antigen) is very sensitive to hydrolysis due to pH differences and other physiochemical factors which might lead to lower detection of antigen in an ELISA. To corroborate the immune titers observed in **Figures 18B – D** and to rule out the anomalies arising due to in-house coating of the antigen, the rabbit sera were screened on a commercial diagnostic plate (Advanced Diagnostics-adi) pre-coated with the Hib-PRP polysaccharide. As described in **Example J-2**, the rabbit immunized sera from the different experimental groups and time-points were diluted 5-fold (1:100, 500, 2500, and 12500) and analyzed for polysaccharide specific IgG antibodies. As seen in **Figure 19A**, the pre-immune sera of all the experimental groups across all the dilutions did not show any significant response and the readings were below the threshold value. The threshold refers to the absorption value got for the 0.5 U/mL calibrator run on the same plate, values below which can be assigned as negatives or false positives.

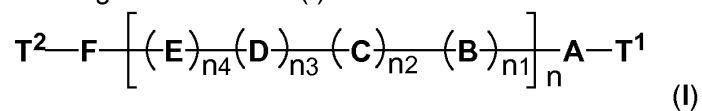
In the post-immune/test sera, the day-14 HiberiX group (inverted triangles), compound **94** with (triangles) and without (squares) Alhydrogel showed an immune titer lower than the threshold value even at the highest dilution of 1:100 (**Figure 19B**). However, the PBS control group (filled circles) showed a higher signal than the threshold value at the highest dilution of 1:100 only in the day-14 time point but was lower than the threshold in all other time points of day-21, and -35. This was a similar

trend observed in the in-house coated antigen plates (**Figure 18B – D**). The adjuvanted compound **94** (triangles) and the HiberiX group (inverted triangles) in the day-21 and-35 time points showed a saturation up to dilutions of 1:500 and 1:2500, respectively. The effect of the adjuvant and boosting response was clearly visible in the compound **94** with Alhydrogel group as the titers significantly increase from 1:2500 in the day-21 time point to 1:12500 in the day-35 time point. Clearly at day-35, the group immunized with adjuvanted compound **94** showed comparable response to HiberiX but 1-fold lower. However, in the group immunized with unadjuvanted compound **94**, there were no IgG titers detectable till day-21, but significantly increased up to 1:2500 post second boost. This trend was similar to that observed in ELISA performed using the in-house antigen coated plates (**Figure 18C, D**).

These data demonstrate the immunogenicity of the conjugate **94**. Isotype switching indicates T cell-dependent antibody responses. Serum IgG antibodies were detectable 21 days after the first immunization with the conjugate **94** adjuvanted with Alhydrogel and 35 days after the first immunization with the unadjuvanted conjugate **94**. Antibodies cross-reacting with the natural Hib PRP polysaccharide indicates the potential of these antibodies to bind to *Haemophilus influenzae* bacteria and to confer protection against *Haemophilus influenzae* infection

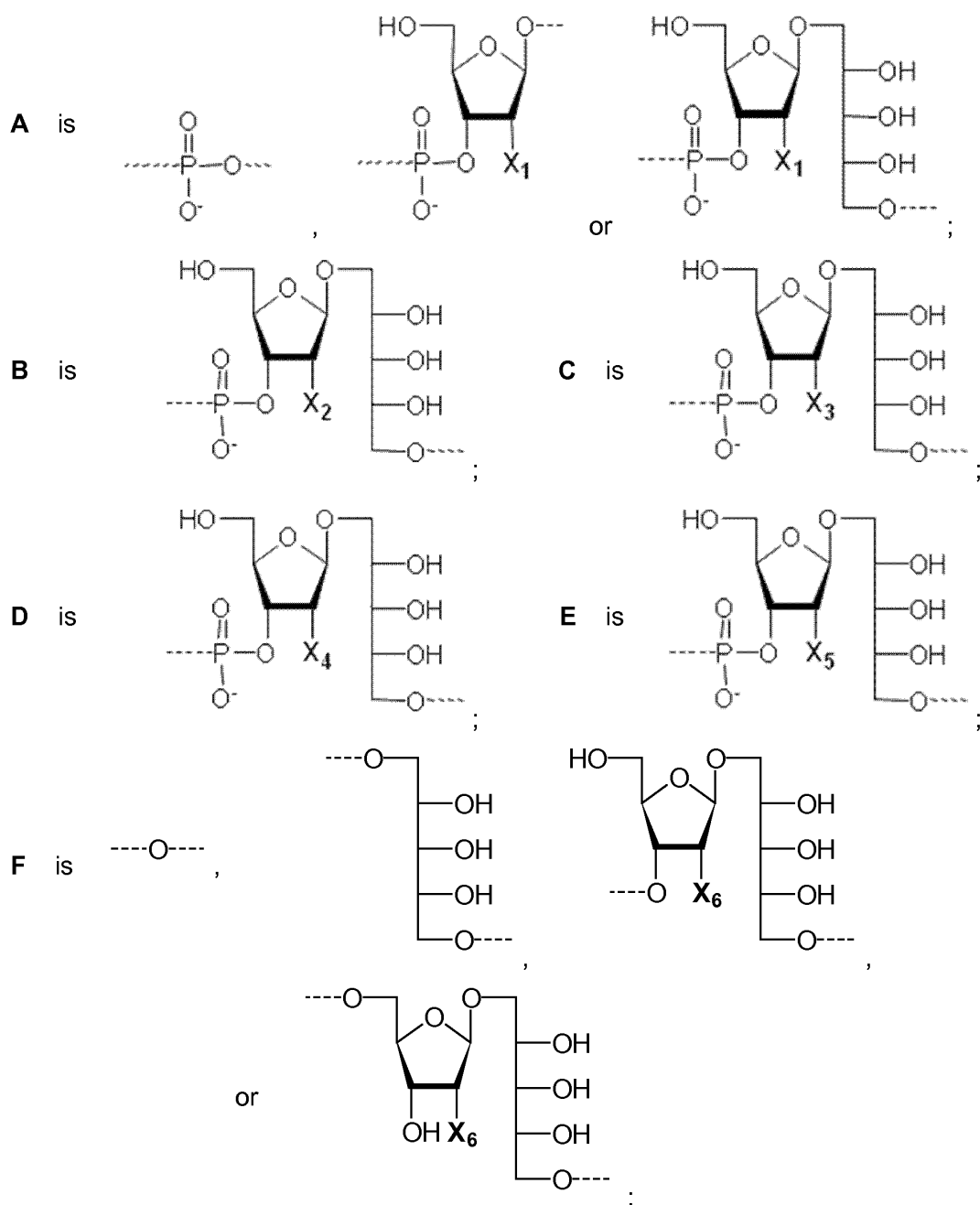
## Claims

1. Saccharide of general formula (I)



5

wherein



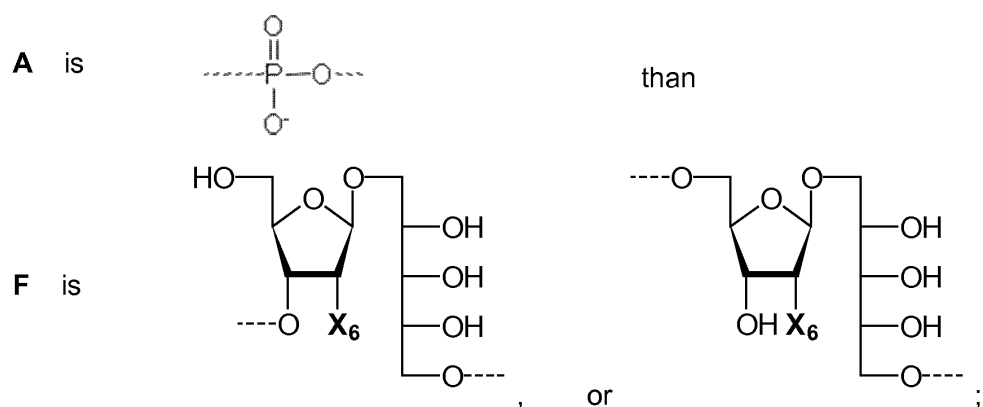
$T^1$  and  $T^2$  represent  $-H$ ,  $-L-NH_2$ ; wherein if  $T^1$  is  $-L-NH_2$ , then  $T^2$  is  $-H$  and if  $T^1$  is  $-H$ , then  $T^2$  is  $-L-NH_2$  or  $-L-COOH$ ;

$X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$ , and at least two of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ , and  $X_6$  are not  $-OH$ ;

$n$  is an integer selected from 1, 2, 3, or 4;

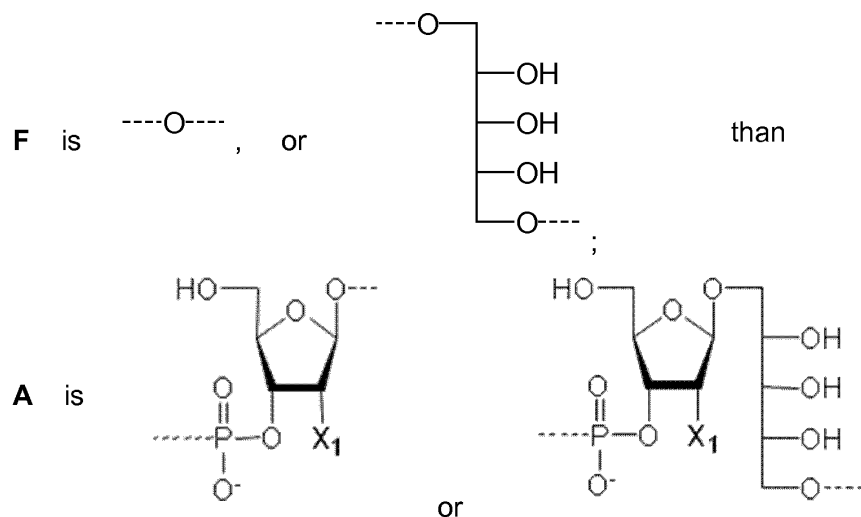
$n_1$ ,  $n_2$ ,  $n_3$  and  $n_4$  are independently of each other selected from 0 and 1 and  $n_1+n_2+n_3+n_4 \geq 1$ ;

under the proviso that if  $n_1+n_2+n_3+n_4 = 1$  and if  $n = 1$  and if



and

under the proviso that if  $n_1+n_2+n_3+n_4 = 1$  and if  $n = 1$  and if

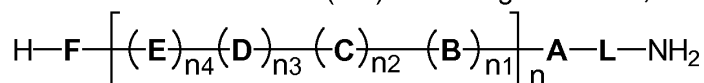


and

- L** is a linker and **L** is selected from:  $-\text{L}^{\text{a}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{e}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{b}}-\text{L}^{\text{e}}-$ ,  $-\text{L}^{\text{a}}-\text{L}^{\text{d}}-\text{L}^{\text{e}}-$ ;  
 wherein  $-\text{L}^{\text{a}}-$  is selected from:  $-(\text{CH}_2)_o-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{C}_2\text{H}_4-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_o-\text{CH}_2-$ ;  
 $-\text{L}^{\text{b}}-$  represents  $-\text{O}-$ , or  $-\text{O}-\text{P}(\text{O})(\text{OH})-\text{O}-$ ;  
 $-\text{L}^{\text{d}}-$  is selected from  $-(\text{CH}_2)_q-$ ,  $-(\text{CF}_2)_q-$ ,  $-(\text{CH}_2-\text{CH}_2-\text{O})_q-\text{C}_2\text{H}_4-$ , and  $-(\text{CH}_2-\text{CH}_2-\text{O})_q-\text{CH}_2-$ ;  
 $-\text{L}^{\text{e}}-$  is selected from:  $-(\text{CH}_2)_{p1}-$ ,  $-(\text{CF}_2)_{p1}-$ ,  $-\text{C}_2\text{H}_4-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$ ,  $-\text{CH}_2-(\text{O}-\text{CH}_2-\text{CH}_2)_{p1}-$  and  $-(\text{CH}_2)_{p1}-\text{O}-(\text{CH}_2)_{p2}-$ ;  
 and *o*, *q*, *p1* and *p2* are independently of each other an integer selected from 1, 2, 3, 4, 5, and 6;

and enantiomers, diastereomers, mixtures of enantiomers, mixtures of diastereomers, anomers, hydrates, solvates of the above mentioned compounds and pharmaceutically acceptable salts thereof.

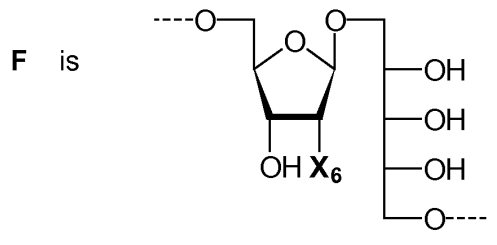
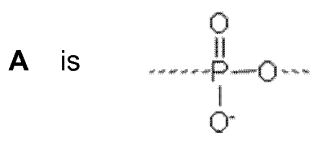
2. Saccharide of the formula (II-1) according to claim 1,



20

(II-1)

wherein



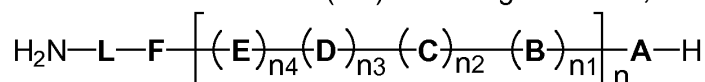
**B**, **C**, **D**, **E**, **L**, **n1**, **n2**, **n3** and **n4** have the meanings as defined in claim 1;

**n** is 1 or 2; and

**X<sub>2</sub>** - **X<sub>6</sub>** are  $-\text{H}$ ,  $-\text{F}$ , or  $-\text{OCH}_3$ .

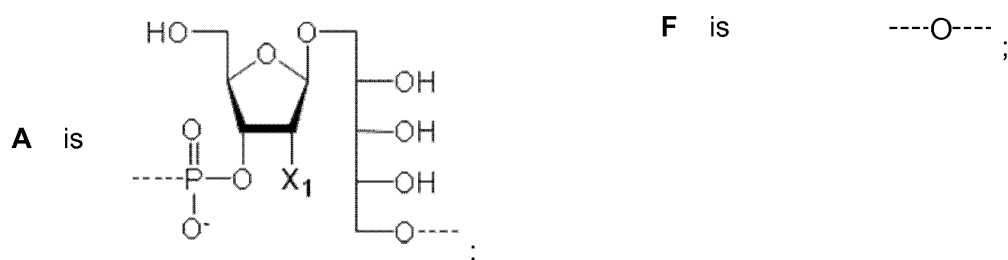
25

3. Saccharide of the formula (II-2) according to claim 1,



(II-2)

wherein

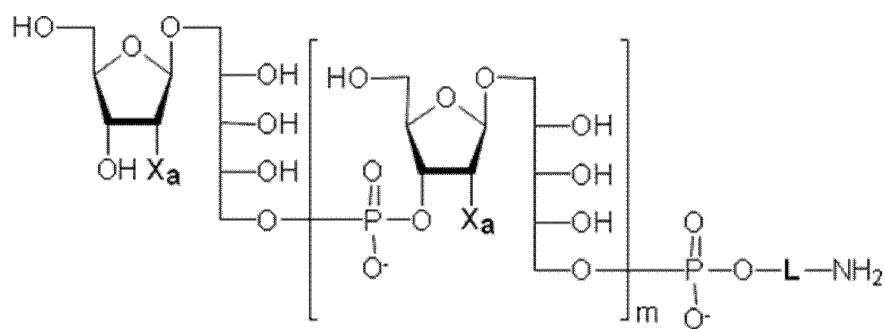


**B, C, D, E, L, n1, n2, n3** and **n4** have the meanings as defined in claim 1;

**n** is 1 or 2; and

**X<sub>1</sub> - X<sub>5</sub>** are -H, -F, or -OCH<sub>3</sub>.

- 5 4. Saccharide of the formula **(III-1)** according to claim 1,



**(III-1)**

wherein

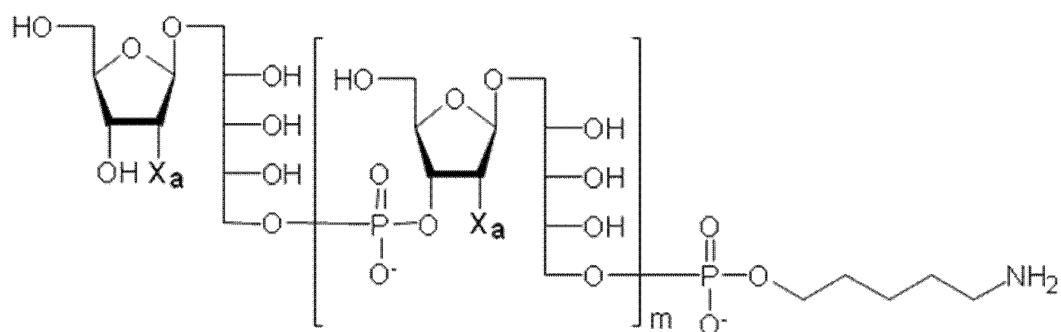
**L** has the meaning as defined in claim 1;

**m** is an integer selected from 1 to 9; and

**X<sub>a</sub>** is -H, -F or -OCH<sub>3</sub>.

15

5. Saccharide of the formula **(IV-1)** according to claim 1,



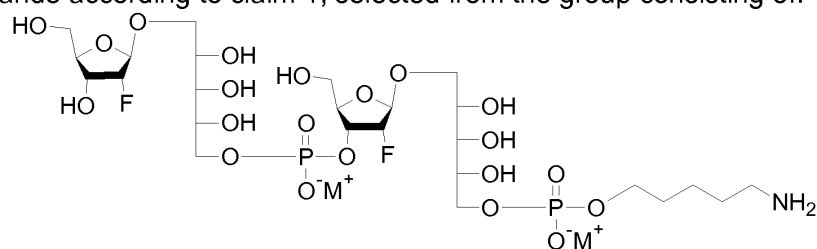
## (IV-1)

wherein

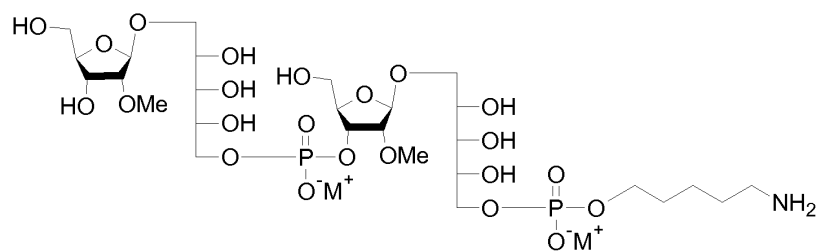
**m** is an integer selected from 1 to 9; and

5 **Xa** is  $-H$ ,  $-F$  or  $-OCH_3$ .

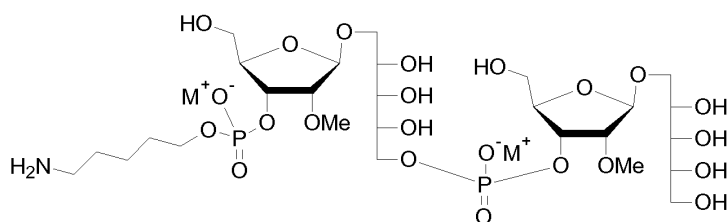
6. Saccharide according to claim 1, selected from the group consisting of:



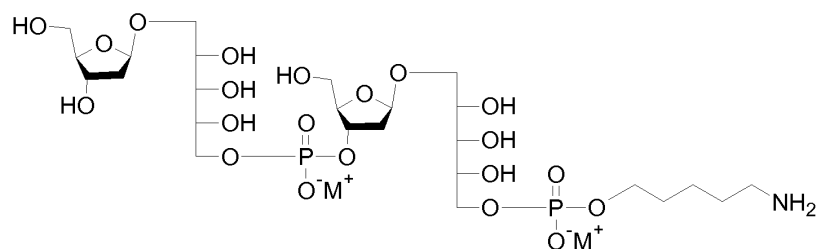
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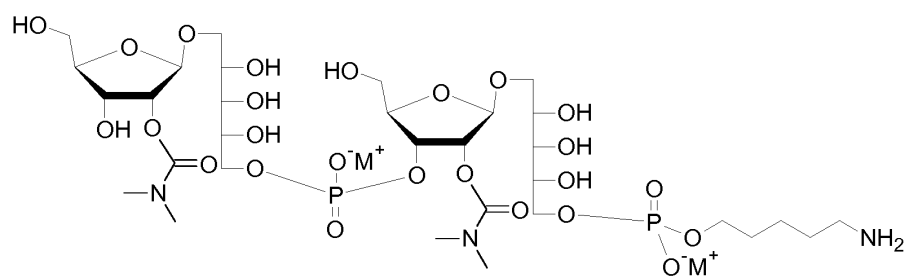
(2b),



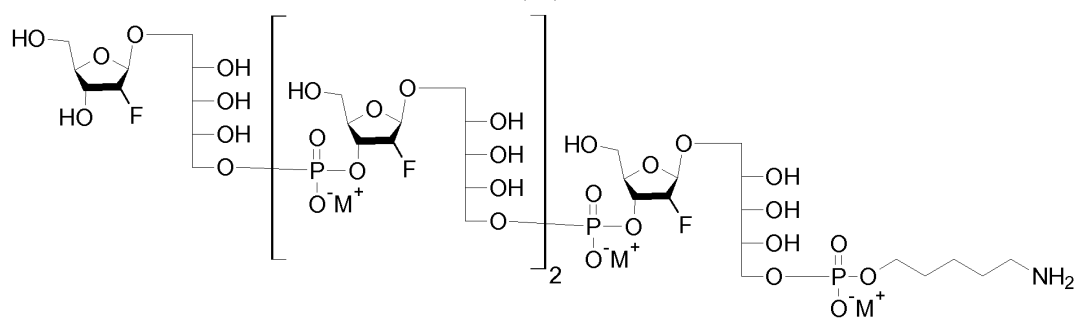
(2b'),



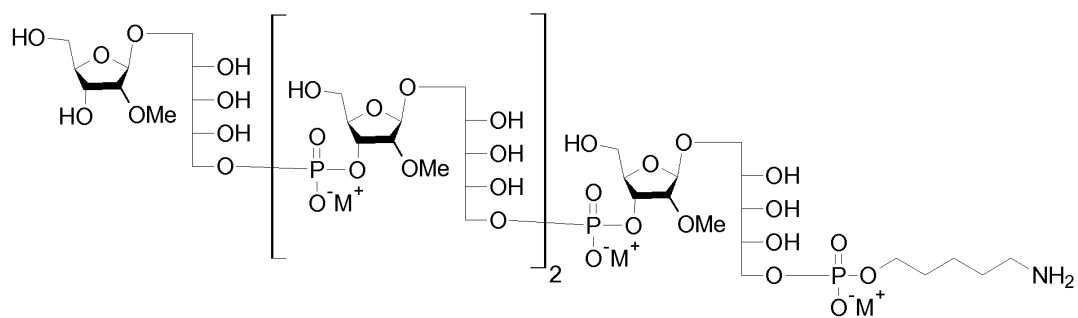
(2c),



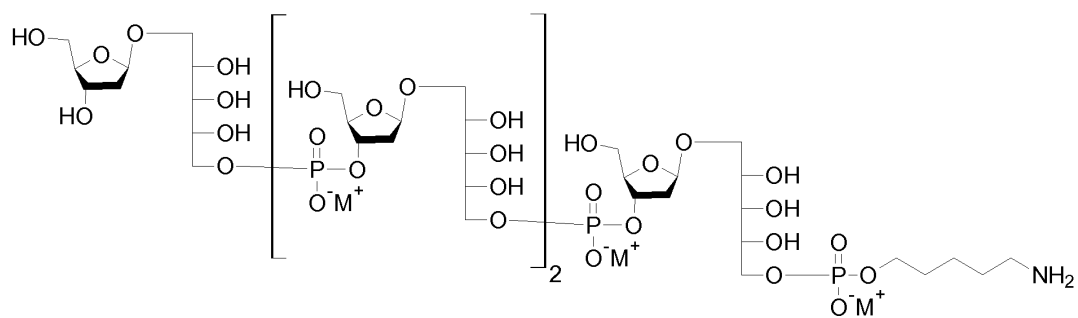
(2f),



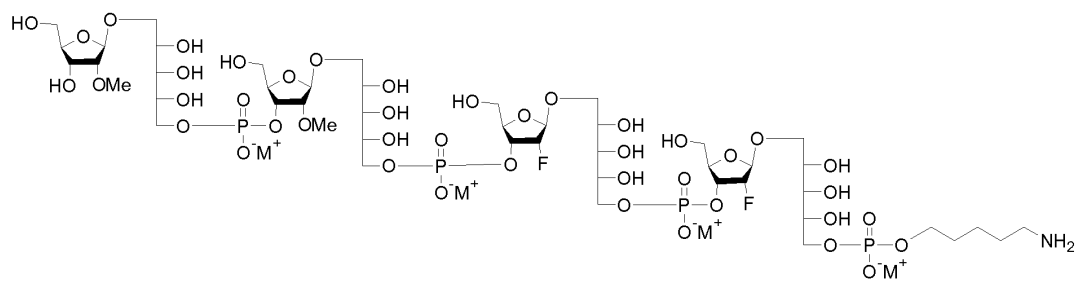
(4a),



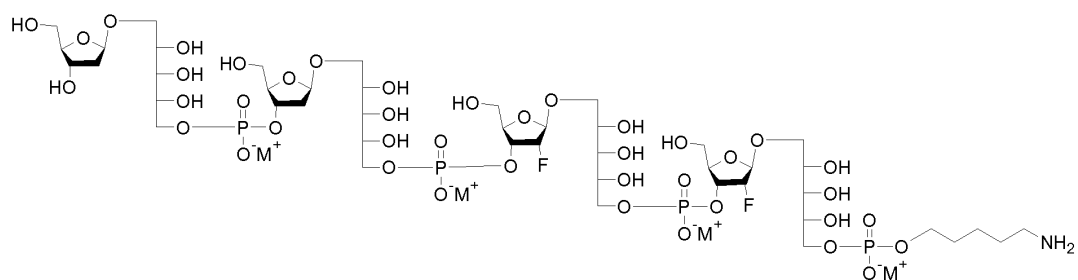
(4b),



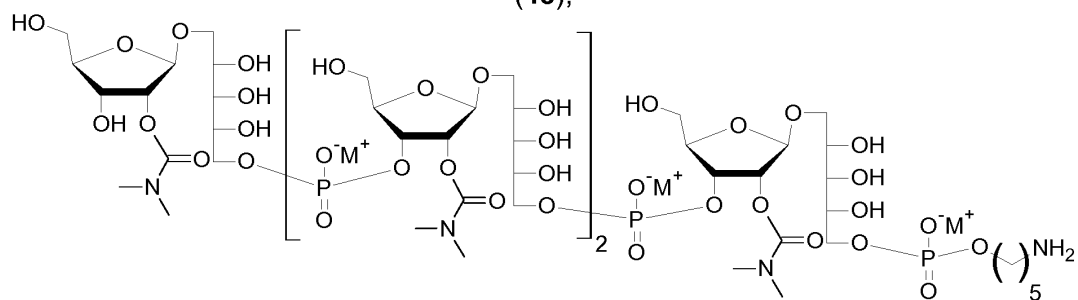
(4c),



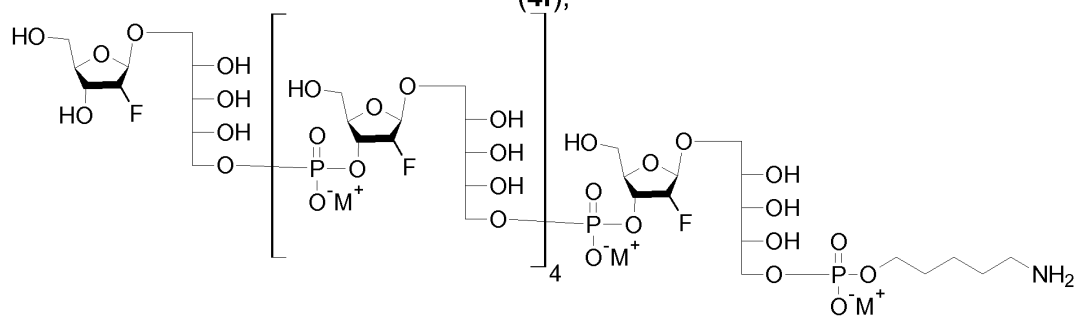
(4d),



(4e),

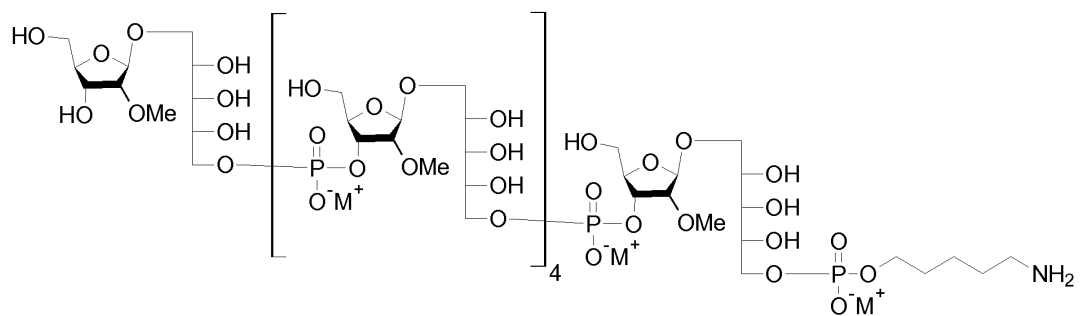


(4f),

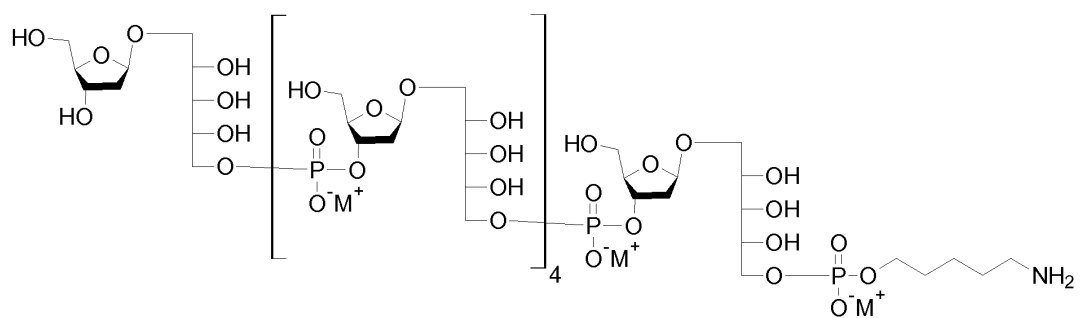


(6a),

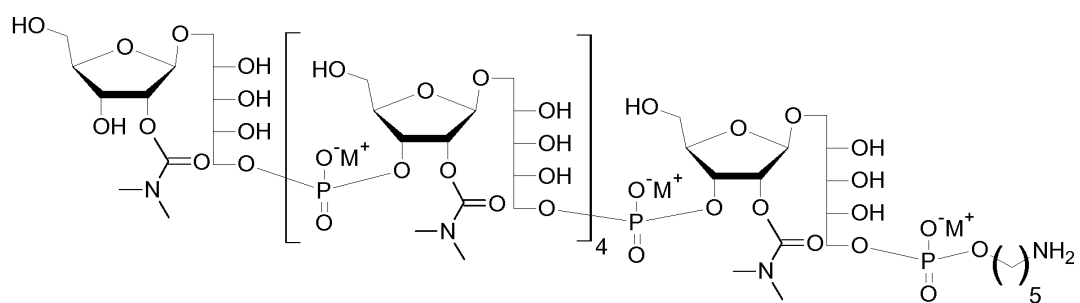
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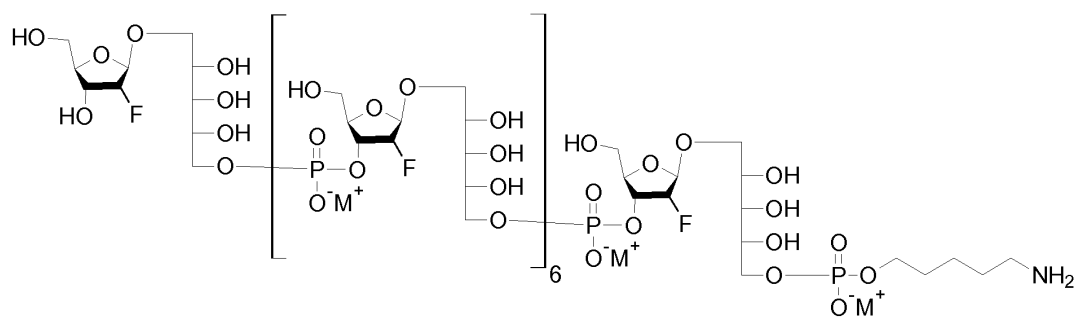
(6b),



(6c),

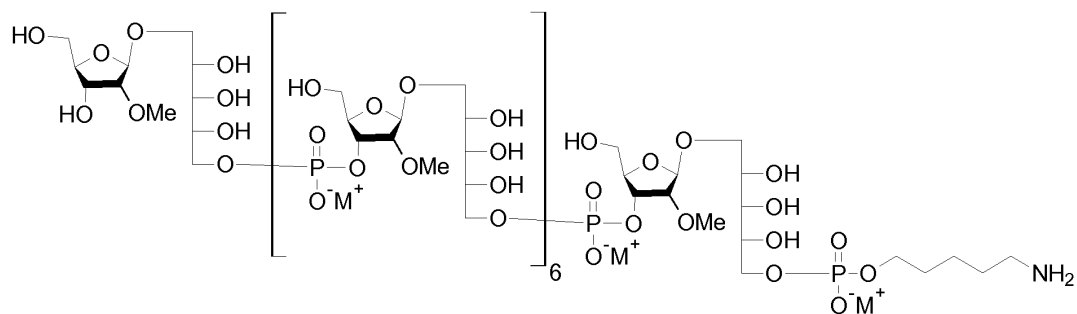


(6f)

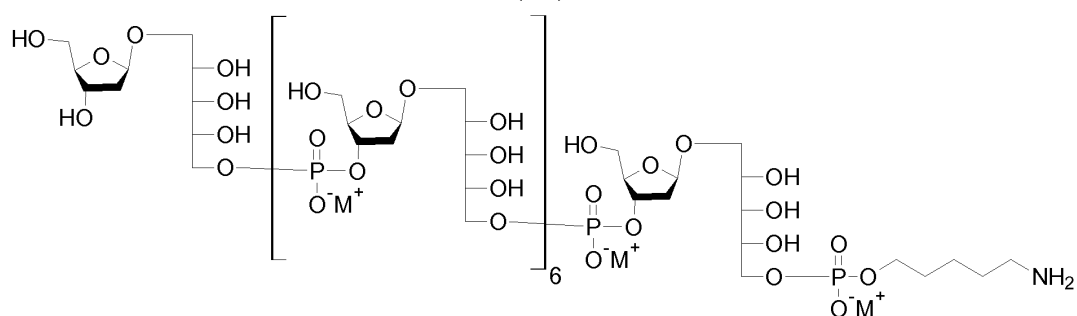


(8a),

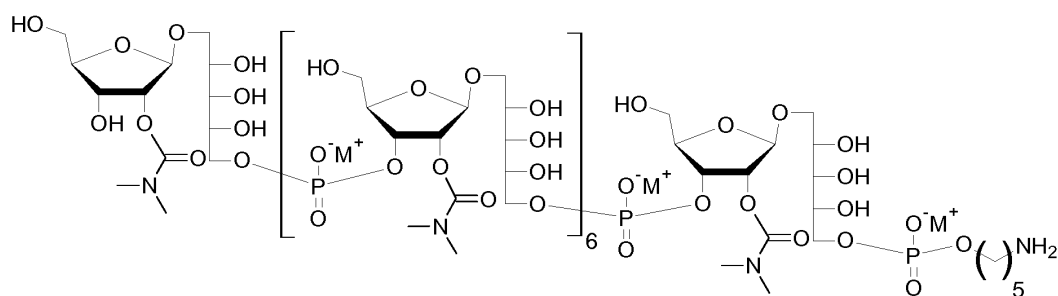
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(8b),



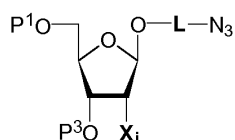
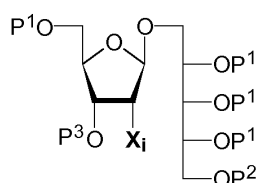
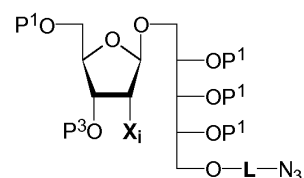
(8c), and

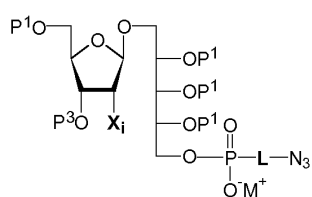
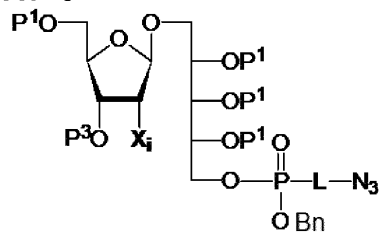
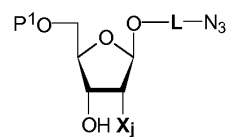
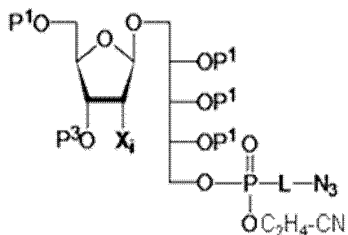
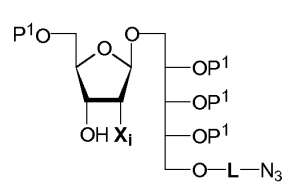
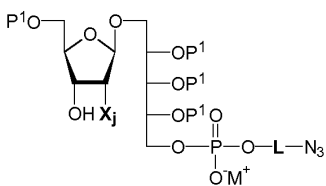
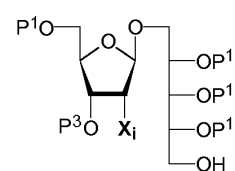
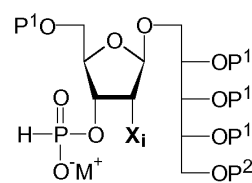
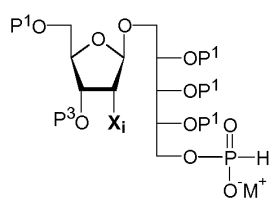
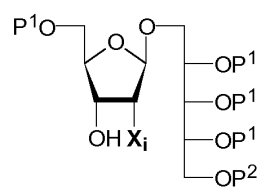
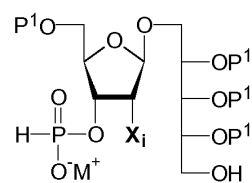
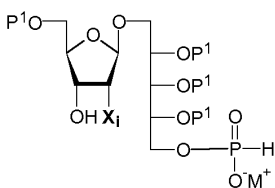
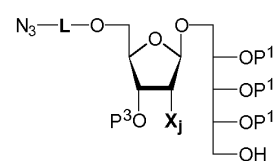
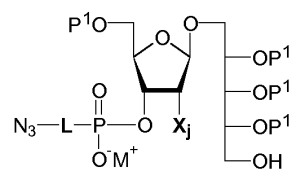
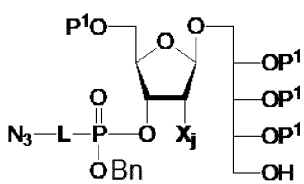
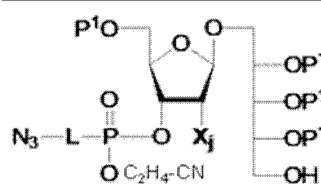


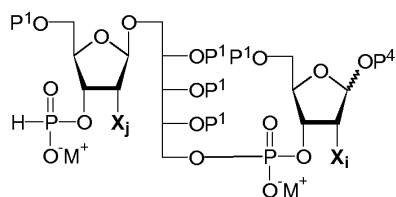
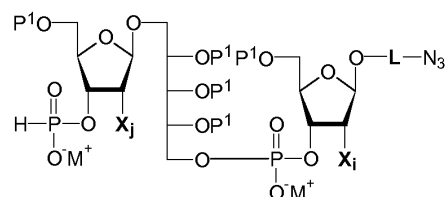
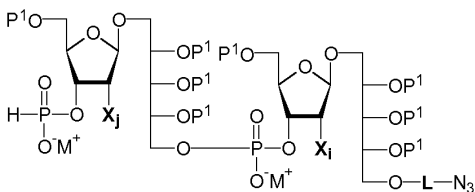
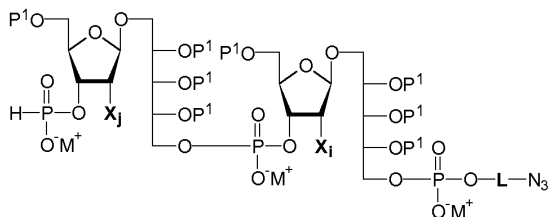
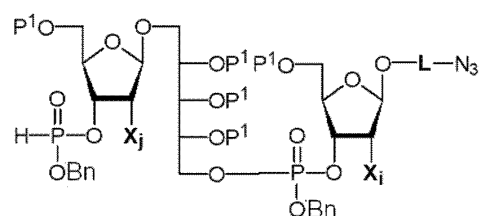
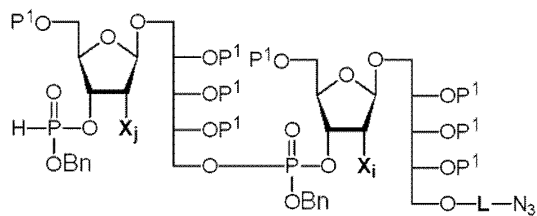
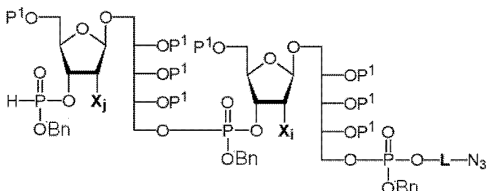
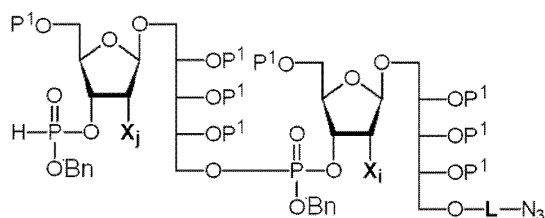
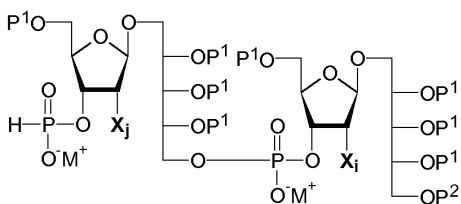
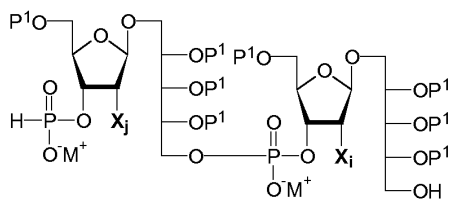
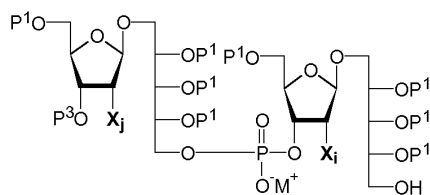
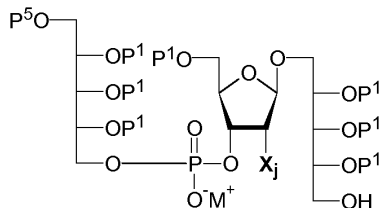
(8f).

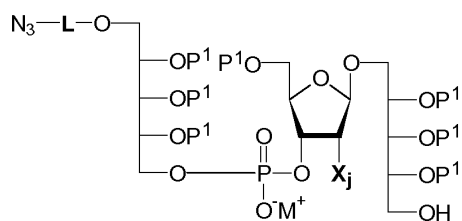
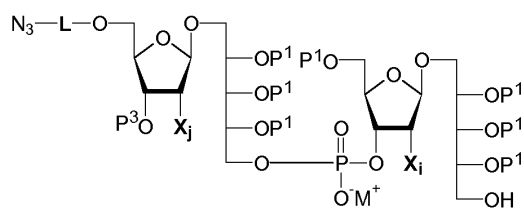
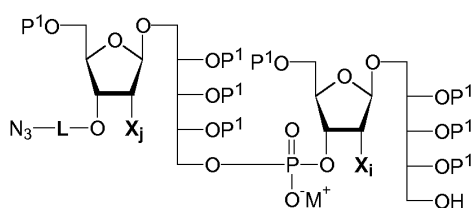
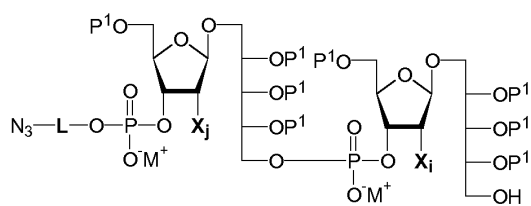
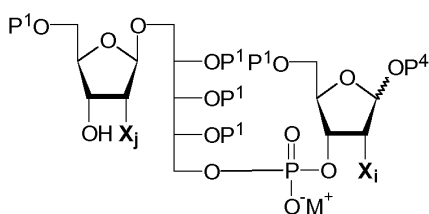
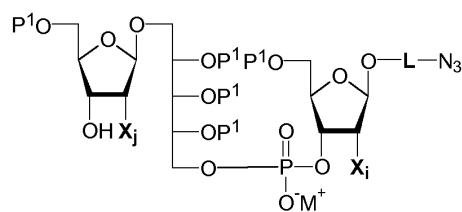
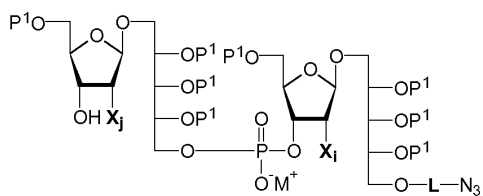
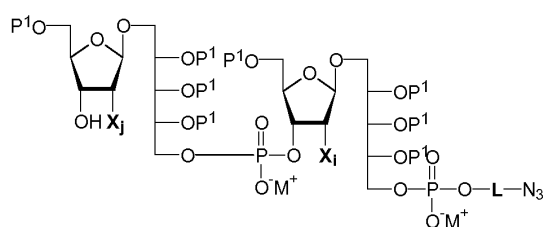
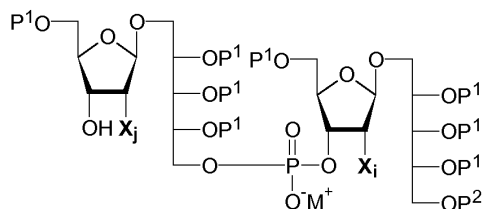
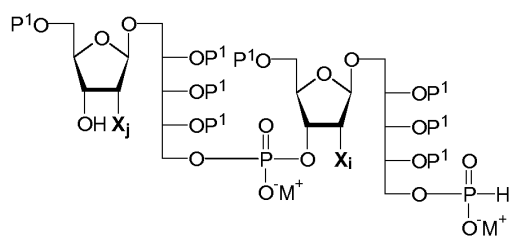
7. Saccharide of the general formula (I) according to any one of claims 1 - 6 conjugated with an immunogenic carrier through the nitrogen atom of the  $-X-L-NH_2$  group.
8. Saccharide according to any of the claims 1 – 7 for use as pharmaceutically active agent in medicine.
9. Saccharide according to any of the claims 1 – 8 for use in raising a protective immune response in a human and/or animal host.

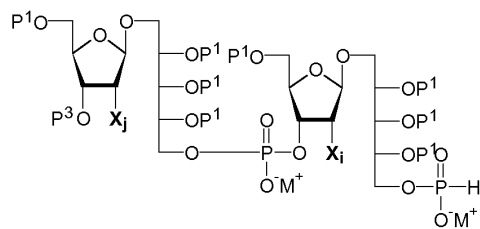
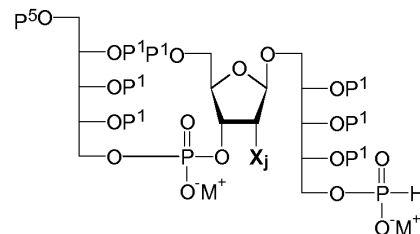
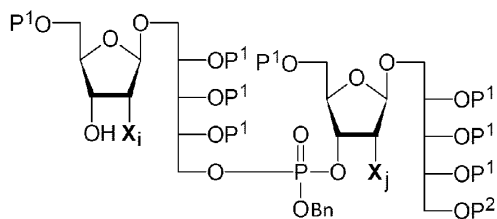
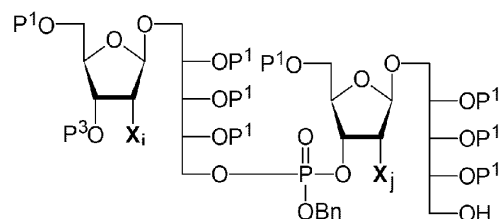
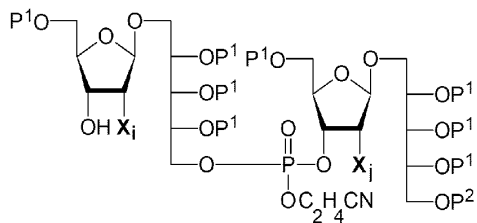
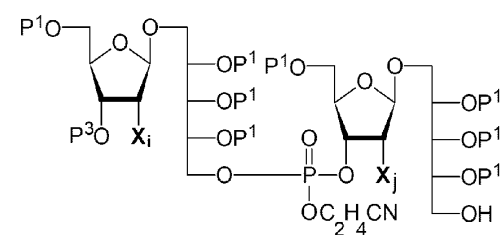
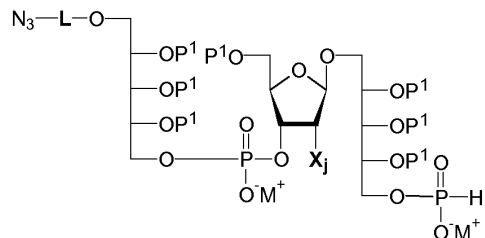
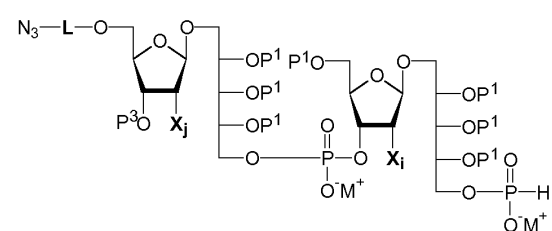
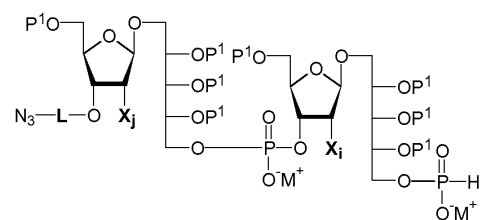
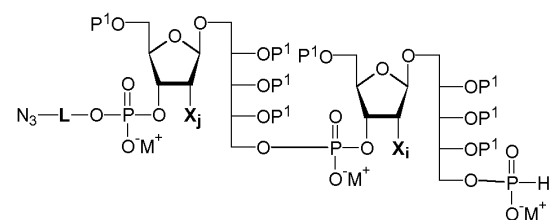
10. Saccharide according to any of the claims 1 – 9 for use in prevention and/or treatment of diseases associated with *Haemophilus influenzae* type b.
11. Saccharide according claim 10, wherein the diseases associated with *Haemophilus influenzae* type b is selected from meningitis, pneumonia, and epiglottitis.
12. Vaccine composition comprising a saccharide according to any of the preceding claims together with at least one pharmaceutically acceptable adjuvant, carrier, cryoprotectant, lyoprotectant, excipient and/or diluent.
13. Vaccine composition according to claim 12 for use in immunization against diseases associated with *Haemophilus influenzae* type b.
14. Vaccine composition according to claim 12 or 13 further comprising at least one of diphtheria antigen, tetanus antigen, pertussis antigen, hepatitis B antigen, inactivated polio vaccine and inactivated rotavirus vaccine.
15. Saccharide according to any one of claims 1 - 6 for use as marker in immunological assays for diagnostics of diseases caused by *Haemophilus influenzae* type b.
16. Intermediate compound for synthesis of the saccharide of the formula (I) is selected from the group consisting of:
- A1\*-2, A1\*-3, A1\*-4, A1\*-5, A1\*-5', A1\*-5'', A1\*-6, A1\*-7, A1\*-8, B1\*-1, B1\*-2, B1\*-3, B1\*-4, B1\*-5, B1\*-6, C1\*-2, C1\*-3, C1\*-3', C1\*-3''A2\*-1, A2\*-2, A2\*-3, A2\*-4, B2\*-1, B2\*-2, B2\*-3, B2\*-4, C2\*-1, C2\*-2, C2\*-3, C2\*-4, A2\*-1', A2\*-2', A2\*-3', A2\*-4', A2\*-2'', A2\*-3'', A2\*-4'', B2\*-1', B2\*-1'', B2\*-1''', B2\*-2', B2\*-3', B2\*-3'', B2\*-3''', B2\*-4', C2\*-1', C2\*-2', C2\*-3' and C2\*-4':**

**A1\*-2****A1\*-3****A1\*-4**

**A1\*-5****A1\*-5'****A1\*-6****A1\*-5''****A1\*-7****A1\*-8****B1\*-1****B1\*-2****B1\*-3****B1\*-4****B1\*-5****B1\*-6****C1\*-2****C1\*-3****C1\*-3'****C1\*-3''**

**A2\*-1****A2\*-2****A2\*-3****A2\*-4****A2\*-2''****A2\*-3''****A2\*-4''****B2\*-4''****B2\*-1****B2\*-2****B2\*-3****B2\*-4**

**C2\*-1****C2\*-2****C2\*-3****C2\*-4****A2\*-1'****A2\*-2'****A2\*-3'****A2\*-4'****B2\*-1'****B2\*-2'**

**B2\*-3'****B2\*-4'****B2\*-1''****B2\*-3''****B2\*-1'''****B2\*-3'''****C2\*-1'****C2\*-2'****C2\*-3'****C2\*-4'**

wherein  $P^1$ ,  $P^2$ ,  $P^3$ ,  $P^4$ , and  $P^5$  represent protecting groups that when bound to  $-O-$  are not identical to  $X_i$  and  $X_j$ ;

$X_i$  and  $X_j$  represent independently of each other  $-H$ ,  $-OH$ ,  $-F$ ,  $-CH_3$ ,  $-C_2H_5$ ,  $-CN$ ,  $-OCH_3$ ,  $-OC_2H_5$ ,  $-OCH(CH_3)_2$ ,  $-OCH_2F$ ,  $-OCF_3$ ,  $-OCO-N(CH_3)_2$ ,  $-O-C_2H_4-O-CH_3$ ,  $-O-CH_2-CF_3$ , wherein

if only one group  $X_i$  or  $X_j$  is present,  $X_i$  or  $X_j$  cannot be  $-OH$ ;

- 5 if both groups  $X_i$  and  $X_j$  are present,  $X_i$  and  $X_j$  cannot be simultaneously  $-OH$ ,

$L$  has the meaning defined in claim 1; and

$M^+$  represents  $Na^+$ ,  $K^+$ ,  $NH_4^+$ , or  $HNEt_3^+$ .