

Conjugates for the Prevention or Treatment of Nicotine Addiction

TECHNICAL FIELD

The present invention relates to nicotine-derived haptens, hapten-spacer conjugates and hapten-carrier conjugates that serve as the antigenic component in anti-nicotine vaccines. The invention also relates to vaccine compositions containing such nicotine-derived hapten-carrier conjugate antigens formulated with adjuvants. Such compositions are used to enhance quit rates or reduce relapse rates in smoking cessation and tobacco/nicotine dependence treatment efforts.

BACKGROUND

Smoking has many serious adverse effects on health and with many government initiatives to reduce or prevent smoking, it has become less socially acceptable. Consequently, many smokers wish to quit the habit, and while many make attempts each year, only a small minority of those who manage to quit do not relapse. The very high failure rate is the result of the addictive nature of nicotine plus the easy availability of cigarettes.

With smoking, or use of nicotine in other forms (e.g., sinus, patches, gum), nicotine enters the bloodstream and rapidly thereafter enters the brain, where it stimulates nicotinic acetylcholine receptors, causing release of dopamine, which in turn activates reward centres. With a smoking quit attempt, there is a loss of the reward response, as well as withdrawal symptoms including a decline in cognitive function. The main reason for relapse is that the loss of reward and the unpleasant withdrawal symptoms can immediately be relieved by smoking.

There are various non-vaccine therapies for smoking cessation. Nicotine replacement therapy, such as nicotine containing chewing gum or skin patches, may help wean smokers off cigarettes but they do not break the addiction cycle that nicotine causes. Another approach is the use of drugs that target nicotinic acetylcholine receptors, such as varenicline. Such drugs, which reduce the rewards normally encountered by smoking, have been relatively successful in aiding smoking cessation, however relapse rates are high after drug treatment ends since a lapse (e.g., smoking a single cigarette) can easily turn into a full relapse with reactivation of reward centres.

More recent nicotine cessation strategies have focused on vaccines that stimulate the immune system to produce anti-nicotine antibodies that bind to nicotine in the bloodstream, thus reducing the amount and rate that nicotine can enter the brain. This in turns prevents reward centres from being activated and helps break the addiction cycle. Since antibodies induced by vaccines can be long-living, anti-nicotine vaccines are useful both to assist in smoking cessation as well as prevention of relapse. Additionally, since the antibodies act in the periphery, there is no risk of central nervous system (CNS) adverse effects. Examples of such vaccines are described in WO 00/32239, WO 02/49667, WO 03/82329 and US 2006/111271. Nicotine derivatives are described in EP-A-421762, WO 01/70730, WO 01/80844 and US 2005/119480. Further nicotine derivatives have been identified under registry numbers 136400-02-7, 250683-10-4, 861023-80-5 and 861025-04-9. Nicotine haptens are described in WO 99/61054, WO 02/58635, WO 03/82329, WO 2005/40338 and EP-A-1849780.

SUMMARY

The present invention relates to nicotine-derived haptens, hapten-spacer conjugates, and conjugation methods which can be used to prepare immunogenic hapten-carrier conjugates for use in vaccines designed to enhance quit rates or reduce relapse rates in smoking cessation treatment efforts. The invention also relates to vaccine formulations containing the above-mentioned conjugates together with adjuvants or excipients, which are used for immunization of smokers in order to elicit antibodies against the haptens, which in turn will also recognize and specifically bind to nicotine. The invention further relates to a method to enhance quit rates or reduce relapse rates in smoking cessation treatment efforts which comprises administering the hapten-carrier conjugate to smokers wishing to quit. In other embodiments the vaccine could be used in non-smokers to prevent them from becoming addicted to nicotine if they were subsequently exposed to it by smoking or other means.

The nicotine-derived hapten-carrier conjugates of the invention may have the advantage that they are more immunogenic, are more specific, are more stable, or have other more useful properties than the nicotine-derived hapten-carrier conjugates known in the art.

The nicotine-derived hapten-carrier conjugates of the invention may be more immunogenic antigens than other known nicotine-derived hapten-carrier conjugates for use in anti-nicotine vaccines. As well, the vaccine formulations containing the nicotine-

derived hapten-carrier conjugates of the invention as antigen together with adjuvants may be more immunogenic than other anti-nicotine vaccine formulations, which are typically adjuvanted with aluminium hydroxide, and result in higher quit rates and lower relapse rates amongst patients who are dependent on nicotine/tobacco and wish to quit smoking. Due to better immunogenicity inherent within the antigen, as well as enhanced by the adjuvants, the vaccine formulations of the invention may also achieve higher anti-nicotine antibody titres more quickly and with fewer doses, resulting in improved compliance compared to the vaccine formulations known in the art.

The synthetic routes to the nicotine-derived hapten compounds of the invention may have the advantage that they provide an increased overall synthetic yield (preferably up to a 20 fold increase in overall synthetic yield), involve a reduced number of synthetic steps, result in increased purity of the resulting nicotine-derived haptens (e.g. >99% purity) or have other more useful properties than the synthetic routes to the nicotine-derived hapten compounds known in the art.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of immunization of mice with vaccines comprising nicotine-derived hapten-conjugates of the invention together with adjuvants, on anti-nicotine antibody levels in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived haptens (from Preparations 4, 7, 8, and 12) conjugated to diphtheria toxoid (DT; 10 µg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555, a 21-mer TLR9 agonist containing immunostimulatory CpG motifs (50 µg). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA.

Figure 2 shows the effect of immunization of mice with anti-nicotine vaccines containing conjugate antigens that comprise haptens of the invention on avidity of resulting anti-nicotine antibodies in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived haptens (from Preparations 4, 7, 8 and 12) conjugated to diphtheria toxoid (DT; 10 µg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg). Avidity Index corresponds to concentration of ammonium thiocyanate required to elute 50% of anti-nicotine antibodies from nicotine-BSA coated plates and requirement of higher concentrations indicates with higher avidity antibodies.

Figure 3 shows the effect of immunization of mice with anti-nicotine vaccines containing conjugate antigens that comprise haptens of the invention on distribution of intravenously (IV) administered ^3H -nicotine in brain and blood. BALB/c mice (n = 6 per group) were immunized with nicotine-derived haptens (from Preparations 4, 7, 8 and 12) conjugated to diphtheria toxoid (DT; 10 μg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). At 2 wks after the last boost, ^3H -nicotine (0.05mg/kg nicotine containing 3 μCi ^3H -nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ^3H quantified and plasma/brain ratio of ^3H determined.

Figure 4 shows the interaction with nicotine of anti-nicotine antibodies following immunization of mice with vaccines obtained from haptens of the invention. BALB/c mice (n = 12 per group) were immunized with nicotine-derived haptens (from Preparations 4, 7, 8 and 12) conjugated to diphtheria toxoid (DT; 10 μg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). At 2 wks after the last boost, the interaction of anti-nicotine antibodies with nicotine was demonstrated by competitive ELISA.

Figure 5 shows the specificity of anti-nicotine antibodies following immunization of mice with vaccines containing haptens of the invention. BALB/c mice (n = 12 per group) were immunized with nicotine-derived haptens (from Preparations 4 and 12) conjugated to diphtheria toxoid (DT; 10 μg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). At 2 wks after the last boost, specificity of anti-nicotine antibodies to nicotine, cotinine, acetylcholine and varenicline was determined by competitive ELISA.

Figure 6 shows the effect of immunization of mice with anti-nicotine vaccines using different spacers to conjugate the nicotine-derived hapten of the invention to the carrier on anti-nicotine antibody levels in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 4; 5'-aminopropyl nicotine) conjugated to diphtheria toxoid (DT; 10 μg) with conjugates being using different linkers (Preparations 24-34) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and 50 μg CpG 24555. Anti-nicotine IgG Ab levels in plasma were measured by ELISA.

Figure 7 shows the effect of immunization of mice with anti-nicotine vaccines using different spacers to conjugate the nicotine-derived hapten of the invention to the carrier on levels and avidity of anti-nicotine antibodies in plasma and on the distribution of ^3H -nicotine in plasma and brain. BALB/c mice ($n = 12$ per group) were immunized with nicotine-derived hapten (Preparation 4; 5'-aminopropyl nicotine) conjugated to diphtheria toxoid (DT; $10\ \mu\text{g}$) with conjugates being using different linkers (Preparations 24-34) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: $40\ \mu\text{g Al}^{3+}$) and CpG 24555 ($50\ \mu\text{g}$). At 2 wks post 3rd immunization, anti-nicotine IgG Ab levels in plasma were measured by ELISA and avidity was measured by ammonium thiocyanate assay. ^3H -nicotine (0.05mg/kg nicotine containing $3\ \mu\text{Ci } ^3\text{H-nic}$) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ^3H quantified and plasma/brain ratio of ^3H determined.

Figure 8 shows the effect of immunization of mice with anti-nicotine vaccines using different spacers to conjugate the nicotine-derived hapten of the invention to the carrier on anti-nicotine antibody levels in plasma at various time points. BALB/c mice ($n = 12$ per group) were immunized with nicotine-derived hapten (Preparation 12; 5'-aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; $10\ \mu\text{g}$) with conjugates being using different linkers (Preparations 24-34) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: $40\ \mu\text{g Al}^{3+}$) and CpG 24555 ($50\ \mu\text{g}$). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA

Figure 9 shows the effect of immunization of mice with anti-nicotine vaccines using different spacers to conjugate the nicotine-derived hapten of the invention to the carrier on levels and avidity of anti-nicotine antibodies in plasma and on the distribution of ^3H -nicotine in plasma and brain. BALB/c mice ($n = 12$ per group) were immunized with nicotine-derived hapten (Preparation 12; 5'-aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; $10\ \mu\text{g}$) with conjugates being using different linkers (Preparations 24-34) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: $40\ \mu\text{g Al}^{3+}$) and CpG 24555 ($50\ \mu\text{g}$). At 2 wks post 3rd immunization, anti-nicotine IgG Ab levels in plasma were measured by ELISA and avidity was measured by ammonium thiocyanate assay. ^3H -nicotine (0.05mg/kg nicotine containing $3\ \mu\text{Ci } ^3\text{H-nic}$) was administered by IV injection, blood collected,

animals perfused, brains removed, levels of ^3H quantified and plasma/brain ratio of ^3H determined.

Figure 10 shows the effect of succinylation of the hapten-carrier conjugates of the invention on anti-nicotine antibody levels in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 4; 5'-aminopropylnicotine) conjugated to diphtheria toxoid (DT; 10 μg) with conjugates being prepared using 2 different conditions, each with or without a succinic anhydride step, by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA.

Figure 11 shows the effect of succinylation of the hapten-carrier conjugates of the invention, on distribution of ^3H -nicotine in blood and brain. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 4; 5'-aminopropylnicotine) conjugated to diphtheria toxoid (DT; 10 μg) with conjugates being prepared using 2 different conditions, each with or without a succinic anhydride step, by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). At 2 wks after last boost, ^3H -nicotine (0.05mg/kg nicotine containing 3 μCi ^3H -nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ^3H quantified in brain and plasma.

Figure 12 shows the effect of immunization of mice with anti-nicotine vaccines using different spacers to conjugate the nicotine-derived hapten of the invention to the carrier on the anti-nicotine antibody levels in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 12; 5'-aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; 10 μg) or CRM₁₉₇ (10 μg) with conjugates being using different linkers (Preparations 24-34) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and CpG 24555 (50 μg). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA.

Figure 13 shows the effect of immunization of mice with anti-nicotine vaccines on the ^3H -nicotine distribution in mice. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 12; 5'-aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; 10 μg) or CRM₁₉₇ (10 μg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and

CpG 24555 (50 µg). At two weeks post the third immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in brains relative to control animals was determined.

5 **Figure 14** shows the effect of immunization of mice with anti-nicotine vaccines on the anti-nicotine antibody levels in plasma at various time points. BALB/c mice (n = 12 per group) were immunized with nicotine-derived hapten (Preparation 12; 5'aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; 10 µg) or CRM₁₉₇ (10 µg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium
10 hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg). A range of different hapten loadings were evaluated. Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA.

Figure 15 shows the effect of immunization of mice with anti-nicotine vaccines on the ³H-nicotine distribution in mice. BALB/c mice (n = 12 per group) were immunized
15 with nicotine-derived hapten (Preparation 12; 5'aminoethoxy nicotine) conjugated to diphtheria toxoid (DT; 10 µg) or CRM₁₉₇ (10 µg) by intra-muscular vaccination (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg). A range of different hapten loadings were evaluated. At two weeks
20 post the third immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in brains relative to control animals was determined.

Figures 16-18 show the effect of immunization of mice with anti-nicotine vaccines on anti-nicotine antibody levels and avidity in plasma at various time points.
25 BALB/c mice (n = 10 per group) were immunized with nicotine-derived hapten (Preparation 12; 5'aminoethoxy nicotine) conjugated to CRM₁₉₇ (10 µg) by intra-muscular vaccination in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg), or in the presence of ISCOMATRIX (IMX; 0.1 to 3.0 Units). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA (day
30 21 and 28) and avidity was measured by inhibition ELISA. Figure 16 [i] shows results 3 weeks post 1st dose; and [ii] shows results 1 week post 2nd does. Figure 17 shows avidity (IC₅₀) 1 week post 2nd dose. Figure 18 shows sequestration of nicotine in plasma (top); and uptake of nicotine into brain (bottom).

Figures 19 and 20 show the effect of conjugation conditions of the nicotine-derived hapten-carrier conjugates of Table 6 on anti-nicotine antibody levels and corresponding IC₅₀ values. BALB/c mice (n = 10 per group) were immunized with the nicotine-derived hapten by intra-muscular vaccination in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg). Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA and avidity was measured by inhibition ELISA.

Figures 21 and 22 show distribution of ³H-nicotine in blood and brain for the hapten-carrier conjugates of Table 6. BALB/c mice (n = 10 per group) were immunized with the nicotine-derived hapten by intra-muscular vaccination in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and CpG 24555 (50 µg). At one week post the second immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in blood and brains relative to control animals was determined.

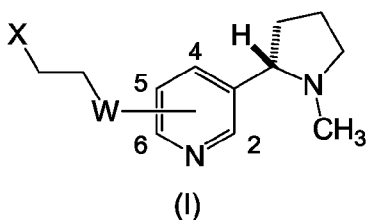
Figure 23 shows the results of testing for binding of the hapten-carrier conjugates with differing percent monomeric carrier protein to CpG/Alhydrogel. Binding was determined by incubating CpG/Alhydrogel with a known amount of hapten-carrier conjugate and then measuring the concentration of conjugate left in solution after incubation. The % decrease in concentration of conjugate is equivalent to % conjugate binding to the CpG/Alhydrogel. BALB/c mice (n = 10 per group) were immunized with 10 µg of different conjugates by intra-muscular injection in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 10 µg CpG 24555. At one week post the second immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in blood and brains relative to control animals was determined.

SEQUENCE LISTING

SEQ ID NO:1 is the nucleotide sequence of immunostimulatory oligonucleotide ODN CpG 24555.

DETAILED DESCRIPTION

In one aspect of the invention relates to a hapten of the formula (I):



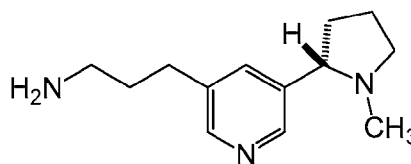
wherein W is -CH₂- or -O-; and X is -NH₂ or -SH.

In one embodiment, W is in position 2, 5 or 6 of the pyridine ring.

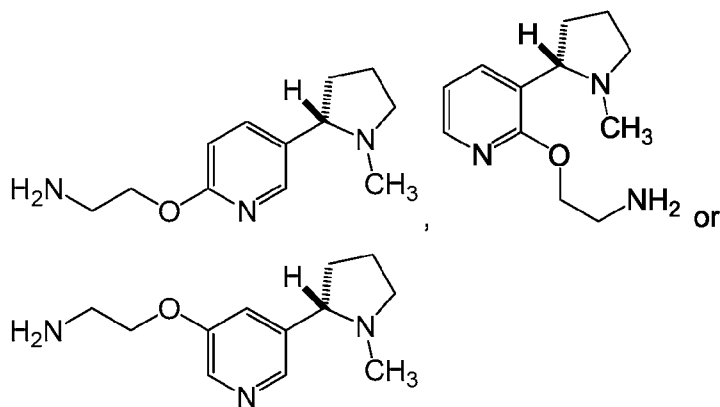
5 In another embodiment, W is in position 5 of the pyridine ring.

In another embodiment, W is -O-.

In another embodiment, W is -O-; and W is in position 5 of the pyridine ring.

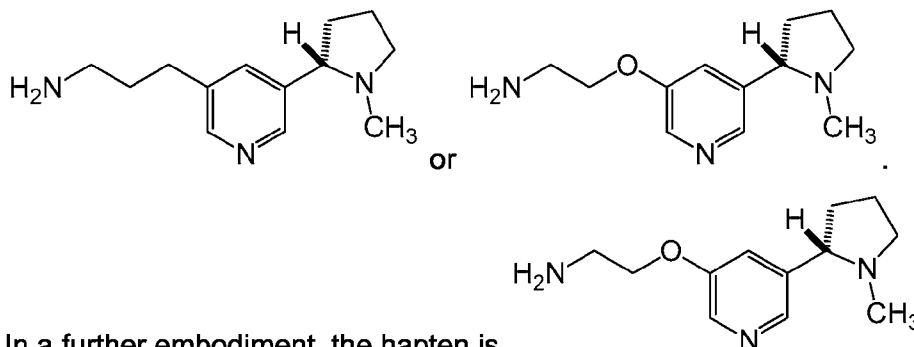


In a further embodiment, the haptin is



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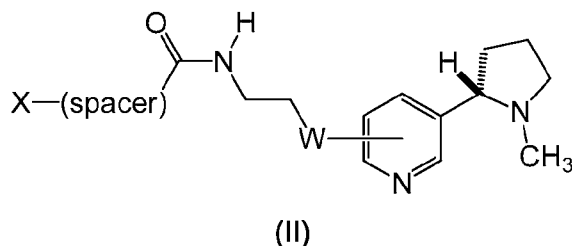
In another embodiment, the haptin is



In a further embodiment, the haptin is

In a second aspect, the invention relates to a haptin-spacer conjugate of the

15 formula (II):



wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; and X is -NH₂ or -SH.

As used herein, an 'alkylene group' is meant a -(CH₂)_n- group in which n is the required number of carbon atoms. As used herein, an 'alkylene group interrupted by 1 to 4 oxygen atoms' is, for example, -CH₂CH₂OCH₂-, -CH₂CH₂OCH₂CH₂OCH₂- or -CH₂CH₂OCH₂CH₂OCH₂CH₂OCH₂CH₂OCH₂CH₂-. As used herein, an 'alkylene group interrupted by 1 to 4 oxygen atoms and interrupted by a -N(H)C(O)-' is, for example, -CH₂CH₂CH₂OCH₂CH₂OCH₂CH₂OCH₂CH₂CH₂NHCOCH₂CH₂-.

In one embodiment, W is in position 2, 5 or 6 of the pyridine ring.

In another embodiment, W is in position 5 of the pyridine ring.

In a further embodiment, W is -O-.

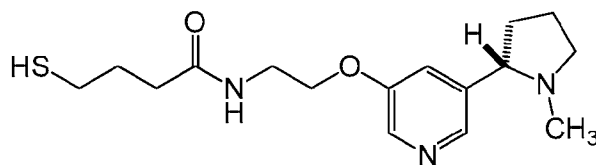
In another embodiment, W is -O-; and W is in position 5 of the pyridine ring.

In one embodiment, -(spacer)- is a C₁-C₆ alkylene group.

In another embodiment, -(spacer)- is a C₁-C₁₀ alkylene group interrupted by 1 to 4 oxygen atoms.

In yet another embodiment, -(spacer)- is a C₁-C₁₂ alkylene group interrupted by 3 oxygen atoms and interrupted by a -N(H)C(O)-.

In one embodiment, the haptens-spacer conjugate is



The following further embodiments are envisaged:

(i) a haptens-spacer conjugate of formula (II) as described above, wherein W is

-O-;

(ii) a haptens-spacer conjugate of formula (II) as described above, wherein W is

-CH₂-;

(iii) a hapten-spacer conjugate of formula (II) as described above or in embodiments (i) and (ii), wherein X is -SH.

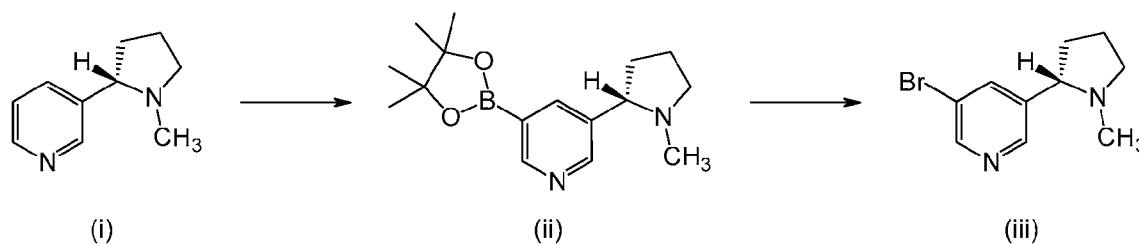
(iv) a hapten-spacer conjugate of formula (II) as described above or in embodiments (i) to (iii), wherein W is in position 2, 5 or 6 of the pyridine ring;

5 (v) a hapten-spacer conjugate of formula (II) as described above or in embodiments (i) to (iv), wherein W is in the 5 position of the pyridine ring;

(vi) a hapten-spacer conjugate of formula (II) as described above or in embodiments (i) to (v), wherein -(spacer)- is a C₁-C₈ alkylene group, a C₆ cycloalkylene group, or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally
10 interrupted by -N(H)C(O)-; and

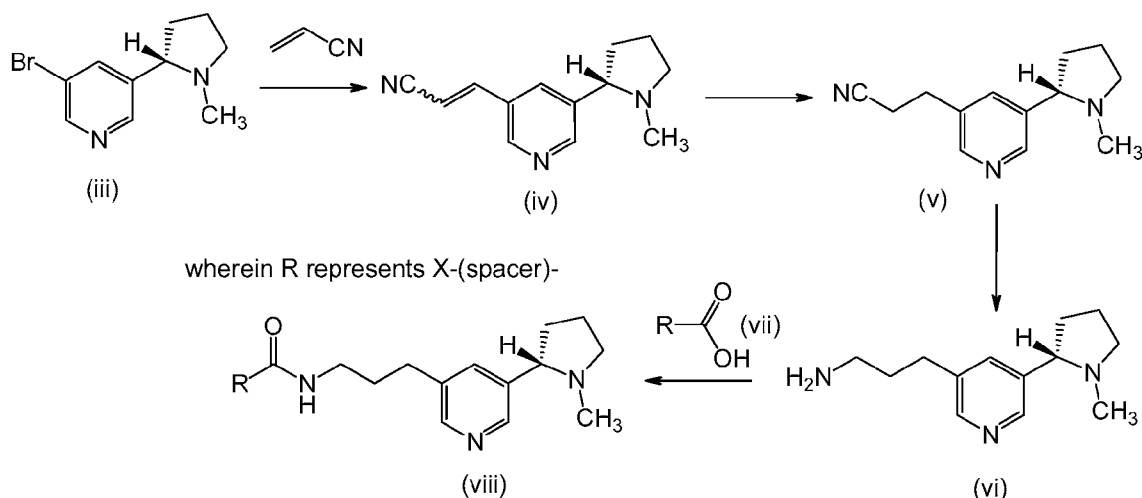
(vii) a hapten-spacer conjugate of formula (II) as described above or in embodiments (i) to (vi), wherein -(spacer)- is a C₁-C₈ alkylene group.

In the following schemes, which depict general methods for obtaining the compounds of formula (I), the substituents are as defined above for the compounds of
15 formula (I) or derivatives thereof, unless otherwise stated:



Scheme 1

Boronate ester (ii) can be formed from the reaction of (S)-(-)-nicotine (i) with a suitable iridium catalyst, typically methoxy(cyclooctadiene)iridium(I) dimer, a ligand,
20 such as 4,4'-di-tert-butyl-2,2'-dipyridyl, and a boron source, such as bis(pinacolato)diboron or 4,4,5,5-tetramethyl-1,3,2-dioxaborolane, in a suitable solvent, such as 1,4-dioxane or THF, at a temperature between room temperature and reflux. Boronate ester (ii) can then be converted to bromide (iii) using copper (II) bromide in a suitable solvent system, such as methanol/water or ethanol/water, at a temperature of,
25 typically, between 60°C and reflux.

**Scheme 2**

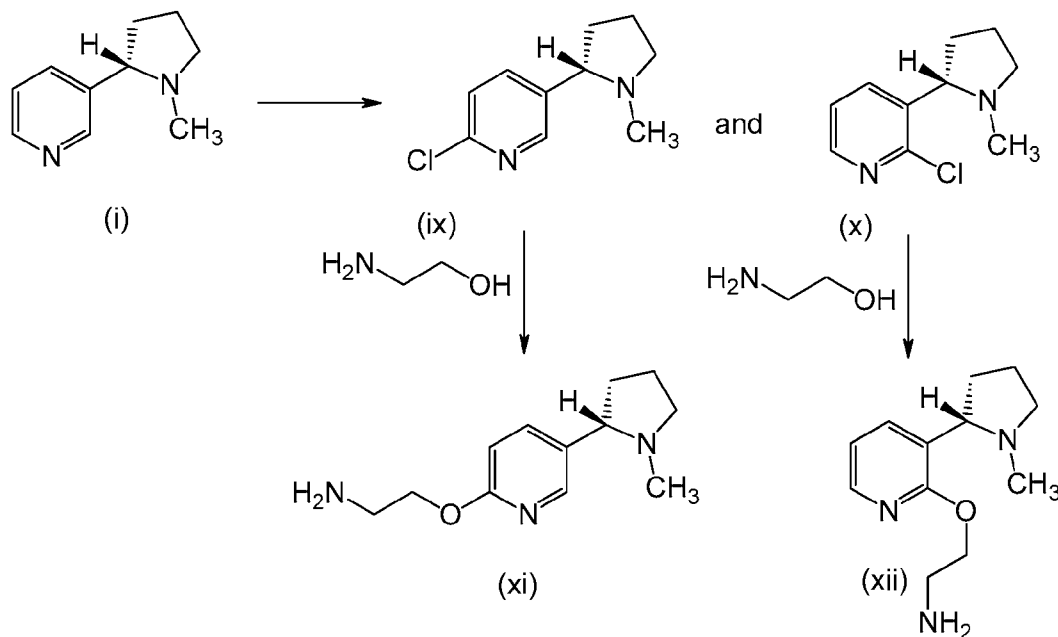
Bromide (iii) can then be converted to unsaturated cyanide (iv) with acrylonitrile, under palladium coupling conditions, using a suitable palladium source, such as palladium (II) acetate or tetrakis(triphenylphosphine)palladium, in the presence of a
 5 suitable phosphine ligand, such as tri(*o*-tolyl)phosphine or trifurylphosphine, in the presence of a suitable base, such as sodium carbonate, triethylamine or *N*-diisopropylethylamine, in a suitable solvent, such as acetonitrile or 1,4-dioxane, at a temperature, typically, around reflux.

Hydrogenation of (iv) to give (v) is typically carried out using a suitable catalyst, such as palladium on carbon, palladium hydroxide on carbon or platinum on activated charcoal, under a hydrogen atmosphere in a suitable solvent, such as methanol, ethanol or ethyl acetate, at a temperature typically around room temperature.

Reduction of nitrile (v) to amine (vi) is typically carried out using a suitable
 15 catalyst, such as Raney Nickel, under a hydrogen atmosphere (typically around 50-100 psi pressure) in a suitable solvent, such as methanol or ethanol, in the presence of concentrated ammonia, at a temperature of typically around 40-70 °C.

The formation of amides of type (viii) can be carried out under standard literature conditions. The acid (vii) can be converted to an acid chloride using a suitable
 20 chlorinating agent, such as oxalyl chloride or thionyl chloride, in a suitable solvent, such as dichloromethane or toluene, optionally in the presence of catalytic DMF, at a suitable temperature, typically between 0°C and room temperature. The acid chloride can then be reacted with the amine (vi) in the presence of a base, such as triethylamine or diisopropylethylamine, in a suitable solvent, such as dichloromethane or toluene, at a
 25 temperature of between 0°C and room temperature. Alternatively the acid (vii) can be

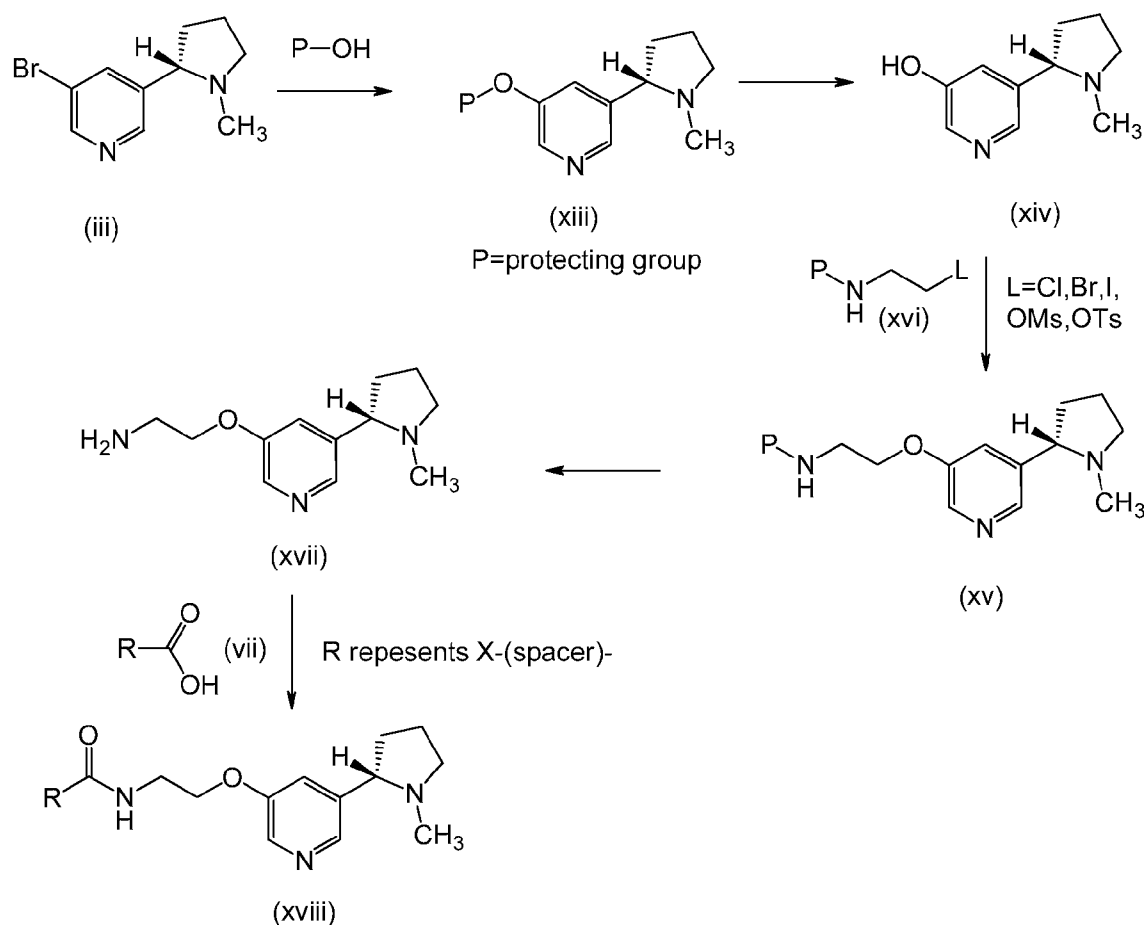
converted to a suitable activated species with a coupling agent, such as T_3P , EDCI.HCl, EDCI.MeI, HBTU, HATU, PyBop, DCC, or CDI, in a suitable solvent, such as dichloromethane or DMF. In the presence of EDCI.HCl or EDCI.MeI, HOBT is optionally added. A suitable base, such as triethylamine or diisopropylethylamine, is also used and the reaction is typically carried out at room temperature.



Scheme 3

Deprotonation of (i) can be carried out with a suitable base, such as the super-base $nBuLi-LiDMAE$ (formed by reaction of *n*-butyllithium with dimethylaminoethanol), in a suitable solvent, such as hexane, toluene, hexane/toluene or hexane/THF, at a suitable temperature, typically of between $-78\text{ }^{\circ}\text{C}$ and $0\text{ }^{\circ}\text{C}$. The resulting anion can be quenched with a suitable chlorine source, such as hexachloroethane or *N*-chlorosuccinimide, at a temperature of between $-78\text{ }^{\circ}\text{C}$ and room temperature, to give the two chloropyridine analogues (ix) and (x).

Chloropyridine analogues (ix) and (x) can be converted to amines (xi) and (xii) using ethanolamine, preferably as solvent and reactant, and using a suitable strong base, such as sodium hydride or potassium *tert*-butoxide, at a temperature of typically between $50\text{--}100\text{ }^{\circ}\text{C}$.

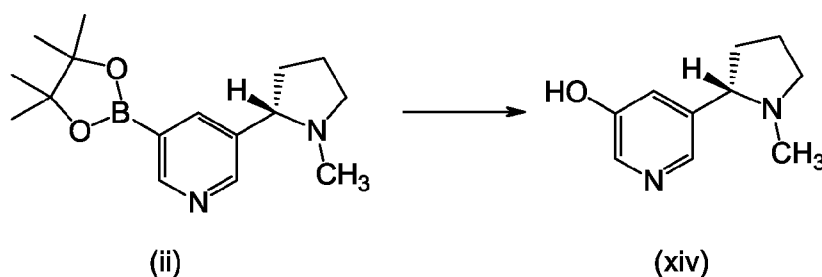


Scheme 4

Bromide (iii) can be reacted with a protecting group carrying alcohol (for example benzyl alcohol or, preferably, p-methoxybenzylalcohol) using a suitable base, typically sodium hydride, in a suitable solvent, such as DMF or NMP, at a temperature of typically around 90-130 °C. Removal of the protecting group to give (xiv) can be carried out using standard literature methods (for example, for the p-methoxybenzylalcohol, a suitable acid such as trifluoroacetic acid can be used).

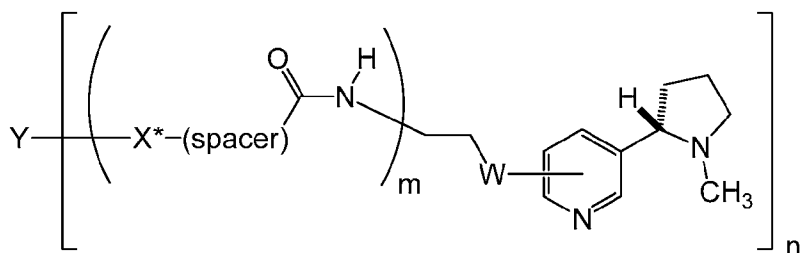
Alcohol (xiv) can be converted to protected amine (xv) (the protecting group is preferably BOC) using a suitable alkylating agent (xvi), such as a halide, mesylate or tosylate, and a base, such as potassium carbonate or caesium carbonate, in a suitable solvent, such as acetonitrile or DMF, at a temperature of typically between 80 °C and reflux. Deprotection of the amine can be carried out, using standard literature methods, to give (xvii) (for example, for the case of the BOC protecting group, the deprotection can be carried out using a suitable acid source such as trifluoroacetic acid or hydrogen chloride in a suitable solvent such as 1,4-dioxane, THF or dichloromethane).

Formation of amides of type (xviii) can be carried out under standard literature conditions. The acid (vii) can be converted to an acid chloride using a suitable chlorinating agent, such as oxalyl chloride or thionyl chloride, in a suitable solvent, such as dichloromethane or toluene, optionally in the presence of catalytic DMF, at a suitable temperature, typically of between 0°C and room temperature. The acid chloride can then be reacted with the amine (xvii) in the presence of a base, such as triethylamine or diisopropylethylamine, in a suitable solvent, such as dichloromethane or toluene, at a temperature of between 0°C and room temperature. Alternatively the acid (vii) can be converted to a suitable activated species with a coupling agent, such as T₃P, EDCI.HCl, EDCI.MeI, HBTU, HATU, PyBop, DCC, or CDI, in a suitable solvent, such as dichloromethane or DMF. In the presence of EDCI.HCl or EDCI.MeI, HOBT is optionally added. A suitable base, such as triethylamine or diisopropylethylamine, is also used and the reaction is typically carried out at room temperature. Alternatively, the amine (xvii) can be reacted with acid anhydrides or lactones to prepare further derivatives of general structure (xviii). For example, γ -butyrolactone or γ -thiobutyrolactone can be used as the acyl source in this step, or for example an anhydride such as succinic or phthalic anhydride to provide derivatives (xviii).

**Scheme 5**

Alcohol (xiv) can also be prepared via boronate ester (ii) using a suitable oxidising agent, typically hydrogen peroxide, and a suitable acid, such as acetic acid, in a suitable solvent, such as THF or 1,4-dioxane.

In a third aspect, the invention relates to a hapten-carrier conjugate of formula (III):

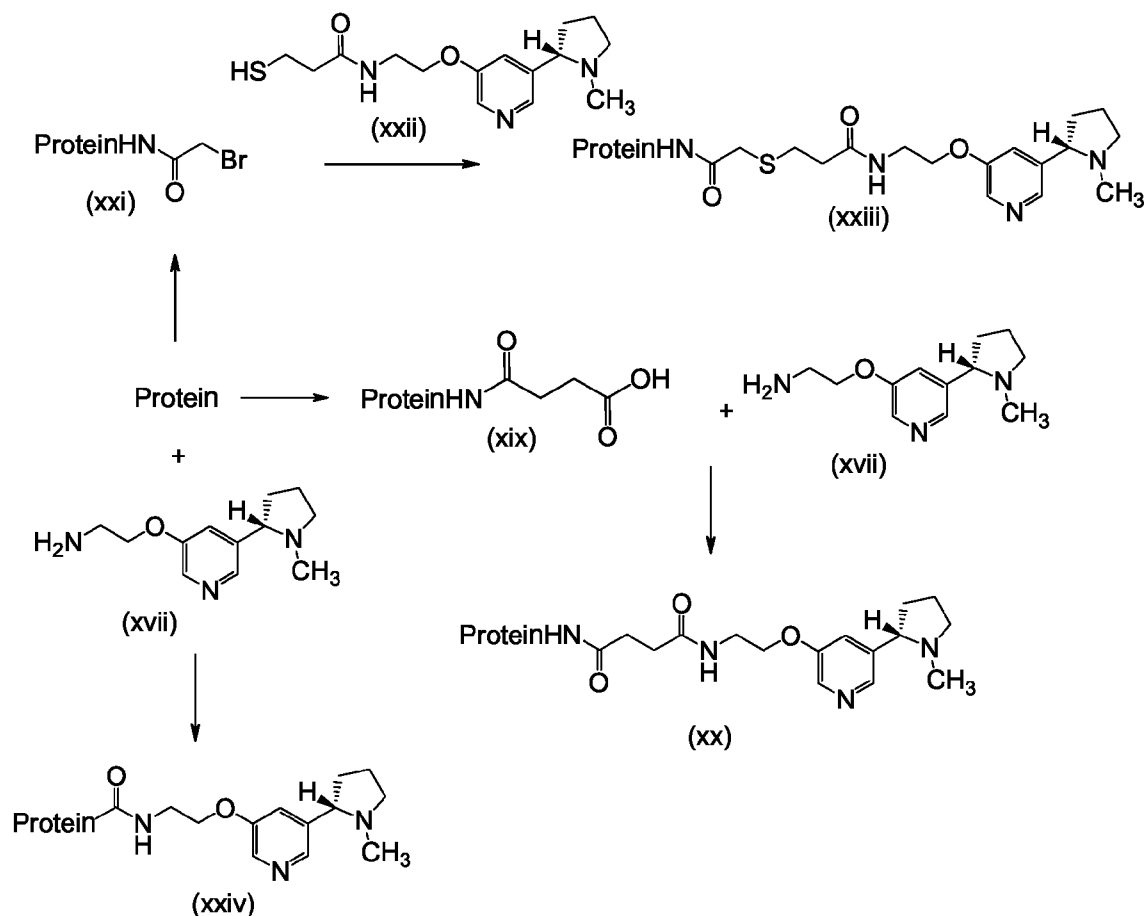


(III)

wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by -N(H)C(O)-, or a C₃-C₁₀ cycloalkylene group; m is 0 or 1; X* is -N(H)- or -S-; n is an integer from 1 to 1000; and Y is an optionally modified carrier protein selected from bacterial toxoids, immunogenic substances, viruses, virus-like particles, protein complexes, proteins, polypeptides, liposomes and immuno-stimulating complexes.

In certain embodiments, Y is a diphtheria toxoid or CRM₁₉₇.

For attachment of the haptens to the carrier proteins, the following methods are illustrative. The carrier protein, such as diphtheria toxoid (DT) or CRM₁₉₇, for example, can be activated by treatment with an anhydride, for example succinic anhydride, to produce a derivatized version of the carrier protein (xix). This derivative can then be coupled to a hapten (xvii) in the presence of a standard coupling reagent by conversion to a suitable activated species with, for example, T₃P, EDCI.HCl, EDCI.Mel, HBTU, HATU, PyBop, DCC, or CDI, in a suitable solvent or buffer (such as Dulbeccos' Phosphate Buffered Saline). In the presence of EDCI.HCl or EDCI.Mel, HOBT or *N*-hydroxysuccinimide (or sulfated versions thereof) is optionally added and the reaction is typically carried out at room temperature to provide the conjugates (xx). Alternatively, the succinylation/derivatization step may be omitted, and direct coupling of the hapten to free carboxyl groups on the carrier protein can be carried out using the above methods to provide the conjugates (xxiv). Alternatively, the carrier protein may be treated with an alternative derivatizing reagent such as bromoacetic acid *N*-hydroxysuccinimide to give a derivatized species (xxi), which may be treated with a thiol-containing hapten (xxii) to provide the conjugate (xxiii).

**Scheme 6**

The following embodiments are envisaged:

- (i) a hapten-carrier conjugate of formula (III) as described above, wherein W is
5 -O-;
- (ii) a hapten-carrier conjugate of formula (III) as described above, wherein W is
-CH₂-;
- (iii) a hapten-carrier conjugate of formula (III) as described above or in
embodiments (i) and (ii), wherein W is in position 2, 5 or 6 of the pyridine ring;
- 10 (iv) a hapten-carrier conjugate of formula (III) as described above or in
embodiments (i) to (iii), wherein W is in the 5 position of the pyridine ring;
- (v) a hapten-carrier conjugate of formula (III) as described above or in
embodiments (i) to (iv), wherein -(spacer)- is a C₁-C₈ alkylene group, a C₆ cycloalkylene
group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally
15 interrupted by -N(H)C(O)-;

(vi) a hapten-carrier conjugate of formula (III) as described above or in embodiments (i) to (iv), wherein -(spacer)- is C₁-C₈ alkylene;

(vii) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (vi), wherein m is 0;

5 (viii) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (vii), wherein the carrier is an optionally modified protein selected from tetanus toxoid, diphtheria toxoid or derivatives thereof such as non-toxic mutant diphtheria toxoid CRM₁₉₇, keyhole limpet hemocyanin (KLH), hemocyanine, albumin, outer membrane protein complex (OMPC) from *Neisseria meningitidis*, the B subunit of
10 heat-labile *Escherichia coli*, recombinant exoprotein A from *Pseudomonas aeruginosa* (rEPA) and virus-like particles such as those assembled from recombinant coat protein of bacteriophage Qb;

(ix) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (viii), wherein the carrier is a protein selected from diphtheria toxoid
15 and CRM₁₉₇, which are optionally modified;

(x) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (ix), wherein the carrier is optionally modified CRM₁₉₇;

(xi) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (x), wherein n is an integer in the range of 1 to 40;

20 (xii) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (x), wherein n is an integer in the range of 10 to 18;

(xiii) a hapten-carrier conjugate of formula (III) as described above or according to embodiments (i) to (xii) or as described above, wherein the carrier protein is a modified succinylated protein.

25 Diphtheria toxin is converted to diphtheria toxoid by incubation at 37°C in the presence of formaldehyde and other excipients for between 4 to 6 weeks. This treatment creates a highly cross-linked protein of heterogeneous molecular weight that renders the protein non-toxic but retains its immunogenicity. The use of this protein as a vaccine carrier protein is well documented, and has been used as an anti-
30 gonadotrophin releasing factor (GnRF) vaccine for pigs, as an alternative to surgical castration (Improvac™, Pfizer). It is also commercially available in an unconjugated form as a human vaccine against diphtheria as part of the DTaP vaccine to treat diphtheria, tetanus and acellular pertussis respectively.

CRM₁₉₇ is a genetically detoxified version of diphtheria toxin, rendered non-toxic by a single point mutation of a glycine residue at position 52 for a glutamic acid residue. The mutation removes the ability of the protein to bind to NAD⁺, and as such, the protein is enzymatically inactive. Due to the absence of cross-linking, the protein is a more homogenous molecular weight product than diphtheria toxoid, a formaldehyde inactivated preparation of diphtheria toxin. CRM₁₉₇ has also been used as a carrier protein for the commercially available anti-pneumococcal vaccine treatment (Pneumovax®, Pfizer).

In a fourth aspect, the invention relates to a method of making nicotine-derived hapten-carrier conjugates as described above, comprising coupling an optionally modified carrier protein with a nicotine-derived hapten of formula (I) or a hapten-spacer conjugate of formula (II), as described above.

In certain embodiments, the attachment of the haptens to the carrier proteins can be done in a way which minimizes the number of carrier proteins which are linked together. In certain embodiments, the number of carrier proteins linked together is less than 5%, less than 10%, less than 15%, less than 20%, less than 25% or less than 30% of the total number of carrier proteins.

In a preferred embodiment, the invention relates to a method of making a nicotine-derived hapten carrier conjugate of formula (III), as described above, wherein X* is -NH-, comprising treating an optionally modified carrier protein with sulfo-N-hydroxysuccinimide, followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, then adding a hapten of formula (I) or a hapten-spacer conjugate of formula (II), as described above, wherein X is -NH₂.

In an alternative embodiment, the invention relates to a method of making a hapten carrier conjugate of formula (III) as described above, wherein X* is -NH-, comprising treating the carrier protein with succinic anhydride to give a modified succinylated carrier protein; treating the modified succinylated carrier protein with sulfo-N-hydroxysuccinimide, followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, then adding a hapten of formula (I) or a hapten-spacer conjugate of formula (II), as described above, wherein X is -NH₂.

In an alternative embodiment, the invention relates to a method of making a hapten carrier conjugate of formula (III), as described above, wherein X* is -S-, comprising treating the carrier protein with bromoacetic acid N-hydroxysuccinimide

ester, then adding a hapten of formula (I) or a hapten-spacer conjugate of formula (II), as described above, wherein X is -SH.

In a fifth aspect, the invention relates to vaccines (or vaccine compositions) comprising a plurality of hapten-carrier conjugates of formula (III), as defined above,
5 and one or more adjuvants.

Examples of suitable adjuvants are those known to enhance antibody responses to antigens, including the antibody responses against the nicotine hapten when it is coupled to a carrier molecule. Adjuvants are well known in the art (J.C. Aguilar, E.G. Rodríguez, Review: Vaccine Adjuvants Revisited, 2007, Vaccine, 25, 3752-3762). The
10 adjuvant may act by one or more mechanisms including direct innate immune activation, creating a depot, or acting as a delivery vehicle for the antigen. The adjuvant that acts by direct innate immune activation may be an agonist for a Toll-like receptor (TLR) including, but not limited to, stabilized poly I:C that activates TLR3, a derivative of lipopolysaccharide such as monophosphoryl-lipid A (MPL) or Glycopyranosyl Lipid
15 Adjuvant (GLA) that activate via TLR4, flagellin that activates via TLR5, small molecules of the imidazoquinoline family such as Imiquimod or resiquimod that activate via TLR7 or TLR8 or both TLR7 and TLR8, oligoribonucleotides (ORN) that activate via TLR7 and/or TLR8, and oligodeoxynucleotides (ODN) containing CpG motifs that activate via TLR9. The CpG ODN TLR9 agonists may be of the A-Class, B-Class, C-Class or P-
20 Class, with or without halogenation of the 5'T known as an E modification, and may be made with a wholly phosphodiester backbone, a wholly phosphorothioate backbone, a chimeric backbone, a "semi-soft" backbone that is wholly phosphorothioate except between the cytosines and guanosines of the CpG motif. The adjuvant that acts by direct innate immune activation may act through a non-TLR mechanism, such as QS21
25 or other saponins.

The adjuvant may be an aluminium salt that act as both a depot system as well as an innate immune activator via the inflammasome. The aluminium salt is preferentially selected from aluminium hydroxide or aluminium phosphate. The aluminium hydroxide is preferentially Alhydrogel original or Alhydrogel'85.

30 The adjuvant that acts through both immune activation and delivery vehicle may be an immune stimulatory complex (ISCOM) such as ISCOMATRIX.

The adjuvant that has delivery vehicle properties may be macromolecular complexes, nanocapsules, nanoparticles, microspheres, microparticles, or virosomes and these may have moieties on their surfaces for the purpose of targeting to specific

cell types. The adjuvant may be a lipid-based system including oil-in-water emulsions, water-in-oil emulsions, micelles, mixed micelles, and liposomes. Liposomes may be unilamellar or multilamellar. The emulsion may be squalene based such as MF-59.

The adjuvant may be a virosome.

5 Preferred adjuvants are selected from CpG oligodeoxynucleotides (CpG ODN), aluminium salts, QS21 and ISCOMS.

Preferred CpG ODN are of the B Class that preferentially activate B cells. In aspects of the invention, the CpG ODN has the nucleic acid sequence

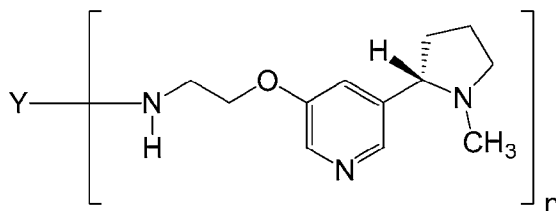
5' T*C*G*T*C*G*T*T*T*T*C*G*G*T*G*C*T*T*T*T 3' (SEQ ID NO:1) wherein *
10 indicates a phosphorothioate linkage. The CpG ODN of this sequence is known as CpG 24555.

As used herein, the term "oligodeoxynucleotide" (ODN) means multiple nucleotides (i.e., molecules comprising a deoxyribose sugar linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine
15 (e.g., cytosine (C) or thymidine (T)) or a substituted purine (e.g., adenine (A) or guanine (G)). Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by nucleic acid synthesis). Oligonucleotides having phosphorothioate linkages are relatively resistant to degradation in vivo (e.g., via endo- and exo-nucleases), providing enhanced activity in
20 vivo.

Methods for synthesis and chemical modification of oligonucleotides are known to the skilled person and are described, for example in Uhlmann E. *et al.* (1990), Chem. Rev. 90:543; "Protocols for Oligonucleotides and Analogs" Synthesis and Properties & Synthesis and Analytical Techniques, S. Agrawal, Ed., Humana Press, Totowa, USA
25 1993; Crooke, S.T. *et al.* (1996) Annu. Rev. Pharmacol. Toxicol. 36:107-129; and Hunziker J. *et al.*, (1995), Mod. Synth. Methods 7:331-417. The oligonucleotides of the invention can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S. L., and Caruthers, M. H., (1981) Tet. Let. 22:1859); nucleoside H-phosphonate method
30 (Garegg *et al.*, (1986) Tet. Let. 27:4051-4054; Froehler *et al.*, (1986) Nucl. Acid Res. 14:5399-5407; Garegg *et al.*, (1986) 27:4055-4058; Gaffney *et al.*, (1988) Tet. Let. 29:2619-2622). These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These oligonucleotides are referred to as synthetic oligonucleotides. Modified backbones such as phosphorothioates may be

synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863, and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (e.g. Uhlmann, E. and Peyman, A., Chem. Rev. 90:544, 1990; Goodchild, J., Bioconjugate Chem. 1:165, 1990).

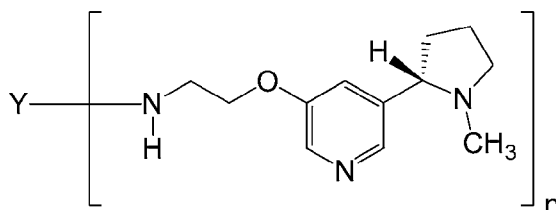
The most preferred adjuvants are CpG 24555 (as described in co-pending application number PCT/IB2009/055444, hereby incorporated by reference in its entirety) used together with an aluminium hydroxide salt such as Alhydrogel. Thus, in one embodiment there is provided a vaccine (or vaccine composition) comprising a hapten-carrier conjugate of formula (III) as defined above, and CpG 24555. In a further embodiment there is provided a vaccine (or vaccine composition) comprising a hapten-carrier conjugate of formula (III) as described above, CpG 24555 and an aluminium hydroxide salt. In another embodiment there is provided a vaccine (or vaccine composition) comprising CpG 24555 and a plurality of hapten-carrier conjugates of formula (IV):



(IV)

wherein, Y is diphtheria toxoid or CRM₁₉₇ and n is an integer in the range of 1 to 40.

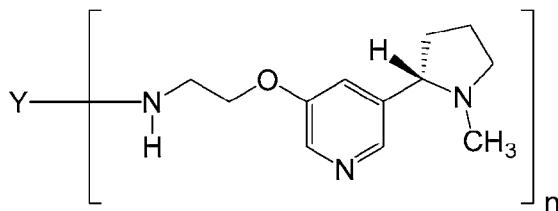
In yet another embodiment there is provided a vaccine (or vaccine composition) comprising CpG 24555, an aluminium hydroxide salt, and a plurality of hapten-carrier conjugates of formula (IV):



(IV)

wherein, Y is diphtheria toxoid or CRM₁₉₇ and n is an integer in the range of 1 to 40.

In yet another embodiment there is provided a vaccine (or vaccine composition) comprising an aluminium hydroxide salt (e.g. Alhydrogel), and a plurality of hapten-carrier conjugates of formula (IV):



(IV)

wherein, Y is diphtheria toxoid or CRM₁₉₇ and n is an integer in the range of 1 to 40.

In certain embodiments, n is an integer in the range of 1 to 30 inclusive. In certain embodiments, n is an integer in the range of 5 to 23 inclusive. In certain embodiments, n is an integer in the range of 10 to 18 inclusive. In certain embodiments, n is 10. In certain embodiments, n is 11. In certain embodiments, n is 12. In certain embodiments, n is 13. In certain embodiments, n is 14. In certain embodiments, n is 15. In certain embodiments, n is 16. In certain embodiments, n is 17. In certain embodiments, n is 18.

By varying the coupling conditions which form the hapten-carrier conjugate, see Exemplification below, the cross-coupling of carrier proteins can be minimized. When carrier proteins cross-couple, the resulting hapten-carrier conjugate comprises multiple carrier proteins covalently or non-covalently bound together and is referred to herein as a "high molecular mass species." In contrast, a "low molecular mass species," as used herein, refers to hapten-carrier conjugates wherein a portion of the carrier protein is lost during the preparation of the conjugate. In certain embodiments it has been observed that high molecular weight species lead to a less effective vaccine. Therefore, in certain embodiments, the present invention relates to any of the aforementioned vaccine compositions wherein less than 5%, less than 10%, less than 15%, less than 20%, less than 25% or less than 30% of the carrier proteins which are a part of the hapten-carrier conjugates are cross-coupled (i.e. are high molecular mass species).

In certain embodiments, the present invention relates to any of the aforementioned vaccine compositions wherein at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the carrier proteins which are part of the hapten-carrier conjugates are not cross-coupled.

In certain embodiments, more than 70%, more than 75%, more than 80%, more than 85%, more than 90% or more than 95% of the antigenic components of any one of the aforementioned vaccine compositions are hapten-carrier conjugates of formula IV.

In certain embodiments, the present invention relates to any of the
5 aforementioned vaccine compositions wherein at least 70%, at least 75%, at least 80%,
at least 85%, at least 90% or at least 95% or the carrier proteins have molecular
weights between 50,000 Daltons and 70,000 Daltons. In certain embodiments, the
present invention relates to any of the aforementioned vaccine compositions wherein at
least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% or the
10 carrier proteins have molecular weights of about 58,000 Daltons.

The vaccine compositions of the present invention may optionally contain one or
more pharmaceutically acceptable excipients. Suitable excipients include sterile water,
salt solutions and buffers. In one embodiment, the hapten-carrier conjugate is
solubilised in an aqueous, saline solution at a pharmaceutically acceptable pH. The
15 vaccine composition may also optionally contain at least one auxiliary agent, such as
dispersion media, coatings, surfactants, preservatives and stabilizers. The vaccine
composition of the present invention preferably is sterile.

The vaccine composition of the present invention will be generally administered
for both priming and boosting doses. It is expected that an initial series will include
20 several doses that will be spaced several weeks apart with additional boosting doses
being spaced months or years apart, or at such times where the levels of circulating
antibody fall below a desired level that has been shown clinically to correlate with
enhanced quit rates. Preferably the vaccine composition of the present invention is
administered as an initial series of 3 to 6 doses administered over 6 to 24 weeks and
25 boosting doses are administered every 3 to 12 months thereafter.

The vaccine composition of the present invention may be administered by a
parenteral route, for example via intramuscular (IM), intradermal (ID) or subcutaneous
(SC) injection, or, if using an appropriate device, by topical administration to the skin
(transdermal) or a mucosal surface.

30 In certain embodiments, hapten carrier conjugates and/or vaccines of the
invention can be administered intradermally (ID) through the use of microneedle devices
In one aspect, the invention provides a microneedle device comprising one or more
microneedles that is coated with, contains, or is effective to deliver a nicotine-derived
hapten-carrier conjugate or a vaccine composition of the invention. In certain

embodiments, the one or more microneedles can be prepared as patches wherein the microneedles allow simple non-invasive administration to the skin. Another aspect of the invention contemplates the use of microneedle technology for delivery of a nicotine-derived hapten-carrier conjugate or a vaccine composition of the invention.

5 Microneedle delivery technologies can facilitate delivery of vaccine compositions containing nicotine-derived hapten-carrier conjugate to specific and desired skin depths using arrays of short (e.g., less than 4 mm in length) needle(s). Such microneedles pierce the stratum corneum and underlying layers of the epidermis to present drug directly into the epidermis or adjacent dermis. Due to the small size of microneedles,
10 application is relatively pain-free, with minimal (if any) bleeding or application site reaction. Herein, the term "microneedle" refers to an elongated structure that is sufficiently long to penetrate through the stratum corneum skin layer and into the epidermal/dermal layer, but sufficiently short to not result in substantial pain due to activation of nerve endings.

15 Microneedles that facilitate transdermal delivery are described in Prausnitz, Advanced Drug Delivery Reviews 56 (2004) 581-587; Zahn et al., Biomed. Microdevices 6 (2004) 183-190; Shirkhandeh J. Materials Sci. 16 (2005) 37-45; Park, J. Controlled Release 104 (2005) 51-66; US Patent Nos. 3,964,482, 6,503,231, 6,745,211; 6,611,707; 6,334,856; and US Patent Application Publication Nos. 2005/0209565,
20 2004/0106904, 2004/0186419, 2002/0193754 and 2010/0196445; all of which are hereby incorporate by reference in their entireties. Suitable microneedles have been fabricated from many materials, including silicon, metals, and polymers. Davis et al. describe the mechanics of microneedle insertion into the skin (Davis et al., J. Biomech. 37 (2004) 1155-1163).

25 Solid or hollow microneedles can be used in the embodiments described herein. In one embodiment, the microneedles for use in the invention are solid. For example, channels can be made by penetrating the skin with a microneedle array, followed by removal of the needles and subsequent application of the drug (see, e.g., Martanto et al., Pharm. Res. 21 (2004) and McAllister et al., PNAS 100 (2003) 13755-13760). The
30 formulation comprising therapeutic agent according to the invention may be applied to the microneedle-treated site as a gel, hydrogel, topical cream, salve, ointment, or other topical formulation; and/or by using delivery devices such as bandages, occlusive bodies, patches, and/or the like.

In another embodiment, solid (non-porous) microneedles are coated with a nicotine-derived hapten-carrier conjugate or a vaccine composition according to the invention prior to application to the skin. The epidermis is then punctured using the microneedles, which are kept in contact with the skin surface for a sufficient period of
5 time, allowing diffusion of the hapten-carrier conjugate or a vaccine composition into the surrounding skin tissue. (For administration of a therapeutic agent in such a fashion, see Prausnitz, *Advanced Drug Delivery Reviews* 56 (2004) 581-587). In another embodiment, hollow (porous) microneedles are used, which contain channels that allow storage of a hapten-carrier conjugates or a vaccine composition of the invention. Upon
10 application to skin, the hapten-carrier conjugate or vaccine composition diffuses into the skin tissue by diffusion or pressure-driven flow. (For administration of a therapeutic agent in such a fashion, see Zahn et al., *Biomed. Microdevices* 6 (2004) 183-190).

In yet another embodiment, in contrast to conventional hollow needle technologies, certain aspects of the invention relate to microneedles formed from a solid
15 matrix of dissolvable and/or biodegradable material which can be used to deliver vaccine compositions of the invention. For examples of solid microneedles see U.S. Patent and Published Application Nos. 6,945,952, 7,611,481, 2002/0082543, 2005/0197308 and 2008/0269685, all of which describe microneedle arrays made of biodegradable polymers and are hereby incorporated by reference in their entireties.

20 In certain embodiments, the solid microneedles can be composed of fast-dissolving and/or swelling materials, including thermoforming polymer materials that are synthetically and/or naturally derived. In certain embodiments, the solid microneedles can be formed from suitable biocompatible, biodegradable polymers such as poly(lactide)s, poly(glycolide)s, poly(lactide-co-glycolide)s, polyanhydrides,
25 polyorthoesters, polyetheresters, polycaprolactones, polyesteramides, poly(butyric acid), poly(valeric acid), polyurethanes and copolymers and blends thereof. In other embodiments, the solid microneedles can be formed from non-biodegradable polymers such as polyacrylates, polymers of ethylene-vinyl acetates and other acyl substituted cellulose acetates, non-degradable polyurethanes, polystyrenes, polyvinyl chloride,
30 polyvinyl fluoride, poly(vinyl imidazole), chlorosulphonate polyolefins, polyethylene oxide, blends and copolymers thereof.

Microneedles can be used alone or as arrays of more than one microneedle. Various sizes of arrays are suitable for use with the invention. In one embodiment, 1-10 microneedles are used. In other embodiments, a microneedle array comprising 10-25,

10-50, 25-50, 25-200, 25-100, or 50-100 needles is used. An array of microneedles can vary on the basis of several factors, including but not limited to, length, diameter, interneedle distance, sharpness, and the total number of microneedles used. In an exemplary embodiment, an array of microneedles comprises a 10x10 matrix. In another
5 embodiment, an array of microneedles comprises a 20x20 matrix. In some embodiments, the distance between each microneedle in an array is from approximately 100 μm to approximately 400 μm . In an embodiment, the particular dimensions of the array can be chosen depending on the desired enhancement of skin permeability.

In some embodiments, a microneedle has a length from 20 μm to approximately
10 1000 μm , for example from approximately 50 μm to approximately 150 μm , or from approximately 150 μm to approximately 500 μm , or from 500 μm to approximately 1000 μm , or from 600 μm to approximately 800 μm . In other embodiment, a microneedle is approximately 50, 100, 250, 500, 600, 700, 800, 900 or 1000 μm in length. In still other embodiments, the microneedle is at least 50, 100, 250, 500, 600, 700, 800, 900 or 1000
15 μm in length. In other embodiments, the microneedle is less than 50, 100, 250, 500, 600, 700, 800, 900 or 1000 μm in length. In one embodiment, the microneedle is approximately 700 μm in length. In some embodiments, the microneedle penetrates skin at a depth of approximately 400 μm to approximately 700 μm . In an embodiment, the microneedle penetrates skin at a depth approximately corresponding to the bottom
20 of the stratum corneum. In another embodiment, the microneedle penetrates skin to approximately the top of the dermal layer. In still other embodiments, the microneedle penetrates skin up to a depth approximately in between the bottom of the stratum corneum and the top of the dermal layer. The microneedle may be any of a variety of diameters as needed to maintain efficacy. In some embodiments, the outer diameter of
25 a microneedle can be from approximately 20 μm to approximately 100 μm . In other embodiments, the outer diameter of a microneedle can be from approximately 10 μm to approximately 50 μm . The inner diameter of a hollow microneedle can be from approximately 5 μm to approximately 70 μm in some embodiments. In addition the outer or inner diameters of a microneedle can be up to 10, 20, 30, 40, 50, 60, 70, 80, 90, or
30 100 μm . Any combination of the above microneedle dimensions may be used as necessary with the systems and methods described herein.

A microneedle can be manufactured from a variety of materials, including but not limited to, silicon, a metal, a polymer, and glass. In some embodiments, silicon microneedles are used. Silicon microneedles, whether solid or hollow, can be etched

from silicon wafers. For example, the location of each microneedle is marked and the surrounding silicon is etched away, resulting in an array of microneedles attached to a common base. In some embodiments, the thickness of a silicon wafer is between approximately 300-600 μm .

5 In other embodiments, microneedles are made of a metal, including but not limited to nickel, titanium, and alloys such as stainless steel. In some embodiments, metal microneedles are made from epoxy molds which are then electroplated with a chosen metal, while the epoxy mold is subsequently etched away. The resulting microneedles may either be reusable or disposable. Microneedles may also be obtained
10 from commercial sources, including Zosano Pharma, Inc., Corium and 3M. Both Zosano and 3M have developed coated microneedle-containing patches. Corium has developed biodegradable microneedles. 3M has developed hollow microneedles (e.g. Intanza® / IDflu®).

 The hapten-carrier conjugate or vaccine composition can be applied to
15 microneedles using a variety of methods. In one embodiment, a solution comprising the hapten-carrier conjugate or vaccine composition is prepared and the solution is deposited onto/within the microneedles, followed by drying of the solution. Alternatively, the microarray is dipped into a solution comprising the hapten-carrier conjugate or vaccine composition, resulting in coating of the microneedles with the hapten-carrier
20 conjugate or vaccine composition. When additional proteins or components are to be coated onto or within the microneedles of the invention, additional coating steps may be performed. Alternatively, several or all components of the solution are mixed into one solution which is then deposited onto/within the microneedle. Dip coating, spray coating, or other techniques known in the art may be used, for example those described in PCT
25 Pub. No. WO 2006/138719, which is hereby incorporated by reference in its entirety. Coatings may be solid or semi-solid.

 The amount of hapten-conjugated carrier protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Suitable dosage ranges are 0.01 to
30 10 mg/dose, preferably 0.1 to 1.0 mg/dose. It generally takes a person two or more weeks to generate antibodies against a foreign antigen after a single vaccine dose, and it generally requires several vaccine doses administered over several weeks to induce high sustained antibody titers such as those desired for an anti-nicotine vaccine to aid in smoking cessation. The production of antibodies in a person's blood can be monitored

by using techniques that are well-known to the skilled artisan, such as ELISA, radioimmunoassay, surface plasma resonance, and Western blotting methods.

Thus, in a sixth aspect, the present invention relates to a hapten-carrier conjugate, or a vaccine composition as defined above, for use as a medicament.

5 In yet a further aspect, the present invention relates to a hapten-carrier conjugate, or a vaccine composition as defined above, for use in increasing quit rates and reducing relapse rates in smokers wishing to quit, ex-smokers wishing to avoid relapse, or for the prevention of nicotine addiction.

10 In yet a further aspect, the present invention relates to a nicotine-derived hapten-carrier conjugate, or a vaccine composition, as defined above, for the manufacture of a medicament for the treatment or prevention of nicotine dependence in a person in need of such treatment.

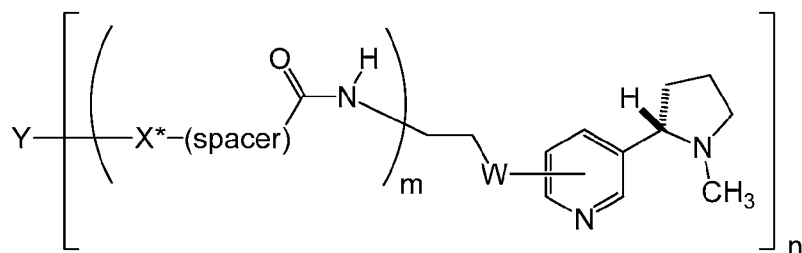
15 In yet a further aspect, the present invention related to a nicotine-derived hapten-carrier conjugate, or a vaccine composition, as defined above, for use in treating, or preventing, nicotine addiction in a person in need of such treatment.

20 In yet a further aspect, the present invention relates to a treatment method for aiding smoking cessation in smokers wishing to quit, or preventing relapse in smokers who have quit, or preventing nicotine addiction in person who might be exposed to smoking or nicotine from another source, the method comprising administering to the person an effective amount of a hapten-carrier conjugate, or a vaccine composition, as defined above.

25 In one embodiment, the method further comprises administering to the person another non-vaccine smoking cessation product. Suitable products include pharmacotherapy products that targets nicotinic acetylcholine receptors, such as varenicline, or bupropion, optionally in sustained-release form. Other products that can be used in the method of the invention include clonidine and nortriptyline. Additional suitable products include nicotine replacement therapy (NRT) products, in the form, for example, of a patch (16 h and 24 h), a gum, a nasal spray or an inhaler.

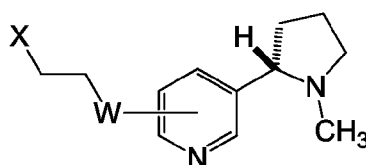
30 In yet a further aspect, the present invention relates to a method of making a hapten carrier conjugate according to the invention, comprising coupling an optionally modified carrier protein with a hapten according to the invention or a hapten-spacer conjugate according to the invention.

In yet a further aspect, the present invention relates to a method of making a hapten carrier conjugate of formula (III):



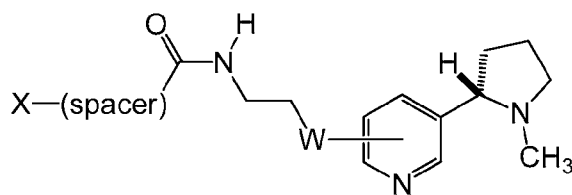
(III)

wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; X* is -NH-; m is 0 or 1; n is an integer from 1 to 1000; and Y is an optionally modified carrier protein selected from bacterial toxoids, immunogenic substances, viruses, virus-like particles, protein complexes, proteins, polypeptides, liposomes and immuno-stimulating complexes; comprising treating an optionally modified carrier protein with sulfo-N-hydroxysuccinimide, followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, then adding either a hapten of the formula (I):



(I)

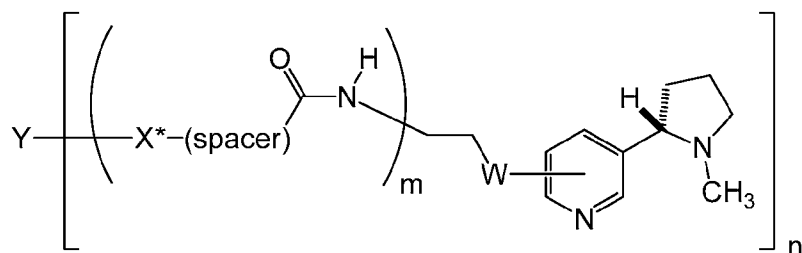
wherein W is -CH₂- or -O-; and X is -NH₂; or a hapten-spacer conjugate according to formula (II):



(II)

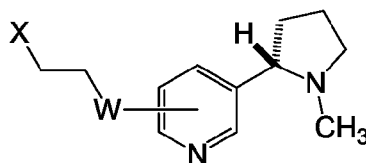
wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; and X is -NH₂.

In yet a further aspect, the present invention relates to a method of making a conjugate of formula (III):



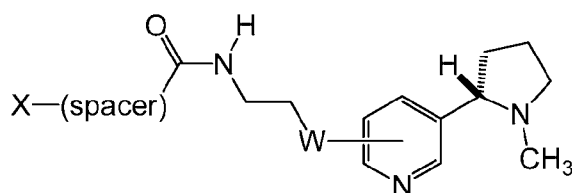
(III)

wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; X* is -NH-; m is 0 or 1; n is an integer from 1 to 1000; and Y is an optionally modified carrier protein selected from bacterial toxoids, immunogenic substances, viruses, virus-like particles, protein complexes, proteins, polypeptides, liposomes and immuno-stimulating complexes; comprising treating the carrier protein with succinic anhydride to give a modified succinylated carrier protein; treating the modified succinylated carrier protein with sulfo-N-hydroxysuccinimide, followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, then adding either a hapten of the formula (I):



(I)

wherein W is -CH₂- or -O-; and X is -NH₂; or a hapten-spacer conjugate according to formula (II):



(II)

wherein W is -CH₂- or -O-; -(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; and X is -NH₂.

BIOLOGICAL ASSAYS

Anti-nicotine antibody ELISA. The levels of anti-nicotine antibodies in mouse plasma were quantified using an ELISA assay as follows. Since the nicotine molecule is not suitable for coating to ELISA plates, it was therefore linked to a larger molecule (bovine serum albumin) having bioadhesive properties. The nicotine derivative (rac-trans3'-thio methyl nicotine) was conjugated to bovine serum albumin and the nicotine-BSA conjugate obtained was used to coat 96 well Immuno Maxi-Sorp ELISA plates (VWR) (100 µL/well) at a final concentration of 1 µg/mL in carbonate buffer (Sigma-Aldrich), and incubated overnight at 4°C. The plates were then aspirated and washed with phosphate buffered saline containing 0.05% Tween[®] 20 (Sigma-Aldrich, P3563). Plates were blocked with 200 µL of blocking buffer (carbonate buffer + 10% Bovine Calf Serum, Fisher) at 37°C for 1 hour and then washed as above. Samples and reference plasma were serially diluted in dilution buffer (1X PBS with 0.05% Tween[®] 20 + 10% BCS) and added to the plates (100 µL/well). The plates were incubated at 37°C for 2 hours. They were washed again and then incubated with goat Anti-mouse IgG-HRP (Southern Biotech), diluted with dilution buffer (1X PBS with 0.05% Tween[®] 20 + 10% BCS) for 1 hour at 37°C. The plates were then washed again and incubated with O-phenylenediamine dihydrochloride (OPD) substrate (1 x 5 mg OPD tablet dissolved in Phosphate Citrate Buffer, Sigma-Aldrich) in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 µL of 4N sulphuric acid (VWR) to each well and read at 450 nm using an automated plate reader. The samples were quantified as the highest plasma dilution that resulted in an antibodiesorbance value (OD 450) two times greater than that of non-immune plasma, with a cut-off value of 0.05.

Measurement of avidity of anti-nicotine antibodies. The avidity of anti-nicotine antibodies was measured using an ammonium thiocyanate elution based ELISA method as follows. 96 well Immuno Maxi-Sorp ELISA plates (VWR) were coated (100 µL/well) with a Nicotine-BSA antigen solution at a concentration of 1 µg/mL in carbonate buffer (Sigma-Aldrich) and incubated overnight at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween[®] 20 (Sigma-Aldrich), and then blocked with 200 µL carbonate buffer + 10% bovine calf serum (Fisher) for 1 hour at 37°C. The plates were washed again. Plasma samples, previously determined to contain anti-nicotine antibodies, were diluted in PBS containing 0.05% Tween[®] 20 + 10% bovine calf serum (BCS) to achieve optimal antibodiesorbance values of approximately 1.0 at 450 nm, and were then added to the plates at 100

$\mu\text{L}/\text{well}$. The plates were incubated for 2 hours at 37°C and then washed. Next, elution was performed by adding ammonium thiocyanate (NH_4SCN) ($100 \mu\text{L}/\text{well}$) in concentrations ranging from 0 to 2.0 M diluted in PBS/0.05% Tween[®] 20 and incubated for 15 min at room temperature. The plates were then washed and antibody binding was detected using goat anti-mouse IgG-HRP (Southern Biotech) diluted with dilution buffer (1X PBS with 0.05% Tween[®] 20 + 10% BCS) for 1 hour at 37°C . The plates were then washed again and incubated with O-phenylenediamine dihydrochloride (OPD) substrate (1 x 5 mg OPD tablet dissolved in Phosphate Citrate Buffer, Sigma-Aldrich) in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 μL of 4N sulphuric acid (VWR) to each well and read at 450 nm using an automated plate reader. The results were then expressed as the percent reduction in binding of antigen-antibody (% reduction in OD) in the presence of NH_4SCN and plotted against the molar concentration of NH_4SCN . The avidity index was calculated as the concentration of NH_4SCN required to produce 50% reduction in binding.

Evaluation of nicotine distribution in plasma and brain. The effect of immunization on nicotine distribution in the plasma and brain was determined by administering to animals (pre-immunized with an anti-nicotine vaccine) 0.05 mg/kg of (-) nicotine hydrogen tartrate containing $3\mu\text{Ci } ^3\text{H-nicotine}$ in 100 μL of PBS over 10 seconds via tail vein infusion. Blood was obtained 5 min later via cardiac puncture and the plasma was collected. The mouse was immediately perfused with PBS by injecting 20mL into the left ventricle of the heart over 1 to 2 min and the brain was harvested and weighed. The brain was digested at $\sim 50^{\circ}\text{C}$ for 72 hours in 1mL digestion buffer (100mM sodium chloride, 25mM Tris, 25mM EDTA, 0.5% Igepal CA-630 and 0.3mg/mL proteinase K per 100mg tissue). 100 μL aliquots of brain digest or plasma were mixed with 5mL liquid scintillation fluid and levels of radiolabelled nicotine were determined by liquid scintillation counting.

Competition ELISA to determine specificity of antibodies induced by anti-nicotine vaccines. The specificity of anti-nicotine antibodies was determined using a competition ELISA as follows. 96 well Immuno Maxi-Sorp ELISA plates (VWR) were coated (100 $\mu\text{L}/\text{well}$) with a Nicotine-BSA antigen solution at a concentration of 1 $\mu\text{g}/\text{mL}$ in carbonate buffer (Sigma-Aldrich) and incubated overnight at 4°C . The plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween[®] 20 (Sigma-Aldrich), blocked with 200 μL carbonate buffer + 10% bovine calf serum (Fisher) for 1 hour at 37°C and then washed again. During this incubation, plasma samples,

previously determined to contain anti-nicotine antibodies, were diluted in PBS containing 0.05% Tween[®] 20 + 10% bovine calf serum (BCS) to achieve optimal antibodiesorbance values of approximately 1.0 to 1.5 at 450 nm, and different inhibitors (nicotine, cotinine, acetylcholine, varenicline) were diluted serially starting at 20,000

5 μ M. Equal volumes (65 μ L) of diluted samples and selected inhibitor were added to wells of a non-coated 96 well plate and allowed to incubate for 1 hour at 37°C. Following incubation, the plasma /inhibitor mixtures were added at 100 μ L/well to the previously blocked Nicotine-BSA coated plates. The plates were incubated for 30 min at 37°C and then washed again. Antibody binding was detected using goat anti-mouse

10 IgG-HRP (Southern Biotech) diluted with dilution buffer (1X PBS with 0.05% Tween[®] 20 + 10% BCS) for 1 hour at 37°C. The plates were then washed again and incubated with O-phenylenediamine dihydrochloride (OPD) substrate (1 x 5 mg OPD tablet dissolved in Phosphate Citrate Buffer, Sigma-Aldrich) in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 μ L of 4N sulphuric acid (VWR) to each

15 well and read at 450 nm using an automated plate reader. OD readings at 450 nm were plotted against the molar concentration of inhibitor and the 50% inhibition was extrapolated for each sample tested.

EXEMPLIFICATION

20 The present invention is illustrated by but not limited to the following preparations and examples, in which the following abbreviations are used:

Ammonia	Concentrated solution of ammonia in water possessing a specific gravity of 0.880
CDCl ₃	Chloroform-d1
Celite [®]	Filtration agent
CI	Chemical ionisation
DCM	Dichloromethane
dPBS	Dulbecco's Phosphate buffered saline
DMF	N,N-Dimethylformamide
ES+	Electrospray ionisation positive scan
EtOAc	Ethyl acetate

Et ₃ N	Triethylamine
h	Hour
¹ H NMR	Proton Nuclear Magnetic Resonance
[Ir(cod)(OMe)] ₂	bis(η ⁴ -1,5-cyclooctadiene)-di-μ-methoxy-diiridium(I)
LCMS	Liquid chromatography-mass spectroscopy
MeCN	Acetonitrile
MeOH	Methanol
MgSO ₄	Magnesium sulfate
min	Minute
m/z	Mass spectrum peak
Pd/C	Palladium on charcoal
T3P	propanephosphonic anhydride, 50% w/v solution in ethyl acetate

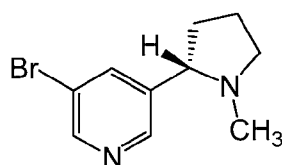
¹H NMR spectra were in all cases consistent with the proposed structures.

Characteristic chemical shifts (δ) are given in parts-per-million downfield from tetramethylsilane using conventional abbreviations for designation of major peaks: e.g. s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; brm, broad multiplet; brt, broad triplet.

LCMS, unless otherwise indicated, was performed under the following conditions: Waters XBridge C18 5nm, 2.1x30mm column, 0:100 to 95:5 over 3.1 min, MeCN:(10mM (NH₄)₂HCO₃ aq).

10 PREPARATIONS

Preparation 1: (S)-3-bromo-5-(1-methylpyrrolidin-2-yl)pyridine (or 5-bromonicotine)

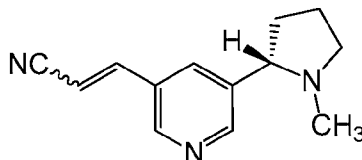


This compound was prepared following the method described in J. Am. Chem. Soc., 2007, 50, 15434-15435.

Bis(pinacolato)diboron (7.16 g, 28.21 mmol) was dissolved in 1,4-dioxane (60 mL) then degassed by bubbling argon through. (S)-(-)-Nicotine (6.48 mL, 40.3 mmol) was added, followed by 4,4'-di-tert-butyl-2,2'-dipyridyl (650 mg, 2.42 mmol). Degassing was continued for 10 min then methoxy(cyclooctadiene)iridium(I) dimer (753 mg, 1.21 mmol) was added. The reaction mixture was heated at reflux temperature for 18 hours. The solvent was evaporated and the residue dissolved in MeOH (100 mL). Copper (II) bromide (27.0 g, 120.9 mmol) in water (100 mL) was added and the reaction mixture heated at 80 °C for 3 hours. Ammonia solution (10% aq., 100 mL) was added to the reaction mixture which was then extracted with ethyl acetate and dried over MgSO₄. The solvent was evaporated and the crude product was purified by flash chromatography (5% MeOH in DCM) to give the title compound as an orange oil (6.14 g, 63%).

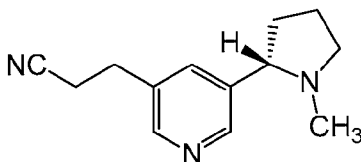
¹H NMR (400 MHz, CDCl₃) δ = 1.68-1.70 (m, 1H), 1.80-1.82 (m, 1H), 1.92-1.94 (m, 1H), 1.99-2.02 (m, 1H), 2.03 (s, 3H), 2.20-2.34 (m, 1H), 3.08 (t, 1H), 3.20 (t, 1H), 7.86 (s, 1H), 8.42 (s, 1H), 8.54 (s, 1H).

Preparation 2: (S)-3-(5-(1-methylpyrrolidin-2-yl)pyridin-3-yl)acrylonitrile



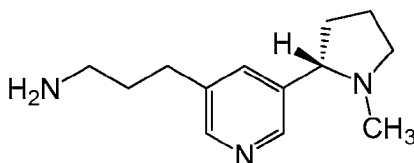
A mixture of (S)-3-bromo-5-(1-methylpyrrolidin-2-yl)pyridine (preparation 1) (300 mg, 1.24 mmol), palladium (II) acetate (14 mg, 0.06 mmol), tri(o-tolyl)phosphine (75 mg, 0.25 mmol) and triethylamine (0.35 mL, 2.48 mmol) in MeCN (10 mL) was degassed by bubbling argon through. Acrylonitrile (0.12 mL, 1.87 mmol) was then added and the reaction mixture heated at reflux temperature for 18 hours. The reaction mixture was cooled to room temperature, filtered through Celite, and the solvents evaporated to give a brown solid. The crude product was purified by flash chromatography (2% [20% ammonia in MeOH] in DCM) to give the title compound as a red-brown oil (188 mg, 71%, mixture of *cis* and *trans* isomers).

¹H NMR (400 MHz, CDCl₃) δ = 1.60-1.79 (m, 1H), 1.80-1.90 (m, 1H), 1.90-2.04 (m, 1H), 2.17 and 2.19 (2 x s, 3H), 2.16-2.40 (m, 2H), 3.11-3.30 (m, 2H), 5.57 and 6.00 (2 x d, 1H), 7.16 and 7.40 (2 x d, 1H), 7.80 and 8.26 (2 x s, 1H), 8.57 (s, 1H), 8.60 and 8.71 (2 x s, 1H).

Preparation 3: (S)-3-(5-(1-methylpyrrolidin-2-yl)pyridin-3-yl)propanenitrile

(S)-3-(5-(1-Methylpyrrolidin-2-yl)pyridin-3-yl)acrylonitrile (preparation 2) (185 mg, 0.87 mmol) and 5% Pd/C (50 mg) in MeOH (5 mL) were stirred under an atmosphere of hydrogen for 72 hours. The reaction mixture was filtered through Celite then the solvent evaporated. The crude product was purified by flash chromatography (5% [20% ammonia in MeOH] in DCM) to give the title compound as a pale yellow oil (141 mg, 75%).

¹H NMR (400 MHz, CDCl₃) δ = 1.66-1.78 (m, 1H), 1.78-1.89 (m, 1H), 1.89-2.02 (m, 1H), 2.17 (s, 3H), 2.17-2.25 (m, 1H), 2.31 (q, 1H), 2.65 (t, 2H), 2.97 (t, 2H), 3.10 (t, 1H), 3.24 (t, 1H), 7.61 (d, 1H), 8.38 (s, 1H), 8.44 (s, 1H).

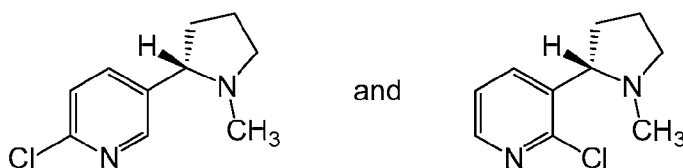
Preparation 4: (S)-3-(5-(1-methylpyrrolidin-2-yl)pyridin-3-yl)propan-1-amine

(S)-3-(5-(1-Methylpyrrolidin-2-yl)pyridin-3-yl)propanenitrile (preparation 3) (140 mg, 0.65 mmol) and Raney nickel (50 mg) in 20% ammonia in MeOH (50 mL) were stirred under 50 psi hydrogen at 50 °C for 4 hours. The reaction mixture was filtered through Celite then the solvent evaporated. The crude product was purified by flash chromatography (7% [20% ammonia in MeOH] in DCM) to give the title compound as a pale yellow oil (126 mg, 88%).

¹H NMR (400 MHz, CD₃OD) δ = 1.70-1.85 (m, 3H), 1.85-2.03 (m, 2H), 2.15 (s, 3H), 2.35 (q, 2H), 2.59-2.78 (m, 4H), 3.12-3.37 (m, 2H), 7.70 (s, 1H), 8.31 (br s, 2H).

LCMS: 1.91 min (97%), *m/z* = 220.16 [M+H]⁺

Preparations 5 and 6: (S)-2-chloro-5-(1-methylpyrrolidin-2-yl)pyridine (or 6-chloronicotine) and (S)-2-chloro-3-(1-methylpyrrolidin-2-yl)pyridine (or 2-chloronicotine)



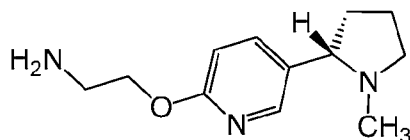
These compounds were prepared following the method described in Eur. J. Org. Chem., 2006, 3562-3565.

Butyl lithium (2M in hexanes, 83 mL, 166 mmol) was added to a solution of dimethylaminoethanol (9.3 mL, 92.5 mmol) in hexanes (70 mL) at -20°C . (S)-(-)-
5 Nicotine (5.0 g, 30.8 mMol) in hexanes (30 mL) was added and the reaction mixture stirred at -20°C for 1 hour. The green solution was then cooled to -78°C and hexachloroethane (29.1 g, 123 mmol) was added. The reaction mixture was stirred for 30 min at -78°C then quenched by the addition of ammonium chloride (sat. aq.). DCM and 2M HCl (aq.) were added. The aqueous layer was washed with DCM ($\times 2$) and then
10 basified by the addition of ice / ammonia (aq.). The aqueous layer was extracted with DCM ($\times 2$), dried over MgSO_4 and evaporated. The crude products were purified by flash chromatography (20% ethyl acetate in hexanes) to give 6-chloronicotine (preparation 5) (2.2 g, 36%) and 2-chloronicotine (preparation 6) (120 mg, 2%), both as yellow oils.

6-chloronicotine: ^1H NMR (300 MHz, CDCl_3) δ = 1.58-2.01 (m, 3H), 2.14 (s, 3H),
15 2.10-2.23 (m, 1H), 2.28 (q, 1H), 3.05 (t, 1H), 3.19 (dt, 1H), 7.26 (d, 1H), 7.64 (dd, 1H), 8.27 (d, 1H).

2-chloronicotine: ^1H NMR (300 MHz, CDCl_3) δ = 1.45-1.60 (m, 1H), 1.76-1.98 (m, 2H), 2.22 (s, 3H), 2.31-2.49 (m, 2H), 3.24 (t, 1H), 3.55 (t, 1H), 7.20-7.29 (m, 1H), 7.93 (d, 1H), 8.24 (dd, 1H).

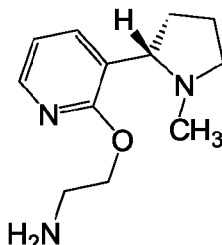
20 **Preparation 7: (S)-2-(5-(1-methylpyrrolidin-2-yl)pyridin-2-yloxy)ethanamine**



Sodium hydride (60% in oil, ~500 mg) was added to 6-chloronicotine (preparation 5) (690 mg, 3.5 mmol) in ethanolamine (3 mL). After 15 min at room temperature the reaction mixture was heated at 80°C for 16 hours. The reaction mixture was partitioned
25 between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate, dried over MgSO_4 and evaporated. The crude product was purified by flash chromatography (5% [20% ammonia in MeOH] in DCM) to give the title compound as a colourless oil (44 mg, 6%).

^1H NMR (400 MHz, CD_3OD) δ = 1.71-2.00 (m, 3H), 2.17 (s, 3H), 2.17-2.23 (m, 1H), 2.31 (q, 1H), 2.99 (t, 2H), 3.09 (t, 1H), 3.20 (t, 1H), 4.26 (t, 2H), 6.83 (d, 1H), 7.68 (d, 1H), 8.02 (d, 1H).

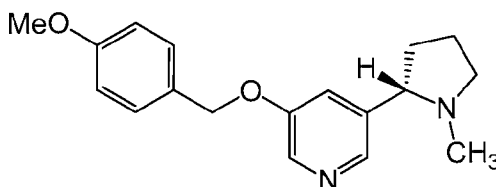
LCMS: 2.02 min (100%), m/z = 222.1 $[\text{M}+\text{H}]^+$

Preparation 8: (S)-2-(3-(1-methylpyrrolidin-2-yl)pyridin-2-yloxy)ethanamine

Sodium hydride (60% in oil, ~200 mg) was added to 2-chloronicotine (preparation 6) (120 mg, 0.61 mmol) in ethanolamine (2 mL). After 15 min at room temperature the reaction mixture was heated at 80 °C for 16 hours. The reaction mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate, dried over MgSO₄ and evaporated. The crude product was purified by flash chromatography (5% [20% ammonia in MeOH] in DCM) to give the title compound as a colourless oil (8.5 mg, 6%).

¹H NMR (400 MHz, CD₃OD) δ = 1.60-1.70 (m, 1H), 1.81-1.99 (m, 2H), 2.20 (s, 3H), 2.23-2.40 (m, 2H), 3.03 (t, 2H), 3.19 (t, 1H), 3.53 (t, 1H), 4.27-4.41 (m, 2H), 6.96 (d, 1H), 7.74 (d, 1H), 8.00 (d, 1H).

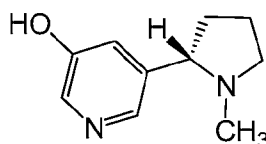
LCMS: 2.03 min (99%), *m/z* = 222.1 [M+H]⁺

Preparation 9: (S)-3-(4-methoxybenzyloxy)-5-(1-methylpyrrolidin-2-yl)pyridine

(plus 4 or 6 isomer)

Sodium hydride (60% in oil, 650 mg, 32.5 mmol) was added to 4-methoxybenzyl alcohol (1.4 g, 11.6 mmol) in DMF (10 mL). After 30 min at room temperature 5-bromonicotine (preparation 1) (1.4 g, 11.6 mmol) in DMF (2 mL) was added. The reaction mixture was heated at 90 °C for 16 hours. The reaction mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate, the organic layers combined, dried over MgSO₄ and evaporated. The crude product was purified by flash chromatography (2.5% → 5% MeOH in DCM) to give the title compound as a yellow oil, mixed with ~30% of another isomer (402 mg, 23%).

Preparation 10: (S)-5-(1-methylpyrrolidin-2-yl)pyridin-3-ol (or 5-hydroxynicotine)



Preparation 10a: (S)-3-(4-Methoxybenzyloxy)-5-(1-methylpyrrolidin-2-yl)pyridine
5 (preparation 9) (400 mg, 1.34 mmol) was stirred in trifluoroacetic acid (2 mL) and DCM (5 mL) for 2 hours. The reaction mixture was evaporated, then co-evaporated with DCM (×5) and 20% ammonia in MeOH. The crude product was purified by flash chromatography (10% MeOH in DCM) to give the title compound as a yellow oil (225 mg, 94%).

10 ^1H NMR (400 MHz, CDCl_3) δ = 2.20-2.39 (m, 3H), 2.48-2.59 (m, 1H), 2.76 (s, 3H), 3.83 (br s, 1H), 4.38 (br s, 1H), 7.42 (d, 1H), 8.16 (s, 1H), 8.20 (s, 1H).

LCMS: 1.26 min (100%), m/z = 179.1 $[\text{M}+\text{H}]^+$

Preparation 10b: (S)-Nicotine (54mL, 336.2mmol) and 4,4'-di-tertbutyl-2,2'-
dipyridyl (5.41g, 20.2mmol) were added successively to a solution of
15 bis(pinacolato)diboron (59.8g, 235.5mmols) in 1,4-dioxane (218mL). The resulting solution was degassed under vacuum, at room temperature, for 15 to 20 minutes and then released to nitrogen. The procedure was then repeated twice more.

$[\text{Ir}(\text{cod})(\text{OMe})]_2$ (6.7g, 10.1mmol) was charged to the reaction vessel and the
reaction mixture was degassed under vacuum, at room temperature, for 5 minutes, then
20 released to nitrogen. The procedure was then repeated twice more.

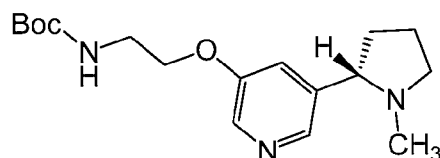
The resulting solution was heated at 85°C for 16 hours, after which time analysis indicated complete reaction. The reaction mixture was cooled to room temperature and concentrated to dryness under reduced pressure, at 50 to 55°C. The resulting orange residue was dissolved in tetrahydrofuran (740mL) and the solution was cooled to
25 between 0 and 5°C. Acetic acid (52.1mL, 908.8mmol) was charged to the vessel, followed by slow addition of hydrogen peroxide solution (30%, 43.1mL, 454.4mmol), maintaining the temperature at between 0 and 10°C. The resulting mixture was stirred for 16 hours at room temperature, after which time analysis indicated complete reaction.

The reaction mixture then was cooled to between 0 and 5°C, and quenched by
30 the addition of aqueous sodium metabisulphite solution (30%, 50mL). The pH of the resulting mixture was adjusted by the addition of concentrated ammonium hydroxide solution (130mL). The layers of the resulting biphasic mixture were separated and the

aqueous layer was re-extracted with tetrahydrofuran (300mL). The combined organic layers were concentrated to give the crude compound as an orange gum. This was purified by column chromatography on silica gel (MeOH in DCM, 5 to 20%) to remove the majority of impurities, followed by further column chromatography (100% THF) to remove isomeric impurities. Pure 5-hydroxynicotine was isolated as a yellow solid (26g, 48% yield).

^1H NMR (400 MHz, CDCl_3) δ = 1.70-2.05 (m, 4H), 2.21 (s, 3H), 2.31 (m, 1H), 3.11 (t, 1H), 3.25 (m, 1H), 7.32 (d, 1H), 8.01 (s, 1H), 8.05 (s, 1H).

Preparation 11: (S)-tert-butyl 2-(5-(1-methylpyrrolidin-2-yl)pyridin-3-yloxy)ethylcarbamate

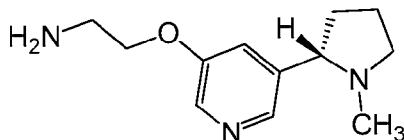


(S)-5-(1-Methylpyrrolidin-2-yl)pyridin-3-ol (preparation 10) (175 mg, 0.98 mmol), tert-butyl 2-bromoethylcarbamate (550 mg, 2.45 mmol) and potassium carbonate (676 mg, 4.9 mmol) in DMF (7 mL) were heated at 90 °C for 16 hours. The reaction mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate, dried over MgSO_4 and evaporated. The crude product was purified by flash chromatography (5% → 10% MeOH in DCM) to give the title compound as a yellow oil (108 mg, 34%).

^1H NMR (400 MHz, CDCl_3) δ = 1.42 (s, 9H), 1.70-2.00 (m, 3H), 2.17 (s, 3H), 2.09-2.20 (m, 1H), 2.20-2.30 (q, 1H), 3.15-3.26 (m, 2H), 3.45 (t, 2H), 4.08 (t, 2H), 7.43 (d, 1H), 8.07 (s, 1H), 8.13 (s, 1H).

LCMS: 2.62 min (100%), m/z = 322.15 $[\text{M}+\text{H}]^+$

Preparation 12: (S)-2-(5-(1-methylpyrrolidin-2-yl)pyridin-3-yloxy)ethanamine



Preparation 12a: (S)-tert-butyl 2-(5-(1-Methylpyrrolidin-2-yl)pyridin-3-yloxy)ethylcarbamate (preparation 11) (105 mg, 0.33 mmol) was stirred in trifluoroacetic acid (1 mL) and DCM (5 mL) for 1.5 h. The reaction mixture was evaporated, then co-evaporated with DCM (×5) and 20% ammonia in MeOH. The crude product was

purified by flash chromatography (5% [20% ammonia in MeOH] in DCM) to give the title compound as a yellow oil (62 mg, 85%).

^1H NMR (400 MHz, CDCl_3) δ = 1.70-2.01 (m, 3H), 2.17 (s, 3H), 2.18-2.30 (m, 1H), 2.30-2.40 (m, 1H), 3.03 (t, 2H), 3.14-3.28 (m, 2H), 4.08 (t, 2H), 7.44 (d, 1H), 8.08 (s, 1H), 8.16 (s, 1H).

LCMS: 1.87 min (100%), m/z = 222.12 $[\text{M}+\text{H}]^+$

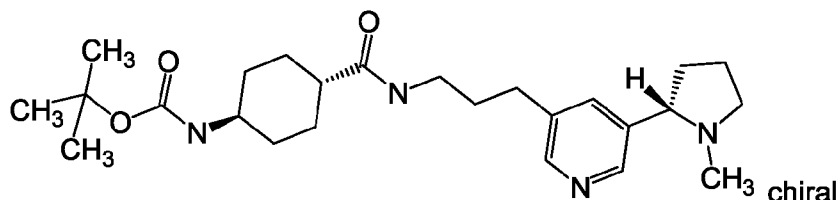
Preparation 12b: Potassium methoxide (3.37g, 46.2mmol), in methanol (40mL), was charged to a solution of 5-hydroxynicotine (preparation 10b) (8.0g, 44.0mmol) in methanol (40mL), at between 0 and 5°C. The resulting mixture was stirred for 90 minutes and then concentrated to dryness under reduced pressure at 45°C.

Boc-1-Amino-2-bromoethane (14.8g, 66.0mmol) in dimethyl formamide (80mL) was heated to 50°C under nitrogen, at which point the previously prepared potassium salt of 5-hydroxynicotine was charged to the vessel, followed immediately by potassium carbonate (6.7g, 48.4mmol).

The resulting mixture was heated at 85°C for 4 hours and then cooled to room temperature. It was concentrated under reduced pressure at 50°C and the resulting orange mixture was stirred in 1,4-dioxane (50mL) for 15 minutes. The solution was then filtered and the liquors were cooled to between 0 and 5°C, then acidified with 4M HCl in 1,4-dioxane (65mL). The mixture was concentrated under reduced pressure at 50°C and the resulting residue was dissolved in tetrahydrofuran (30mL). This mixture was then cooled to between 0 and 5°C, and the pH was adjusted with sodium hydroxide solution (10M, 45mL). The layers of the resulting biphasic mixture were separated and the aqueous layer was re-extracted with tetrahydrofuran (2x 50mL). The combined organic layers were concentrated to give the crude compound as a red oil. The crude product was dissolved in DCM and stirred for 10 minutes, then filtered and the liquors were loaded onto a silica column and purified by eluting with 5 to 30% methanol (containing 20% ammonium hydroxide) in ethyl acetate. The title compound was obtained as a yellow oil, 7.15g (73% yield).

^1H NMR (400 MHz, CDCl_3) δ = 1.65-1.80 (m, 3H), 1.85 (m, 1H), 1.95 (m, 1H), 2.19 (s, 3H), 2.21 (m, 1H), 2.35 (q, 1H), 3.12 (m, 3H), 3.28 (t, 1H), 4.08 (t, 2H), 7.28 (s, 1H), 8.15 (s, 1H), 8.23 (s, 1H).

Preparation 13: tert-butyl {trans-4-[(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)carbamoyl]cyclohexyl}carbamate

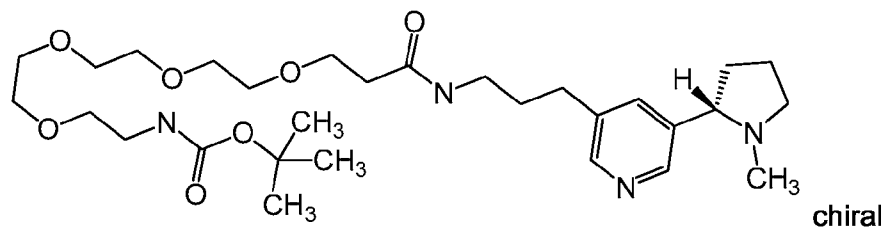


A solution of 3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propan-1-amine
 5 (preparation 4) (100mg, 0.45mmol) in 2-methyltetrahydrofuran (2ml) was treated, with
 stirring, with trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid (132mg,
 0.502mmol) followed by T3P (580μl, 0.91mmol) and then Et₃N (118μl, 0.91mmol). After
 stirring for 3 hours, the solution was treated with T3P (240μL, 0.38mmol). After stirring
 for 18 hours the solution was concentrated *in vacuo* and the residue was taken up in
 10 MeOH and applied to an SCX-2 cartridge which was eluted with MeOH followed by
 ammonia in MeOH (~2M). Product containing fractions were concentrated *in vacuo* and
 the residue purified by column chromatography on silica gel eluting with
 EtOAc:MeOH:NH₃ (gradient from 1:0:0 to 90:10:1) to give the title compound as a gum
 (130mg, 64%).

15 ¹H NMR (400 MHz, CDCl₃) δ = 1.04-1.14 (m, 2H), 1.42 (s, 9H), 1.48-1.59 (m,
 2H), 1.65-1.74 (m, H), 1.79-2.02 (m, 7H), 2.05-2.09 (brm, 2H), 2.13-2.22 (m, 4H), 2.26-
 2.33 (m, H), 2.60-2.64 (t, 2H), 3.04-3.08 (t, H), 3.20-3.30 (m, 3H), 3.40 (brm, H), 4.39
 (brm, H), 5.56 (brt, H), 7.50 (t, H), 8.30-8.31 (d, H), 8.34-8.35 (d, H).

MS, m/z=568 ES+ [M+H]⁺, 567 Cl [M+H]⁺

20 **Preparation 14: tert-butyl (19-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}-
 15-oxo-3,6,9,12-tetraoxa-16-azanonadec-1-yl)carbamate**

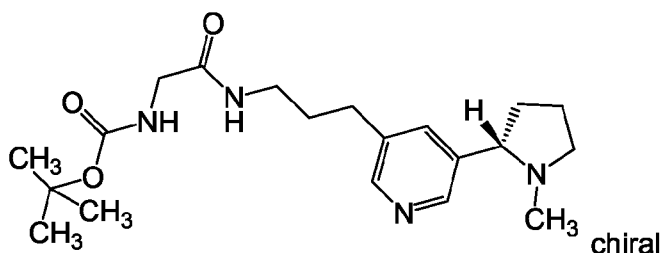


The title compound was prepared by the general method described above for
 preparation 13, using 3-(2-[2-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)-ethoxy]-ethoxy]-
 25 ethoxy)-propionic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic
 acid, to yield a pale yellow gum (180mg, 70%).

^1H NMR (400 MHz, CDCl_3) δ = 1.41 (s, 9H), 1.65-1.76 (m, H), 1.78-1.86 (m, 3H), 1.88-2.01 (m, H), 2.13-2.21 (m, 4H), 2.25-2.32 (m, H), 2.44-2.47 (t, 2H), 2.61-2.65 (t, 2H), 3.03-3.07 (t, H), 3.19-3.29 (m, 6H), 3.49-3.52 (t, 2H), 3.57-3.62 (m, 11H), 3.70-3.73 (t, 2H), 5.21 (brm, H), 6.66 (brm, H), 7.51 (t, H), 8.30-8.31 (d, H),] 8.33 (d, H).

5 MS, m/z =445 ES+ [$\text{M}+\text{H}$] $^+$, 445 Cl [$\text{M}+\text{H}$] $^+$

Preparation 15: *tert*-butyl {2-[(3-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)amino]-2-oxoethyl}carbamate

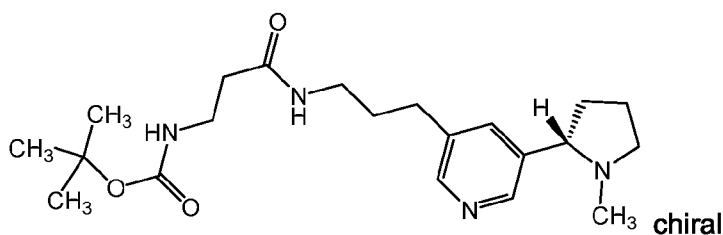


The title compound was prepared by the general method described above for
10 preparation 13, using *tert*-butoxycarbonylamino-acetic acid instead of trans-4-*tert*-
butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (133mg, 77%).

^1H NMR (400 MHz, CDCl_3) δ = 1.45 (s, 9H), 1.66-1.77 (m, H), 1.79-1.90 (m, 3H),
1.91-2.02 (m, H), 2.14-2.23 (m, 4H), 2.27-2.34 (q, H), 2.62-2.66 (m, 2H), 3.04-3.09 (t,
H), 3.21-3.26 (m, H), 3.28-3.34 (m, 2H), 3.75-3.76 (d, 2H), 5.21 (brm, H), 6.29 (brm,
15 H), 7.52 (t, H), 8.31-8.32 (d, H), 8.34-8.35 (d, H).

MS, m/z =377 ES+ [$\text{M}+\text{H}$] $^+$, 377 Cl [$\text{M}+\text{H}$] $^+$

Preparation 16: *tert*-butyl {3-[(3-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)amino]-3-oxopropyl}carbamate

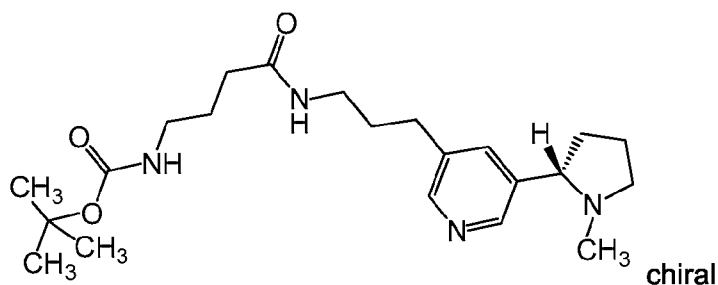


The title compound was prepared by the general method described above for
20 preparation 13, using *tert*-butoxycarbonylamino-propionic acid instead of trans-4-*tert*-
butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (130mg, 73%).

^1H NMR (400 MHz, CDCl_3) δ = 1.41 (s, 9H), 1.67-1.75 (m, H), 1.79-1.88 (m, 3H),
1.91-2.00 (m, H), 2.14-2.23 (m, 4H), 2.27-2.33 (q, H), 2.37-2.40 (t, 2H), 2.62-2.66 (m,
25 2H), 3.04-3.08 (t, H), 3.20-3.30 (m, 3H), 3.37-3.41 (q, 2H), 5.26 (brm, H), 6.00 (brm, H),
7.52 (t, H), 8.31-8.32 (d, H), 8.34 (d, H).

MS, $m/z=391$ ES+ $[M+H]^+$, 391 Cl $[M+H]^+$

Preparation 17: *tert*-butyl {4-[(3-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-4-oxobutyl}carbamate

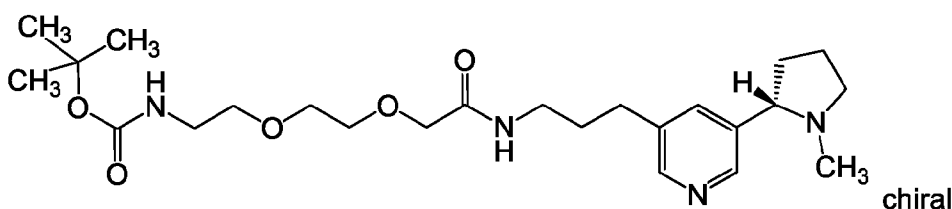


5 The title compound was prepared by the general method described above for preparation 13, using *tert*-butoxycarbonylamino-butanoic acid instead of *trans*-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (140mg, 76%).

^1H NMR (400 MHz, CDCl_3) δ = 1.40 (s, 9H), 1.64-1.72 (m, H), 1.74-1.87 (m, 5H), 1.87-1.89 (m, H), 2.12-2.20 (m, 6H), 2.24-2.31 (q, H), 2.62-2.66 (m, 2H), 3.02-3.06 (t, H), 3.11-3.16 (q, 2H), 3.18-3.29 (m, 3H), 4.91 (brm, H), 6.57 (brm, H), 7.51 (t, H), 8.30 (d, H), 8.32 (d, H).

MS, $m/z=405$ ES+ $[M+H]^+$, 405 Cl $[M+H]^+$

Preparation 18: *tert*-butyl [2-(2-{2-[(3-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-2-oxoethoxy}ethoxy)ethyl]carbamate



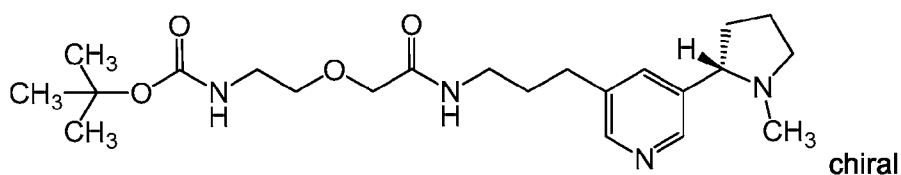
15 The title compound was prepared by the general method described above for preparation 13, using [2-(2-*tert*-Butoxycarbonylamino-ethoxy)-ethoxy]-acetic acid (prepared as described in Angew. Chemie Int. Ed. (2006), 45(30), 4936-4940) instead of *trans*-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a colourless gum (60mg, 28%).

^1H NMR (400 MHz, CDCl_3) δ = 1.42 (s, 9H), 1.67-1.98 (m, 7H), 2.14-2.23 (m, 4H), 2.27-2.33 (q, H), 2.64-2.68 (m, 2H), 3.04-3.09 (t, H), 3.21-3.26 (m, H), 3.29-3.37 (m, 3H), 3.53-3.56 (t, 2H), 3.62-3.68 (m, 4H), 3.98 (s, 2H), 4.99 (brm, H), 6.88 (brm, H), 7.53 (t, H), 8.33 (d, H), 8.35 (d, H).

25 MS, $m/z=465$ ES+ $[M+H]^+$, 465 Cl $[M+H]^+$

Preparation 19: tert-butyl (2-{2-[(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-2-oxoethoxy}ethyl)carbamate

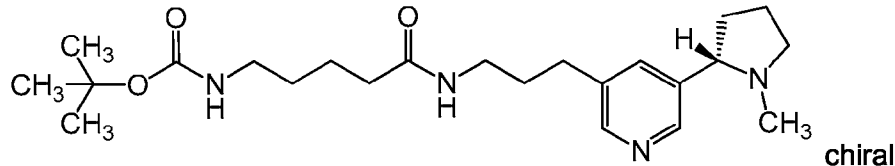
The title compound was prepared by the general method described above for preparation 13, using (2-tert-butoxycarbonylamino-ethoxy)-acetic acid instead of trans-4-tert-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (105mg, 55%).



^1H NMR (400 MHz, CDCl_3) δ = 1.43(s, 9H), 2.16-2.24(m, 5H), 2.31(q, 4H), 2.66(t, 1H), 3.07 (t, 2H), 3.24 (t, 1H), m, 1H), 3.32-3.37 (m, 4H), 3.56 (t, 2H), 3.94(s, 2H), 4.98 (br, 1H), 6.69 (br, 1H), 7.54 (t, 1H), 8.33-8.36 (m, 2H).

MS m/z =421 ES+ $[\text{M}+\text{H}]^+$

Preparation 20: tert-butyl {5-[(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-5-oxopentyl}carbamate

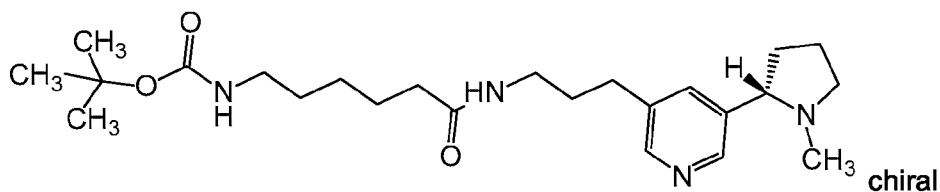


The title compound was prepared by the general method described above for preparation 13, using *tert*-butoxycarbonylamino-pentanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (191mg, 64%).

^1H NMR (400 MHz, CDCl_3) δ = 1.43 (s, 9H), 1.47-1.53 (m, 2H), 1.63-1.75 (m, 3H), 1.80-1.89 (m, 4H), 1.94-2.05 (m, H), 2.16 (s, 3H), 2.16-2.23 (m, 2H), 2.31 (q, H), 2.65 (t, 2H), 3.07 (t, H), 3.14-3.16 (m, 2H), 3.22-3.30 (m, 3H), 5.30 (br, H), 5.80 (br, H), 7.53 (t, H), 8.33 (m, H), 8.35 (m, H).

MS m/z =420 ES+ $[\text{M}+\text{H}]^+$

Preparation 21: tert-butyl {6-[(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-6-oxohexyl}carbamate

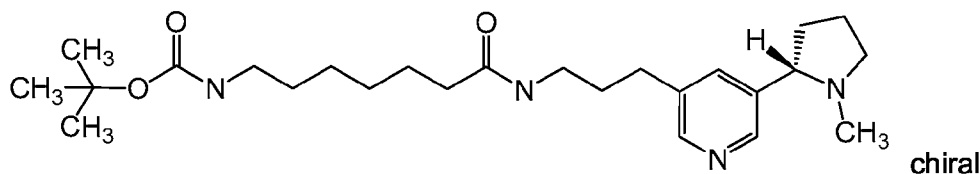


The title compound was prepared by the general method described above for preparation 13, using *tert*-butoxycarbonylamino-hexanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (197mg, 50%).

¹H NMR (400 MHz, CDCl₃) δ = 1.32-1.37 (m, 2H), 1.43 (s, 9H), 1.46-1.54 (m, 2H), 1.61-1.69 (m, 2H), 1.69-1.77 (m, H), 1.81-1.89 (m, 5H), 1.95-2.00 (m, H), 2.14-2.22 (m, 5H), 2.30 (q, H), 2.65 (t, 2H), 3.05-3.13 (m, 3H), 3.22-3.33 (m, 3H), 4.62 (br, H), 5.63 (br, H), 7.53 (t, H), 8.33 (m, H), 8.35 (m, H).

MS m/z=434 ES+ [M+H]⁺

Preparation 22: *tert*-butyl {7-[(3-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) amino]-7-oxoheptyl}carbamate

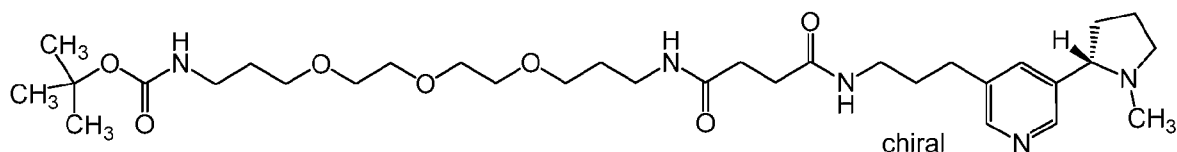


The title compound was prepared by the general method described above for preparation 13, using *tert*-butoxycarbonylamino-heptanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (204mg, 55%).

¹H NMR (400 MHz, CDCl₃) δ = 1.31-1.35 (m, 4H), 1.44 (s, 9H), 1.44-1.49 (m, 2H), 1.60-1.67 (m, 2H), 1.70-1.77 (m, H), 1.81-1.89 (m, 5H), 1.94-1.99 (m, H), 2.13-2.23 (m, 5H), 2.30 (q, H), 2.65 (t, 2H), 3.05-3.12 (m, 3H), 3.22-3.33 (m, 3H), 4.37 (br, H), 5.68 (br, H), 7.53 (t, H), 8.33 (m, H), 8.35 (m, H).

MS m/z=448 ES+ [M+H]⁺

Preparation 23: *tert*-butyl (22-{5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}-15,18-dioxo-4,7,10-trioxa-14,19-diazadocos-1-yl)carbamate



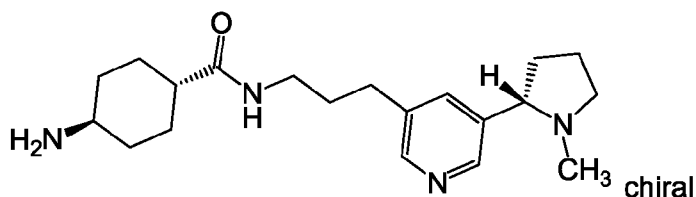
The title compound was prepared by the general method described above for preparation 13, using N-(3-{2-[2-(3-*tert*-butoxycarbonylamino-propoxy)-ethoxy]-ethoxy}-propyl)-succinamic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (284mg, 53%).

¹H NMR (400 MHz, CDCl₃) δ = 1.43 (s, 9H), 1.71-1.77 (m, 9H), 1.87-2.02 (m, 4H), 2.16-2.22 (m, 4H), 2.30 (q, H), 2.63 (t, 2H), 3.06 (t, H), 3.19-3.28 (m, 5H), 3.33-

3.37 (m, 2H), 3.51-3.65 (m, 12H), 5.09 (br, H), 6.59 (br, H), 6.64 (br, H), 7.52 (t, H), 8.32 (m, H), 8.35 (m, H).

MS m/z =622 ES⁺ [M+H]⁺

Preparation 24: trans-4-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) cyclohexanecarboxamide

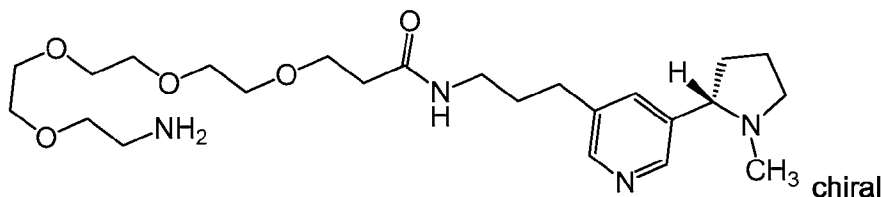


A solution of tert-butyl {trans-4-[(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)carbamoyl]cyclohexyl}carbamate (preparation 13) (130mg, 0.29mmol) in DCM (3mL) was treated with trifluoroacetic acid (1mL). The resulting solution was stirred at ambient temperature for 2 hours after which time it was concentrated *in vacuo*. The residue was dissolved in MeOH and applied to an SCX-2 cartridge and eluted with MeOH followed by ammonia in MeOH (2M). The fractions containing product were concentrated *in vacuo* to give the title compound as a colourless gum (98mg, 97%).

¹H NMR (400 MHz, CDCl₃) δ = 1.02-1.12 (m, 2H), 1.45-1.76 (m, 5H), 1.77-2.03 (m, 9H), 2.12-2.21 (m, 4H), 2.25-2.32 (q, H), 2.59-2.68 (m, 3H), 3.02-3.06 (t, H), 3.19-3.29 (m, 3H), 5.66 (br, H), 7.49 (t, H), 8.29 (d, H), 8.33 (d, H).

MS, m/z = 345 and 367 ES⁺ [M+H]⁺ and [M+Na]⁺, 345 Cl [M+H]⁺

Preparation 25: 1-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)-3,6,9,12-tetraoxapentadecan-15-amide



20

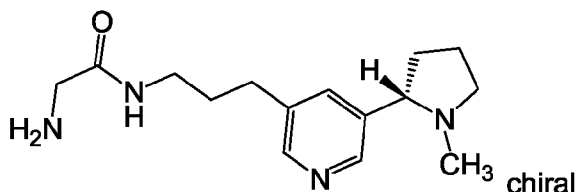
The title compound was prepared by the general method described above for preparation 24, starting from preparation 14, to yield a colourless gum (149mg, 100%).

¹H NMR (400 MHz, CDCl₃) δ = 1.64-1.75 (m, H), 1.77-1.99 (m, 7H), 2.12-2.21 (m, 4H), 2.25-2.32 (q, H), 2.44-2.46 (t, 2H), 2.60-2.64 (t, 2H), 2.82-2.85 (t, 2H), 3.02-3.06 (t, H), 3.19-3.29 (m, 3H), 3.47-3.50 (t, 2H), 3.59-3.61 (m, 11H), 3.69-3.72 (t, 2H), 6.76 (brm, H), 7.50 (t, H), 8.30-8.31 (d, H), 8.33 (d, H).

25

MS, m/z = 467 and 489 ES⁺ [M+H]⁺ and [M+Na]⁺, 467 Cl [M+H]⁺

Preparation 26: N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)glycinamide

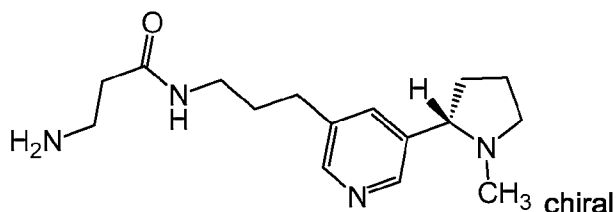


The title compound was prepared by the general method described above for preparation 24, starting from preparation 15, to yield a colourless gum (99mg, 100%).

^1H NMR (400 MHz, CDCl_3) δ = 1.66-2.01 (m, 7H), 2.13-2.22 (m, 4H), 2.27-2.33 (q, H), 2.62-2.66 (t, 2H), 3.05-3.09 (t, H), 3.21-3.26 (t, H), 3.29-3.34 (m, 4H), 7.35 (brm, H), 7.53 (t, H), 8.32 (d, H), 8.33-8.34 (d, H).

MS, m/z = 277 and 291 ES^+ $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$, 277 Cl^- $[\text{M}+\text{H}]^+$

Preparation 27: N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)-beta-alaninamide

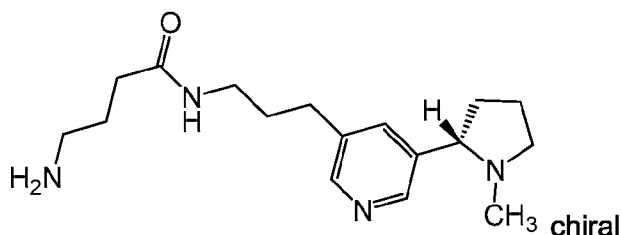


The title compound was prepared by the general method described above for preparation 24, starting from preparation 16, to yield a colourless gum (99mg, 100%).

^1H NMR (400 MHz, CDCl_3) δ = 1.65-1.76 (m, H), 1.77-2.00 (m, 6H), 2.13-2.22 (m, 4H), 2.26-2.32 (m, 3H), 2.62-2.66 (t, 2H), 2.98-3.01 (t, 2H), 3.03-3.08 (t, 2H), 3.20-3.31 (m, 3H), 7.26 (brm, H), 7.51 (t, H), 8.31-8.32 (d, H), 8.33-8.34 (d, H).

MS, m/z = 291 and 313 ES^+ $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$, 291 Cl^- $[\text{M}+\text{H}]^+$

Preparation 28: 4-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) butanamide

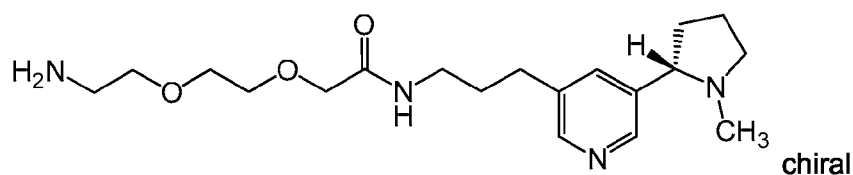


The title compound was prepared by the general method described above for preparation 24, starting from preparation 17, to yield a colourless gum (104mg, 99%).

^1H NMR (400 MHz, CDCl_3) δ = 1.65-2.00 (m, 9H), 2.13-2.32 (m, 7H), 2.61-2.65 (t, 2H), 2.72-2.76 (t, 2H) 3.03-3.07 (t, H) 3.20-3.30 (m, 3H), 6.22 (brm, H), 7.50 (t, H), 8.30-8.31 (d, H) 8.33-8.34 (d, H).

MS, m/z = 305 and 327 ES^+ $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$, 305 Cl $[\text{M}+\text{H}]^+$

5 **Preparation 29: 2-[2-(2-aminoethoxy)ethoxy]-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)acetamide**

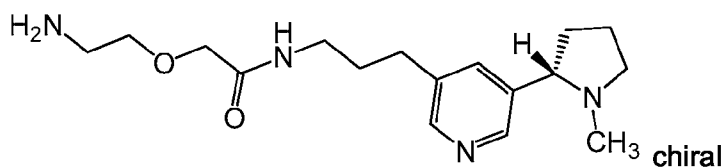


The title compound was prepared by the general method described above for preparation 24, starting from preparation 18, to yield a colourless gum (49mg, 100%).

10 ^1H NMR (400 MHz, CDCl_3) δ = 1.67-1.78 (m, H), 1.79-2.02 (m, 4H), 2.15-2.34 (m, 7H), 2.64-2.67 (t, 2H), 2.88-2.91 (t, 2H), 3.06-3.10 (t, 1H), 3.22-3.27 (t, 1H), 3.31-3.36 (q, 2H), 3.54-3.57 (t, 2H), 3.63-3.70 (m, 4H), 3.99 (s, 2H), 7.02 (dm, 2H), 7.54 (t, H), 8.33-8.34 (d, H), 8.35 (d, H).

MS, m/z = 365 and 387 ES^+ $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$, 365 Cl $[\text{M}+\text{H}]^+$

15 **Preparation 30: 2-(2-aminoethoxy)-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl)acetamide**

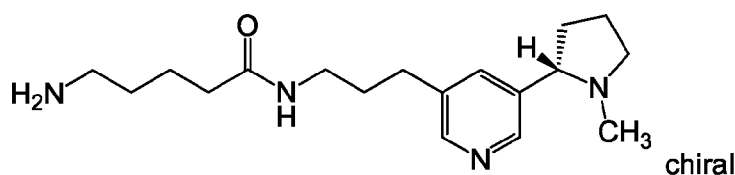


The title compound was prepared by the general method described above for preparation 24, starting from preparation 19, yielding a pale yellow gum (87mg, 100%).

20 ^1H NMR (400 MHz, CDCl_3) δ = 1.70-1.97 (m, 6H), 2.17 (s, 3H), 2.18-2.23 (m, H), 2.31 (q, H), 2.67 (t, 2H), 2.94 (m, 2H), 3.08 (t, H), 3.26 (m, H), 3.35 (q, 2H), 3.56 (t, 2H), 3.98 (s, 2H), 7.35 (br, H), 7.55 (t, H), 8.34 (d, H), 8.35 (d, H).

MS, m/z = 321 ES^+ $[\text{M}+\text{H}]^+$

25 **Preparation 31 5-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) pentanamide**

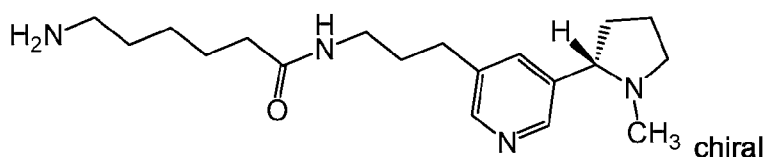


The title compound was prepared by the general method described above for preparation 24, starting from preparation 20, yielding a pale yellow gum (78mg, 54%).

^1H NMR (400 MHz, CDCl_3) δ = 1.46-1.52 (m, H), 1.64-1.72 (m, 3H), 1.81-2.02 (m, 7H), 2.15-2.24 (m, 5H), 2.31(q, H), 2.65 (t, 2H), 2.72 (t, H), 3.07 (t, H), 3.20-3.32 (m, 4H), 5.79 (br, H), 6.25 (br, H), 7.53 (s, H), 8.33 (m, H), 8.35 (m, H).

MS, m/z = 319 ES^+ $[\text{M}+\text{H}]^+$

Preparation 32: 6-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) hexanamide

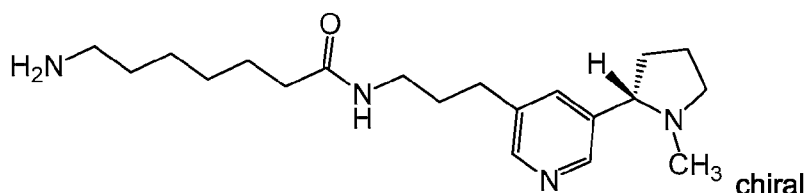


The title compound was prepared by the general method described above for preparation 24, starting from preparation 21, yielding a pale yellow gum (70mg, 46%).

^1H NMR (400 MHz, CDCl_3) δ = 1.32-1.41 (m, 2H), 1.50-1.57 (m, H), 1.59-1.70 (m, 4H), 1.81-1.85 (m, 3H), 1.87-2.01 (m, 2H), 2.15 (s, 3H), 2.15-2.3 (m, 3H), 2.30 (q, H), 2.63 (t, 2H), 2.765 (t, H), 3.06 (t, H), 3.19-2.29 (m, 3H).

MS, m/z = 333 ES^+ $[\text{M}+\text{H}]^+$

Preparation 33: 7-amino-N-(3-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}propyl) heptanamide

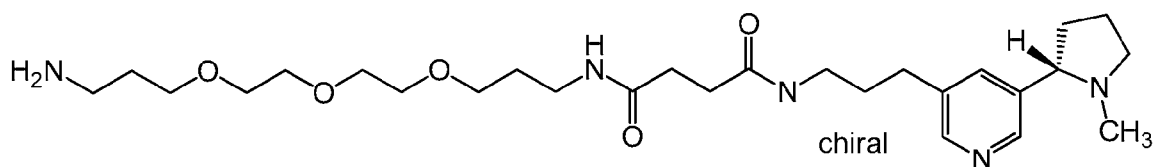


The title compound was prepared by the general method described above for preparation 24, starting from preparation 22, yielding a pale yellow gum (59mg, 37%).

^1H NMR (400 MHz, CDCl_3) δ = 1.32-1.29 (m, 3H), 1.46-1.50 (m, H), 1.61-1.66 (m, 2H), 1.70-1.74 (m, H), 1.82-1.89 (m, 3H), 1.94-2.04 (m, 3H), 2.17 (s, 3H), 2.15-2.23 (m, 3H), 2.32 (q, H), 2.65 (t, 2H), 2.71 (t, H), 3.08 (t, H), 3.18-3.33 (m, 4H), 5.58 (br, H), 5.65 (br, H), 7.53 (d, H), 8.34 (m, H), 8.36 (m, H).

MS, m/z = 347 ES^+ $[\text{M}+\text{H}]^+$

Preparation 34: N-(3-{2-[2-(3-aminopropoxy)ethoxy]ethoxy}propyl)-N'-{(3-{5-[(2S)-1-methyl pyrrolidin-2-yl]pyridin-3-yl}propyl)succinamide

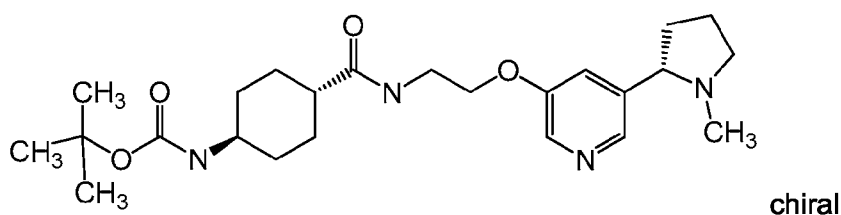


The title compound was prepared by the general method described above for preparation 24, starting from preparation 23, yielding a pale yellow gum (120mg, 51%).

^1H NMR (400 MHz, CDCl_3) δ = 1.71-1.87 (m, 6H), 1.90-2.02 (m, H), 2.16 (s, 3H), 2.17-2.24 (m, H), 2.30 (q, H), 2.40-2.51 (m, 6H), 2.61-2.67 (m, 2H), 2.87 (t, 2H), 3.22-3.29 (m, 3H), 3.31 (q, 2H), 3.51-3.65 (m, 12H), 6.85 (br, H), 6.95 (br, H), 7.53 (s, H), 8.33 (m, H), 8.35 (m, H).

MS, m/z = 522 ES^+ $[\text{M}+\text{H}]^+$

Preparation 35: tert-butyl (trans-4-[(2-{5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy) ethyl]carbamoyl)cyclohexyl)carbamate



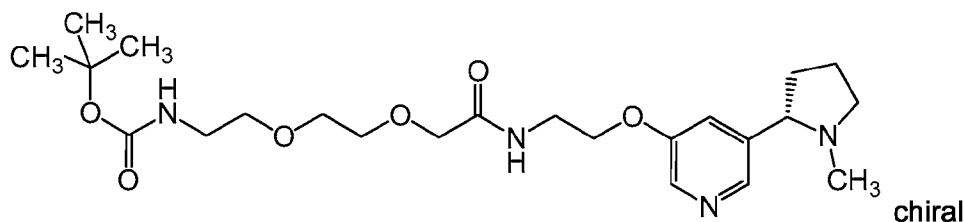
A solution of 2-[(5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl)oxy]ethanamine (preparation 12) (55mg, 0.25mmol) in 2-methyltetrahydrofuran (2ml) was treated, with stirring, with trans-4-tert-butoxycarbonyl-cyclohexane carboxylic acid (91mg, 0.373mmol) followed by T3P (317 μl , 0.498mmol) and the Et₃N (118 μl , 0.91mmol). After stirring for 3 hours, the solution was treated with T3P (69 μL , 0.498mmole). After stirring for 18 hours the solution was concentrated *in vacuo* and the residue was taken up in MeOH and applied to an SCX-2 cartridge which was eluted with methanol followed by ammonia in MeOH (~2M). Product containing fractions were concentrated *in vacuo* and the residue purified by column chromatography on silica gel eluting with EtOAc:MeOH:NH₃ (gradient from 1:0:0 to 90:10:1). This material was then dissolved in DCM and treated with PS-Isocyanate resin for 3 hours then filtered and evaporated *in vacuo* to give the title compound as a pale yellow gum (35mg, 35%);

^1H NMR (400 MHz, CDCl_3) δ = 1.14-1.25 (m, 2H), 1.42 (d, 9H), 1.47-1.58 (m, 2H), 1.75-1.85 (m, 3H), 1.87-2.06 (m, 4H), 2.09-2.18 (m, 1H), 2.21 (s, 3H), 2.23-2.32 (m, 1H),

2.38-2.45 (q, 1H), 3.24-3.29 (m, 3H), 3.55-3.58 (t, 2H), 4.11-4.14 (t, 2H), 7.45-7.43 (m, 1H), 8.10 (d, 1H), 8.15-8.16 (d, 1H).

MS m/z = 447 ES⁺ [M+H]⁺, 447 CI [M+H]⁺

Preparation 36: tert-butyl {2-[2-(2-{[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]amino}-2-oxoethoxy)ethoxy]ethyl}carbamate

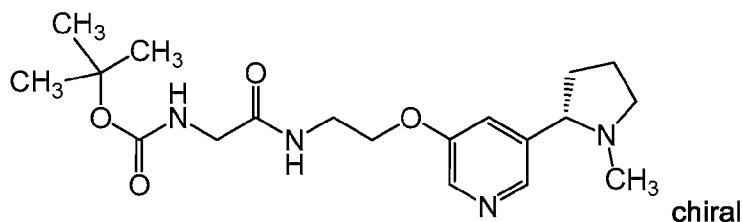


The title compound was prepared by the general method described above for preparation 35, using [2-(2-tert-Butoxycarbonylamino-ethoxy)-ethoxy]-acetic acid (prepared as described in Angew. Chemie Int. Ed. (2006), 45(30), 4936-4940) instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (50mg, 43%).

¹H NMR (400 MHz, CDCl₃) δ = 1.41 (s, 9H), 1.72-1.81 (m, 1H), 1.84-2.01 (m, 2H), 2.18 (s, 3H), 2.21-2.30 (m, 1H), 2.33-2.40 (q, 1H), 3.16-3.26 (m, 4H), 3.50-3.52 (t, 2H), 3.62-3.69 (m, 6H), 4.01 (s, 2H), 4.16-4.19 (t, 2H), 7.44-7.45 (m, 1H), 8.10 (d, 1H), 8.15-8.16 (d, 1H).

MS m/z = 467 ES⁺ [M+H]⁺, 467 CI [M+H]⁺

Preparation 37: tert-butyl (2-{[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] amino}-2-oxoethyl)carbamate

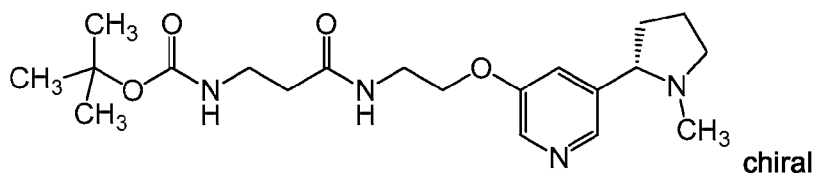


The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-acetic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (50mg, 53%).

¹H NMR (400 MHz, CDCl₃) δ = 1.42 (s, 9H), 1.87-2.09 (m, 3H), 2.30-2.38 (m, 4H), 2.60-2.67 (q, 1H), 3.37-3.43 (brt, 1H), 3.52-3.56 (brt, 1H), 3.61-3.64 (t, 2H), 3.70 (s, 2H), 4.14-4.17 (t, 2H), 7.49-7.50 (m, 1H), 8.14-8.15 (d, 1H), 8.20-8.21 (d, 1H).

MS m/z = 379 ES⁺ [M+H]⁺, 379 CI [M+H]⁺

Preparation 38: tert-butyl (3-([2-((5-((2S)-1-methylpyrrolidin-2-yl)pyridin-3-yl)oxy)ethyl] amino)-3-oxopropyl)carbamate

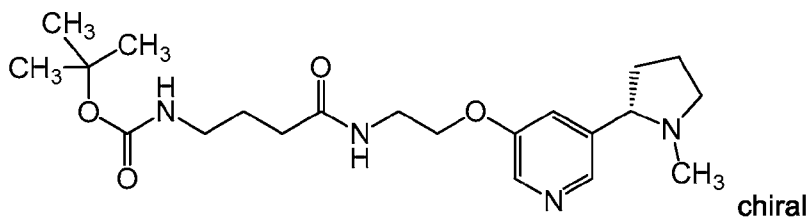


The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-propionic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (70mg, 72%).

^1H NMR (400 MHz, CDCl_3) δ = 1.39 (s,9H), 1.75-1.84 (m,1H), 1.87-2.04 (m,2H), 2.21 (s,3H), 2.23-2.31 (m,1H), 2.37-2.47 (m,3H), 3.24-3.33 (m,4H), 3.58-3.61 (t,2H), 4.12-4.15 (t,2H), 7.44-7.45 (m,1H), 8.10-8.11 (d,1H), 8.16-8.17 (d,1H).

MS m/z = 393 ES+ $[\text{M}+\text{H}]^+$, 393 CI $[\text{M}+\text{H}]^+$

Preparation 39: tert-butyl (4-([2-((5-((2S)-1-methylpyrrolidin-2-yl)pyridin-3-yl)oxy)ethyl] amino)-4-oxobutyl)carbamate

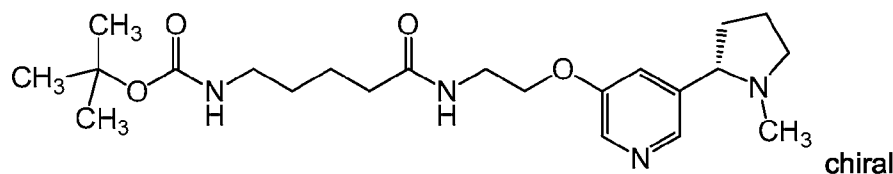


The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-butanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (43mg, 42%).

^1H NMR (400 MHz, CDCl_3) δ = 1.41 (s,9H), 1.70-1.78 (m,2H), 1.96-2.12 (m,3H), 2.21-2.24 (t,2H), 2.33-2.42 (m,4H), 2.69-2.76 (q,1H), 3.02-3.07 (m,2H), 3.43-3.49 (brt,1H), 3.58-3.61 (t,2H), 3.64-3.69 (brt,1H), 4.15-4.18 (t,2H), 7.51-7.52 (m,1H), 8.16-8.17 (d,1H), 8.23-8.24 (d,1H).

MS m/z = 407 ES+ $[\text{M}+\text{H}]^+$, 407 CI $[\text{M}+\text{H}]^+$

Preparation 40: tert-butyl (5-([2-((5-((2S)-1-methylpyrrolidin-2-yl)pyridin-3-yl)oxy)ethyl] amino)-5-oxopentyl)carbamate

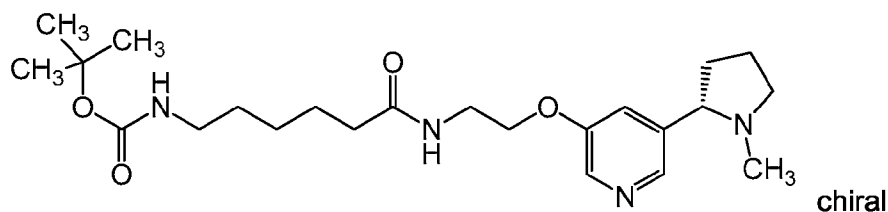


The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-pentanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (49mg, 47%).

¹H NMR (400 MHz, CDCl₃) δ = 1.41-1.50 (m, 11H), 1.57-1.65 (m, 2H), 2.01-2.18 (m, 3H), 2.20-2.24 (t, 2H), 2.37-2.44 (m, 1H), 2.46 (s, 3H), 2.78-2.85 (q, 1H), 3.00-3.05 (m, 2H), 3.49-3.55 (m, 1H), 3.58-3.61 (t, 2H), 3.76-3.80 (brt, 1H), 4.15-4.16 (t, 2H), 7.55-7.56 (m, 1H), 8.18-8.19 (d, 1H), 8.25-8.26 (d, 1H).

MS m/z = 421 ES+ [M+H]⁺, 421 CI [M+H]⁺

Preparation 41: *tert*-butyl (6-([2-({5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] amino)-6-oxohexyl)carbamate

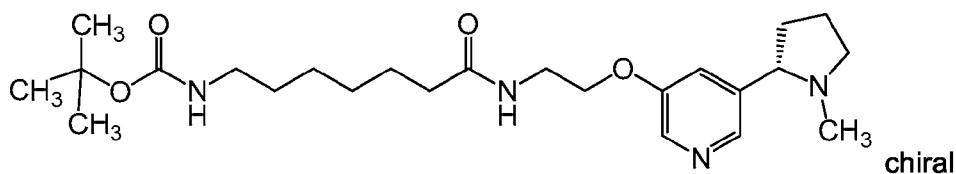


The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-hexanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (65mg, 60%).

¹H NMR (400 MHz, CDCl₃) δ = 1.29-1.35 (m, 2H), 1.42-1.49 (m, 11H), 1.57-1.65 (m, 2H), 2.05-2.15 (m, 3H), 2.19-2.23 (t, 2H), 2.38-2.46 (m, 1H), 2.48 (s, 3H), 2.82-2.89 (m, 1H), 2.96-3.00 (t, 2H), 3.53-3.61 (m, 3H), 3.62-3.66 (brt, 1H), 4.15-4.18 (t, 2H), 7.55-7.56 (m, 1H), 8.19-8.20 (d, 1H), 8.26-8.27 (d, 1H).

MS m/z = 435 ES+ [M+H]⁺, 435 CI [M+H]⁺

Preparation 42: *tert*-butyl (7-([2-({5-[(2*S*)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] amino)-7-oxoheptyl)carbamate



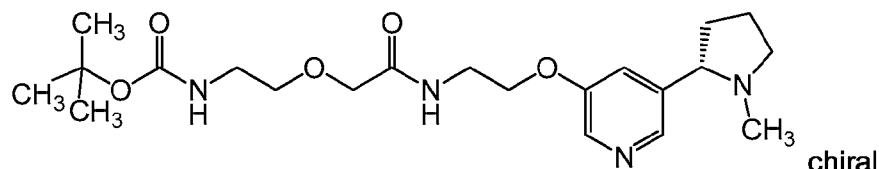
The title compound was prepared by the general method described above for preparation 35, using *tert*-butoxycarbonylamino-heptanoic acid instead of trans-4-*tert*-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (67mg, 60%).

¹H NMR (400 MHz, CDCl₃) δ = 1.28-1.34 (m, 4H), 1.39-1.45 (m, 11H), 1.55-1.64 (m, 2H), 2.03-2.15 (m, 3H), 2.19-2.22 (t, 2H), 2.38-2.46 (m, 1H), 2.48 (s, 3H), 2.81-2.88

(m,1H), 2.97-3.00 (t,2H), 3.51-3.57 (m,1H), 3.58-3.61 (t,2H), 3.80-3.84 (m,1H), 4.15-4.18 (t,2H), 7.55-7.57 (m,1H), 8.19 (d,1H), 8.16 (d,1H).

MS m/z = 449 ES⁺ [M+H]⁺, 449 CI [M+H]⁺

Preparation 43: tert-butyl [2-(2-([2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] amino)-2-oxoethoxy)ethyl]carbamate

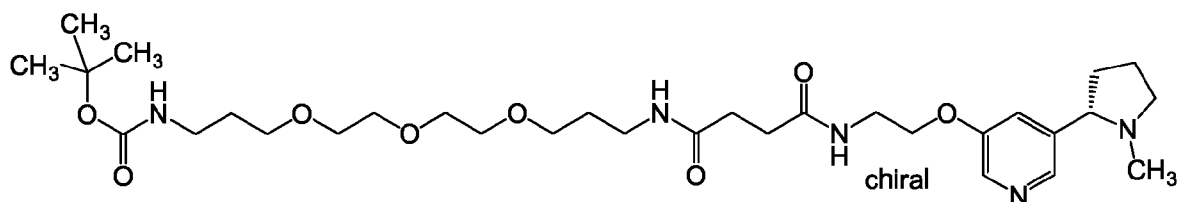


The title compound was prepared by the general method described above for preparation 35, using (2-tert-butoxycarbonylamino-ethoxy)-acetic acid instead of trans-4-tert-butoxycarbonyl-cyclohexane carboxylic acid, to yield a pale yellow gum (42mg, 42%).

¹H NMR (400 MHz, CDCl₃) δ = 1.42 (s,9H), 2.15-2.32 (m,3H), 2.43-2.52 (s,1H), 2.61 (s,3H), 3.03-3.10 (m,1H), 3.24-3.29 (m,2H), 3.52-3.55 (t,2H), 3.66-3.69 (m,3H), 3.98 (s,2H), 4.06-4.13 (m,1H), 4.21-4.24 (t,2H), 7.62-7.63 (m,1H), 8.24 (d,1H), 8.32-8.33 (d,1H).

MS m/z = 423 ES⁺ [M+H]⁺, 423 CI [M+H]⁺

Preparation 44: tert-butyl [21-(5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl)oxy)-15,18-dioxo-4,7,10-trioxa-14,19-diazahenicos-1-yl]carbamate

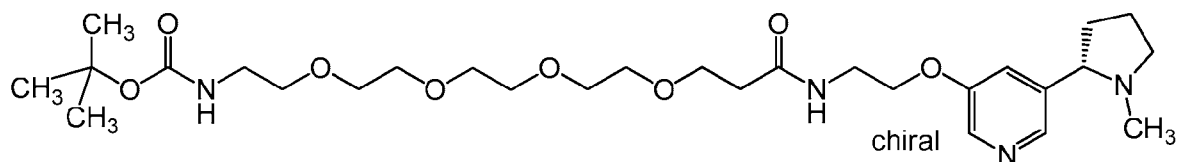


The title compound was prepared by the general method described above for preparation 35, using N-(3-{2-[2-(3-tert-butoxycarbonylamino-propoxy)-ethoxy]-ethoxy}-propyl)-succinamic acid instead of trans-4-tert-butoxycarbonyl-cyclohexane carboxylic acid, to yield an orange gum (80mg, 51%).

¹H NMR (400 MHz, CDCl₃) δ = 1.42 (s,9H), 1.68-1.76 (m,4H), 1.97-2.15 (m,3H), 2.35-2.52 (m,9H), 2.71-2.80 (m,1H), 3.09-3.14 (m,2H), 3.20-3.26 (m,2H), 3.48-3.51 (t,5H), 3.55-3.64 (m,10H), 3.66-3.76 (m,1H), 4.14-4.17 (t,2H), 7.53-7.54 (m,1H), 8.17-8.18 (d,1H), 8.24-8.25 (d,1H).

MS m/z = 625 ES⁺ [M+H]⁺, 625 CI [M+H]⁺

Preparation 45: tert-butyl [18-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)-15-oxo-3,6,9,12-tetraoxa-16-azaoctadec-1-yl]carbamate

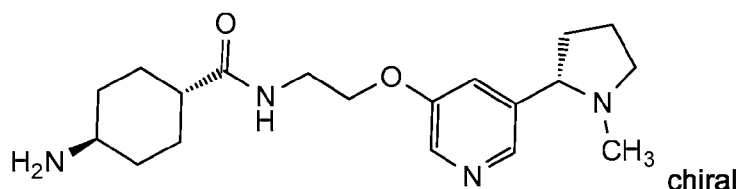


The title compound was prepared by the general method described above for preparation 35, using 3-(2-[2-[2-(2-tert-Butoxycarbonylamino-ethoxy)-ethoxy]-ethoxy]-ethoxy)-propionic acid instead of trans-4-tert-butoxycarbonyl-cyclohexane carboxylic acid, to yield an orange gum (80mg, 56%).

^1H NMR (400 MHz, CDCl_3) δ = 1.42 (s,9H), 2.03-2.20 (m,3H), 2.38-2.50 (m,6H), 2.85-2.91 (m,1H), 3.19-3.22 (m,2H), 3.47-3.50 (t,2H), 3.53-3.63 (m,15H), 3.71-3.74 (t,2H), 3.84-3.92 (m,1H), 4.17-4.19 (t,2H), 7.58-7.59 (m,1H), 8.20-8.21 (d,1H), 8.28 (d,1H).

MS m/z = 570 ES+ $[\text{M}+\text{H}]^+$, 570 CI $[\text{M}+\text{H}]^+$

Preparation 46: trans-4-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy) ethyl]cyclohexanecarboxamide

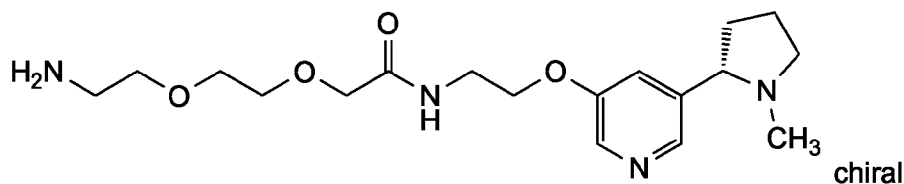


A solution of tert-butyl (trans-4-[[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]carbonyl]cyclohexyl)carbamate (preparation 35) (35mg, 0.078mmol) in DCM (3mL) was treated with trifluoroacetic acid (1mL). The resulting solution was stirred at ambient temperature overnight, after which time it was concentrated *in vacuo*. The residue was dissolved in MeOH and applied to an SCX-2 cartridge and eluted with MeOH followed by ammonia in MeOH (2M). The fractions containing product were concentrated *in vacuo* to give the title compound as an orange gum (17mg, 62%).

^1H NMR (400 MHz, CDCl_3) δ = 1.16-1.26 (m,2H), 1.47-1.57 (m,2H), 1.71-2.01 (m,8H), 2.13-2.30 (m,4H), 2.33-2.40 (q,1H), 2.68-2.76 (m,1H), 3.18-3.26 (m,2H), 3.56-3.59 (t,2H), 4.12-4.14 (t,2H), 7.43-7.44 (m,1H), 8.09-8.10 (d,1H), 8.14-8.15 (d,1H).

MS m/z = 347 ES+ $[\text{M}+\text{H}]^+$, 347 CI $[\text{M}+\text{H}]^+$

Preparation 47: 2-[2-(2-aminoethoxy)ethoxy]-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]acetamide

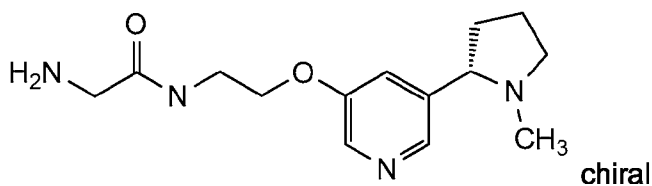


The title compound was prepared by the general method described above for preparation 46, starting from preparation 36, to yield an orange gum (37mg, 95%).

¹H NMR (400 MHz, CDCl₃) δ = 1.72-1.81 (m, 1H), 1.84-2.03 (m, 2H), 2.18 (s, 3H),
 5 2.22-2.30 (m, 1H), 2.34-2.41 (q, 1H), 2.83-2.86 (t, 2H), 3.19-3.26 (m, 2H), 3.54-3.57 (t, 2H),
 3.65-3.71 (m, 6H), 4.02 (s, 2H), 4.16-4.19 (t, 2H), 7.44-7.45 (m, 1H), 8.10-8.11 (d, 1H),
 8.15 (d, 1H).

MS m/z = 367 ES⁺ [M+H]⁺, 367 Cl [M+H]⁺

Preparation 48: N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]glycinamide

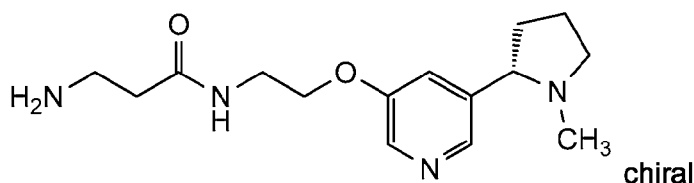


The title compound was prepared by the general method described above for preparation 46, starting from preparation 37, to yield an orange gum (37mg, 100%).

¹H NMR (400 MHz, CDCl₃) δ = 1.74-1.84 (m, 1H), 1.87-2.05 (m, 2H), 2.20 (s, 3H),
 15 2.23-2.32 (m, 1H), 2.38-2.44 (q, 1H), 3.23-3.28 (m, 2H), 3.37 (s, 2H), 3.63-3.66 (t, 2H),
 4.15-4.17 (t, 2H), 7.45-7.47 (m, 1H), 8.10-8.11 (d, 1H), 8.15-8.16 (d, 1H).

MS m/z = 279 ES⁺ [M+H]⁺, 279 Cl [M+H]⁺

Preparation 49: N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]-beta-alaninamide



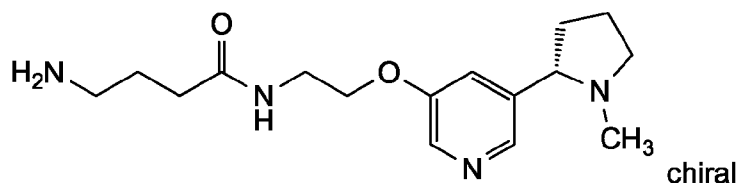
20

The title compound was prepared by the general method described above for preparation 46, starting from preparation 38, to yield an orange gum (52mg, 100%).

¹H NMR (400 MHz, CDCl₃) δ = 1.72-1.81 (m, 1H), 1.84-2.03 (m, 2H), 2.18 (s, 3H),
 2.21-2.30 (m, 1H), 2.33-2.44 (m, 3H), 2.93-2.96 (t, 2H), 3.16-3.26 (m, 2H), 3.60-3.62
 25 (t, 2H), 4.13-4.16 (t, 2H), 7.43-7.44 (m, 1H), 8.10 (d, 1H), 8.14-8.15 (d, 1H).

MS m/z = 293 ES⁺ [M+H]⁺, 293 CI [M+H]⁺

Preparation 50: 4-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] butanamide

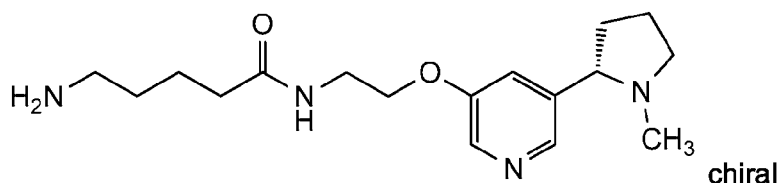


5 The title compound was prepared by the general method described above for preparation 46, starting from preparation 39, to yield an orange gum (28mg, 88%).

¹H NMR (400 MHz, CDCl₃) δ = 1.73-1.82 (m, 3H), 1.84-2.03 (m, 2H), 2.18 (s, 3H), 2.21-2.30 (m, 3H), 2.33-2.40 (q, 1H), 2.68-2.72 (t, 2H), 3.18-3.26 (m, 2H), 3.58-3.61 (t, 2H), 4.12-4.15 (t, 2H) 7.43-7.44 (m, 1H), 8.10 (d, 1H), 8.14-8.15 (d, 1H).

10 MS m/z = 307 ES⁺ [M+H]⁺, 307 CI [M+H]⁺

Preparation 51: 5-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] pentanamide

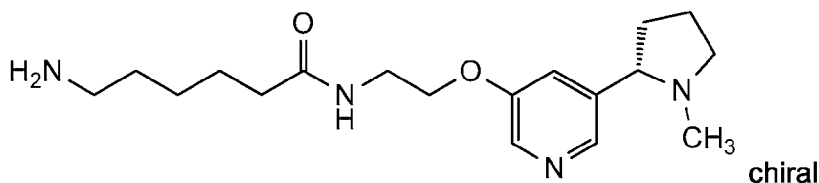


15 The title compound was prepared by the general method described above for preparation 46, starting from preparation 40, to yield an orange gum (30mg, 81%).

¹H NMR (400 MHz, CDCl₃) δ = 1.48-1.56 (m, 2H), 1.61-1.68 (m, 2H), 1.71-1.81 (m, 1H), 1.85-2.03 (m, 2H), 2.18 (s, 3H), 2.22-2.30 (m, 3H), 2.33-2.40 (q, 1H), 2.68-2.72 (t, 2H), 3.18-3.26 (m, 2H), 3.58-3.61 (t, 2H), 4.12-4.15 (t, 2H), 7.43-7.44 (m, 1H), 8.10 (d, 1H), 8.14-8.15 (d, 1H).

20 MS m/z = 321 ES⁺ [M+H]⁺, 321 CI [M+H]⁺

Preparation 52: 6-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] hexanamide

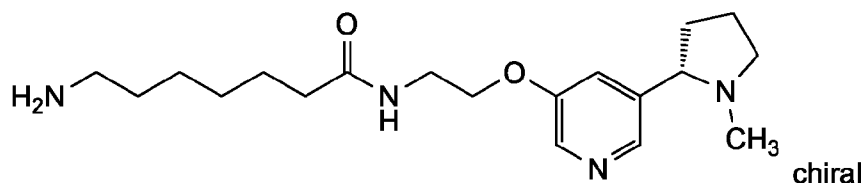


25 The title compound was prepared by the general method described above for preparation 46, starting from preparation 41, to yield an orange gum (38mg, 76%).

^1H NMR (400 MHz, CDCl_3) δ = 1.31-1.39 (m,2H), 1.48-1.55 (m,2H), 1.59-1.66 (m,2H), 1.72-1.81 (m,1H), 1.84-2.03 (m,2H), 2.18 (s,3H), 2.21-2.30 (m,3H), 2.33-2.40 (q,1H), 2.65-2.69 (t,2H), 3.18-3.26 (m,2H), 3.57-3.60 (t,2H), 4.15-4.12 (t,2H), 7.43-7.44 (m,1H), 8.09-8.10 (d,1H), 8.14-8.15 (d,1H).

5 MS m/z = 335 ES+ $[\text{M}+\text{H}]^+$, 335 Cl $[\text{M}+\text{H}]^+$

Preparation 53: 7-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] heptanamide

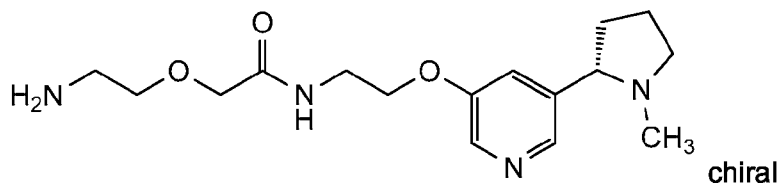


The title compound was prepared by the general method described above for
10 preparation 46, starting from preparation 42, to yield an orange gum (37mg, 72%).

^1H NMR (400 MHz, CDCl_3) δ = 1.32-1.37 (m,4H), 1.44-1.51 (m,2H), 1.57-1.65 (m,2H), 1.72-1.82 (m,1H), 1.83-2.03 (m,2H), 2.18-2.30 (m,6H), 2.33-2.40 (q,1H), 2.65-2.68 (t,2H), 3.18-3.26 (m,2H), 3.57-3.60 (t,2H), 4.12-4.15 (t,2H), 7.43-7.44 (m,1H), 8.09-8.10 (d,1H), 8.14-8.15 (d,1H).

15 MS m/z = 349 ES+ $[\text{M}+\text{H}]^+$, 349 Cl $[\text{M}+\text{H}]^+$

Preparation 54: 2-(2-aminoethoxy)-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy) ethyl]acetamide

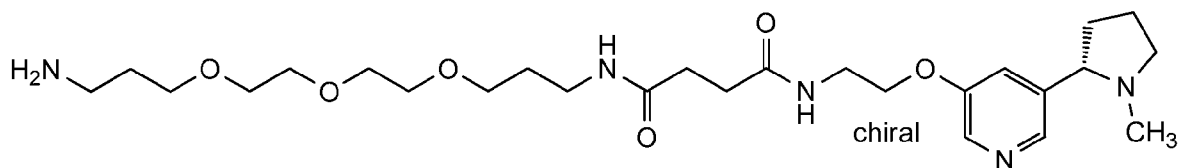


The title compound was prepared by the general method described above for
20 preparation 46, starting from preparation 43, to yield an orange gum (27mg, 84%).

^1H NMR (400 MHz, CDCl_3) δ = 1.72-1.82 (m,1H), 1.84-2.03 (m,2H), 2.18 (s,3H), 2.22-2.30 (m,1H), 2.34-2.40 (q,1H), 2.86-2.89 (t,2H), 3.18-3.26 (m,2H), 3.55-3.58 (t,2H), 3.66-3.68 (t,2H), 4.00 (s,2H), 4.16-4.19 (t,2H), 7.44-7.45 (m,1H), 8.10 (d,1H), 8.15 (d,1H).

25 MS m/z = 323 ES+ $[\text{M}+\text{H}]^+$, 323 Cl $[\text{M}+\text{H}]^+$

Preparation 55: N-(3-[2-[2-(3-aminopropoxy)ethoxy]ethoxy]propyl)-N'-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]succinamide

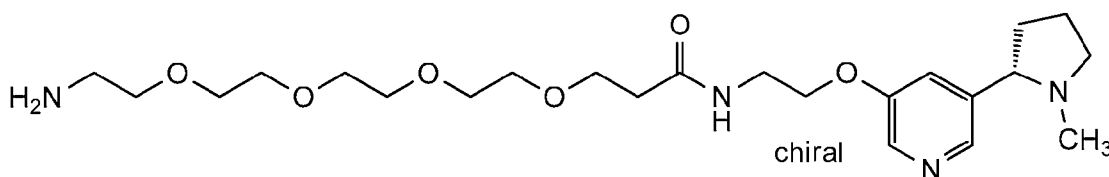


The title compound was prepared by the general method described above for preparation 46, starting from preparation 44, to yield an orange gum (59mg, 88%).

¹H NMR (400 MHz, CDCl₃) δ = 1.70-1.80 (m, 5H), 1.86-2.03 (m, 2H), 2.18 (s, 3H), 2.21-2.30 (m, 1H), 2.33-2.40 (q, 1H), 2.44-2.52 (m, 4H), 2.76-2.80 (t, 2H), 3.18-3.26 (m, 4H), 3.48-3.51 (t, 2H), 3.55-3.63 (m, 12H), 4.11-4.14 (t, 2H), 7.43-7.44 (m, 1H), 8.10 (d, 1H), 8.15 (d, 1H).

MS m/z = 524 ES⁺ [M+H]⁺, 524 CI [M+H]⁺

Preparation 56: 1-amino-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl]-3,6,9,12-tetraoxapentadecan-15-amide

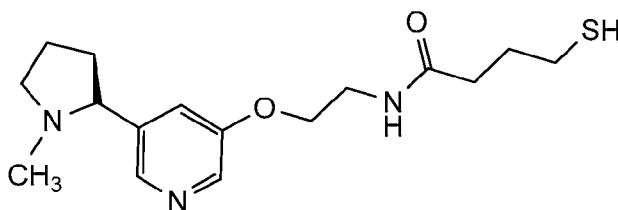


The title compound was prepared by the general method described above for preparation 46, starting from preparation 45, to yield an orange gum (55mg, 85%).

¹H NMR (400 MHz, CDCl₃) δ = 1.71-1.81 (m, 1H), 1.84-2.03 (m, 2H), 2.18 (s, 3H), 2.22-2.30 (m, 1H), 2.33-2.40 (q, 1H), 2.45-2.48 (t, 2H), 2.78-2.80 (t, 2H), 3.18-3.25 (m, 2H), 3.50-3.53 (t, 2H), 3.58-3.62 (m, 14H), 3.71-3.74 (t, 2H), 4.13-4.15 (t, 2H), 7.44-7.45 (m, 1H), 8.10 (d, 1H), 8.15-8.16 (d, 1H).

MS m/z = 469 ES⁺ [M+H]⁺, 469 CI [M+H]⁺

Preparation 57: 4-Mercapto-N-[2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethyl] butanamide.



A solution of 2-({5-[(2S)-1-methylpyrrolidin-2-yl]pyridin-3-yl}oxy)ethanamine (preparation 12) (1100mg, 4.971mmol), in water (10ml), was treated with γ -thiobutyrolactone (861ul, 9.94mmol) and the reaction mixture was heated to 60°C

overnight. It was then evaporated *in vacuo* to leave a colourless gum, which was pre-adsorbed onto silica from MeOH. This was then chromatographed on a 40g ISCO column, eluting with EtOAc to EtOAc:MeOH:NH₃ 80:20:1. The appropriate fractions were evaporated *in vacuo* to give the title compound as a colourless gum, 690mg. Yield =43%.

¹H NMR (400Mhz, CD₃OD): δ=1.77-2.06 (m, 6H), 2.22-2.35 (m, 6H), 2.41-2.51 (m, 3H), 3.26-3.32 (m, H), 3.58-3.61 (t, 2H), 4.13-4.15 (t, 2H), 7.45-7.47 (m, H), 8.11-8.12 (d, H), 8.17 (d, H). MS m/z=324 ES+ [M+H]⁺ and 322 CI- [M-H]⁻

10 EXAMPLES

Example 1. The hapten of preparation 4 was coupled to diphtheria toxoid protein using the following procedure.

a) The hapten was obtained as a yellow oil and stored at 2–8 °C until the day of use (for up to one week, longer storage was at -20°C). This oil was dissolved in either Dulbeccos' Phosphate Buffered Saline without Ca or Mg (DPBS) or deionised water at 50 mg hapten per ml solution. The result was a yellow-brown suspension or solution.

b) Separately, an aliquot of concentrated diphtheria toxoid (DT) was obtained and using a gel filtration desalting column (Bio-Rad), the buffer of the DT was changed to DPBS. The eluate from the column was a 4ml solution at approximately 11 mg/ml as determined by the Bradford assay (Coomassie Brilliant Blue reagent, Pierce Chemical) using a bovine serum albumin standard curve. An aliquot of this was diluted with further DPBS to give a 4ml solution at 5 mg/ml protein in DPBS.

c) This aliquot of DT was reacted with succinic anhydride to create a modified succinylated diphtheria toxoid. The 4ml aliquot of DT in DPBS was combined with 80mg succinic anhydride as a solid, and placed on a tube roller to provide gentle agitation at room temperature for 3 h. It was noted that using this method, the end product was not a clear solution. At the end of the 3 h reaction time, the aliquot was applied to a gel filtration desalting column and eluted into DPBS, to remove unreacted small molecule components. This increased the volume of the sample to 6ml total.

d) 28 mg of sulfo-N-hydroxysuccinimide (s-NHS), as a solid, were added to the 6ml succinylated DT aliquot, and s-NHS was allowed to dissolve by inverting the tube and gentle mixing. Then 28 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing. The reaction mixture (succinylatedDT/EDC/s-NHS)

was incubated at room temperature for 10 min, 200 µl of the hapten solution prepared above were added and the reaction mixture was briefly stirred using a Vortex to mix.

e) The mixture was reacted for 3 to 4 h with gentle agitation on a tube roller, at room temperature. At the end of the reaction time, the aliquot was applied to a gel
5 filtration desalting column and eluted into DPBS, to remove unreacted small molecule components. This increased the volume of the conjugate sample to 8ml total. This 8 ml eluate was then concentrated using 3k MWCO centrifugal ultrafilters (Millipore) to approx 3.0 - 3.5 ml.

f) Using a bovine serum albumin standard, the final conjugate was analysed for
10 protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay, and the final concentration determined as 2.3 mg/ml. The conjugate was analysed for hapten incorporation using the ultraviolet spectroscopy in the 250 – 270 nm region versus an unconjugated control normalised for protein content, and was found to more strongly antibodiesorb in this region compared with the native toxoid. The conjugate
15 was also analysed by SDS-PAGE electrophoresis and for endotoxin content using the LAL assay. Finally, a 100 µg sample of the conjugate was hydrolysed and analysed using reversed-phase HPLC to determine the number of haptens incorporated per protein molecule. The hapten – DT conjugate was found to have 19 haptens per protein monomer

20 g) Finally the sample was sterile filtered through a 0.22 µm syringe filter and aseptically subdivided into 0.5 ml aliquots. These aseptic aliquots were stored at -80 °C until shipping to the study location on dry ice.

Example 2. The hapten of preparation 12 was coupled to diphtheria toxoid protein using the general procedure described above for example 1, with the following
25 changes: in step d), 186 µl of the hapten solution were added to the reaction mixture; and in step f), the final concentration was determined as 3.3 mg/ml, and the hapten – DT conjugate was found to have 13 haptens per protein monomer.

Example 3. The hapten of preparation 7 was coupled to diphtheria toxoid protein using the general procedure described above for example 1, with the following
30 changes in steps c, e and f.

In step c), a 3 ml portion of the aliquot was applied to a gel filtration desalting column and eluted into DPBS, to remove unreacted small molecule components, whereby the volume of the purified sample was increased to 4ml total; step d) was performed as follows: 80 µl of the hapten solution were added to the 4ml succinylated

DT aliquot, and inverted gently to mix. Then, 28 mg of sulfo-N-hydroxysuccinimide (s-NHS) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing. Finally, 28 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing.

In step e), the mixture was reacted for 2 h, the volume of the conjugate sample was increased to 6 ml total after elution, and the 6 ml eluate was then concentrated using 10k MWCO centrifugal ultrafilters (Millipore) to approx 2.5 - 3.0 ml.

In step f), the final concentration was determined as 2.4 mg/ml, and the hapten – DT conjugate was found to have 14.5 haptens per protein monomer.

Example 4. The hapten of preparation 8 was coupled to diphtheria toxoid protein using the general procedure described above for example 1, with the following changes: step d) was performed as follows: 120 µl of the hapten solution were added to the 6ml succinylated DT aliquot. The mixture was stirred briefly with a Vortex. Then 28 mg of sulfo-N-hydroxysuccinimide (s-NHS) were added, as a solid, and allowed to dissolve by inverting the tube and gentle mixing. Finally 28 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing; and in step f), the final concentration was determined as 2.5 mg/ml, and the hapten – DT conjugate was found to have 19.3 haptens per protein monomer.

Example 5. The hapten-spacer conjugates from preparations 24 to 34 and 46 to 56 were coupled to diphtheria toxoid protein as described in the method below.

a) The hapten-spacer conjugates were obtained as a yellow oil and stored at 2 to 8 °C until the day of use (for up to one week, longer storage was at -20°C). This oil was dissolved in Dulbeccos' Phosphate Buffered Saline without Ca or Mg (DPBS) at 50 mg hapten per ml solution. The result was a yellow-brown suspension or solution. Preparation 33 did not dissolve fully in deionised water, and so was further dissolved to 50% methanol to a final concentration of 25 mg hapten-spacer conjugate per ml solution

b) Separately, an aliquot of concentrated diphtheria toxoid (DT) was obtained and using a gel filtration desalting column (Bio-Rad), the buffer of the DT was changed to DPBS. The eluate from the column was a 4ml solution at approximately 11 mg/ml as determined by the Bradford assay (Coomassie Brilliant Blue reagent, Pierce Chemical) using a bovine serum albumin standard curve. An aliquot of this was diluted with further DPBS to give a 2ml solution at 5 mg/ml protein in DPBS.

c) This aliquot of DT was reacted with succinic anhydride to create a modified succinylated diphtheria toxoid. The 2ml aliquot of DT in DPBS was combined with 40mg succinic anhydride as a solid, and placed on a tube roller to provide gentle agitation at room temperature for 3 hours. It was noted that using this method, the end product was not a clear solution. At the end of the 3 hour reaction time, the aliquot was applied to a gel filtration desalting column and eluted into DPBS, to remove unreacted small molecule components. This increased the volume of the sample to 3ml total.

d) 9 mg of sulfo-N-hydroxysuccinimide (s-NHS), as a solid, were added to the 3ml DT aliquot, and the s-NHS was allowed to dissolve by inverting the tube and gentle mixing. Then 9 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing. 50 µl of the hapten-spacer conjugate solution prepared above (100ul for preparation 33) were added to the reaction mixture (succinylated DT/EDC/s-NHS)

e) The mixture was reacted for 3 to 4 hours, with gentle agitation, on a tube roller, at room temperature. At the end of the reaction time, the aliquot was applied to a gel filtration desalting column and eluted into DPBS, to remove unreacted small molecule components. This increased the volume of the conjugate sample to 4ml total.

f) Using a bovine serum albumin standard, the final conjugate was analysed for protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay, and the final concentration was determined as indicated in table 1. The conjugate was analysed for hapten incorporation using ultraviolet spectroscopy in the 250 to 270 nm region versus an unconjugated control, normalised for protein content, and was found to more strongly antibodiesorb in this region compared with the native toxoid. The conjugate was also analysed by SDS-PAGE electrophoresis and for endotoxin content using the LAL assay. Finally, a 100 µg sample of the conjugate was hydrolysed and analysed using reversed-phase HPLC to determine the number of haptens incorporated per protein molecule. The hapten loading for the tested samples is detailed below in tables 1 and 2.

g) Finally the sample was sterile filtered through a 0.22 µm syringe filter and aseptically subdivided into 1.0 ml aliquots. These aseptic aliquots were stored at -80°C until shipping to the study location on dry ice.

Table 1

Conjugate	protein conc. (mg/ml)	Hapten load
DT-hapten-spacer preparation 24	1.69	11
DT-hapten-spacer preparation 25	1.82	21
DT-hapten-spacer preparation 26	1.84	22
DT-hapten-spacer preparation 27	2.22	16
DT-hapten-spacer preparation 28	1.91	16
DT-hapten-spacer preparation 29	1.60	19
DT-hapten-spacer preparation 30	1.81	14
DT-hapten-spacer preparation 31	2.18	15
DT-hapten-spacer preparation 32	1.62	15
DT-hapten-spacer preparation 33	1.51	11
DT-hapten-spacer preparation 34	1.78	14

Table 2

Conjugate	protein conc. (mg/ml)	Hapten load
DT-hapten-spacer preparation 46	1.39	3.6
DT-hapten-spacer preparation 47	1.56	23.6
DT-hapten-spacer preparation 48	1.43	22.8
DT-hapten-spacer preparation 49	1.41	19.2
DT-hapten-spacer preparation 50	1.58	19.7
DT-hapten-spacer preparation 51	1.48	14.2
DT-hapten-spacer preparation 52	1.57	11
DT-hapten-spacer preparation 53	1.60	12.1
DT-hapten-spacer preparation 54	1.89	29.3
DT-hapten-spacer preparation 55	1.32	12.6
DT-hapten-spacer preparation 56	1.45	20.9

Example 6. Haptens with a reactive amine group can also be conjugated to diphtheria toxoid without the addition of succinic anhydride. An example of the hapten of preparation 12 conjugated to diphtheria toxoid is detailed below, but this can also be performed with preparations 4, 7, 8, 24-34 and 46-56.

5 a) The hapten was obtained as a yellow oil and stored at 2 to 8 °C until the day of use (for up to one week, longer storage was at -20°C). This oil was dissolved in Dulbeccos' Phosphate Buffered Saline without Ca or Mg (DPBS) at 50 mg hapten per ml solution. The result was a yellow-brown suspension or solution.

10 b) Separately, an aliquot of concentrated diphtheria toxoid (DT) was obtained and using a gel filtration desalting column (Bio-Rad), the buffer of the DT was changed to DPBS. The eluate from the column was a 4ml solution at approximately 11 mg/ml as determined by the Bradford assay (Coomassie Brilliant Blue reagent, Pierce Chemical) using a bovine serum albumin standard curve. An aliquot of this was diluted with further DPBS to give a 2ml solution at 5 mg/ml protein in DPBS.

15 c) 9 mg of sulfo-N-hydroxysuccinimide (s-NHS), as a solid, were added to the 2ml DT aliquot, and the s-NHS was allowed to dissolve by inverting the tube and gentle mixing. Then 9 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to the mixture, as a solid, and allowed to dissolve by inverting the tube and gentle mixing. 50 µl of the hapten solution prepared above were added to the
20 reaction mixture (DT/EDC/s-NHS). At this stage the pH was adjusted down to pH 5.6 with dropwise addition of 1M HCl.

 d) The mixture was reacted for 3 to 4 hours with gentle agitation, on a tube roller, at room temperature. At the end of the reaction time, the aliquot was applied to a gel filtration desalting column and eluted into DPBS, to remove unreacted small molecule
25 components. This increased the volume of the conjugate sample to 3ml total.

 e) Using a bovine serum albumin standard, the final conjugate was analysed for protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay, and the final concentration determined as 3.01 mg/ml. The conjugate was analysed for hapten incorporation using ultraviolet spectroscopy in the 280 nm region versus an
30 unconjugated control normalised for protein content, and was found to more strongly antibodiesorb in this region compared with the native toxoid. The conjugate was also analysed by SDS-PAGE electrophoresis and for endotoxin content using the LAL assay. Finally, a 100 µg sample of the conjugate was hydrolysed and analysed using reversed-

phase HPLC to determine the number of haptens incorporated per protein molecule.

The hapten – DT conjugate was found to have 11.6 haptens per protein monomer

g) Finally the sample was sterile filtered through a 0.22 µm syringe filter and aseptically subdivided into 1.0 ml aliquots. These aseptic aliquots were stored at -80 °C
5 until shipping to the study location on dry ice.

Example 7. Preparation 12 can also be coupled to CRM₁₉₇, as well as to diphtheria toxoid. An example of such as conjugation is given below. Although not shown, the same process can also be applied to other amine containing haptens such as 4, 7, 8, 24-34 and 46-56.

10 a) The hapten from preparation 12, was obtained as a yellow oil and stored at -20°C. This oil was dissolved in Dulbeccos' Phosphate Buffered Saline without Ca or Mg (DPBS) at 50 mg hapten per ml solution. The result was a yellow-brown suspension or solution.

b) Separately, a 20 ml aliquot of concentrated CRM₁₉₇ (5.91 mg/ml) was thawed
15 from -80°C to 2-8°C. An 8.62 ml aliquot of thawed material was taken and diluted with 1.38 ml of DPBS to give a 10ml solution at 5 mg/ml protein in DPBS.

c) 500 µl of the hapten solution prepared above was added to the 10ml CRM₁₉₇ solution, this was added slowly and dropwise then left to react on a tube roller for 5 mins.

20 d) After this time, 90 mg of sulfo-N-hydroxysuccinimide (s-NHS) as a solid, was then added to the 10ml CRM₁₉₇ solution. The s-NHS was allowed to dissolve by inverting the tube with gentle mixing. The solution was then left to react on a tube roller for 5mins.

e) After 5 mins, 90 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
25 hydrochloride (EDC) was added to the CRM₁₉₇ solution, again as a solid, and this was allowed to dissolve by inverting the tube with gentle mixing. At this stage the pH was adjusted down to pH 5.6 with dropwise addition of 1M HCl.

f) The reaction mixture was then transferred to a cold room (2-8°C), and left to react on a tube roller for 6 hours.

30 g) At the end of the reaction time the sample was desalted into dPBS using 5 x NAP25 columns to remove excess un-reacted reagents (ON: 2ml, OFF: 3ml). The final volume was increased to 15ml.

h) Using a bovine serum albumin standard, the final conjugate was analysed for protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay, and

the final concentration determined as 2.88 mg/ml. The conjugate was analysed for hapten incorporation using ultraviolet spectroscopy in the 280 nm region versus an unconjugated control normalised for protein content, and was found to more strongly antibodiesorb in this region compared with the native toxoid. The conjugate was also analysed by SDS-PAGE electrophoresis and for endotoxin content using the LAL assay. Finally, a 100 µg sample of the conjugate was hydrolysed and analysed using reversed-phase HPLC to determine the number of haptens incorporated per protein molecule. The hapten – CRM₁₉₇ conjugate was found to have 8.0 haptens per protein monomer

i) Finally the sample was sterile filtered through a 0.22 µm syringe filter and aseptically subdivided into 1.0 ml aliquots. These aseptic aliquots were stored at -80 °C until shipping to the study location on dry ice.

Example 8. It was found that by increasing the sNHS/EDC concentration of the reaction mix, the amount of hapten loaded onto the carrier protein could be controlled. An example of a conjugation of preparation 7 to diphtheria toxoid is shown below. The method is as described for example 6 with the following changes in steps c and e.

In step c): at this stage the amount of sulfo-N-hydroxysuccinimide (s-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) added to the 2ml aliquot of desalted DT can be varied. In this experiment, 9 mg of sNHS and EDC was added to one aliquot (this is referred to as condition 2) and 36mg of sNHS and EDC was added to another aliquot (this is referred to as condition 4). The samples were not pH adjusted down to pH 5.6 with dropwise addition of 1M HCl.

In step e): for the conjugate made using condition 2, the final concentration was determined as 3.14 mg/ml, and the hapten – DT conjugate was found to have 8.3 haptens per protein monomer. For the conjugate made using condition 4, the final concentration was determined as 2.46 mg/ml, and the hapten – DT conjugate was found to have 13.2 haptens per protein monomer.

Example 9. Preparation of thiolated hapten-spacer conjugates onto CRM₁₉₇ and formalin inactivated Diphtheria Toxoid (DT) via bromoacetic acid N-hydroxysuccinimide ester (BAANS) conjugation chemistry.

a) 3ml of CRM₁₉₇ (17.73mg at 5.91mg/ml) and 3ml of DT (24mg at 8mg/ml) were thawed and desalted into 100mM Phosphate buffer, pH 8.0, using 10DG desalting columns (Pierce) (on: 3ml, off: 4ml). The concentration was adjusted to 4mg/ml using the same buffer.

b) A 1ml aliquot of the desalted CRM₁₉₇ and DT samples was taken and cooled to 2-8 °C.

c) 20mg of the hapten-spacer conjugate of preparation 57 was dissolved in 300ul DMSO and 700ul dPBS to a final concentration of 20mg/ml.

5 d) 500ul (10mg) of the hapten-spacer conjugate solution was added to 500ul of prewashed TCEP ((tris(2-carboxyethyl)phosphine)) gel (Pierce) and incubated for 3 hours with agitation at room temperature. The remaining hapten solution was frozen at -20°C for long term storage.

10 e) 90 minutes into step (d), 10mg of BAANS (Sigma) was dissolved in 500ul DMSO to a final concentration of 20mg/ml.

f) 1.5mg (75ul) of BAANS was added to 1ml of DT/CRM₁₉₇, slowly and dropwise (at 4mg/ml). It was reacted for 90 mins at <11°C. (0.375mg BAANS added per mg CRM₁₉₇/DT).

15 g) After 90mins, still operating at <11°C, the bromoacetylated CRM₁₉₇/DT was desalted into cold 100mM sodium carbonate/bicarbonate buffer, pH 9.1, using a NAP10 column (1ml on, 1.5ml off).

20 h) The hapten-spacer conjugate was aspirated from the TCEP gel, and 250ul (5mg) of the hapten-spacer conjugate was added into both activated CRM₁₉₇/DT samples, again slowly and dropwise, with mixing to prevent concentration gradients from forming.

i) After the hapten-spacer conjugate was added, the pH was checked and adjusted to 9.1 with dropwise addition of 0.1M NaOH/HCl if required.

25 j) The reaction mixture was kept in the antibodiesence of light, with mixing, for 18 hours.

k) After this time, 2ul N-acetyl cysteamine (NAC) was added to each reaction mixture and reacted, again in antibodiesence of light, for 3 hours with agitation (0.5ml per g CRM₁₉₇/DT).

30 l) After reaction, it was desalted into Dulbecco's PBS using a 10DG desalting column (BioRad) (3ml on, 4ml off).

m) Finally the sample was sterile filtered through a 0.22 µm syringe filter and aseptically aliquoted. These aseptic aliquots were stored at 2-8°C until shipping to the study location.

n) Using a bovine serum albumin standard, the final conjugate was analysed for protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay and

the final concentration was determined as 1.59 mg/ml. The conjugate was also analysed by SDS-PAGE electrophoresis and for endotoxin content using the LAL assay. Finally, a 150 µg sample of the conjugate was hydrolysed and analysed using reversed-phase HPLC to determine the number of haptens incorporated per protein molecule.

- 5 The hapten – CRM₁₉₇ conjugate was found to have 11.6 haptens per protein monomer

Example 10. It was found that by increasing the BAANS concentration of the reaction mix, the amount of hapten/hapten-spacer conjugate loaded onto the carrier protein could be controlled. An example of a conjugation of preparation 57 to CRM₁₉₇ is shown below.

- 10 a) 9ml of CRM₁₉₇ (53.19mg at 5.91mg/ml) was thawed and desalted into 100mM phosphate buffer, pH 8.0, using 10DG desalting columns (BioRad) (on: 3ml, off: 4ml). The concentration was adjusted to 4mg/ml using the same buffer.

b) 30mg of the hapten-spacer conjugate of preparation 57 was dissolved in 1.5ml DMSO (final conc. 20 mg/ml).

- 15 c) 1ml TCEP gel (Pierce) was prepared by washing twice in dulbecco's PBS, before 1.5ml of the hapten-spacer conjugate containing solution was added. This was then incubated at 2-8°C on a rotating platform for 2 hours.

- d) After 1 hour of the TCEP incubation, 10 ml of 4mg/ml CRM₁₉₇ solution was split into 5 x 2ml aliquots in separate reaction vessels, and stored in a cold room for 30 mins to adjust the temperature of the solution to 2-8°C. All future steps were performed at 2-8°C

- e) At the same time, a stock solution of BAANS was prepared by dissolving 20mg BAANS in 1ml DMSO. The 5 reaction vessels were labelled as either condition 1, 2, 3, 4 or 5. After the temperature of the CRM₁₉₇ aliquots was adjusted to 2-8°C, varying amounts of BAANS solution (50ul, 100ul, 150ul, 225ul and 300ul) was added to the 5 x 2ml reaction vessels, as indicated in table 2. The BAANS solution was added slowly (drop-wise) and with mixing.

- f) The aliquots were reacted for 30mins at 2-8°C on a rotating platform.

- g) After the 30 min incubation, 5 x 2ml bromoacetylated CRM₁₉₇ was desalted into 100mM sodium carbonate /bicarbonate buffer pH 9.1 using NAP25 (Gibco) columns (ON: 2ml, OFF: 3ml).

- h) The TCEP gel was spun out from the hapten samples and 5.6mg (280ul) of the hapten-spacer conjugate was added (slowly, dropwise) with mixing, to each of the

desalted bromoacetylated CRM₁₉₇ samples. The reaction vessels were covered in foil, to protect the contents from light.

- i) The mixtures were reacted for 18 hours on a rotating platform
- j) Unreacted BrAc groups were quenched by adding 0.5ml NAC per g CRM₁₉₇ (therefore 4ul per reaction) to the reaction mixture. The mixture was allowed to react for 3 hours on a rotating platform (still covered in foil to protect from light)
- k) Finally, each reaction was desalted into dulbecco's PBS using DG10 columns (ON:3ml, OFF: 4ml). Final volume was 4ml.
- l) The samples were sterile filtered through a 0.22 µm syringe filter and aseptically aliquoted. These aseptic aliquots were stored at 2-8°C until shipping to the study location.
- m) Using a bovine serum albumin standard, the final conjugates were analysed for protein content using the Bradford (Pierce Coomassie Brilliant Blue reagent) assay and the final concentration was determined as shown in Table 3. The conjugates were also analysed by SDS-PAGE electrophoresis, and for endotoxin content, using the LAL assay. Finally, a 450 µg sample of each conjugate was hydrolysed and analysed using reversed-phase HPLC (N=3) to determine the number of haptens incorporated per protein molecule (hapten load data shown in table 5).

Table 3

conjugate	CRM ₁₉₇ (mg)	BAANS (mg)	Amount of BAANS solution added (20mg/ml)	Molar excess compared to CRM ₁₉₇
CRM ₁₉₇ - preparation 57 cond.1	8	1	50ul	30
CRM ₁₉₇ - preparation 57 cond.2	8	2	100ul	60
CRM ₁₉₇ - preparation 57 cond.3	8	3	150ul	90
CRM ₁₉₇ - preparation 57 cond.4	8	4.5	225ul	135
CRM ₁₉₇ - preparation 57 cond.5	8	6	300ul	180

Table 4

conjugate	protein conc. (mg/ml)
CRM ₁₉₇ - preparation 57 cond.1	1.76
CRM ₁₉₇ - preparation 57 cond.2	1.79
CRM ₁₉₇ - preparation 57 cond.3	1.73
CRM ₁₉₇ - preparation 57 cond.4	1.57
CRM ₁₉₇ - preparation 57 cond.5	1.63

Table 5

Sample	Rep	Total Load	Unconj	Conjugated	Mean load
CRM ₁₉₇ - preparation 57 cond.1	1	6.1	ND	6.1	6.7
	2	8.5	ND	8.5	
	3	5.5	ND	5.5	
CRM ₁₉₇ - preparation 57 cond.2	1	7.8	ND	7.8	9.1
	2	10.8	ND	10.8	
	3	8.7	ND	8.7	
CRM ₁₉₇ - preparation 57 cond.3	1	9.1	ND	9.1	11.9
	2	12.5	ND	12.5	
	3	14	ND	14	
CRM ₁₉₇ - preparation 57 cond.4	1	12.5	ND	12.5	14.5
	2	16.8	ND	16.8	
	3	14.2	ND	14.2	
CRM ₁₉₇ - preparation 57 cond.5	1	12.2	ND	12.2	15.1
	2	15.5	ND	15.5	
	3	17.6	ND	17.6	

Example 11. BALB/c mice (Charles River, Montreal, QC.) (n = 12 per group) were immunized with 10 µg of the conjugates of examples 1 to 4 by intra-muscular

injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. Anti-nicotine IgG antibody levels in plasma were measured by ELISA as described above.

The results are shown in Figure 1, from which it can be seen that a strong anti-nicotine antibody response is obtained, for each tested conjugate, 4 weeks after the priming injection, which response is sustained or increased 2 weeks after each of the first and second boosting injections.

Example 12. BALB/c mice (n = 12 per group) were immunized with 10 µg of the conjugates of examples 1 to 4 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. Avidity indexes were calculated at various time-points. The Avidity Index corresponds to the concentration of ammonium thiocyanate required to elute 50% of anti-nicotine antibodies from Nicotine-BSA coated plates as described above.

The results are shown in Figure 2, from which it can be seen that the antibodies in mice by the tested conjugates exhibit a high avidity 4 weeks after the priming injection, which is sustained or increased 2 weeks after each of the first and second boosting injections.

Example 13. BALB/c mice (n = 6 per group) were immunized with 10 µg of the conjugates of examples 1 to 4 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. At 2 weeks after the last boost, ³H-nicotine (0.05mg/kg nicotine containing 3 µCi ³H-nic) was administered by intravenous injection, blood was collected, the animals were perfused, the brains were removed, and the levels of ³H quantified and the brain/plasma ratio of ³H determined as described above.

The results are shown in Figure 3, from which it can be seen that the plasma/brain ratio of the tested conjugates is significantly greater than that of control animals, indicating that the antibodies induced by the tested conjugates are specific for nicotine and have sequestered nicotine in the blood and prevented nicotine uptake into the brain.

Example 14. BALB/c mice (n = 12 per group) were immunized with 10 µg of the conjugates of examples 1 to 4 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. At 2 weeks after the second boost, interaction of anti-nicotine antibodies with nicotine was demonstrated by competition ELISA as described above.

The results are shown in Figure 4, from which it can be seen that the antibodies induced by the tested conjugates recognize and bind to nicotine.

Example 15. BALB/c mice (n = 12 per group) were immunized with 10 µg of the conjugates of examples 1 and 2 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. At 2 weeks after the second boost, the specificity of anti-nicotine antibodies to nicotine, cotinine, acetylcholine and varenicline was determined by competition ELISA as described above.

The results are shown in Figure 5, from which it can be seen that there is a dose-dependent inhibition of binding of anti-nicotine antibodies to nicotine-coated ELISA plates as amount of added nicotine is increased. However, no such effect is observed with varenicline, cotinine or acetylcholine, indicating that the antibodies induced by the tested conjugates are specific for nicotine and not for cotinine, varenicline or acetylcholine.

Example 16. BALB/c mice (n = 12 per group) were immunized with 10 µg of the conjugates of example 5 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA as described above.

The results are shown in Figures 6, 7, 8 and 9 from which it can be seen that a very strong anti-nicotine antibody response is obtained, for each tested conjugate, 4 weeks after the priming injection, which response is increased 2 weeks after boost. Furthermore, both levels of anti-nicotine antibodies as well as avidity of anti-nicotine antibodies vary depending on spacer used. In addition, all tested conjugates result in higher plasma/brain ratios than in control animals, indicating that antibodies induced by tested conjugates can sequester nicotine in the blood and prevent its uptake into brain to a greater extent than in control animals.

Example 17. BALB/c mice (n = 12 per group) were immunized with 10 µg of the conjugates of Example 6 and 7 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 50 µg CpG 24555. Anti-nicotine antibody levels (total IgG) in plasma were measured by ELISA. At 2 weeks after the last boost, ³H-nicotine (0.05mg/kg nicotine containing 3 µCi ³H-nic) was administered by intravenous injection, blood was collected, the animals were

perfused, the brains were removed, and the levels of ^3H quantified and the %change in levels of ^3H relative to control animals was determined as described above.

The results are shown in Figures 12 (anti-nicotine antibody levels) and 13 (%change in levels of ^3H) from which it can be seen that an immune response is obtained, for each tested conjugate, which response increased in a dose dependent manner. This indicates that both DT and CRM₁₉₇ are suitable carriers for the nicotine-derived haptens. In addition, all tested conjugates result in lower levels of ^3H -nicotine entering the brain than in control animals, indicating that antibodies induced by tested conjugates can sequester nicotine in the blood and prevent its uptake into brain to a greater extent than in control animals.

Example 18. BALB/c mice (n = 12 per group) were immunized with 10 μg of the conjugates of Example 6, 7 and 8 by intra-muscular injection (on days 0, 28, 42) in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and 50 μg CpG 24555. Anti-nicotine IgG Ab levels in plasma were measured by ELISA. At 2 weeks after the last boost, ^3H -nicotine (0.05mg/kg nicotine containing 3 μCi ^3H -nic) was administered by iv injection, blood was collected, the animals were perfused, the brains were removed, and the levels of ^3H quantified and the %change in levels of ^3H relative to control animals was determined as described above.

The results are shown in Figures 14 (anti-nicotine antibody levels) and 15 (%change in levels of ^3H) from which it can be seen that an immune response is obtained, for each tested conjugate, which response increased with hapten loading. In addition, all tested conjugates result in lower levels of ^3H -nicotine entering the brain than in control animals, indicating that antibodies induced by tested conjugates can sequester nicotine in the blood and prevent its uptake into brain to a greater extent than in control animals.

Example 19. BALB/c mice (n = 10 per group) were immunized with 10 μg of the conjugate of Example 7 by intra-muscular injection in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 μg Al^{3+}) and 10 μg CpG 24555, or in the presence of ISCOMATRIX (IMX; 0.1 to 3.0 Units). Anti-nicotine IgG Ab levels in plasma were measured by ELISA (day 21 and 28), and avidity by inhibition ELISA. The results are shown in Figures 16 and 17 from which it can be seen that an immune response (Ab levels and avidity) is obtained with the use of CpG 24555 and aluminium hydroxide as combination adjuvant, or with ISCOMATRIX as sole adjuvant, which response increased with ISCOMATRIX dose.

At 1 wk post 2nd immunization, ^3H -nicotine (0.05mg/kg nicotine containing 3 μCi ^3H -nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ^3H quantified and % change in ^3H -nicotine in blood and brains relative to control animals was determined. Results are shown in Figure 18 from which it can be seen that the use of CpG 24555 and aluminum hydroxide as combination adjuvants or ISCOMATRIX as sole adjuvant result in lower levels of ^3H -nicotine entering the brain than in control animals, indicating that antibodies induced by tested conjugates can sequester nicotine in the blood and prevent its uptake into brain to a greater extent than in unimmunized control animals.

Example 20. The samples described in Table 6 below were prepared as follows.

a) Frozen CRM₁₉₇ (200 ml at 5.9 mg/ml) was thawed overnight at 4°C.

b) Once thawed, the CRM₁₉₇ was concentrated from 200 ml to 100 ml by ultrafiltration (UF) using a Kwick Start polyethersulfone (PES) membrane with a 10 kD molecular weight cutoff.

c) The concentrated CRM₁₉₇ was diafiltered (DF) 8 TOVs (turn over volumes) using 50 mM MOPS (3-(N-morpholino)propanesulfonic acid), 50 mM NaCl, pH 7.2. The post UF/DF CRM₁₉₇ was filtered using a SARTOPORE 2 150 sterile capsule filter.

d) The concentration of the CRM₁₉₇ was determined by measuring absorbance at A₂₈₀ using an extinction coefficient of 0.942. The concentration was determined to be 9.65 mg/ml.

f) The post UF/DF CRM₁₉₇ was diluted to 7.18 mg/ml from 9.65 mg/ml using 50 mM MOPS, 50 mM NaCl, pH 7.2.

g) In a separate vessel, 6 M HCl solution (7.46 mL) was slowly added to 7460 mg of the hapten of preparation 12 while cooled in an ice bath. Then 50 mM MOPS, 50 mM NaCl, pH 7.2 buffer was added (2.00 mL) and the pH was checked by pH paper. The pH was approximately 9. 6 M HCl was added in small increments until a pH of 7.5 was achieved (1.70 mL HCl added). The total volume of the solution was 18.65 mL resulting in a concentration of 400 mg/mL of the hapten of preparation 12.

h) In a separate vessel, 7000 mg of sulfo-N-hydroxysuccinimide (sNHS) was dissolved in 50 mM MOPS, 50 mM NaCl, pH 7.2 solution (14 mL) and 19.25 M NaOH solution was added in small increments until a pH of 7.13 was achieved (1.44 mL of NaOH added). The total volume of the solution was 18.50 mL resulting in a concentration of 378 mg/mL of sNHS.

i) In a separate vessel, 8000 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was dissolved in 5 mL of 50 mM MOPS, 50 mM NaCl, pH 7.2. The total volume of the solution was 12.0 mL resulting in a concentration of 666 mg/mL of EDC. EDC solution was made last, just prior to addition to the conjugation reactions.

j) The post UF/DF CRM₁₉₇, the hapten of preparation 12, sNHS, and EDC solutions were combined in such a way as to generate the 24 samples listed in Table 6. For the 24 samples, the correctly calculated amounts of the solutions were mixed together in the following manner: UF/DF CRM₁₉₇, the hapten of preparation 12, and sNHS were combined and the pH of the mixture was checked. For samples number 10 and 18, the pH was further adjusted using 1 N NaOH to bring the pH to approximately 7.5. Lastly, EDC was added to the samples. Each reaction tube was briefly vortexed and incubated at 15°C in a water bath for 18 hrs.

k) After 18 hours of incubation, samples were buffer exchanged using Amicon ultra centrifugal concentrators with a 30 kD molecular weight cutoff. The buffer used was 20 mM potassium phosphate, 20 mM histidine, pH 7.0. Protein concentration was determined using a commercially available micro BCA kit.

l) 200 mg/ml sucrose was dissolved in water and added to the each of the 24 conjugated samples at a 1:1 (volume) ratio. Subsequently, PS80 was added to a final concentration of 0.2 mg/ml. The samples were stored at 4°C for short term storage, -20°C for long term storage.

Table 6

Sample	sNHS: CRM ₁₉₇ molar ratio	Hapten of Prep 12: CRM ₁₉₇ molar ratio	EDC: CRM ₁₉₇ molar ratio	CRM ₁₉₇ (mg/mL)	IC ₅₀	Efficacy
1	2600	2550	3000	3.0	7.95	41.0
2	5000	2550	3000	3.0	4.85	46.0
3	3800	4672	3000	3.0	9.05	50.0
4	3800	3258	4634	3.0	10.1	33.0
5	3800	3258	3408	4.6	8.05	38.0

6	200	2550	3000	3.0	5.65	43.0
7	1400	428	3000	3.0	18.25	26.0
8	1400	1842	1366	3.0	5.3	48.0
9	1400	1842	2592	1.4	4.75	45.0
10	3800	428	3000	3.0	20.4	21.0
11	3800	1842	1366	3.0	5.6	32.0
12	3800	1842	2592	1.4	4.65	33.0
13	1400	4672	3000	3.0	8.43	32.0
14	2600	3964	1366	3.0	3.87	26.0
15	2600	3964	2592	1.4	6.84	41.0
16	1400	3258	4634	3.0	7.97	32.0
17	2600	1136	4634	3.0	15.81	27.0
18	2600	2550	4226	1.4	12.16	36.0
19	1400	3258	3408	4.6	19.82	20.0
20	2600	1136	3408	4.6	18.37	33.0
21	2600	2550	1774	4.6	10.12	42.0
22	2600	2550	3000	3.0	8.51	40.0
23	2600	2550	3000	3.0	8.3	46.0
24	2600	2550	3000	3.0	16.71	44.0

The analytical methodology utilized to examine the samples 1-24 are detailed below.

SELDI-MS. Surface-enhanced laser desorption ionization (SELDI) is an ionization method in mass spectrometry that is used for the analysis of protein mixtures. The assay reports net mass gain to the CRM₁₉₇ scaffold through the conjugation process. The change in mass is reported as adducts to the scaffold. Ideally, the adducts should equate with the Hapten specific Epitope Density assay. Adducts = $(\text{Mass}_{\text{Conjugate}} - \text{Mass}_{\text{Scaffold}}) / \text{Mass}_{\text{Hapten}}$. It is noted that adducts levels in excess of the

ED value indicates that mass other than hapten is added to the scaffold; and that samples high in adduct are also high in HMMS.

Size Exclusion Chromatography. A size exclusion assay was developed to resolve HMMS (high molecular mass species), Dimer, Monomer and LMMS (low molecular mass species) towards understanding process parameters that impact relative abundance of these components. The method reports relative area percents for the HMMS peak group, Dimer, Monomer and the LMMS peak group. It is noted that process stoichiometry and ratio's of the starting materials impacted the apparent distribution of peak area between HMMS, Dimer, Monomer and LMMS.

Epitope Density. A reversed phase liquid chromatography assay coupled to an acid hydrolysis sample preparation was developed to determine the amount of hapten conjugated to the CRM₁₉₇ carrier protein. The hapten molecule is conjugated via an amide bond to the CRM₁₉₇ scaffold; this bond is hydrolyzed to release hapten along with the substituent amino acids according to standard hydrolysis chemistry. The amount of conjugated hapten 7 is a measure of process consistency, product quality and efficacy.

The results of the characterization of samples 1-24 are shown in Table 7. This data shows a strong relationship between Efficacy and Hapten epitope density coupled to CRM₁₉₇. In addition, it shows that optimal epitope density values correlate with high monomer, low HMMS and low adducts.

Table 7

Sample	Acid Hydrolysis Epitope Density	SELDI-MS Apparent Epitope Density	High Molecular Mass Species (HMMS) %	Dimer %	Monomer %	Low Molecular Mass Species (LMMS) %
1	18.6	19.9	0.0	0.0	100.0	0.0
2	13.0	15.8	0.0	0.0	100.0	0.0
3	14.3	12.2	0.0	0.0	94.1	5.9
4	27.1	30.4	1.9	0.0	95.1	3.0
5	20.8	24.8	1.3	0.0	98.7	0.0

6	15.2	17.4	0.0	0.0	100.0	0.0
7	13.1	34.3	20.0	20.4	49.8	9.7
8	11.5	12.8	0.0	0.0	100.0	0.0
9	4.8	12.7	0.0	0.0	100.0	0.0
10	5.4	30.5	10.2	9.2	72.1	8.5
11	6.7	6.8	1.6	6.3	84.8	7.3
12	9.0	12.9	0.0	0.0	100.0	0.0
13	17.4	23.9	5.0	11.8	83.2	0.0
14	4.5	4.8	4.6	4.5	79.5	11.3
15	7.9	10.3	0.3	0.0	99.7	0.0
16	25.0	31.7	0.0	0.0	100.0	0.0
17	15.6	41.6	7.7	10.2	82.1	0.0
18	7.0	21.9	0.0	0.0	100.0	0.0
19	30.0	36.3	0.0	0.0	100.0	0.0
20	17.5	42.2	5.0	0.0	86.3	8.7
21	10.5	12.1	0.0	0.0	99.3	0.7
22	13.2	16.7	14.4	0.0	57.0	28.6
23	18.1	25.0	0.0	0.0	100.0	0.0
24	16.1	23.6	0.0	0.0	100.0	0.0

Example 21. BALB/c mice (n = 10 per group) were immunized with 10 µg of samples 1-24 (see Example 20, Tables 6 and 7) by intra-muscular injection in the

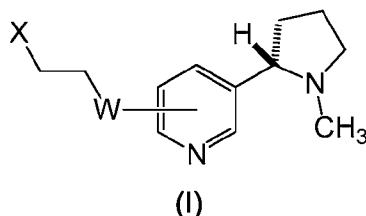
presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 10 µg CpG 24555. Anti-nicotine IgG Ab levels in plasma were measured by ELISA (day 21 and 28), and avidity by inhibition ELISA. The results are shown in Figures 19 and 20 from which it can be seen that an immune response is obtained, for each tested conjugate, which response increased with % monomer and which was optimal with a hapten load of between 10 to 18 haptens per unit carrier.

At 1 wk post 2nd immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in blood and brains relative to control animals was determined. Results are shown in Figures 21 and 22 from which it can be seen that all tested conjugates result in lower levels of ³H-nicotine entering the brain than in non-immunized control animals, indicating that antibodies induced by tested conjugates can sequester nicotine in the blood and prevent its uptake into brain to a greater extent than in control animals, and that efficacy increased with % monomer (Figure 21) and was optimal with a hapten load of between 10 to 18 haptens per unit carrier (Figure 22).

Example 22. Samples 1-24 (see Example 20, Tables 6 and 7) were tested for binding to Alhydrogel. BALB/c mice (n = 10 per group) were also immunized with 10 µg of these different conjugates by intra-muscular injection in the presence of aluminium hydroxide (alum; Alhydrogel-85: 40 µg Al³⁺) and 10 µg CpG 24555. At 1 wk post 2nd immunization, ³H-nicotine (0.05 mg/kg nicotine containing 3 µCi ³H-nic) was administered by IV injection, blood collected, animals perfused, brains removed, levels of ³H quantified and % change in ³H-nicotine in blood and brains relative to control animals was determined. Results are shown in Figure 23, from which it can be seen that conjugates with a higher % monomer content, have a higher % binding to Alhydrogel and that this correlates with a greater efficacy as demonstrated by a greater reduction in the amount of ³H-nicotine in the brain.

CLAIMS

1. A haptén of the formula (I):

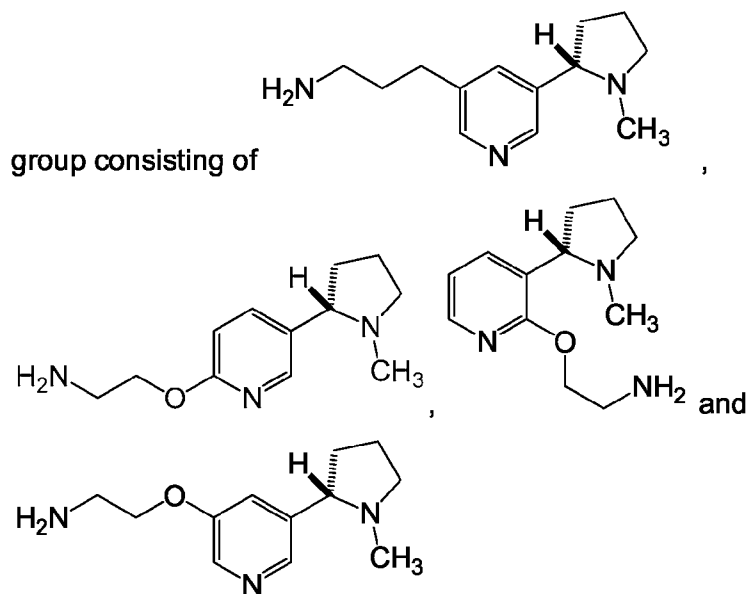


wherein

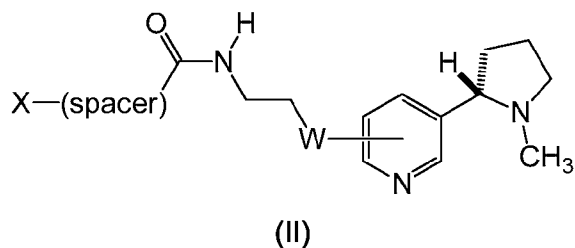
W is -CH₂- or -O-; and

X is -NH₂ or -SH.

2. The haptén according to claim 1, wherein W is in position 2, 5 or 6 of the pyridine ring.
3. The haptén according to claim 1 or claim 2, wherein W is -O- and W is in position 5 of the pyridine ring.
4. The haptén according to claim 1 or claim 2, wherein the haptén is selected from the



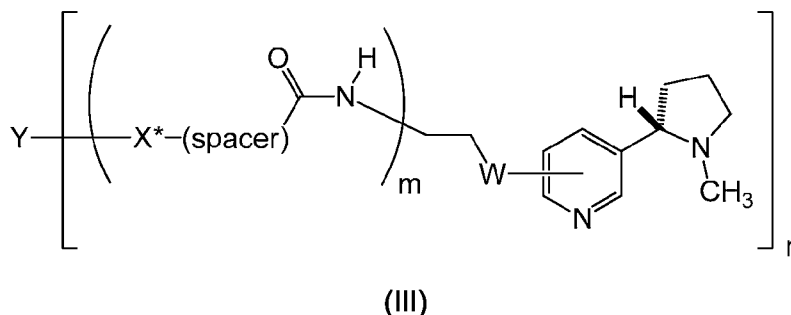
5. A haptén-spacer conjugate of the formula (II):



wherein W is -CH₂- or -O-;

-(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-; and
X is -NH₂ or -SH.

6. The hapten-spacer conjugate according to claim 5, wherein X is -SH.
7. The hapten-spacer conjugate according to claim 5, wherein X is -NH₂.
8. The hapten-spacer conjugate according to any of claims 5 to 7, wherein -(spacer)- is a C₁-C₈ alkylene group.
9. The hapten-spacer conjugate according to any of claims 5 to 8, wherein W is -O-; and W is in position 5 of the pyridine ring.
10. A hapten-carrier conjugate of formula (III):



wherein

W is -CH₂- or -O-;

-(spacer)- is a C₁-C₈ alkylene group, a C₃-C₁₀ cycloalkylene group or a C₁-C₁₂ alkylene group interrupted by 1 to 4 oxygen atoms and optionally interrupted by a -N(H)C(O)-;

X* is -NH- or -S-;

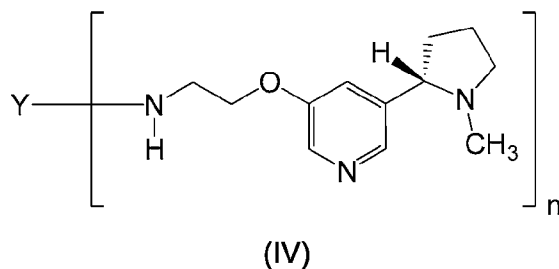
m is 0 or 1;

n is an integer from 1 to 1000; and

Y is an optionally modified carrier protein selected from bacterial toxoids, immunogenic substances, viruses, virus-like particles, protein complexes, proteins, polypeptides, liposomes and immuno-stimulating complexes.

11. The hapten-carrier conjugate according to claim 10, wherein W is in position 2, 5 or 6 of the pyridine ring.
12. The hapten-carrier conjugate according to claim 10 or claim 11, wherein W is -O- and W is in position 5 of the pyridine ring.
13. The hapten-carrier conjugate according to claim 12, wherein m is 0 and X* is -NH-.

14. The hapten-carrier conjugate according to any of claims 10 to 13, wherein the carrier protein is an optionally modified protein selected from derivatives of tetanus toxin (e.g., chemically inactivated tetanus toxoid), derivatives of diphtheria toxin (e.g., chemically inactivated diphtheria toxoid or the non-toxic genetic mutant CRM₁₉₇), keyhole limpet hemocyanin (KLH), hemocyanine, outer membrane protein complex (OMPC) from *Neisseria meningitidis*, the B subunit of heat-labile *Escherichia coli*, recombinant exoprotein A from *Pseudomonas aeruginosa* (rEPA) and other virus-like particles such as those assembled from recombinant coat protein of bacteriophage Qb, hepatitis B surface antigen, hepatitis B core antigen or a virosome.
15. The hapten-carrier conjugate of formula (III) according to any of claims 10 to 14, wherein the carrier is a protein selected from diphtheria toxoid and CRM₁₉₇, which are optionally modified.
16. The hapten-carrier conjugate of formula (III) according to any of claims 10 to 15, wherein the carrier protein is a modified succinylated protein.
17. The hapten-carrier conjugate according to any of claims 10 to 16, wherein n is an integer in the range of 10 to 18 inclusive.
18. A vaccine composition comprising:
a plurality of hapten-carrier conjugates according to any of claims 10 to 17; and
one or more adjuvants.
19. The vaccine composition according to claim 18, wherein one of the one or more adjuvants is CpG 24555.
20. The vaccine composition according to claim 18, wherein one of the one or more adjuvants is an aluminum salt.
21. The vaccine composition according to claim 20, wherein the aluminium salt is aluminum hydroxide.
22. The vaccine composition according to claim 21, wherein the adjuvants are CpG 24555 and aluminium hydroxide.
23. The vaccine composition according to claim 20, wherein one of the one or more adjuvants is an immune stimulatory complex (ISCOM).
24. The vaccine composition according to claim 23, wherein the immune stimulatory complex is ISCOMATRIX.
25. A vaccine composition comprising CpG 24555 and a plurality of hapten-carrier conjugates of the formula (IV):

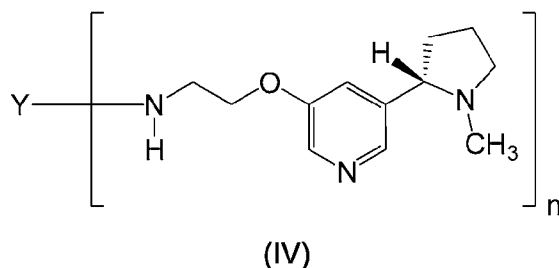


wherein

Y is diphtheria toxoid or CRM₁₉₇; and

n is an integer in the range of 1 to 40 inclusive.

26. A vaccine composition comprising CpG 24555, aluminium hydroxide, and a plurality of hapten-carrier conjugates of the formula (IV):

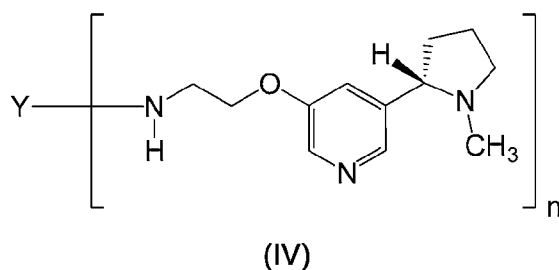


wherein

Y is diphtheria toxoid or CRM₁₉₇;

and n is an integer in the range of 1 to 40 inclusive.

27. A vaccine composition comprising ISCOMATRIX and a plurality of hapten-carrier conjugates of the formula (IV):



wherein

Y is diphtheria toxoid or CRM₁₉₇;

and n is an integer in the range of 1 to 40 inclusive.

28. The vaccine composition of any one of claims 25 to 27, wherein n is an integer in the range of 10 to 18 inclusive.

29. The vaccine composition of any one of claims 18 to 28, wherein at least 70% of the plurality of hapten-carrier conjugates comprise carrier proteins (Y) which are not high molecular weight species.
30. A hapten-carrier conjugate according to claims 10 to 17, or a vaccine composition according to claims 18 to 29, for use as a medicament.
31. A hapten-carrier conjugate according to claims 10 to 17, or a vaccine composition according to claims 18 to 29, for use in increasing quit rates in smokers wishing to quit and reducing relapse rates in ex-smokers who have successfully quit through vaccination with an anti-nicotine vaccine or through previous treatment with a pharmacotherapy or by self-quit; or for use in treating, or preventing, nicotine dependence in a person in need of such treatment.
32. A method for aiding smoking cessation in smokers wishing to quit or preventing relapse in ex-smokers who have successfully quit through vaccination with an anti-nicotine vaccine or through previous treatment with a pharmacotherapy or by self-quit, or preventing nicotine dependence in a person in need of such treatment, the method comprising administering to the person the vaccine composition according to claims 18 to 29.