

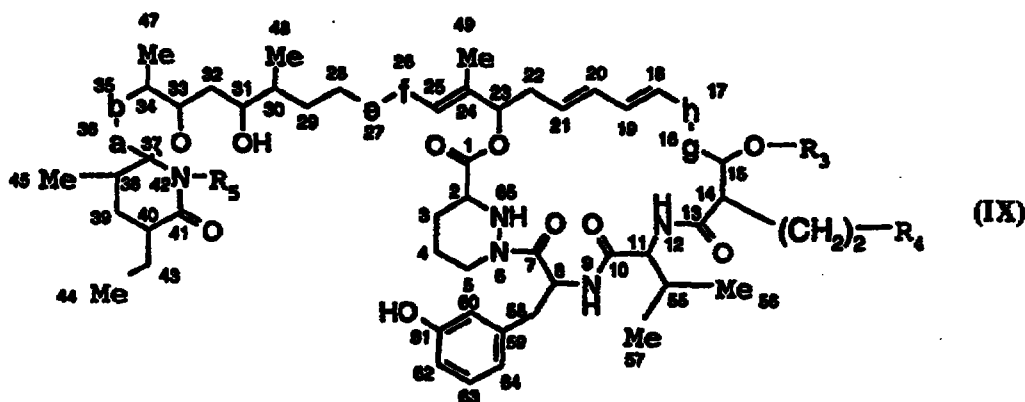
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| <p>(51) International Patent Classification <sup>6</sup> :<br/>C07K 5/062, C07D 309/10, 309/30,<br/>491/10, C12P 21/02, A61K 38/05, 31/35,<br/>31/445, G01N 33/68 // (C12P 21/02,<br/>C12R 1:465) (C07D 491/10, 311:00,<br/>221:00)</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | <p>A1</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | <p>(11) International Publication Number: WO 97/02285</p> <p>(43) International Publication Date: 23 January 1997 (23.01.97)</p> |
| <p>(21) International Application Number: PCT/EP96/02952 ✓</p> <p>(22) International Filing Date: 4 July 1996 (04.07.96)</p> <p>(30) Priority Data:<br/>9513596.8 4 July 1995 (04.07.95) ✓ GB<br/>9515495.1 28 July 1995 (28.07.95) ✓ GB</p> <p>(71) Applicant (for all designated States except AT DE US): SANDOZ LTD. [CH/CH]; Lichtstrasse 35, CH-4002 Basle (CH).</p> <p>(71) Applicant (for DE only): SANDOZ-PATENT-GMBH [DE/DE]; Humboldtstrasse 3, D-79539 Lörrach (DE).</p> <p>(71) Applicant (for AT only): SANDOZ-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H. [AT/AT]; Brunner Strasse 59, A-1230 Vienna (AT).</p> <p>(72) Inventors; and<br/>(75) Inventors/Applicants (for US only): FEHR, Theodor [CH/CH]; Gempenring 40, CH-4143 Dornach (CH). OBERER, Lukas [CH/CH]; Schlossgasse 17, CH-4456 Tenniken (CH). QUESNIAUX RYFFEL, Valerie [FR/CH]; Schützenweg</p> | <p>7, CH-4123 Allschwil (CH). SANGLIER, Jean-Jacques [CH/CH]; Rebeggassli 10, CH-4123 Allschwil (CH). SCHULER, Walter [DE/DE]; Rheinstrasse 21, D-79639 Grenzach-Wyhlen (DE). SEDRANI, Richard [LU/CH]; Herrengrabenweg 15, CH-4054 Basle (CH).</p> <p>(74) Common Representative: SANDOZ LTD.; Patents &amp; Trade-marks Div., Lichtstrasse 35, CH-4002 Basle (CH).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SI, SG, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AR, EP patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published<br/>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> |                                                                                                                                  |

(54) Title: MACROLIDES



## (57) Abstract

A novel class of macrolides in which i) positions 2 to 6 inclusive of the macrocyclic ring are provided by a piperidazinyl carboxylic acid residue; and/or ii) positions 7 to 9 inclusive of the macrocyclic ring are provided by an aromatic  $\alpha$ -amino acid residue; and/or iii) positions 10 to 12 inclusive of the macrocyclic ring are provided by an aliphatic  $\alpha$ -amino acid residue, preferably comprising two, or especially all three of the characteristic structural features i), ii) and iii), more especially a compound of formula (IX) is provided having immunosuppressant and anti-inflammatory properties and protected and ring-open forms thereof.

- ☐ Description : paged ..... to paged .....  
☒ Claim(s) : paged ..62.. to paged ..75..  
☐ Drawing(s) : paged ..... to paged .....  
☐ Abstract : paged ..... to paged .....

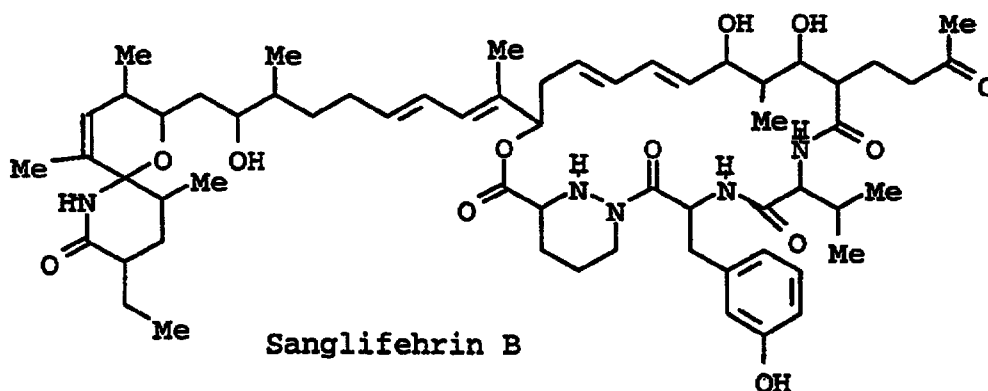
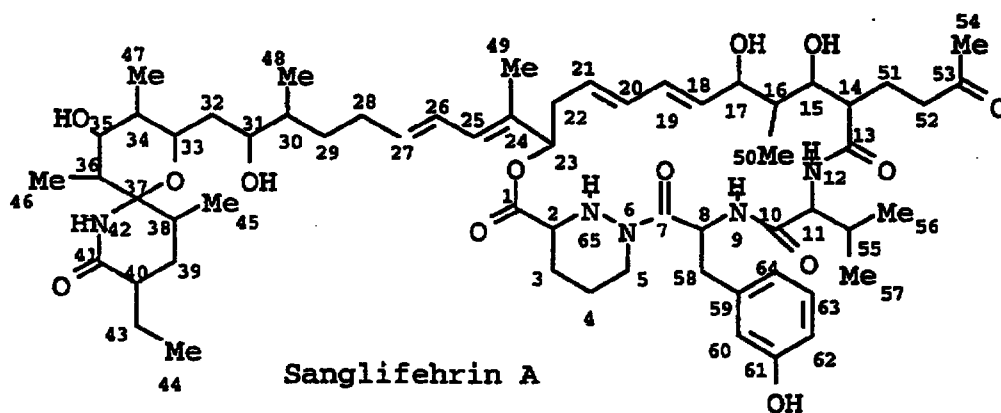
WO 97/02285

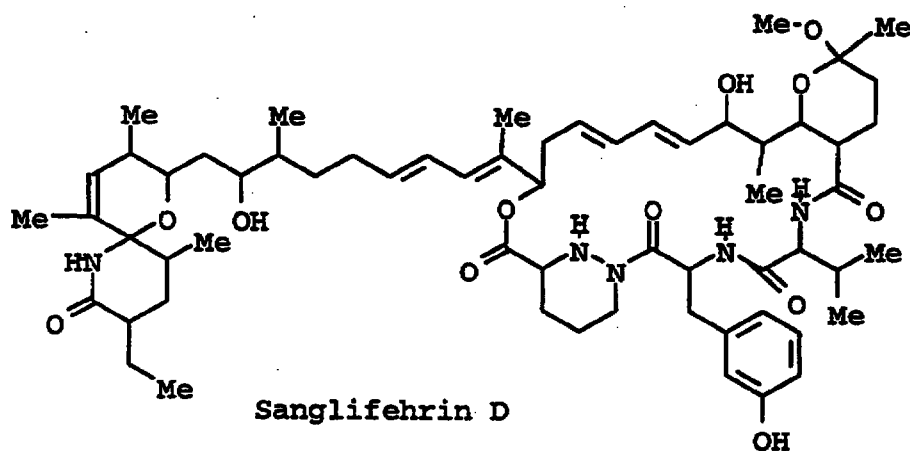
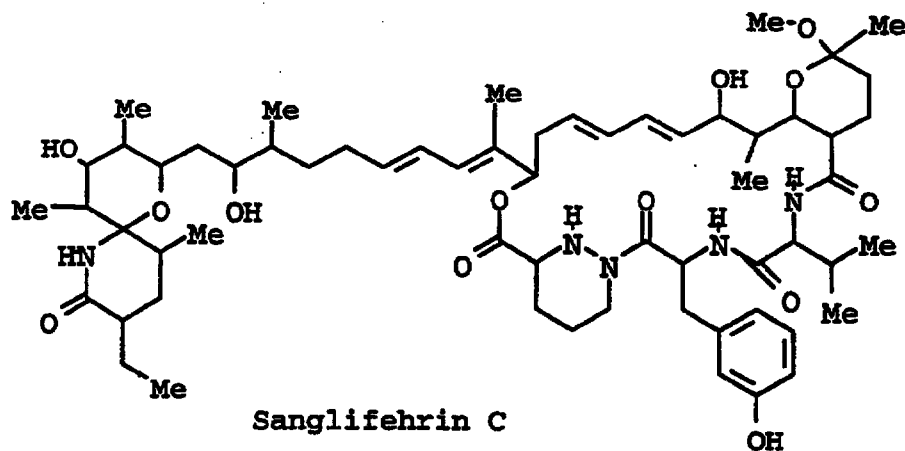
PCT/EP96/02952

# MACROLIDES

The present invention relates to a novel class of macrolides having valuable pharmaceutical and related activity. For convenience compounds of this novel macrolide class are referred to herein collectively as "Sanglifehrins".

The first of the Sanglifehrins were isolated from actinomycete fermentation broths. These are the Sanglifehrins A through D of formulae





As can be seen the macrocyclic ring of Sanglifehrins A to D is of entirely novel structure characterised in that i) positions 1 to 6 comprise a 3-carboxypiperidazinyl carboxylic acid residue, ii) positions 7 to 9 an aromatic  $\alpha$ -amino acid residue, and iii) positions 10 to 12 an aliphatic  $\alpha$ -amino acid residue. The remainder of the macrocyclic ring is comprised by an hydroxy carboxylic acid residue, providing, in the case of Sanglifehrins A to D a further 11 carbon atoms in the primary macrocyclic ring.

In accordance with conventional practice in macrolide chemistry the atoms of the Sanglifehrin primary macrocyclic ring are numbered as indicated above for Sanglifehrin A, starting with the carbon atom of the carbonyl group of the macrocyclic lactone linkage as position 1.

Sanglifehrins A to D are also characterised by the presence of a novel bicyclic spiro system attached at the 23 position of the macrocyclic ring via a hydrocarbyl linker group.

Sanglifehrins A to D may be subjected to extensive chemical manipulation to obtain yet further macrolides of the Sanglifehrin class. Such manipulations include cleavage of the macrocyclic ring, in particular at the lactone oxy-group, cleavage of the linker group between the macrolide and spiro ring systems, and manipulation, e.g. protection, derivatisation or other chemical modification of substituent groupings; for example, as hereinafter described. Further means of modification will be apparent to those skilled in the art.

In accordance with the invention it has been found that Sanglifehrins, in particular those in which the spiro ring system is present, as in the case of Sanglifehrins A to D, have a characteristic and entirely novel profile in terms of their biological activity. In particular they have been found to exhibit the following combination of activities:

- cyclophilin binding activity;
- immunosuppressive activity;
- inhibition of both B-cell and T-cell proliferation
- they do not, however, have FK binding protein binding activity or calcineurin inhibiting activity.

The Sanglifehrins can accordingly be seen as providing an exciting and novel class of immunosuppressant and antiinflammatory compounds. In particular the Sanglifehrins have an activity profile that differs from that of previously known immunosuppressant and antiinflammatory compounds such as cyclosporins and macrolides, e.g. of the rapamycin and FK 506 class, indicating that the Sanglifehrins have a different mode of action than such previous compounds. Thus the Sanglifehrins provide a novel category of drug substance both in terms of structure and activity which may be anticipated to materially extend the bounds of immunosuppressive and/or antiinflammatory therapy; for example, to avoid or reduce undesirable side effects of

previous immunosuppressive and antiinflammatory therapies and/or to improve or extend such therapy to new disease areas or new patient categories.

Sanglifehrins, e.g. in which the macrolide ring is in ring-opened form, in which the 26 and 27 positions in the hydrocarbyl linker between the macrolide and spiro ring systems are both hydroxy substituted, or in which the spiro residue attached to the macrocyclic ring has been cleaved or truncated, generally lack some or all of the combination of Sanglifehrin characteristic activities. For example, Sanglifehrins in which the spiro residue is cleaved typically possess cyclophilin binding activity but do not possess significant immunosuppressive activity. As will be apparent to those skilled in the art, however, such compounds provide valuable components, intermediates or key building blocks for the preparation of further novel Sanglifehrins, and hence further extend the therapeutic potential of the Sanglifehrin class.

In that its presence appears material to the biological activity, e.g. of the Sanglifehrins A to D, the bicyclic spiro system too may be viewed as providing a structural component with key biological significance, useful as a structural component for further derivatisation or modification both in relation to the production of further Sanglifehrins or for application in the derivatisation or modification of other drug substances; for example, to modify the activity of other immunosuppressive drug substances of the macrolide class.

As indicated, the Sanglifehrins represent a novel class of macrolide compounds of entirely novel and wholly characteristic structure.

Accordingly in a first aspect the invention provides:

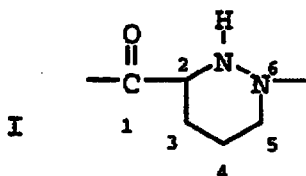
a macrolide in which

- i) positions 2 to 6 inclusive of the macrocyclic ring are provided by a piperidazinyl carboxylic acid residue; and/or
- ii) positions 7 to 9 inclusive of the macrocyclic ring are provided by an aromatic  $\alpha$ -amino acid residue; and/or

iii) positions 10 to 12 inclusive of the macrocyclic ring are provided by an aliphatic  $\alpha$ -amino acid residue, in free or protected form, or a salt thereof.

Suitably the macrolides of the invention comprise two, especially all three of the characteristic structural features i), ii) and iii).

The piperidazinyl carboxylic acid residue is suitably a 1,2 piperidazin-3-carboxy-1-yl residue of which the carboxy moiety occupies the 1-position, and the 1-nitrogen atom the 6-position of the macrocyclic ring, e.g. a residue of formula I



wherein the assigned numbers represent the position of the atoms of the residue in the macrocyclic ring. This residue may be ring substituted or unsubstituted. Suitably it is unsubstituted.

The  $\alpha$ -amino moiety of the aromatic  $\alpha$ -amino acid residue suitably occupies the 9-position of the macrocyclic ring. Suitably the aromatic  $\alpha$ -amino acid is a phenylalanine, especially 3-OH-phenylalanine, residue in free or protected form.

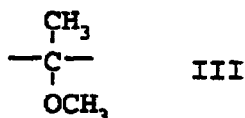
The  $\alpha$ -amino moiety of the aliphatic  $\alpha$ -amino acid residue suitably occupies the 12-position of the macrocyclic ring. Suitably the aliphatic  $\alpha$ -amino acid residue is a valine residue in free or protected form.

The remainder of the macrocyclic ring suitably comprises a hydroxy carboxylic acid residue, the oxy-moiety of which completes the macrocyclic lactone linkage and the carbonyl moiety of which forms an amide linkage with the  $\alpha$ -amino group at position 12 of the macrocyclic ring. The said hydroxy carboxylic acid residue suitably has a chain length of from 6 to 20, more suitably 11 carbon atoms. It

$$\begin{array}{ccccccccccccccc} & & & & & & R_1 & R_2 & R_3 & R_4 & & & & & \\ & & & & & & | & | & | & | & & & & & \\ \text{---} & \text{CH} & \text{---} & \text{CH}_2 & \text{---} & \text{CH} & \text{=CH} & \text{---} & \text{CH} & \text{=CH} & \text{---} & \text{CH} & \text{---} & \text{C} & \text{---} & \text{CH} & \text{---} & \text{CH} & \text{---} & \text{CO} & \text{---} \\ & | & & & & & & & & & & & & | & & & & & & & & \\ & \text{O} & & & & & & & & & & & & \text{CH}_3 & & & & & & & & \\ & | & & & & & & & & & & & & & & & & & & & \\ & & & & & & & & & & & & & & & & & & & & \end{array}$$

II

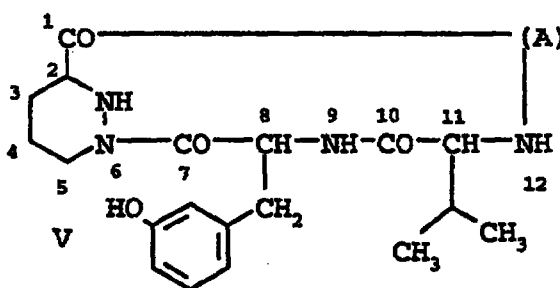
**R<sub>3</sub> and R<sub>4</sub> together represent a structure of formula III**



Preferred macrolides in accordance with the invention are accordingly those comprising a macrocyclic ring of formula IV

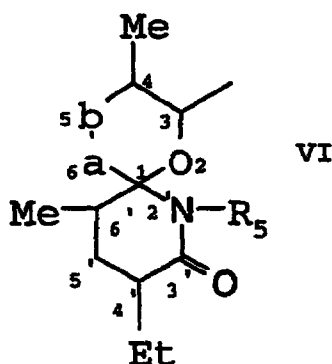


6



in free or protected form, or salt thereof

Generally in the Sanglifehrins, as in Sanglifehrins A to D, the macrocyclic ring is substituted at the carbon atom adjacent the oxy moiety of the lactone bridge. Typically this substituent comprises a 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue, e.g. of the formula VI



wherein

-a-b- is  $-(\text{Me})\text{C}=\text{CH}-$  or  $-(\text{Me})\text{CH}-\text{CH}(\text{OH})-$  and

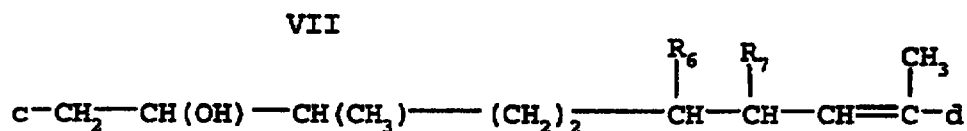
$\text{R}_5$  is H or Me

(wherein Me and Et represent methyl and ethyl respectively)

in free or protected form, or salt thereof linked to the macrolide ring via a linker comprising a linear sequence of from 6 to 11, typically 9, carbon atoms between the spiro residue and the macrolide ring.

The linker group may be substituted or unsubstituted and/or contain one or more unsaturated linkages in particular cumulative double bonds along its length. Suitably the linker group may be methyl substituted, e.g. by two methyl groups. Suitably the linker group may be further substituted by hydroxy, e.g. by 3 hydroxy

substituents, and/or may be ethylenically unsaturated, e.g. contain two carbon-carbon double bonds. More suitably the linker group comprises a 1-methyl-7-methyl-nonanoyl-9-yl, especially a 1-methyl-7-methyl-1-nonenoyl-9-yl or a 1-methyl-7-methyl-1,3-nonadienoyl-9-yl, residue optionally substituted, e.g. in the 3, 4, and/or 8 position. Preferably the linker group is a group of formula VII



wherein

c represents linkage to the spiro residue;

d represents linkage to the macrocyclic ring and

R<sub>6</sub> and R<sub>7</sub> are each OH or together represent an additional bond,

in free or protected form.

The linker group will generally be attached to the macrocyclic ring at the carbon atom immediately adjacent to the lactone oxy group, i.e. when the macrocyclic ring comprises an 11-oxy-endecanoyl-11-yl residue, at the 11 position of thereof.

Accordingly the invention provides compounds of formula VIII



wherein

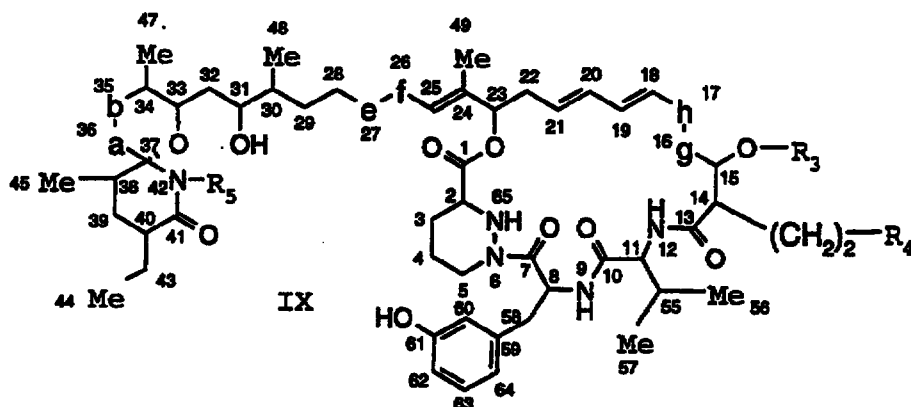
S represents a spiro bicyclo residue as previously defined;

L represents a linker group as previously defined, and

M represents a macrolide ring as previously defined,

in free or protected form, or salt thereof.

Particular compounds of the invention are those of formula IX



wherein

-a-b- is as defined above;

-e-f- is -CH(OH)-CH(OH)- or -CH=CH-;

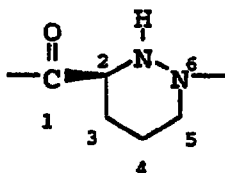
-g-h- is as defined above for -a-b-, and

R<sub>3</sub>, R<sub>4</sub> and R<sub>5</sub> are as defined above,

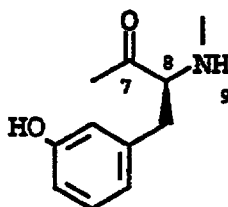
in free or protected form or salt thereof

The compounds of formulae I to IX contain asymmetric carbon atoms and thus may exist in a number of epimeric forms. All of the possible epimers as well as diastereoisomeric mixtures thereof are encompassed in the invention. However, compounds of formulae VIII and IX in which the macrolide ring is in ring-closed form and which are of appropriate stereochemistry typically possess activities which are characteristic of Sanglifehrins, as hereinbefore referred to. Epimers which possess sanglifehrin characteristic activities are preferred. In general, e.g. for pharmaceutical use in accordance with the invention, epimers which possess sanglifehrin characteristic activities in pure or substantially pure form (i.e. free or substantially free of epimers which lack sanglifehrin characteristic activities), e.g. comprising at least 90%, e.g. at least 95% of active epimer (i.e. comprising less than 10%, e.g. less than 5% of inactive epimer) will be preferred.

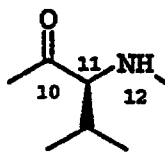
Preferably the 3-carboxypiperidazinyl carboxylic acid residue i) at positions 1 to 6 of the macrocyclic ring has the following conformation:



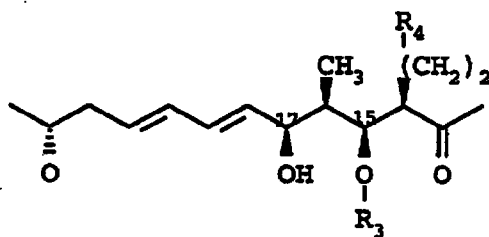
Preferably the aromatic amino acid ii) at positions 7 to 9 of the macrocyclic ring has the L configuration, e.g. is of configuration



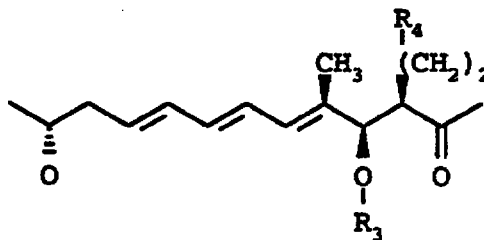
Preferably the aliphatic amino acid iii) at positions 10 to 12 of the macrocyclic ring has the L configuration, e.g. is of configuration



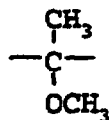
When the remainder of the macrocyclic ring comprises a residue of formula II, it preferably has the configuration



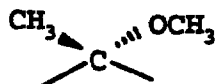
or



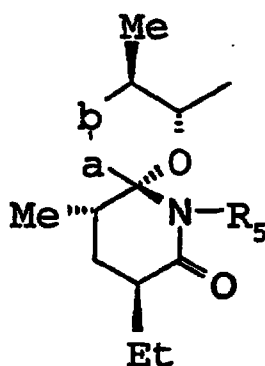
When  $R_3$  and  $R_4$  together represent



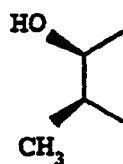
it preferably has the configuration



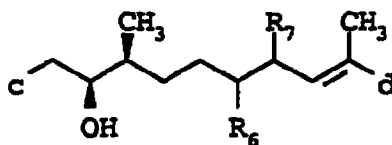
Preferably the 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue has the configuration



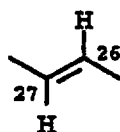
wherein when -a-b- is -(Me)CH-CH(OH)-, it preferably has the configuration



When the linker is of formula VII, it is preferably of configuration



When  $R_6$  and  $R_7$  are each OH, the configuration at positions 26 and 27 is preferably either 26(S), 27(S) or 26(R), 27(R). When  $R_6$  and  $R_7$  together represent an additional bond, the configuration at positions 26 and 27 is preferably

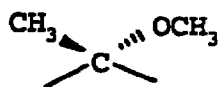


The chemical structure is a complex molecule with numerous numbered atoms (1-62) and various functional groups. Key features include:

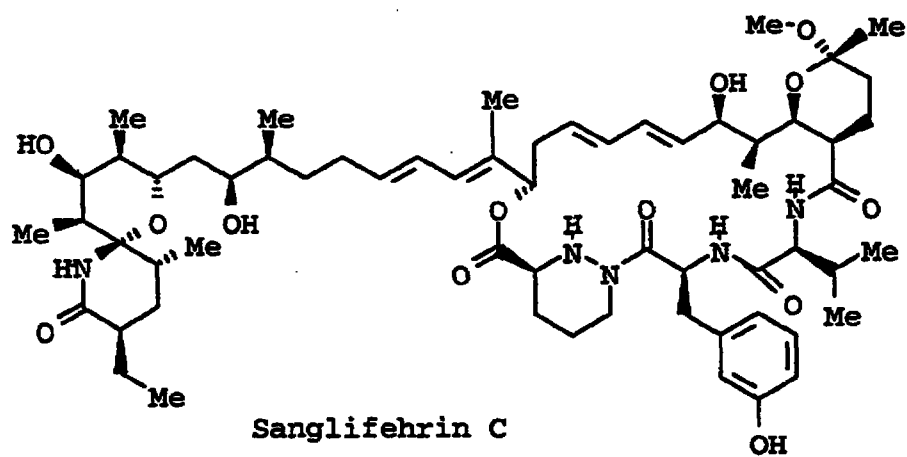
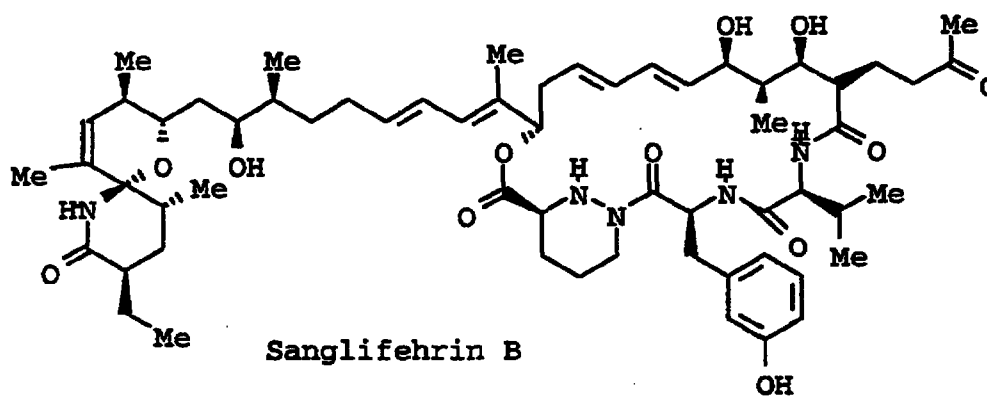
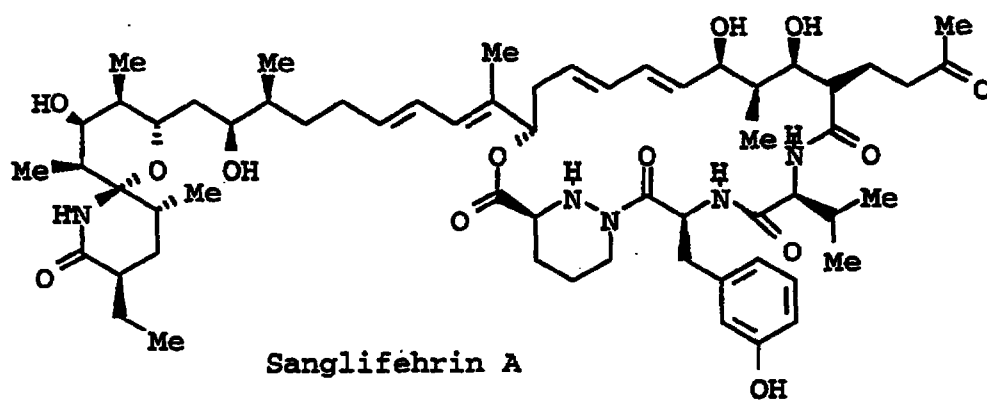
- Top Left:** A chain of atoms including a methyl group (Me) at 47, a hydroxyl group (OH) at 31, and a methyl group (Me) at 48. Atoms 32, 33, 34, 35, 36, and 37 are also labeled.
- Top Center:** A chain of atoms including a methyl group (Me) at 49, a carbonyl group (C=O) at 25, and a methyl group (Me) at 20. Atoms 21, 22, 23, 24, and 26 are also labeled.
- Top Right:** A chain of atoms including a methyl group (Me) at 17, a carbonyl group (C=O) at 16, and a methyl group (Me) at 15. Atoms 18, 19, and 20 are also labeled.
- Bottom Left:** A ring system including a methyl group (Me) at 45, a methyl group (Me) at 44, and a methyl group (Me) at 43. Atoms 38, 39, 40, 41, and 42 are also labeled.
- Bottom Center:** A ring system including a methyl group (Me) at 56, a methyl group (Me) at 55, and a methyl group (Me) at 57. Atoms 58, 59, 60, 61, and 62 are also labeled.
- Bottom Right:** A chain of atoms including a methyl group (Me) at 14, a carbonyl group (C=O) at 13, and a methyl group (Me) at 12. Atoms 15, 16, and 17 are also labeled.

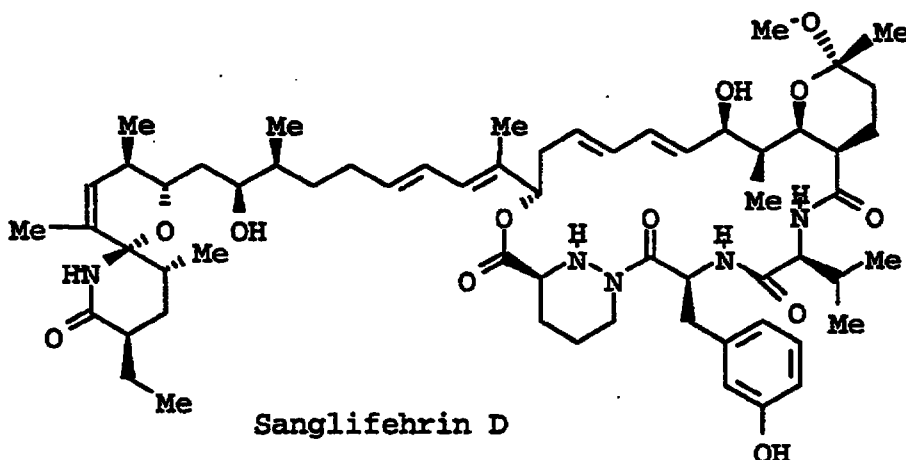
C[C@H](O)[C@H](O)CCC(C)(O)CCC(=C)C

12

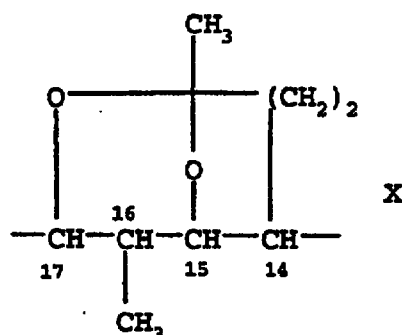


Preferably Sangliffehrins A to C have the following configurations

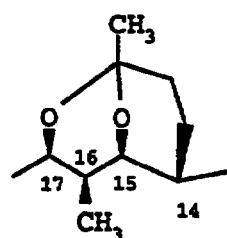




The compounds of the invention may be in free or protected form, e.g. in protected forms as described in "Protective Groups in Organic Synthesis" by T. W. Greene and P. G. M. Wuts, 2nd Edition, 1991, John Wiley & Sons Inc., New York. In particular OH groups may be in protected form, e.g. in the form of silyl ethers (for instance as described in pages 68-86 of Greene and Wuts *ibid.*), esters (see e.g. pages 87-103 of Greene and Wuts *ibid.*) and carbonates (see e.g. pages 104-111 of Greene and Wuts *ibid.*). Such protected forms also include internally protected forms; for example, in the case of macrolides of formula IX, wherein -g-h- is -CH(CH<sub>3</sub>)-CH(OH)-, the protected form wherein the 14 to 17 positions of the macrocyclic ring comprise a residue of formula X:



e.g. of configuration



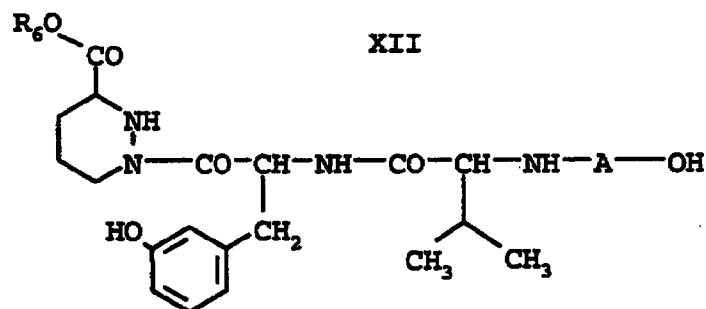
Also for example, 1,3 diols present in Sanglifehrins may be protected as appropriate ring structures, e.g. as described on pages 118-142 of Greene and Wuts *ibid.*

Compounds of the invention also exist in salt form. Examples of suitable pharmaceutically acceptable salts for use in accordance with the invention include acid and base addition salts as appropriate having regard to the particular substituents present in the compound.

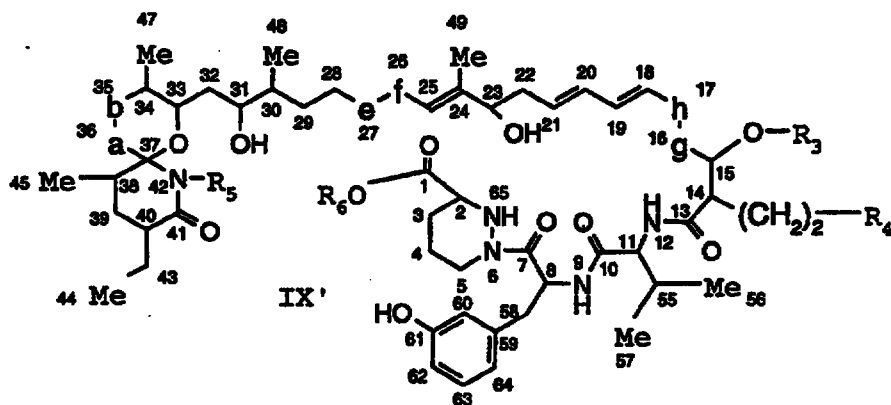
As previously indicated the macrocyclic ring of the compounds of the invention can be cleaved, in particular at the lactone oxy group, to provide compounds wherein the macrocyclic ring is in ring-open form. Generally cleavage of the lactone oxy group proceeds by hydrolysis (solvolysis), e.g. to provide compounds of formula XI:



e.g. of formula XII



e.g. a compound of formula IX'



wherein X, Y, Z, A, R<sub>3</sub>, R<sub>4</sub> and R<sub>5</sub> are as defined above and R<sub>6</sub> is H or C<sub>1-4</sub> alkyl, e.g. methyl.

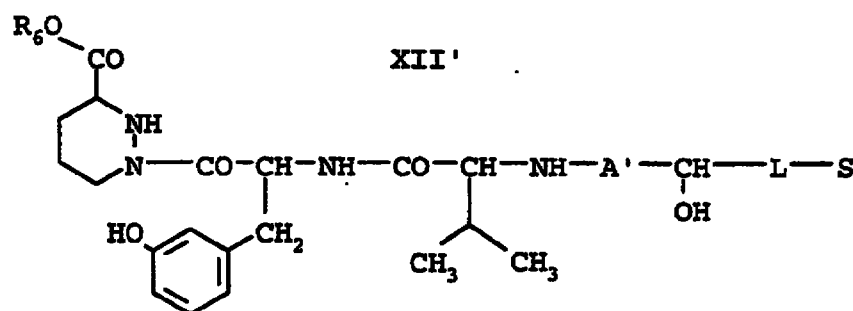
Such ring-opened forms provide intermediate means for modification of the basic Sanglifehrin macrocyclic ring system and are also part of the present invention.

Accordingly in a further aspect the present invention provides:

- a macrolide as hereinbefore defined in ring-opened form, said ring-opened macrocycle being in free or protected form, or salt thereof;
- a compound R<sub>6</sub>O - X - Y - Z - A - OH as defined above, in free or protected form, or salt thereof;

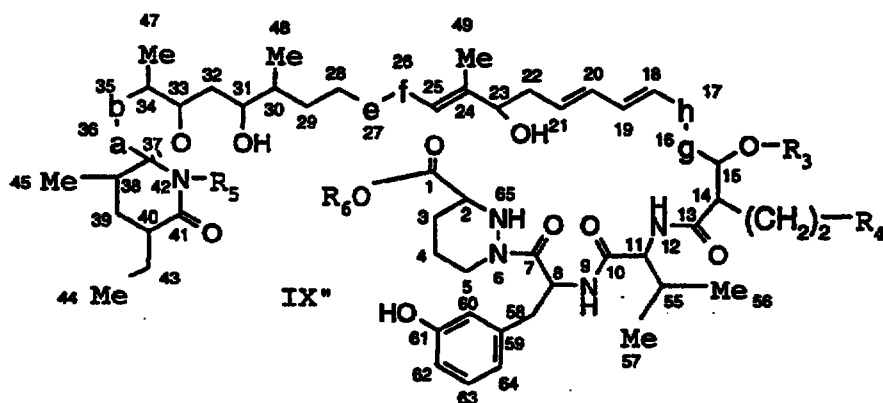
- a compound  $R_6O - X - Y - Z - A' - CH(OH) - L - S$  wherein  $- A' - CH(OH) -$  is a hydroxy carboxylic acid residue, e.g. a residue of formula II, as defined above, and the other symbols are as defined above, in free or protected form, or a salt thereof;

a compound of formula XII'



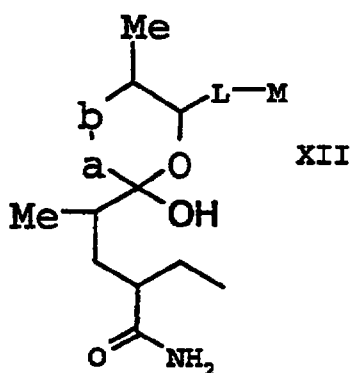
in free or protected form, or salt thereof;

- a compound of formula IX''



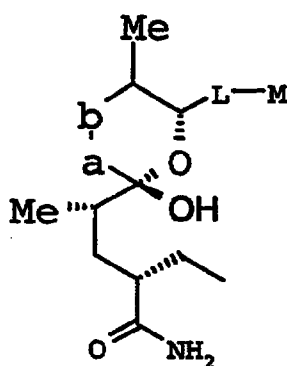
in free or protected form or a salt thereof.

The invention also includes compounds in which the 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexane ring system is in ring-opened form, e.g. a compound of formula XII

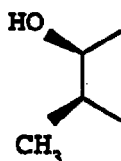


wherein a, b, L and M are as defined above, in free or protected form, or a salt thereof.

The ring-opened compounds of the invention are preferably of conformation as the preferred conformations identified above for ring-closed compounds. The ring-opened spiro bicyclo ring system of compounds of formula XII is preferably of conformation:



wherein when -a-b- is -(Me)CH-CH(OH)-, it preferably has the configuration:

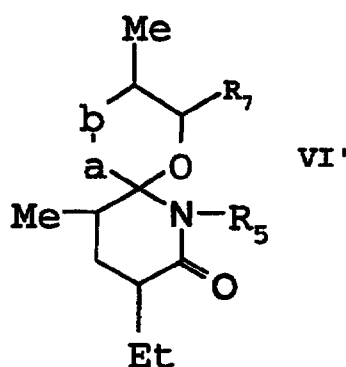


Macrolides in accordance with the invention having a spiro bicyclo residue attached to the macrocyclic ring may also be subjected to cleavage of the intervening

linker group, e.g. in relation to formula IX, in particular at the linkage between residues 26 and 27 to yield separate novel spiro bicyclo compounds and further macrolides. As also previously indicated these compounds too are useful as intermediates, the spiro bicyclic moiety of the Sanglifehrins in particular having an integral functional role in the biological activity of the Sanglifehrins as a class.

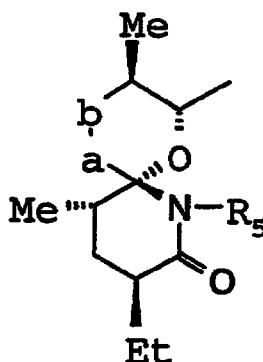
Accordingly the present invention provides:

- A 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclohexane, in free or protected form, or a salt thereof, in particular a compound of formula VI'

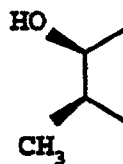


wherein R<sub>7</sub> is H, an optionally protected OH group, a reactive functional group, or a -CH<sub>2</sub>-CH(OH)-CH(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>-CHO group or the delta lactol equivalent thereof, in free or protected form or salt thereof.

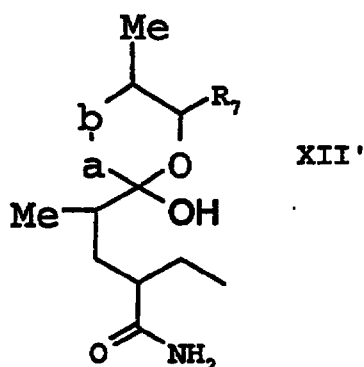
Preferably the compound of formula VI' has the following configuration



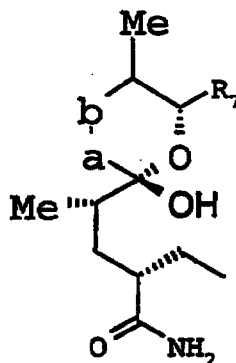
wherein when -a-b- is -(Me)CH-CH(OH)-, it preferably has the configuration:



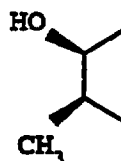
The invention also includes a ring-open 2-oxy-2'-aza-3'-oxo-3'-yl-spirobicyclohexane, in free or protected form, or a salt thereof, in particular a compound of formula XII'



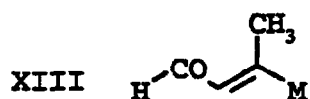
wherein a, b and R<sub>7</sub> are as previously defined, in free or protected form, or a salt thereof. The ring-opened spiro bicyclo ring system of compounds of formula XII' is preferably of conformation:



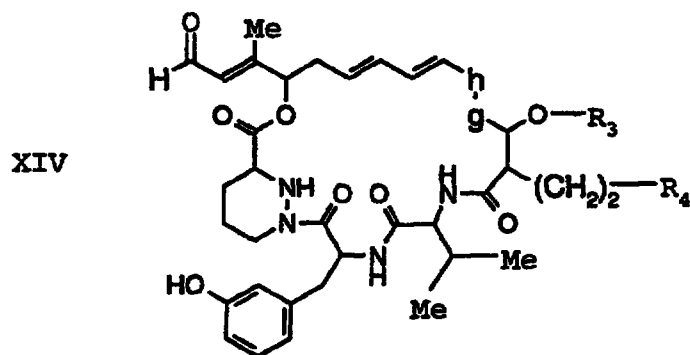
wherein when -a-b- is -(Me)CH-CH(OH)-, it preferably has the configuration:



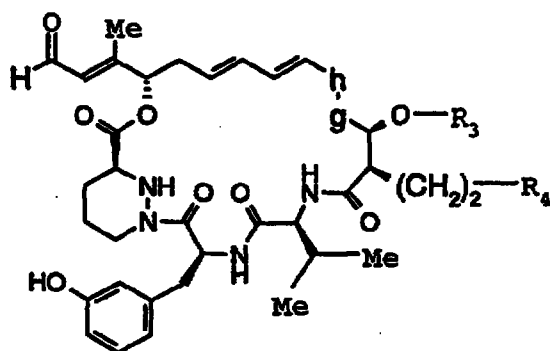
The invention also provides a macrolide of formula XIII



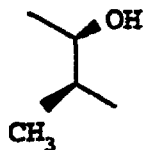
wherein M is a macolide ring as defined above, in particular a macrolide of formula XIV



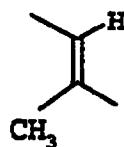
, preferably of conformation



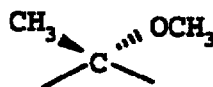
wherein when -g-h- is -(Me)CH-CH(OH)-, it preferably has the configuration:



and when -g-h- is -(Me)C=CH-, it preferably has the configuration:



and when  $R_3$  and  $R_4$  are fused together they are preferably of configuration



in free or protected or ring-opened form, or a salt thereof.

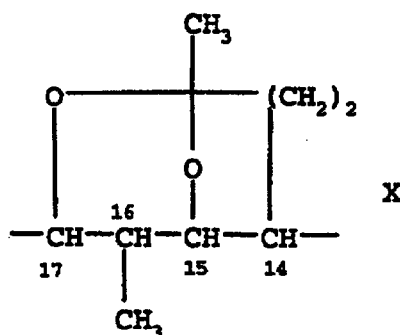
In a further aspect the invention includes the macrolides and compounds of the invention, in particular those which are natural products in substantially purified form, e.g. at least 90%, preferably at least 95%, especially at least 98% pure form.

In addition to the foregoing the present invention also provides a process for the production of any compound of the invention as hereinbefore defined, which process comprises:

- i). for the production of any one of Sanglifehrins A, B, C or D, cultivating a Sanglifehrin A, B, C or D producing actinomycete strain in a culture medium and isolating the desired Sanglifehrin A, B, C or D from the obtained culture broth;
- ii). for the production of Sanglifehrins C and D subjecting Sanglifehrins A and B to cyclisation at positions 15 and 16;
- iii). for the production of Sanglifehrins A and B subjecting Sanglifehrins C and D to ring opening of the lactol ring at positions 15 and 16;
- iv). for the production of a macrolide of formula IX or IX', wherein -g-h- is - $C(CH_3)=CH-$ , dehydrating a compound of formula IX or IX' wherein -g-h- is - $CH(CH_3)-CH(OH)-$  or a protected form thereof;

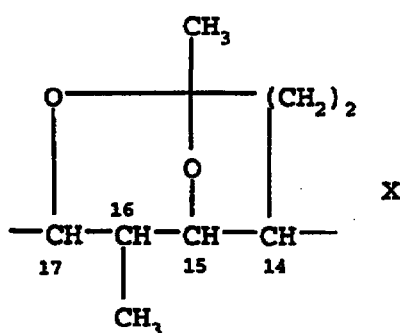
v). for the production of a macrolide of formula IX or IX' wherein  $R_4$  is -CH(OH)-CH<sub>3</sub>, hydrogenating a compound of formula IX or IX' wherein  $R_4$  is -C(O)-CH<sub>3</sub>;

vi). for the production of a macrolide of formula IX or IX' wherein the 14 to 16 positions of the macrolide ring comprise a residue of formula X



causing a compound of formula IX or IX' to undergo internal protection at positions 15 and 17;

vii). for the production of a macrolide of formula IX or IX', causing a compound of formula IX or IX' wherein the 14 to 16 positions of the macrolide ring comprise a residue of formula X



to undergo reversal of internal protection at positions 15 and 17;

viii). for the production of a macrolide of formula IX or IX', in which  $R_5$  is methyl, subjecting a macrolide of formula IX or IX', in which  $R_5$  is H to methylation;

- ix). for the production of a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-protected form, subjecting a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-unprotected form to O-protection;
- x). for the production of a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-unprotected form, subjecting a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-protected form to O-deprotection;
- xi). for the production of a macrolide of formula IX or IX', which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX', in which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-protection;
- xii). for the production of a macrolide of formula IX or IX', which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX' which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-deprotection;
- xiii). for the production of a macrolide of formula IX or IX', in which -e-f- is -CH(OH)-CH(OH)-, subjecting a macrolide of formula IX or IX' in which -e-f- is -CH=CH- to oxidative hydrolysis;
- xiv). for the production of a compound of formula V' or a compound of formula XII, subjecting a compound of formula IX or IX' to cleavage of the linker group between the spiro bicyclo group and the macrocyclic ring.
- xv). for the production of a compound of formula R<sub>6</sub>O - X - Y - Z - A - OH or of formula R<sub>6</sub>O - X - Y - Z - A' - CH(OH) - L - S, subjecting a macrocycle of formula IV or the macrocyclic ring of a compound of formula VIII to ring-opening at the lactone bridge thereof;

xvi). for the production of a macrolide of formula IX or XII in ring-closed form, subjecting a compound of formula  $R_6O - X - Y - Z - A - OH$  or of formula  $R_6O - X - Y - Z - A' - CH(OH) - L - S$  to closure of the macrocyclic ring.

xvii). for the production of a compound of formula XII or XII', subjecting a compound of formula IX or VI' to ring-opening within the spiro bicyclic ring system, and

xix). for the production of a compound of formula IX or VI', subjecting a compound of formula XII or XII' to ring-closure within the spiro bicyclic ring system.

Processes of the invention may be performed, e.g. as described in the examples. As will be appreciated the processes defined above may be applied in any appropriate sequence or combination to obtain other macrolides in free, protected, ring-open and ring-closed form as hereinbefore described.

The macrolides of the invention, e.g. Sanglifehrins A to D, are, or are derived from, natural compounds typically obtained from members of the family Streptomycetaceae.

Microorganisms capable of producing macrolides as hereinbefore defined not previously been identified.

Accordingly in a yet further aspect the present invention provides:

- a macrolide producing actinomycete strain wherein the macrolide is a macrolide in which

i) positions 2 to 6 inclusive of the macrocyclic ring are provided by a piperidazinyI carboxylic acid residue; and/or

- ii) positions 7 to 9 inclusive of the macrocyclic ring are provided by an aromatic  $\alpha$ -amino acid residue; and/or
  - iii) positions 10 to 12 inclusive of the macrocyclic ring are provided by an aliphatic  $\alpha$ -amino acid residue,
- in particular
- a Sanglifehrin A, B, C or D producing actinomycete strain.

Suitably the actinomycete strain is of the family Streptomycetaceae, more suitably of the genus Streptomyces, in particular the strain Streptomyces sp. A92-308110 as hereinafter described, or is derived therefrom, e.g. including mutants, variants, fusants, recombinants or modified forms thereof.

Suitably the strains of the invention are in the form of biologically pure isolates.

For example Streptomyces sp. A92-308110 may be mutated or modified into different forms by conventional techniques, e.g. by UV radiation or by treatment with a chemical mutagen such as N-methyl-N'-nitro-nitrosoguanidine. Recombinant clones may be obtained by protoplast fusion. All such mutants or recombinants or modified forms, capable of producing sanglifehrins, including mutants and recombinants capable of producing increased yields of, sanglifehrins are included within the scope of the present invention.

In a particular embodiment of the invention Sanglifehrins A, B, C, and D, amongst others, are isolated from the novel Streptomyces sp. A92-308110. Samples of Streptomyces sp. A92-308110 were deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on the 3 May 1995 under the terms of the Budapest Treaty and have been assigned deposition number DSM 9954. Samples of Streptomyces sp. A92-308110 may also be obtained from Sandoz Ltd. CH-4002 Basel, Switzerland.

Notice is hereby given that access to samples of DSM 9985 is limited in accordance with the provisions of Rule 28 (4) and (5) EPC.

The isolation of Sanglifehrins A, B, C, and D from cultures of Streptomyces sp. A92-308110 is described in Example 2.

Streptomyces sp. strain A92-308110 belongs to the genus Streptomyces according to the description in Bergey's Manual (Volume 4, 1989, Williams and Wilkins, Baltimore) and The Prokaryotes (1992 Springer Verlag, New York). The cell walls contain LL-diaminopimelic acid. The fatty acids are iso- and anteiso-branched, straight and unsaturated. The sugar spectrum is non distinctive. The vegetative mycelium does not break down into fragments. The aerial mycelium forms long chains of spores.

According to the reference books cited above, the strain designated A92-308110 is a new Streptomyces. A92-308110 grows on various organic and inorganic media and in most cases forms aerial mycelium. The primary substrate mycelium grows as hyphae and is generally beige to greyish-brown. The color of the aerial mycelium belongs to the grey series, number 4, and this mycelium forms long chains of spores which belong to the type spira b.

The ability of Streptomyces sp. A92-308110 to grow on usual biological media, its carbon utilization, and its physiological characteristics are presented in the following tables.

**Table 1 . Growth on various biological media**

| <u>Culture medium</u> | <u>Culture characteristics</u> |
|-----------------------|--------------------------------|
| yeast extract/        | growth: good                   |
| malt agar             | substrate mycelium: brownish   |
|                       | aerial mycelium: grayish-brown |
|                       | soluble pigment: none          |

|                                 |                                                                                                                   |
|---------------------------------|-------------------------------------------------------------------------------------------------------------------|
| oatmeal agar                    | growth: good<br>substrate mycelium: dark brown<br>aerial mycelium: greyish-brown<br>soluble pigment: brownish     |
| glucose-asparagine              | growth: moderate<br>substrate mycelium: brownish<br>aerial mycelium: greyish-brown<br>soluble pigment: none       |
| Inorganic salts/<br>starch agar | growth: moderate<br>substrate mycelium: grey<br>aerial mycelium: greyish-brown<br>soluble pigment: none           |
| Sucrose/<br>nitrate agar        | growth: very poor<br>substrate mycelium: whitish<br>aerial mycelium: poor, greyish brown<br>soluble pigment: none |
| Glycerol/<br>asparagine agar    | growth: moderate<br>substrate mycelium: brownish<br>aerial mycelium: greish-brown<br>soluble pigment: none        |
| Nutrient agar                   | growth: moderate<br>substrate mycelium: beige<br>aerial mycelium: none<br>soluble pigment: brown                  |

**Table 2 : carbon utilization**

|                   |                                                  |
|-------------------|--------------------------------------------------|
| moderate or good: | glucose, fructose, arabinose, xylose, mannose,   |
| poor:             | rhamnose, sucrose, raffinose, cellulose, salicin |
| negative:         | m-inositol                                       |

**Table 3: physiological characteristics**

|                       |                                                                       |
|-----------------------|-----------------------------------------------------------------------|
| nitrate reduction:    | positive                                                              |
| starch hydrolysis:    | moderate on inorganic salts-starch agar,<br>negative on oatmeal agar. |
| tyrosine degradation: | negative                                                              |
| milk peptonisation:   | positive                                                              |
| melanin formation:    | positive                                                              |

growth temperatures: 18-37°C.

Very poor growth at 13°C.

No growth at 45°C.

pH-range: rich growth at pH 5 and 7, good growth at pH 9

NaCl resistance: up to 6%, but reduced growth already at a 2%  
concentration.

Macrolides of the invention, including Sanglifehrins A, B, C and D may be produced by cultivating Streptomyces sp. A92-308110 or a mutant, recombinant or modified form thereof on an appropriate culture medium. Example 1 describes, by way of illustration only of the invention, a procedure for the cultivation of Streptomyces sp. A92-308110.

Thus in further aspects the invention includes:

- a) a biologically pure isolate of strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof which is capable of producing a macrolide of the invention, and
- b) a process for the production of a macrolide of the invention, which comprises cultivating strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof in an appropriate culture medium and optionally recovering the sanglifehrin.

Macrolides in accordance with the invention, e.g. compounds of formula IX; for example, Sanglifehrins A, B, C and D, and their pharmaceutically acceptable salts, hereinafter generically "agents of the invention", exhibit sanglifehrin characteristic activities, i.e. the following combination of activities:

- have cyclophilin binding activity;
- have immunosuppressive activity;
- inhibit proliferation of both B-cells and T-cells;
- but do not have FK binding protein binding activity, and
- do not inhibit calcineurin activity.

These activities and assays to determine these activities are described hereinafter in greater detail. Biological activity of macrolides of the invention, e.g. of formula IX, e.g. of Sanglifehrins A to D, may be demonstrated in standard in vitro and in vivo test methods, e.g. as follows.

1. Primary Humoral Immune Response to Sheep Red Blood Cells (MD, Mishell-Dutton)

Mouse spleen cells (OF 1, female, 8-10 weeks,  $1 \times 10^7$ ) are co-cultured with sheep erythrocytes (SRBC,  $3 \times 10^7$ ) for 3 days in 1 ml final volume in 24 well plates. Lymphocytes are harvested, washed and plated at a density of  $1 \times 10^6$  cells onto soft-agar with fresh antigen (SRBC). Complement (guinea pig serum) is added after a 60-90 minute incubation period and incubation is continued for a further 60 minutes after which the test is evaluated by counting (microscope) the plaques. During the 3 day

incubation, the lymphocytes are sensitized to the antigen (SRBC). When incubated with antigen again, B-lymphocytes secrete specific antibody which binds to the antigen in the vicinity of the secretory lymphocyte. Addition of complement causes lysis of the antibody-coated erythrocytes yielding a plaque. Each plaque represents a single antibody-producing cell.

Inhibition of plaque formation is indicative of pharmaceutical utility. Compounds of the invention, e.g. Sanglifehrins A to D, are active in this assay at a concentration of about ..?... to about ....?.....nM.

#### References:

R.I. Mishell & R.W. Dutton (1966) Immunization of normal mouse spleen cell suspensions in vitro. Science 153: 1004-1006

R.I. Mishell & R.W. Dutton (1967) Immunization of dissociated spleen cell cultures from normal mice. J.Exp.Med. 126:423-442

## 2. Proliferative Response Of Lymphocytes to Allogenic Stimulation

### Two-way MLR (Murine Mixed Lymphocyte Reaction) :

Spleen cells ( $2 \times 10^5$ ) from Balb/c mice (female, 8-10 weeks) are co-incubated for 4 days with  $2 \times 10^5$  spleen cells from CBA mice (female, 8-10 weeks). The allogenic cells induce a proliferative response in the responder spleen cell population which is measured by labelled precursor incorporation into the DNA. Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, have  $IC_{50}$ s in the range from about 30 up to about 200 nM as compared with an  $IC_{50}$  of about 20 nM for cyclosporin A when tested in this assay.

#### Reference:

T. Meo (1979) The MLR in the mouse. In: "Immunological Methods", L. Lefkovits and B. Pernis, Eds., Academic Press, N.Y. pp. 227- 239

3. LPS- stimulated murine B-cells

Spleen cells ( $2 \times 10^5$ ) from CBA mice are incubated for 48 hours with 50  $\mu\text{g/ml}$  LPS plus test compound. Proliferation is measured by labelled precursor incorporation into DNA. Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, inhibit B-cell proliferation and have  $\text{IC}_{50}\text{s}$  in the range from about 40 up to about 100  $\mu\text{M}$ .

*References:*

Greaves, M. and J. Janossy, 1972, Elicitation of selective T and B lymphocyte response by cell surface binding ligands, Transplant Rev., 11: 87

Janossy, G. and M. F. Greaves, 1971, Lymphocyte activation, I, Response of T and B lymphocytes to phytomitogens, Clin. Exp. Immunol. 9: 483-498

4. Cytotoxic and cytostatic activity in vitro using the THP1 cell line

Cytotoxicity is determined using the human monocytic cell line THP1 ( $5 \times 10^4$  cells/well) which are incubated in the presence of  $\text{IFN}\gamma$  (100 U/ml) and LPS (5  $\mu\text{g/ml}$ ) plus test compound (up to 10  $\mu\text{M}$ ) for 24 to 72 h at 37°C. Living cells are quantified using the colourimetric read-out MTT which measures mitochondrial dehydrogenase enzymatic activity in living cells (Mossman 1983). Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, have  $\text{IC}_{50}\text{s}$  of about 1000-5000 nM after 24 h incubation in this assay.

*Reference:*

Mossman T. J. (1983), Rapid colorimetric assay for cellular growth and survival : application to proliferation and cytotoxic assays, *J. Imm. Methods*, 65, 55-63.

#### 5. TNF release by human peripheral blood mononuclear cells

Mononuclear cells are prepared from the peripheral blood of healthy volunteers using Ficoll-Hypaque density separation according to the method of Hansell et al. (1991). Cells ( $10^5$  cells/well in 200  $\mu$ l RPMI 10% FCS by volume) are incubated with serial dilutions of the test compounds for 30 min at 37°C prior to the addition of stimulus. Interferon  $\gamma$  (100 U/ml) and LPS (5 $\mu$ g/ml) are used as stimuli to induce Tumour Necrosis Factor (TNF)  $\alpha$  release by peripheral blood mononuclear cells. After 3 h incubation, the cells are centrifuged (1200 rpm for 10 min) and the supernatants are collected. The amount of TNF present in the cell supernatants is determined using a commercially available enzyme-linked immunosorbent assay kit. Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, have  $IC_{50}$ s in the range from about 200 nm to about 1000 nm when tested in this assay.

#### 6. Cyclophilin Binding Assay

A suitable cyclophilin binding assay is the competitive ELISA test described by Quesniaux in *Eur. J. Immunol.* 1987 17 1359-1365. In this test, the compound to be tested is added during the incubation of cyclophilin (human recombinant cyclophilin A) with coated BSA-cyclosporin A and the concentration required to give a 50% inhibition of the control reaction without competitor is then calculated ( $IC_{50}$ ). An alternative assay is the competitive binding test described by Schneider et al. in *Biochemistry* (1994), 33, 8218-8224, which involves addition of test compound during the incubation of biotinylated cyclophilin (human recombinant cyclophilin A) with coated BSA-cyclosporin A. The amount of biotinylated cyclophilin bound in the presence and absence of a test compound is determined by incubation with streptavidin-coupled alkaline phosphatase. Macrolides of the invention, e.g. compounds of formula IX, e.g.

Sanglifehrin A, B, C and D, have  $IC_{50}$ s in the range from about 10 to about 100 nM, compared with an  $IC_{50}$  of about 80 nM for cyclosporin A when tested in these assays.

Further in vitro assays which may be used to demonstrate the biological activity of Sanglifehrins are IL-2 reporter gene assays and ConA-stimulated spleen cell assays (indicative of effect on T-cell activation).

Macrolides of the invention, e.g. compounds of formula IX, e.g. Sanglifehrin A, B, C and D, do not have FK binding protein binding activity and do not inhibit calcineurin activity when tested in standard tests for these activities.

7. Localised Graft-versus-Host (GvH) Reaction in the rat

[Ford et al., TRANSPL. PROC. 10 (1979) 258].

Spleen cells ( $1 \times 10^7$ ) from 6 week old female Wistar/Furth (WF) rats are injected subcutaneously on day 0 into the left hind-paw of female (F344 x WF) $F_1$  rats weighing about 100g. Animals are treated for 4 consecutive days and the popliteal lymph nodes are removed and weighed on day 7. The difference in weight between the two lymph nodes is taken as the parameter for evaluating the reaction.

Inhibition of GvH reaction in the above test is indicative of pharmaceutical utility. Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, are able to inhibit the GvH reaction by up to about 30% when administered at a dose of about 1 mg/kg s.c..

8. DTH induced by SRBC- $T_H$  cells

Fifty microliters of a 1:1 (v/v) mixture of a  $T_H$  (sheep red blood cell primed) cell clone ( $2 \times 10^6$ ) and a 10 % sheep red blood cell (SRBC) suspension are injected s.c. into the right hind footpad of female C57 BL/6 mice (6-12 weeks old). 50  $\mu$ l of the SRBC cell suspension (diluted 1:1 v/v with PBS) is injected s.c. into the left hind footpad (to

measure non specific increase in footpad swelling due to the injection procedure). Right and left hind footpad thickness is measured 24 hours later.

The percent increase in thickness of the right footpad over the left footpad (z) is calculated. Thickness of right footpad = x; thickness of left footpad = y; % specific increase = z

$$z=((x-y)/y).100$$

Macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, reduce swelling of the DTH mouse by up to about 50% at doses of the order of 5 mg/kg s.c..

#### *References:*

A.T.J. Bianchi, H. Hooijkaas, R. Brenner, R. Tees, A.A. Nordin & M.H. Schreier (1981) Clones of helper T-cells mediate antigen specific, H-2 restricted DTH. *Nature* 290:62-63.

P. Herrmann, M.H. Schreier, J.-F. Borel & C. Feurer (1988) Mast cell degranulation as a major event in the effector phase of delayed-type hypersensitivity induced by cloned helper T cells. *Int. Archs Allergy appl. Immun.* 86: 102- 105

#### 9. Rat/Mouse Heart Allotransplantation

The *in vivo* efficacy of macrolides of the invention is assessed in rat and mouse heart allotransplantation using Alzet osmotic minipumps for s.c. administration. In mouse heart allotransplantation (BALB/c to C3H), macrolides of the invention, e.g. compounds of formula IX and their pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, prolong graft survival at doses of the order of 30 mg/kg/day. In rat heart allotransplantation (DA to Lewis) treatment with suboptimal doses of cyclosporin A in combination with Macrolides of the invention, e.g. compounds of formula IX and their

pharmaceutically acceptable salts, e.g. Sanglifehrins A, B, C and D, prolonged graft survival as exemplified in the table below.

| Cyclosporin A<br>(mg/kg) | Sanglifehrin A<br>(mg/kg) | Graft survival<br>(days) |
|--------------------------|---------------------------|--------------------------|
| 1                        | -                         | 12, 12, 12, 13, 13, 14   |
| 1                        | 10                        | 29, 30, 45, 48, >51, >46 |
| Control (Placebo)        | Control (Placebo)         | Control (Placebo)        |

Agents of the invention are useful as pharmaceuticals, e.g. as immunosuppressive as well as an anti-inflammatory agents.

They are, in particular, useful for the prevention of acute and/or chronic organ or tissue allo- and xenotransplant rejection, e.g. for the treatment of recipients of heart, lung, combined heart-lung, liver, kidney, pancreatic, skin or corneal transplants. They are also indicated for the prevention of graft-versus-host disease, such as following bone marrow transplants.

Agents of the invention are also useful for the treatment of autoimmune disease and of inflammatory conditions, in particular inflammatory conditions with an aetiology including an autoimmune component such as arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans) and rheumatic diseases. Specific auto-immune diseases for which the agents of the invention may be employed include autoimmune haematological disorders (including e.g. haemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, scleroderma, Wegener granulomatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's disease) endocrine ophthalmopathy, Graves disease, sarcoidosis, multiple sclerosis, primary biliary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal and/or allergic keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis, glomerulonephritis (with

and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy) and asthma.

For these and other uses agents of the invention may be administered on their own or together with other immunosuppressant or antiinflammatory agents, including cyclosporins, rapamycins, FK 506, and steroids.

For the above indications the appropriate dosage will, of course, vary depending, and the agent of the invention chosen, for example, on the subject to be treated, the mode of administration and the nature and severity of the condition being treated. However, in general, satisfactory results in animals are obtained at daily dosages of from about 0.01 to 10 mg/kg/day p.o.. In larger mammals, for example humans, an indicated daily dosage is in the range of from about 0.5 to about 500 mg of sanglifehrin administered orally once or, more suitably, in divided dosages two to four times/day.

In organ transplantation in humans, oral doses of 0.1 to 100, preferably 0.3 to 30, more preferably 0.5 to 10, mg/kg of a compound of agent of the invention. When an agent of the invention is given along with other immunosuppressants (e.g. with corticosteroids or with compounds of the cyclosporin or rapamycin class as part of a double, triple or quadruple drug therapy) lower doses (e.g. 0.1 mg/kg/day i.v.; 3 mg/kg/day oral initially) may be used. In particular agents of the invention may be given with other non-steroidal immunosuppressants, e.g. with cyclosporin A, rapamycin or FK 506, with a view to the partial or complete replacement of steroids.

Agents of the invention may be administered by any conventional route, in particular enterally, e.g. orally, for example in the form of solutions for drinking, tablets or capsules or parenterally, for example in the form of injectible solutions or suspensions. Normally for systemic administration oral dosage forms are preferred, although for some conditions, for example for prevention of rejection of liver transplants, an intravenously injectable form is desirable. Compounds may also be administered topically or dermally, e.g. in the form of a dermal cream or gel or like preparation or, for the purposes of application to the eye, in the form of an ocular cream, gel or eye-drop preparation.

Suitable unit dosage forms for oral administration comprise e.g. from 0.5 to 100 mg of the compound per dosage.

In accordance with the foregoing the present invention also provides in a further series of embodiments:

A. A method of effecting immunosuppression in a subject in need of such treatment which method comprises administering to said subject an effective amount of an agent of the invention.

B. A method:

1) for the prevention of acute and/or chronic organ allo- or xenotransplant rejection, for example for the treatment of recipients of organ transplants of any of the particular types listed above; or

2) for the prevention of graft-versus-host disease, for example in recipients of bone marrow transplants; or

3) for the treatment of autoimmune disease or for the treatment of any such disease or condition listed above; or

4) for the treatment of asthma

in a subject in need of such treatment, which method comprises administering to said subject an effective amount of an agent of the invention.

C. An agent of the invention for use as a pharmaceutical, e.g. for use as an immunosuppressant or for use in the treatment of any disease or condition as set forth under B above.

D. A pharmaceutical composition comprising an agent of the invention in association with a pharmaceutically acceptable diluent or carrier.

- E. The use of an agent of the invention for the preparation of a medicament for use as an immunosuppressant or for use in the treatment of any disease or condition as set forth under B above.

In addition macrolides of the invention which possess cyclophilin binding activity, may be useful as reagents in displacement immunoassays for cyclosporins and other cyclophilin binding compounds, for example in the assay procedure described in our copending patent application WO 95/07468. This patent application relates to an assay procedure for determining the concentration of an immunophilin-binding pharmaceutical, e.g. Cyclosporin, in blood; the procedure comprising adding a binding competitor that displaces the pharmaceutical from immunosuppressant-immunophilin complexes in the blood; adding a receptor that binds to the pharmaceutical but not significantly to the binding competitor; separating the receptor-pharmaceutical complex from the sample; and determining the amount of the pharmaceutical. Sanglifehrins may be used as the binding competitor in such assays; for instance, to displace cyclosporins from cyclophilins, thereby releasing the cyclosporin for quantitation, e.g. by a monoclonal antibody which is specific for the cyclosporin.

The invention is further described by way of illustration only in the following Example which refers to the accompanying Figures:

- in which Figure 1 shows the Mass spectrum of the compound of Sanglifehrin B;
- Figure 2 shows the Mass spectrum of the compound of Sanglifehrin A;
- Figure 3 shows the Mass spectrum of the compound of Sanglifehrin D;
- Figure 4 shows the Mass spectrum of the compound of Sanglifehrin C;
- Figure 5 shows the IR spectrum of the compound of Sanglifehrin B;
- Figure 6 shows the IR spectrum of the compound of Sanglifehrin A;
- Figure 7 shows the IR spectrum of the compound of Sanglifehrin D;
- Figure 8 shows the IR spectrum of the compound of Sanglifehrin C;
- Figure 9 shows the NMR spectrum of the compound of formula Sanglifehrin A;
- Figure 10 shows the NMR spectrum of the compound of Sanglifehrin D.
- Figure 11 shows the NMR spectrum of the compound of Sanglifehrin B, and

Figure 12 shows the NMR spectrum of the compound of Sanglifehrin C.

## EXAMPLES

### Culture conditions

Streptomyces sp. A 92-308110 may be cultured at suitable temperatures on various culture media using appropriate nutrients and mineral substances, using aerobic or immersion culture procedures. The fermentation media typically contains a utilisable source of carbon, sources of nitrogen and mineral salts including trace elements, all of which can be added in the form of well defined products or as complex mixtures, for instance as are found in biological products of various origins.

Example 1 describes the original conditions under which compounds of formula I were obtained. Improved yields may be obtained by optimisation of the culture conditions (aeration, temperature, pH, quality and quantity of the carbon and nitrogen sources, quantity of the mineral salts and of the trace elements) and by controlling the fermentation conditions in bioreactors.

### Example 1 Culture of strain A 92-308110.

#### a. Agar starting culture

Agar slant cultures of the strain A 92-308110 are grown for 10 to 14 days at 27°C on the following agar medium:

|                               |       |
|-------------------------------|-------|
| Glucose                       | 10.0g |
| Soluble starch                | 20.0g |
| Yeast extract                 | 5.0g  |
| (Gistex, Gist Brocades)       |       |
| NZ-Amine, Type A (Sheffield)  | 5.0g  |
| Calcium carbonate             | 1.0g  |
| Agar (Bacto)                  | 15.0g |
| Demineralised water to 1000ml |       |

The medium is adjusted to pH 6.6-6.8 with NaOH/H<sub>2</sub>SO<sub>4</sub>, then sterilised for 20 min. at 121°C.

The cultures can be stored at -25°-70°C. A suspension in glycerol-peptone can be stored under liquid nitrogen.

#### **b Preculture**

Spores and mycelium of 10 starting cultures are suspended in 100 ml of a 0.9% salt solution. Two 2 Liter-Erlenmeyer flasks each containing 1 liter of preculture medium are inoculated each with 50 ml of this suspension. The composition of the preculture medium is as follows:

|                                      |        |
|--------------------------------------|--------|
| Glucose techn                        | 7.50g  |
| Glycerin                             | 7.50g  |
| Yeast extract (BBL)                  | 1.35g  |
| Malt extract liquid (Wander)         | 7.50g  |
| Starch soluble                       | 7.50g  |
| NZ-Amine, Type A (Sheffield)         | 2.50g  |
| Soya protein                         | 2.50g  |
| L(-) Asparagine                      | 1.00g  |
| CaCO <sub>3</sub>                    | 0.050g |
| NaCl                                 | 0.050g |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.250g |
| K <sub>2</sub> HPO <sub>4</sub>      | 0.500g |
| MgSO <sub>4</sub> .7H <sub>2</sub> O | 0.100g |
| Trace element solution A             | 1ml    |
| Agar (Bacto)                         | 1g     |
| Demineralised water to 1000ml        |        |

The medium is adjusted to pH 6.8-7.2 with NaOH/H<sub>2</sub>SO<sub>4</sub> and sterilised for 20m at 121°C.

The composition of the trace element solution A is as follows:

|                                      |       |
|--------------------------------------|-------|
| FeSO <sub>4</sub> ·7H <sub>2</sub> O | 5.0g  |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O | 4.0g  |
| MnCl <sub>2</sub> ·4H <sub>2</sub> O | 2.0g  |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O | 0.2g  |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O | 2.0g  |
| H <sub>3</sub> BO <sub>3</sub>       | 0.1g  |
| KI                                   | 0.05g |
| H <sub>2</sub> SO <sub>4</sub> (95%) | 1ml   |
| Demineralised water to 1000 ml       |       |

The precultures are fermented for 24 hr. at 27°C on a rotary shaker at 200 rpm with an eccentricity of 50mm.

c First intermediate culture

Two 75 Liter bioreactors containing each 50 liters of preculture medium are inoculated each with 1 liter of the preculture and fermented for 96 hr. at 27°C. The fermenter is rotated at 150 rpm. Air is introduced at a rate of 0.5 liter per minute per liter medium.

d Second intermediate culture

Two 750 liter fermentation vessels each containing 500 liters of the preculture medium are each inoculated with 50 liter of the first intermediate culture. The second intermediate cultures are incubated for 70 hr at 27°C. The fermenters are rotated at 100 rpm and air introduced at a rate of 0.8 liter per minute per liter medium.

**e. Main culture**

Two 5'000 Liter bioreactors each containing 3'000 liters of the main medium are inoculated respectively with 250 and 300 liters of the second intermediate cultures. The main cultures are incubated during 96 hr at 24°C. The bioreactors are rotated at 45 rpm and air introduced at a rate of 0.5 liter per minute per liter medium.

The composition of the main culture medium is as follows:

|                                      |       |
|--------------------------------------|-------|
| Glucose techn                        | 20g   |
| Malt extract liquid (Wander)         | 2g    |
| Yeast extract (Bacto)                | 2g    |
| Soytone (Bacto)                      | 2g    |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.2g  |
| K <sub>2</sub> HPO <sub>4</sub>      | 0.4g  |
| MgSO <sub>4</sub> .7H <sub>2</sub> O | 0.2g  |
| NaCl                                 | 0.05g |
| CaCl <sub>2</sub> .6H <sub>2</sub> O | 0.05g |
| Trace element solution B             | 1ml   |
| Agar (Bacto)                         | 1g    |
| demineralised water to 1000ml.       |       |

The pH is adjusted to 6.3 with KOH/HCl. The medium is sterilised for 20 min at 121°C.

The composition of the trace element solution B is the following:

|                                                                 |      |
|-----------------------------------------------------------------|------|
| FeSO <sub>4</sub> .7H <sub>2</sub> O                            | 5.0g |
| ZnSO <sub>4</sub> .7H <sub>2</sub> O                            | 4.0g |
| MnCl <sub>2</sub> .4H <sub>2</sub> O                            | 2.0g |
| CuSO <sub>4</sub> .5H <sub>2</sub> O                            | 0.2g |
| (NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> | 0.2g |

|                                          |              |
|------------------------------------------|--------------|
| <b>CoCl<sub>2</sub>.6H<sub>2</sub>O</b>  | <b>1.0g</b>  |
| <b>H<sub>3</sub>BO<sub>3</sub></b>       | <b>0.1g</b>  |
| <b>KJ</b>                                | <b>0.05g</b> |
| <b>H<sub>2</sub>SO<sub>4</sub> (95%)</b> | <b>1ml</b>   |
| <b>Demineralised water to 1000 ml</b>    |              |

**An optimised culture medium for the main culture is as follows:**

|                                                 |              |
|-------------------------------------------------|--------------|
| <b>Soybean meal</b>                             | <b>20.0g</b> |
| <b>Glycerol</b>                                 | <b>40.0g</b> |
| <b>MES</b>                                      | <b>0.1M</b>  |
| <b>Demineralised water to 1000 ml at pH 6.8</b> |              |

**Example 2 - Isolation of Sanglifehrins A, B, C and D from Streptomyces sp. A92-308110**

The first isolation and characterization of the 4 new CBA active metabolites was done from two 3000 l tank fermentations by activity guided fractionation and HPLC and thinlayer chromatographic analysis. CBA (cyclophilin binding assay) as described above was used to test for biological activity.

The two 3000 liter fermentations are processed separately. 1500 liter from each fermentation is stirred with 2000 l ethyl acetate in 4000 liter stainless steel vessel for 20 hours. The separation of the organic phase is done with a Westfalia-Separator typ SA-20. The ethyl acetate extracts are washed twice with 80 liters of water and evaporated to dryness under reduced pressure to give 1.64 and 2 kg extracts. The two crude extracts are defatted by a three step extraction with 40 liter methanol/water 9:1 and 40 liter of hexane. Evaporation to dryness under reduced pressure gives 1.34 kg extract.

The defatted extract is chromatographed in two portions (670 g) on a column of 10 kg Sephadex H in methanol solution. Each portion is dissolved in 3.3 liters of methanol when added to the column. After collection of the first 15 liters eluate as fraction 1 the chromatography is continued by collecting 2 liter fractions.

The most active fractions were 2,3 and 4 and are therefore combined to give 146 g.

This sample is further chromatographed on 1 kg Silicalgel Merck 0.04-0063 mm with methyl-tertiary-butyl-ether (MTBE), MTBE/5 % methanol and MTBE / 10 % methanol. Fractions of 2 liters are collected. Fractions 5 to 9 are the most active ones and are combined to give a sample of 43.8 g. This sample is further separated on a column of 1 kg Silicagel (Merck) 0.04-0.063 mm with a gradient of hexane/acetone 7:3 to acetone. From this chromatography fraction 6 (7.0 g) is further separated on a column of 3 kg Lichroprep RP 18 (Merck) 40-63 um with methanol/water 94:6 (fraction 4-7 2.16 g), then on a column of 100 g Silicagel H with methylenechloride and 3 % methanol (733 mg), a column of 3 kg Lichroprep RP18 with methanol/water 9:1 (621 mg) and then on 100 g Lichroprep RP18 with acetonitrile/water 1:1 to yield 324 mg of

pure Sanglifehrin A (mp 142-145° C (amorphous), ( $\alpha$ )D<sub>25</sub>=-67.30 (c=0.988, methanol)).

Fractions 5 and 7 from the hexane/acetone column are combined (7.1 g) and further purified on a column of 3 kg Lichroprep RP18 40-63  $\mu$ m with methanol/water 9:1 (769 mg), on a column of 100 g silicagel H with MTBE 3 % methanol (309 mg) and finally on 100 g silicagel H with methylenechloride and 3 % methanol to yield 90 mg pure Sanglifehrin B (mp. 117-121° C (amorphous), ( $\alpha$ )D<sub>25</sub>=-52.80 (c=1-128 in methanol)).

Fractions 9 and 10 (2.147 g) of the chromatogramm with methanol/water 94:6 on 3 kg Lichroprep RP18 are further purified on 100 g Silicagel H with methylenechloride/5 % methanol (800 mg) and finally on 3 kg Lichroprep RP18 with methanol/water 9:1 to give 480 mg of Sanglifehrin C (mp. 165-170° C, ( $\alpha$ )D<sub>25</sub>=-35.60 (c=0.736 in methanol).

Fractions 11 and 12 (835 mg) of the chromatogramm with methanol/water 94:6 on 3 kg Lichroprep RP18 are purified on 100 g Silicagel H with MTBE/5 % methanol to give 140 mg of Sanglifehrin D (mp. 137-142° C, amorphous).

Sanglifehrin A, B, C and D were then characterised by UV, IR, Mass and NMR spectroscopy. The results obtained are given in Table 4 below and in the accompanying figures.

Table 4Sanglifehrin A

molecular formula:  $C_{60}H_{91}N_5O_{13}$  (1090.4)

UV (MeOH): 275 (1962), 242 (54500), 197 (75755)

$H^+$  : 275 (1635), 242 (51884),

$OH^-$  : 292 (1973), 242 (60495)

IR-spectra: Figure 6

Mass-spectra: FAB 1096[MH+Li] $^+$  : Figure 2

NMR spectra : Figure 9

Sanglifehrin B

molecular formula:  $C_{60}H_{89}N_5O_{12}$  (1072.4)

UV (MeOH): 273 (4395), 242 (50600), 197 (78577)

IR-spectra : Figure 5

Mass-spectra: FAB 1098[MH+Li] $^+$  : Figure 1

NMR spectra: Figure 11

Sanglifehrin C

molecular formula:  $C_{61}H_{93}N_5O_{13}$  (1104.4)

UV (MeOH): 275 (1876)), 242 (51557), 197 (72643)

$H^+$  : 275 (1391), 242 (50120)

$OH^-$  : 292 (1832), 242 (57960)

IR-spectra : Figure 8

Mass-spectra: FAB 1110[MH+Li] $^+$  : Figure 4

NMR spectra: Figure 12

Sanglifehrin D

molecular formula:  $C_{61}H_{91}N_5O_{12}$  (1086.4)

UV (MeOH): 273 (3194), 242 (47584), 197 (73766)

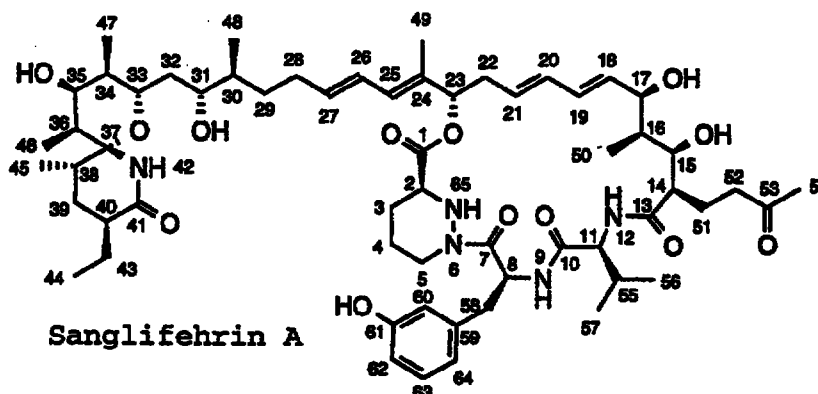
$H^+$  : 273 (3237), 242 (46389)

$OH^-$  : 285 (2600), 242 (52907)

IR-spectra : Figure 7

Mass-spectra: FAB 1092[MH+Li] $^+$  : Figure 3

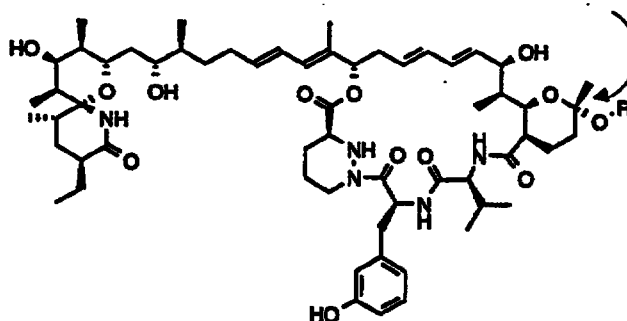
NMR spectra : Figure 10



### Example 3 - Transformation of Sanglifehrin A into Sanglifehrin C

To a stirred, cooled (0°C) solution of 20 mg (18.3  $\mu\text{mol}$ ) of Sanglifehrin A in 0.5 mL of methanol is added one crystal of paratoluenesulfonic acid monohydrate. The resulting yellow solution is stirred for one hour and the reaction is quenched with saturated aqueous sodium bicarbonate solution. The resulting mixture is extracted twice with ethyl acetate. The organic solution is washed twice with saturated brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue is purified by column chromatography on silica gel (95:5 methyl-tert.-butyl ether:methanol) to yield Sanglifehrin C as a white amorphous powder. The latter consisted of a 4:1 mixture of Sanglifehrin C and its C53 epimer, Sanglifehrin C having the (S) configuration as depicted below (R = Me).

4:1 mixture of diastereomers



Alternatively, this transformation can be carried out by using other protic acids (such as pyridinium paratoluenesulfonate, hydrochloric acid or sulfuric acid) or Lewis acids

(such as zinc chloride, magnesium bromide or chloride, titanium tetraisopropoxide or boron trifluoride) in methanol. Use of other alcoholic solvents or cosolvents like ethanol, isopropanol, butanol, allyl alcohol, propargyl alcohol, benzyl alcohol lead in the same manner to analogues where R in the structure above is respectively ethyl, isopropyl, butyl, allyl, propargyl, benzyl.

In the same manner as described above, Sanglifehrin B can be transformed into Sanglifehrin D.

#### **Example 4 - Transformation of Sanglifehrin C into Sanglifehrin A**

A solution of 550 mg (0.50 mmol) of sanglifehrin C in 5 mL of 4:1 THF-water is treated with 0.5 mL of 2N aqueous sulfuric acid and stirred for 1.5 h. The reaction is quenched with saturated aqueous sodium bicarbonate and the resulting mixture is extracted twice with ethyl acetate. The organic solution is washed with saturated aqueous sodium bicarbonate solution and twice with saturated brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue is purified by column chromatography on silica gel (90:10 methyl-tert.-butyl ether:methanol) to yield sanglifehrin A as a white amorphous powder.

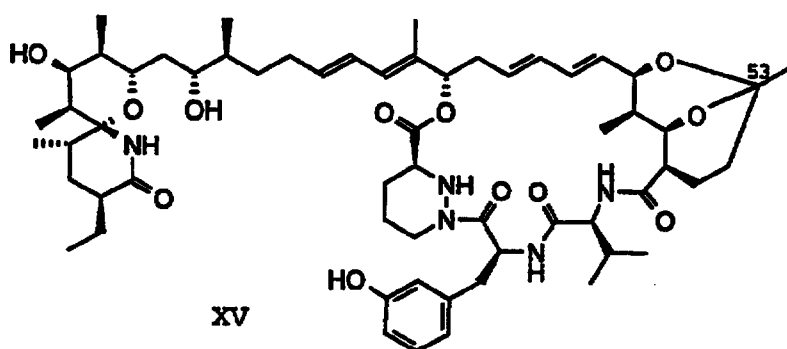
Other inorganic or organic acids can be used in a medium containing water and optionally an organic cosolvent. Suitable acids include hydrochloric acid, paratoluenesulfonic acid or other sulfonic acids, pyridinium paratoluenesulfonate, acetic acid, trifluoroacetic acid, formic acid. Suitable organic cosolvents are acetonitrile, dimethylformamide, dimethylsulfoxide, dioxane.

These reactions are accompanied by the formation of varying amounts of the compound of formula XV, depending among others on the reaction time (for a better procedure leading to the compound of formula XV see Example 5 below).

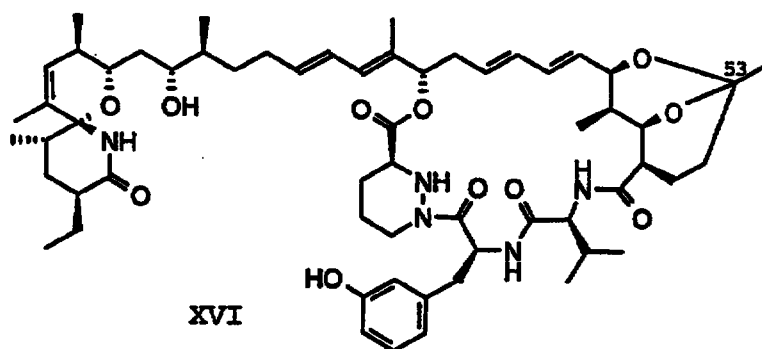
Analogously, sanglifehrin D can be transformed into sanglifehrin B.

#### **Example 5 - Transformation of Sanglifehrin A into the compound of formula XV**

To a stirred, cooled (0°C) solution of 50 mg (46  $\mu$ mol) of sanglifehrin A in 1.9 mL of acetonitrile is added 0.1 mL of hydrogen fluoride - pyridine. The resulting yellow solution is stirred for 1 hour and the reaction is quenched with saturated aqueous sodium bicarbonate. The resulting mixture is extracted twice with ethyl acetate. The organic solution is washed twice with saturated brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue is purified by column chromatography on silica gel (95:5 methyl-tert.-butyl ether:methanol) to yield the compound of formula XV as a white amorphous powder.



In an analogous manner sanglifehrin B can be transformed into the compound of formula XVI. These substances exist as a single epimer at C53, but the absolute configuration has not been unambiguously determined.



Formula XV: MS  $m/z$  1078  $[M+Li]^+$  (rel. intensity 100);  $^1H$  NMR (DMSO) (only characteristic signals listed)  $\delta$  0.40 (3H, d, H-50), 1.20 (3H, s, H-54), 1.69 (3H, s, H-

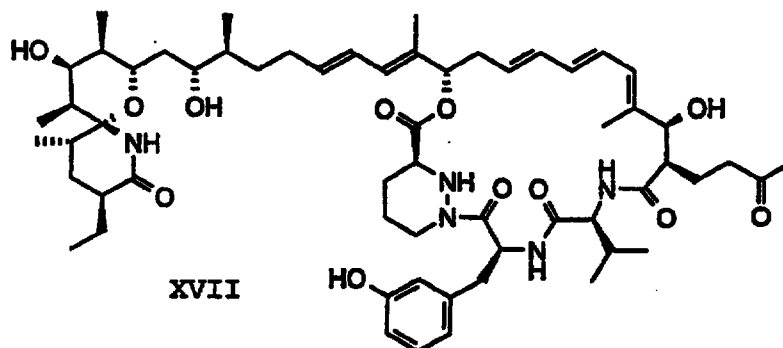
49), 4.20 (1H, t, H-15), 4.58 (1H, dd, H-17), 5.19 (1H, dd, H-18), 5.28 (1H, dd, H-23), 5.62 (1H, m, H-21), 5.67 (1H, m, H-27), 5.99 (1H, d, H-25), 6.03 (1H, dd, H-19), 6.14 (1H, dd, H-20), 6.22 (1H, dd, H-26).

**Example 6 - Transformation of the compound of formula XV into Sanglifehrin A**

To a stirred solution of 54 mg (50  $\mu$ mol) of the compound of formula XV in 0.5 mL of 4:1 THF-water is added 50  $\mu$ L of 2N aqueous sulfuric acid. The resulting solution is stirred at ambient temperature for 12 hours and the reaction is quenched with saturated aqueous sodium bicarbonate solution. This mixture is extracted twice with ethyl acetate. The combined organic solution is washed with saturated aqueous sodium bicarbonate solution and brine, dried over anhydrous sodium sulfated, filtered and concentrated under reduced pressure. Column chromatography of the residue on silica gel (90:10 methyl-tert.-butyl ether:methanol) yields sanglifehrin A as a white amorphous solid.

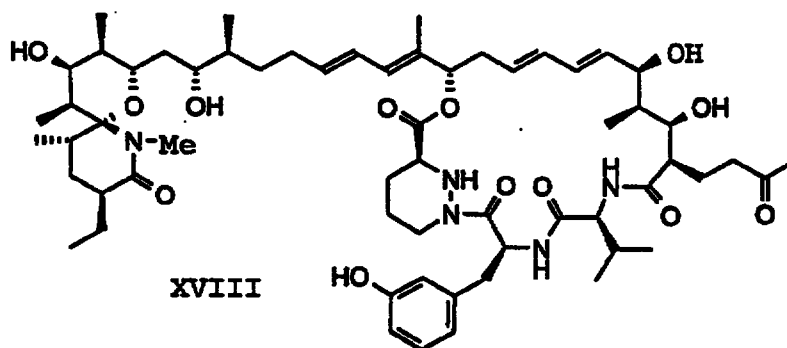
Analogously the compound of formula XVI can be transformed into sanglifehrin B.

The procedures described in examples 3 to 6 can be used as selective intramolecular protection-deprotection sequences. Thus, by the reaction described in example 5, the hydroxyl at position 15 can be selectively protected, which allows the selective manipulation of the remaining free hydroxyls. The procedure in example 5 allows for the selective protection of both the hydroxyls in the 15 and 17 positions. Both procedures can also be used as an intramolecular protection of the C53 ketone. The hydroxyls and the ketone can be regenerated by the reactions described in 4 and 6. Sanglifehrins C and D, as well as the compounds of formulae XV and XVI are therefore important intermediates for the generation of further sanglifehrins.

**Example 7 Preparation of 16-Dehydro-17-Dehydroxy-sanglifehrin A (Formula XVII)**

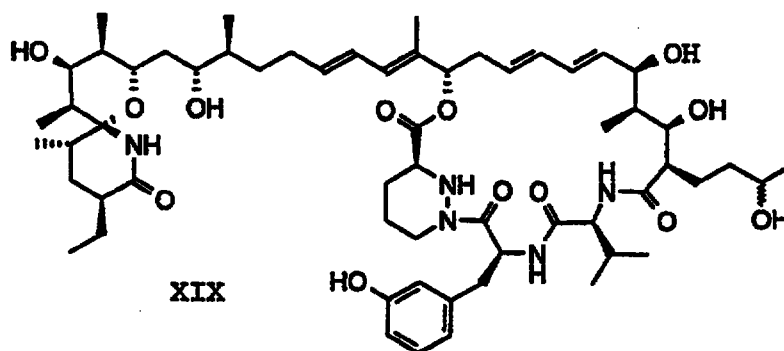
A solution of 54 mg (50  $\mu\text{mol}$ ) of the compound of formula XV and a crystal of paratoluenesulfonic acid monohydrate in 1 mL of 4:1 acetonitrile-water is heated to 80°C for 1.5 hours. The reaction is quenched by the addition of saturated aqueous sodium bicarbonate solution. The resulting mixture is extracted twice with ethyl acetate. The organic layer is washed with saturated aqueous sodium bicarbonate and brine, dried over anhydrous sodium sulfate, filtered and concentrated. The residue is purified by column chromatography on silica gel (90:10 methyl-tert.-butyl ether:methanol), followed by reverse phase chromatography (RP18, 50:50 acetonitrile-water to acetonitrile over 45 minutes) to yield the pure title compound as a white amorphous solid.

MS  $m/z$  1078  $[\text{M}+\text{Li}]^+$  (rel. intensity 100);  $^1\text{H}$  NMR (DMSO) (only characteristic signals listed)  $\delta$  1.58 (3H, s, H-50), 1.71 (3H, s, H-49), 2.08 (3H, s, H-54), 4.03 (2H, d, H-15 and C31-OH), 5.57 (2H, m, H-21 and C35-OH), 5.72 (1H, dt, H-27), 5.96 (1H, d, C15-OH), 6.03 (1H, d, H-25), 6.09-6.28 (4H, m, H-18, H-19, H-20 and H-26), 6.37 (1H, d, H-17).

**Example 8 - Preparation of 42-N-methyl-sanglifehrin A (Formula XVIII)**

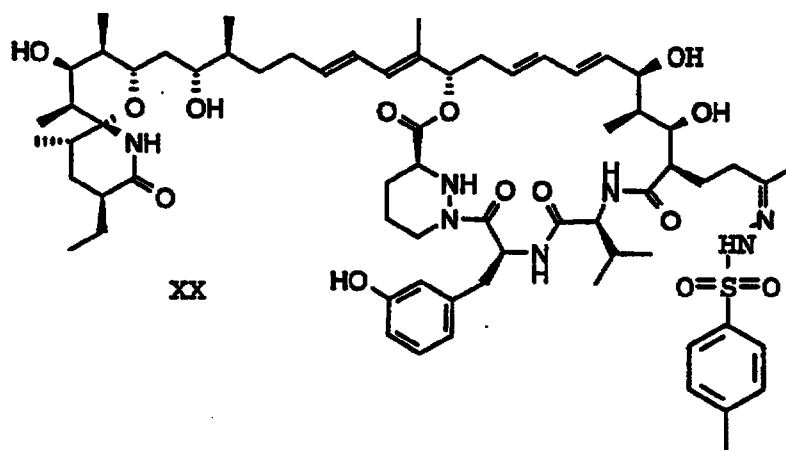
To a stirred, cooled ( $-15^{\circ}\text{C}$ ) solution of 109 mg (0.1 mmol) of sanglifehrin A and 67  $\mu\text{L}$  (0.3 mmol) of 2,6-di-tert.-butylpyridine in 1 mL of methylene chloride is added 16.5  $\mu\text{L}$  of methyl triflate. The mixture is allowed to warm to room temperature and stirring is continued for six hours, after which the reaction is quenched by addition of saturated aqueous sodium bicarbonate solution. The resulting mixture is extracted twice with ethyl acetate. The organic layer is washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. The residue is purified by two successive chromatographies on silica gel (90:10 methyl-tert.-butyl ether:methanol then 95:5 methyl-tert.-butyl ether:methanol) to yield the pure title compound as a white amorphous solid.

MS  $m/z$  1110  $[\text{M}+\text{Li}]^{+}$  (rel. intensity 100);  $^1\text{H}$  NMR (DMSO) (only characteristic signals listed)  $\delta$  1.70 (3H, s, H-49), 2.06 (3H, s, H-54), 3.53 (3H, s, 42 N-Me), 3.98 (1H, d, C31-OH), 4.50 (1H, d, H-65), 4.77 (1H, d, C17-OH), 5.43 (1H, d, C15-OH), 5.49 (1H, d, C35-OH), 7.50 (1H, d, H-12), 8.11 (1H, d, H-9), 9.22 (1H, s, C61-OH).

**Example 9 - Preparation of 53 Dihydro sanglifehrin A (Formula XIX)**

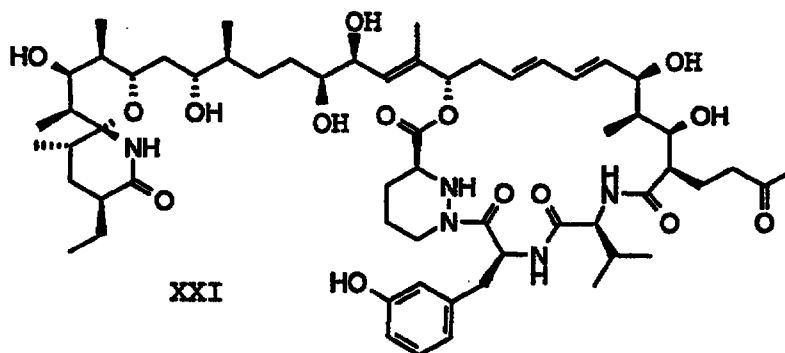
To a stirred, cooled (0°C) solution of 54 mg (50  $\mu$ mol) of sanglifehrin A in 0.5 mL of methanol is added 2.8 mg (75  $\mu$ mol) of sodium borohydride. Stirring is continued for one hour and saturated aqueous sodium bicarbonate is added. The mixture is extracted twice with ethyl acetate. The organic solution is washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. The . The residue is purified by chromatography on silica gel (95:5 methyl-tert.-butyl ether:methanol followed by 90:10 methyl-tert.-butyl ether:methanol) to yield the pure title compound as a white amorphous solid. The isolated product corresponds to a ca. 1:1 mixture of diastereoisomers at C-53)

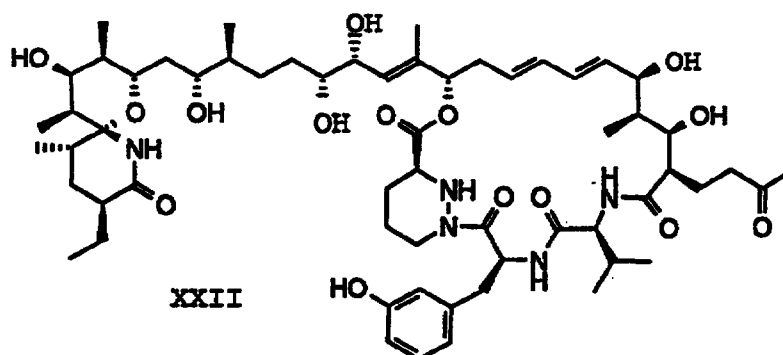
MS  $m/z$  1098  $[M+Li]^+$  (rel. intensity 63), 1104  $[M+2Li-H]^+$  (rel. intensity 100);  $^1H$  NMR (DMSO) (only characteristic signals listed)  $\delta$  0.62 (3H, d, H-50), 1.02 (3H, d, H-54), 3.55 and 3.59 (1H, 2m, H-53).

**Example 10 - Preparation of 53- Tosylhydrazone-sanglifehrin A (formula XX)**

A mixture of 55 mg (50  $\mu\text{mol}$ ) of sanglifehrin A and 23 mg (125  $\mu\text{mol}$ ) of tosylhydrazide in 0.5 mL of methylene chloride is stirred at room temperature for six hours. The solvent is removed and the residue is purified by chromatography on silica gel (90:10 methyl-tert.-butyl ether:methanol) to yield the title compound as a white amorphous powder.

MS  $m/z$  1264  $[\text{M}+\text{Li}]^+$  (rel. intensity 100);  $^1\text{H}$  NMR (DMSO) (only characteristic signals listed)  $\delta$  1.70 (3H, s, H-49), 1.77 (3H, s, H-54), 2.37 (3H, s,  $-\text{NSO}_2\text{C}_6\text{H}_4\text{CH}_3$ ), 6.51 (1H, s, H-60), 6.59 (2H, d, H-62 and H-64), 7.06 (1H, dd, H-63), 7.35 (2H, d, tosyl meta protons), 7.73 (2H, d, tosyl para protons).

**Example 11 - Preparation of 26S,27S-Dihydroxy-sanglifehrin A (Formula XXI) and 26R,27R-Dihydroxy-sanglifehrin A (Formula XXII)**



To a stirred, cooled (0°C) solution of 495 mg (1.5 mmol) of potassium ferricyanide, 207 mg (1.5 mmol) of potassium carbonate, 19.5 mg (0.025 mmol) of (DHQ)<sub>2</sub>PHAL, 65  $\mu$ L (0.005 mmol) of 0.08 M osmium tetroxide in t-butanol and 95 mg (1 mmol) of methyl sulfonamide in 2.5 mL of t-butanol and 5 mL of water is added a solution of 545 mg (0.5 mmol) of sanglifehrin A in 2.5 mL of t-butanol. The resulting biphasic mixture is allowed to warm to room temperature and stirred for three hours. Then 1.08 g (8.6 mmol) of sodium sulfite is added, followed by ethyl acetate and water, and the mixture is vigorously stirred for 15 minutes. The layers are separated and the aqueous layer is extracted twice with ethyl acetate. The combined organic layer is washed with saturated aqueous sodium bicarbonate and brine, dried over anhydrous sodium sulfate, filtered and concentrated. The residue is purified by reverse phase chromatography (RP18, 30:70 acetonitrile-water to acetonitrile over 60 minutes) yielding the 26S,27S-diol as an amorphous powder.

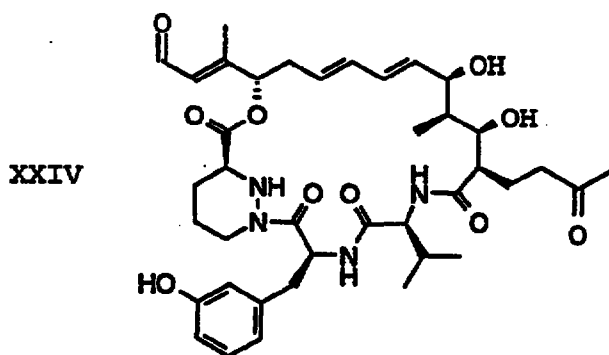
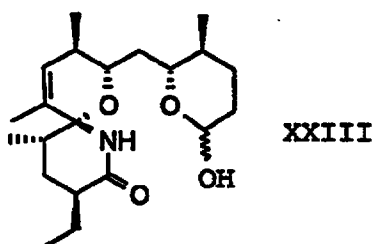
The corresponding 26R,27R-diol is obtained by the above procedure, but using (DHQD)<sub>2</sub>PHAL instead of (DHQ)<sub>2</sub>PHAL.

26S,27S-diol: MS  $m/z$  1130  $[M+Li]^+$  (rel. intensity 100); <sup>1</sup>H NMR (DMSO) (only characteristic signals listed)  $\delta$  1.64 (3H, s, H-49), 2.06 (3H, s, H-54), 3.20 (1H, broad m, H-27), 3.45 (1H, broad m, H-31), 3.94 (3H, m, H-17, H-26 and C31-OH), 4.30 (1H, d, C27-OH), 4.57 (1H, d, C26-OH), 5.20 (1H, t, H-23), 5.33 (1H, d, H-25), 5.57 (3H, m, H-18, H-21 and C35-OH), 6.03 (1H, dd, H-19), 6.14 (1H, dd, H-20).

26R,27R-diol: MS  $m/z$  1130  $[M+Li]^+$  (rel. intensity 100);  $^1H$  NMR (DMSO) (only characteristic signals listed)  $\delta$  1.64 (3H, s, H-49), 2.06 (3H, s, H-54), 3.16 (1H, broad m, H-27), 3.48 (1H, broad m, H-31), 3.94 (3H, m, H-17, H-26 and C31-OH), 4.30 (1H, d, C27-OH), 4.57 (1H, d, C26-OH), 5.20 (1H, dd, H-23), 5.35 (1H, d, H-25), 5.57 (3H, m, H-18, H-21 and C35-OH), 6.03 (1H, dd, H-19), 6.14 (1H, dd, H-20).

### Example 12 - Cleavage of the diol in 26S,27S-Dihydroxy-sanglifehrin A

To a solution of 90 mg (79  $\mu$ mol) of the 26S,27S diol in 0.9 mL of 2:1 THF-water is added 33.7 mg (157  $\mu$ mol) of sodium periodate. Stirring is continued for one hour and saturated aqueous sodium bicarbonate is added. The mixture is extracted twice with ethyl acetate. The organic solution is washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. Purification of the residue on silica gel (95:5 methyl-tert.-butylether:methanol) yields the compounds of formula XXIII (foam) and XXIV (powder).

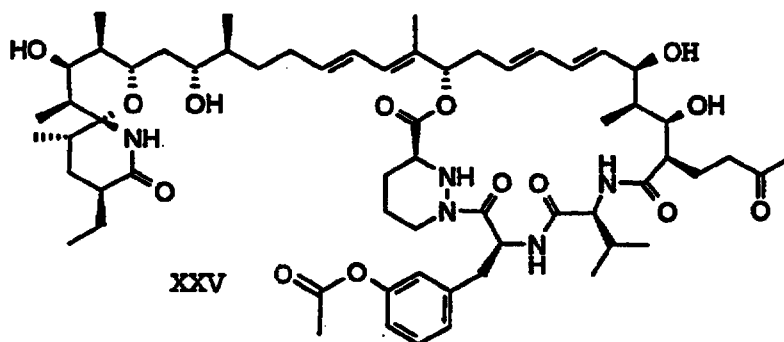


formula XXIII: MS  $m/z$  366  $[M+H-H_2O]^+$  (rel. intensity 100);  $^1H$  NMR (DMSO) (2:1 mixture of  $OH_{ax}:OH_{eq}$  epimers at the anomeric center) (only characteristic signals

listed)  $\delta$  3.54 and 4.08 (1H, 2m, H-31), 3.57 (1H, broad m, H-35), 3.66 (1H, m, H-33), 4.38 (0.67H, ddd, H-27<sub>ax</sub>), 4.95 (0.33H, broad m, H-27<sub>eq</sub>), 5.40 (0.33H, d, C27-OH<sub>eq</sub>), 5.59 (0.33H, d, C35-OH), 5.61 (0.67H, d, C35-OH), 5.96 (0.67H, d, C27-OH<sub>ax</sub>), 7.89 (0.67H, s, NH-42), 7.91 (0.33H, s, NH-42).

formula XXIV: MS  $m/z$  745  $[M+Li]^+$ ;  $^1H$  NMR (DMSO) (only characteristic signals listed)  $\delta$  0.64 (3H, d, H-50), 0.81 (6H, d, H-56 and H-57), 2.06 (3H, s, H-54), 2.17 (4H, s, H-14 and H-49), 3.80 (1H, broad m, H-15), 3.94 (1H, dd, H-17), 5.33 (1H, broad d, H-23), 5.62 (2H, m, H-18 and H-21), 6.89 (1H, d, H-25), 6.10 (1H, dd, H-19), 6.18 (1H, dd, H-20), 10.0 (1H, d, H-26).

**Example 13 - Acetylation of Sanglifehrin A to give 61-O-Acetyl-sanglifehrin A (Formula XXV)**



To a stirred, cooled (0°C) solution of 54 mg (50  $\mu$ mol) of sanglifehrin A and 50  $\mu$ L of pyridine in 0.5 mL of methylene chloride is added 5.2  $\mu$ L (55  $\mu$ mol) of acetic anhydride. The reaction is kept at 0°C for one hour, then allowed to warm to room temperature and stirring is continued for twelve hours. Saturated aqueous sodium bicarbonate is added and the resulting mixture is extracted with ethyl acetate. The organic layer is dried over anhydrous sodium sulfate, filtered and concentrated. The residue is purified by reverse phase chromatography (RP18, 40:60 acetonitrile-water to acetonitrile over 45 minutes) yielding the title compound as an amorphous powder.

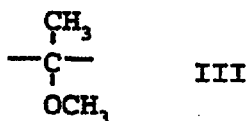
MS  $m/z$  1132  $[M+H]^+$  (rel. intensity 100);  $^1H$  NMR (DMSO) (only characteristic signals listed)  $\delta$  1.68 (3H, s, H-49), 2.06 (3H, s, H-54), 2.25 (3H, s, CH<sub>3</sub>CO<sub>2</sub>), 4.04

(1H, d, C31-OH), 4.67 (1H, d, C2-NH), 4.76 (1H, d, C17-OH), 5.42 (2H, m, H-8 and C15-OH), 5.57 (3H, m, H-18, H-21 and C35-OH), 6.85 (1H, s, H-60), 6.98 (1H, d, H-62), 7.06 (1H, d, H-64), 7.31 (1H, dd, H-63), 7.51 (1H, d, H-12), 7.89 (1H, s, H-42), 8.23 (1H, d, H-9).



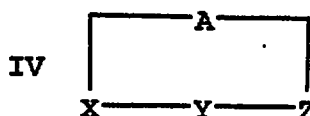


R<sub>3</sub> and R<sub>4</sub> together represent a structure of formula III



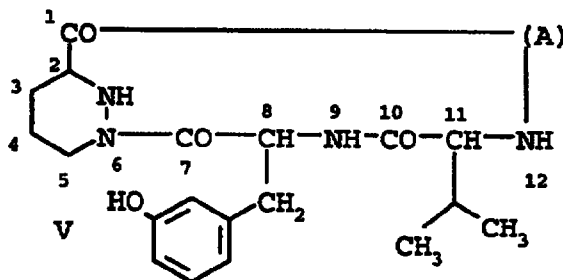
in free or protected form, or salt thereof.

5. A macrolide according to any one of claims 1-5, comprising a macrocyclic ring of formula IV



wherein X, Y and Z are residues i), ii) and iii) as defined in claim 1 and A is a hydroxy carboxylic acid residue as defined in claim 3 or 4, in free or protected form, or salt thereof.

6. A macrolide according to claim 5 comprising a macrocyclic ring of formula V



wherein A is as defined in claim 5, in free or protected form, or salt thereof.

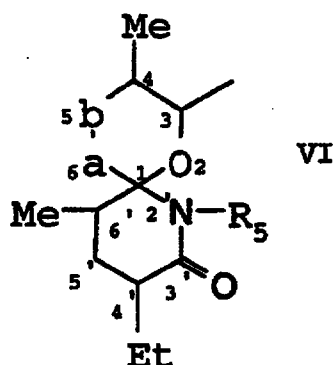
7. A macrolide according to any one of the previous claims, is substituted at the carbon atom adjacent the oxy moiety of the lactone bridge by a 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue.
8. A macrolide according to claim 7 in which the spirobicyclohexanyl residue is of the formula VI





WO 97/02285

PCT/EP96/02952



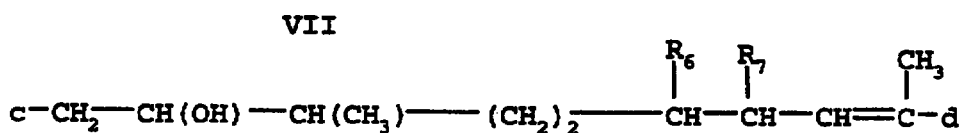
wherein

-a-b- is  $-(\text{Me})\text{C}=\text{CH}-$  or  $-(\text{Me})\text{CH}-\text{CH}(\text{OH})-$  and

$\text{R}_5$  is H or Me

in free or protected form, or salt thereof linked to the macrolide ring via a linker comprising a linear sequence of from 6 to 11, typically 9, carbon atoms between the spiro residue and the macrolide ring.

9. A macrolide according to claim 8, in which the linker between the spiro residue and the macrolide ring is a group of formula VII



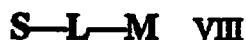
wherein

c represents linkage to the spiro residue;

d represents linkage to the macrocyclic ring and

$\text{R}_6$  and  $\text{R}_7$  are each OH or together represent an additional bond, in free or protected form.

10. A compound of formula VIII



wherein

S represents a 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue;

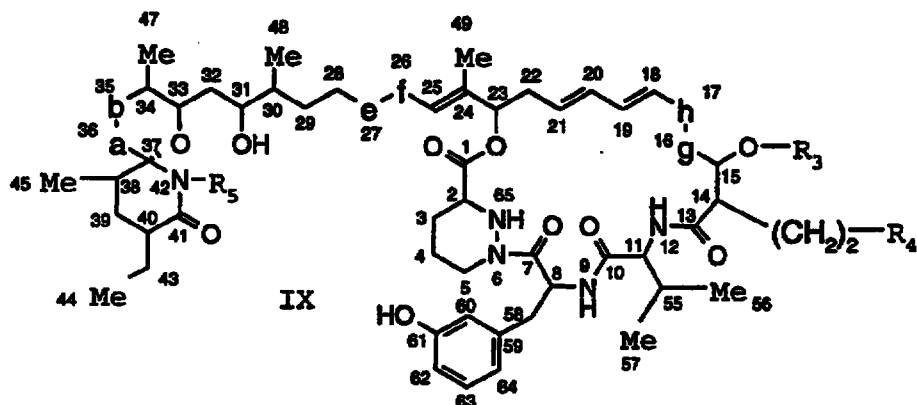




L represents a linker comprising a linear sequence of from 6 to 11, typically 9, carbon atoms, and

M represents a macrolide ring as defined in any one of claims 1 to 6, in free or protected form, or salt thereof.

11. A compound according to claim 10 of formula IX



wherein

-a-b- is as defined above;

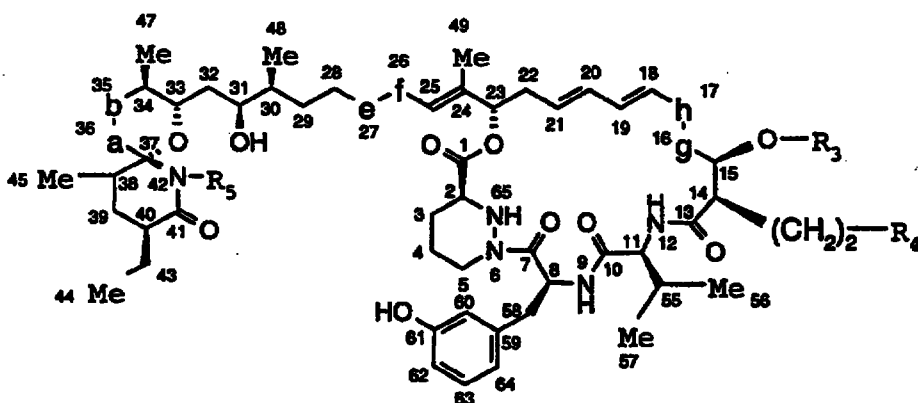
-e-f- is  $-\text{CH}(\text{OH})-\text{CH}(\text{OH})-$  or  $-\text{CH}=\text{CH}-$ ;

-g-h- is as defined above for -a-b-, and

$\text{R}_3$ ,  $\text{R}_4$  and  $\text{R}_5$  are as defined above,

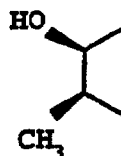
in free or protected form or salt thereof.

12. A compound according to claim 11 the following conformation



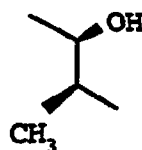
wherein when -a-b- is  $-(\text{Me})\text{CH}-\text{CH}(\text{OH})-$ , it preferably has the configuration:



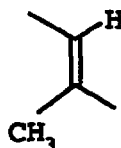


when -e-f- is -CH(OH)-CH(OH)-, it preferably has the (S),(S) configuration or the (R),(R) configuration;

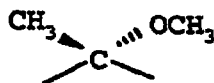
when -g-h- is -(Me)CH-CH(OH)-, it preferably has the configuration:



when -g-h- is -(Me)C=CH-, it preferably has the configuration:



and when R<sub>3</sub> and R<sub>4</sub> are fused together they are preferably is of configuration



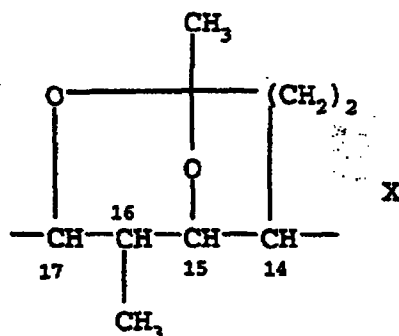
13. A Sanglifehrin selected from the group consisting of Sanglifehrin A, B, C and D.
14. A macrolide according to any one of claims 4 to 9 or a compound according to any one of claims 10 to 12 wherein the 14 to 17 positions of the macrocyclic ring comprise a residue of formula X:



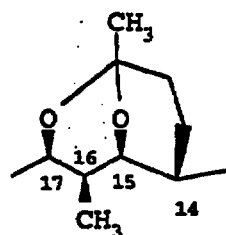


WO 97/02285

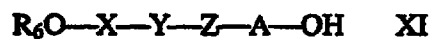
PCT/EP96/02952



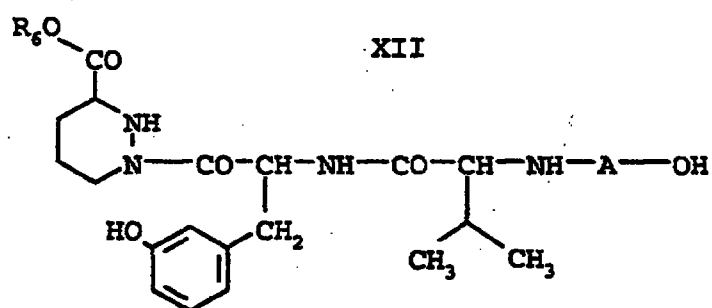
e.g. of configuration



15. A compound of formula XI:

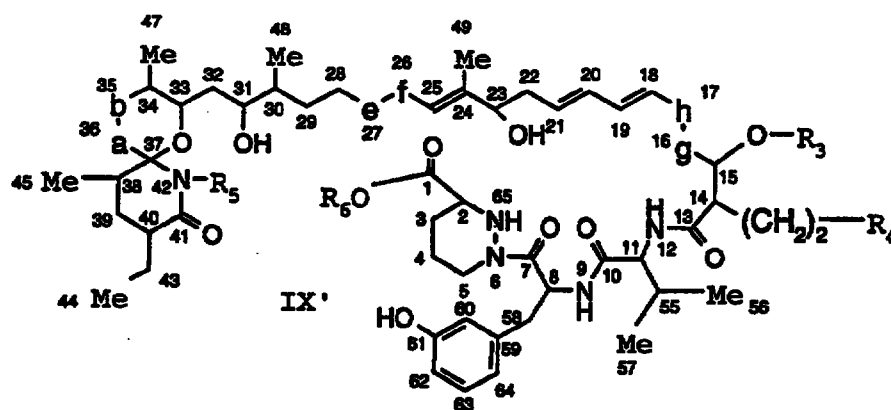


e.g. of formula XII



e.g. a compound of formula IX'

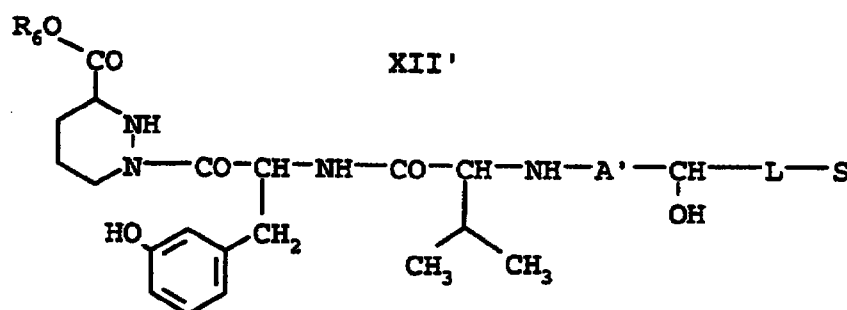




wherein X, Y, Z, A, R<sub>3</sub>, R<sub>4</sub> and R<sub>5</sub> are as defined above and R<sub>6</sub> is H or C<sub>1-4</sub> alkyl, e.g. methyl

in free or protected form, or a salt thereof.

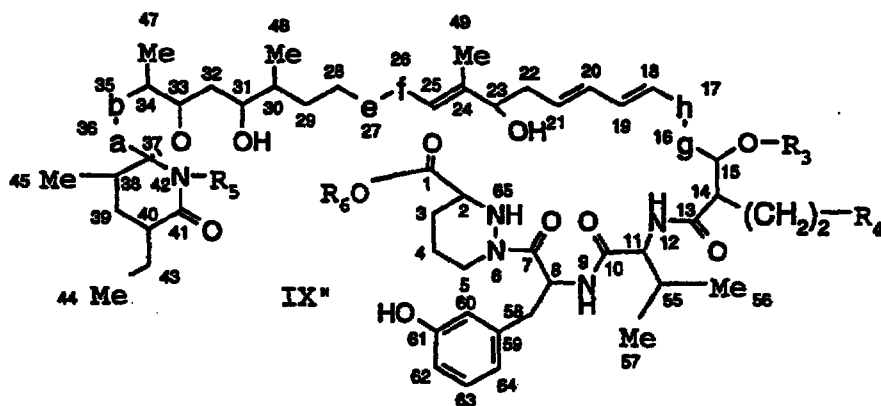
16. A macrolide as defined in any one of claims 1 to 9 in ring-opened form, said ring-opened macrocycle being in free or protected form, or salt thereof;
17. A compound R<sub>6</sub>O - X - Y - Z - A - Ohwherein X, Y, Z and A are as defined in claim 5 and R<sub>6</sub> is H or C<sub>1-4</sub> alkyl, in free or protected form, or salt thereof.
18. A compound R<sub>6</sub>O - X - Y - Z - A' - CH(OH) - L - S wherein - A' - CH(OH) - is a hydroxy carboxylic acid residue as defined in claim 3 and the other symbols are as defined in claim 17, in free or protected form, or a salt thereof.
19. A compound of formula XII'





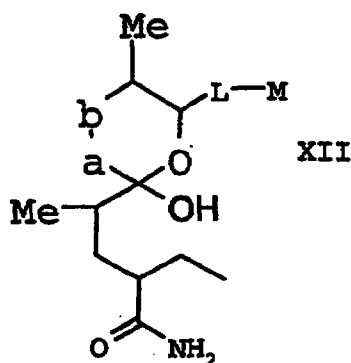
in free or protected form, or salt thereof;

- 20 A compound of formula IX''



in free or protected form or a salt thereof.

21. A compound in which the 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexane ring system is in ring-opened form, e.g. a compound of formula XII



wherein a, b, L and M are as defined above,

in free or protected form, or a salt thereof.

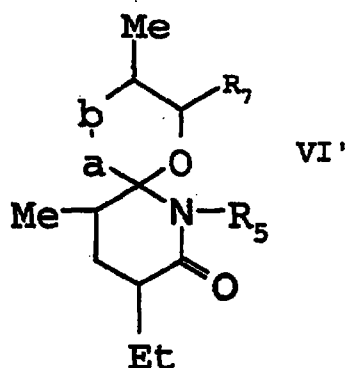
22. A 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexan, in free or protected form, or a salt thereof, in particular a compound of formula VI'





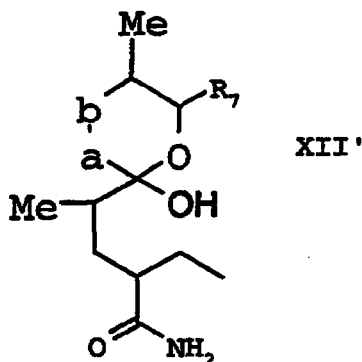
WO 97/02285

PCT/EP96/02952



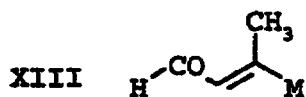
wherein R<sub>7</sub> is H, an optionally protected OH group, a reactive functional group, or a -CH<sub>2</sub>-CH(OH)-CH(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>-CHO group or the delta lactol equivalent thereof, in free or protected form or salt thereof.

23. A ring-open 2-oxy-2'-aza-3'-oxo-3'-yl-spirobicyclo hexan, in free or protected form, or a salt thereof, in particular a compound of formula XII'



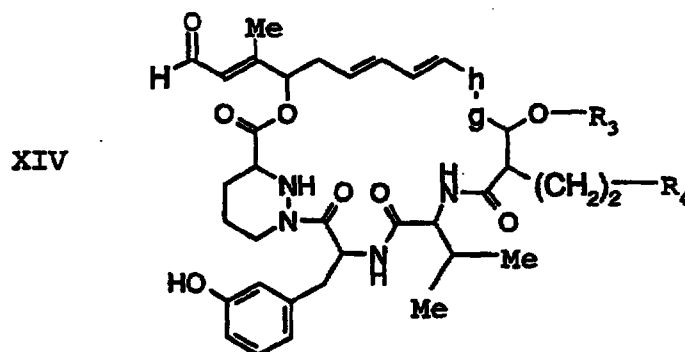
wherein a, b and R<sub>7</sub> are as previously defined, in free or protected form, or a salt thereof.

24. A macrolide of formula XIII



wherein M is a macolide ring as defined above, in particular a macrolide of formual XIV





in free or protected form, or salt thereof.

25 A process for the production of any compound of the invention as hereinbefore defined, which process comprises:

i). for the production of any one of Sanglifehrins A, B, C or D, cultivating a Sanglifehrin A, B, C or D producing actinomycete strain in a culture medium and isolating the desired Sanglifehrin A, B, C or D from the obtained culture broth;

ii). for the production of Sanglifehrins C and D subjecting Sanglifehrins A and B to cyclisation at positions 15 and 16;

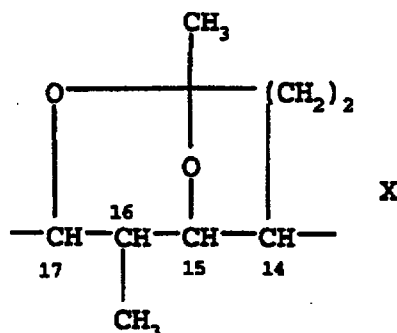
iii). for the production of Sanglifehrins A and B and subjecting Sanglifehrins C and D to ring opening of the lactol ring at positions 15 and 16:

iv). for the production of a macrolide of formula IX or IX', wherein -g-h- is -C(CH<sub>3</sub>)=CH-, dehydrating a compound of formula IX or IX' wherein -g-h- is -CH(CH<sub>3</sub>)-CH(OH)- or a protected form thereof;

v). for the production of a macrolide of formula IX or IX' wherein R<sub>4</sub> is -CH(OH)-CH<sub>3</sub>, hydrogenating a compound of formula IX or IX' wherein R<sub>4</sub> is -C(O)-CH<sub>3</sub>;

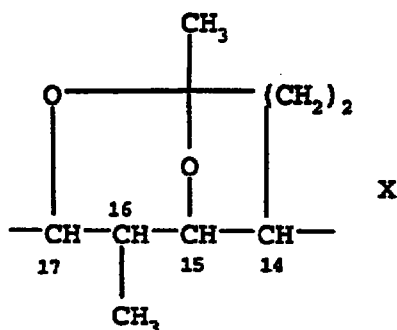
vi). for the production of a macrolide of formula IX or IX' wherein the 14 to 16 positions of the macrolide ring comprise a residue of formula X





causing a compound of formula IX or IX' to undergo internal protection;

vii). for the production of a macrolide of formula IX or IX', causing a compound of formula IX or IX' wherein the 14 to 16 positions of the macrolide ring comprise a residue of formula X



to undergo reversal of internal protection.

viii). for the production of a macrolide of formula IX or IX', in which R<sub>5</sub> is methyl, subjecting a macrolide of formula IX or IX', in which R<sub>5</sub> is H to methylation;

ix). for the production of a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-protected form, subjecting a macrolide of formula IX or IX', in which R<sub>5</sub> is in O-unprotected form to O-protection;

x). for the production of a macrolide of formula IX or IX', in which R<sub>4</sub> is in O-unprotected form, subjecting a macrolide of formula IX or IX', in which R<sub>5</sub> is in O-protected form to O-deprotection;





xi). for the production of a macrolide of formula IX or IX', which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX', in which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-protection;

xii). for the production of a macrolide of formula IX or IX', which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX' which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-deprotection;

xiii). for the production of a macrolide of formula IX or IX', in which -e-f- is -CH(OH)-CH(OH)-, subjecting a macrolide of formula IX or IX' in which -e-f- is -CH=CH- to oxidative hydrolysis;

xiv). for the production of a compound of formula V' or a compound of formula XII, subjecting a compound of formula IX or IX' to cleavage of the linker group between the spiro bicyclo group and the macrocyclic ring.

xv). for the production of a compound of formula  $R_6O - X - Y - Z - A - OH$  or of formula  $R_6O - X - Y - Z - A' - CH(OH) - L - S$ , subjecting a macrocycle of formula IV or the macrocyclic ring of a compound of formula VIII to ring-opening;

xvi). for the production of a macrolide of formula IX or XII in ring-closed form, subjecting a compound of formula  $R_6O - X - Y - Z - A - OH$  or of formula  $R_6O - X - Y - Z - A' - CH(OH) - L - S$  to closure of the macrocyclic ring.





WO 97/02285

PCT/EP96/02952

- xvii). for the production of a compound of formula ????, subjecting a compound of formula ???? to ring-opening within the spiro bicyclic ring system, and
- xix). for the production of a compound of formula ????, subjecting a compound of formula ???? to ring-closure within the spiro bicyclic ring system.
26. a macrolide producing actinomycete strain wherein the macrolide is a macrolide in which
- i) positions 2 to 6 inclusive of the macrocyclic ring are provided by a piperidazinyI carboxylic acid residue; and/or
  - ii) positions 7 to 9 inclusive of the macrocyclic ring are provided by an aromatic  $\alpha$ -amino acid residue; and/or
  - iii) positions 10 to 12 inclusive of the macrocyclic ring are provided by an aliphatic  $\alpha$ -amino acid residue.
27. A biologically pure isolate of strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof which is capable of producing a macrolide of the invention.
28. A process for the production of a macrolide of the invention, which comprises cultivating strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof in an appropriate culture medium and optionally recovering the sanglifehrin.
29. A method of effecting immunosuppression in a subject in need of such treatment which method comprises administering to said subject an effective amount of an agent of the invention.
30. A method:





WO 97/02285

PCT/EP96/02952

- i) for the prevention of acute and/or chronic organ allo- or xenotransplant rejection, for example for the treatment of recipients of organ transplants of any of the particular types listed above; or
- ii) for the prevention of graft-versus-host disease, for example in recipients of bone marrow transplants; or
- iii) for the treatment of autoimmune disease or for the treatment of any such disease or condition listed above; or
- iv) for the treatment of asthma

in a subject in need of such treatment, which method comprises administering to said subject an effective amount of an agent of the invention.

- 31. An agent of the invention for use as a pharmaceutical, e.g. for use as an immunosuppressant or for use in the treatment of any disease or condition as set forth under B above.
- 32. A pharmaceutical composition comprising an agent of the invention in association with a pharmaceutically acceptable diluent or carrier.
- 33. Use of an agent of the invention for the preparation of a medicament for use as an immunosuppressant or for use in the treatment of any disease or condition as set forth under B above.
- 34. Use of a compound according to any one of claims 1-6, 8-10, 15 or 16 as a reagent in a displacement immunoassay for cyclosporins or other cyclophilin binding compounds



1 / 12

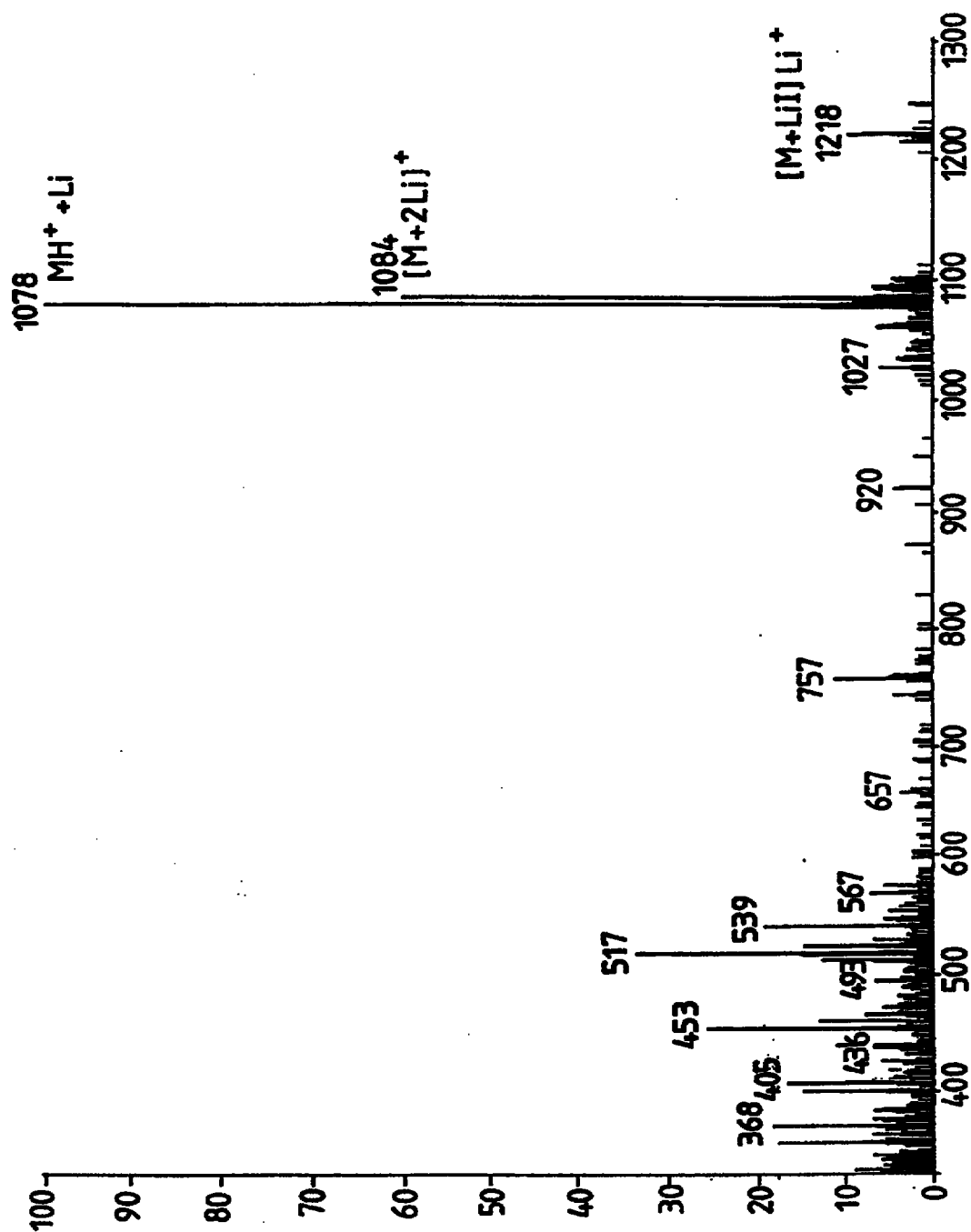


FIG. 1

2 / 12

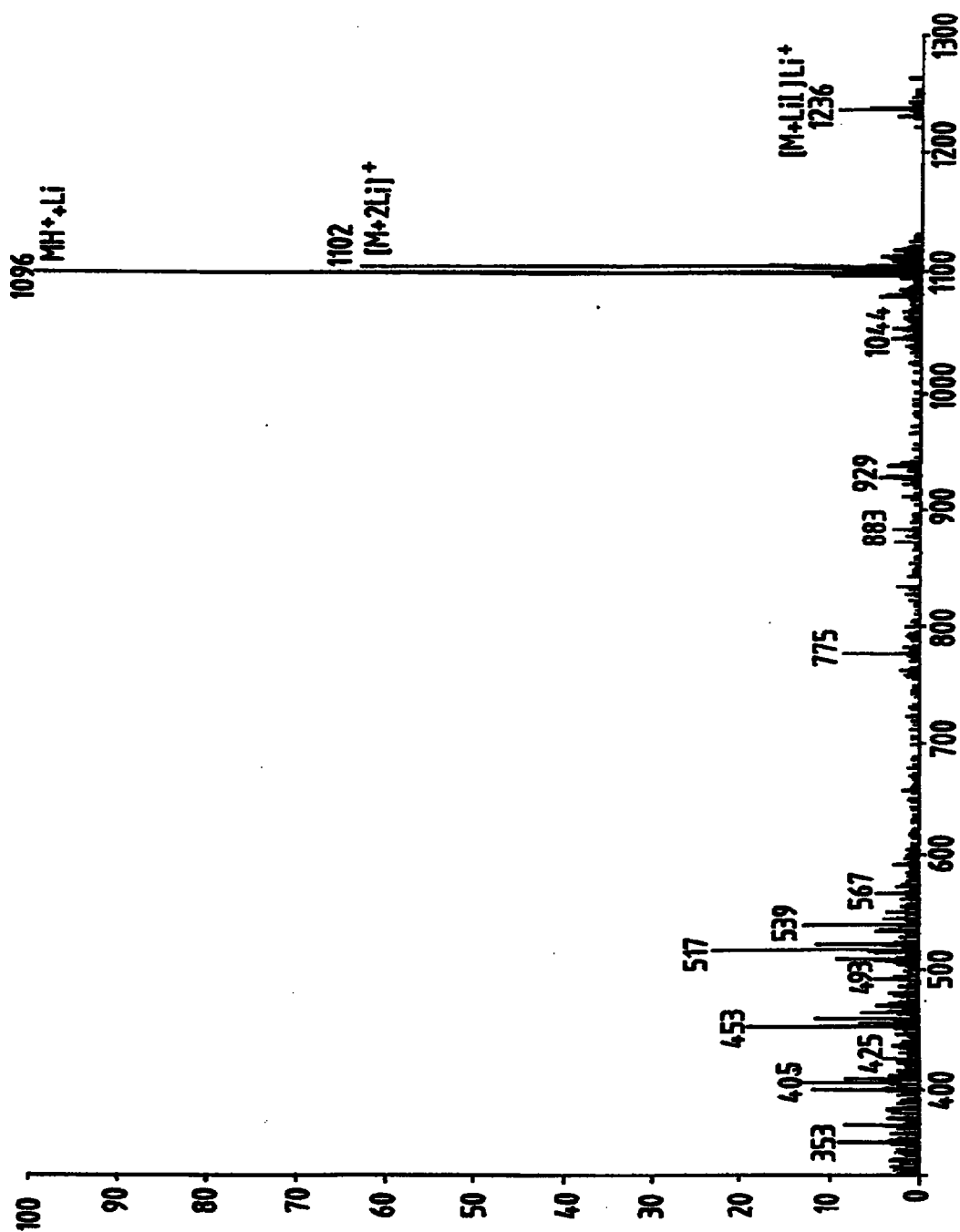


FIG. 2

3 / 12

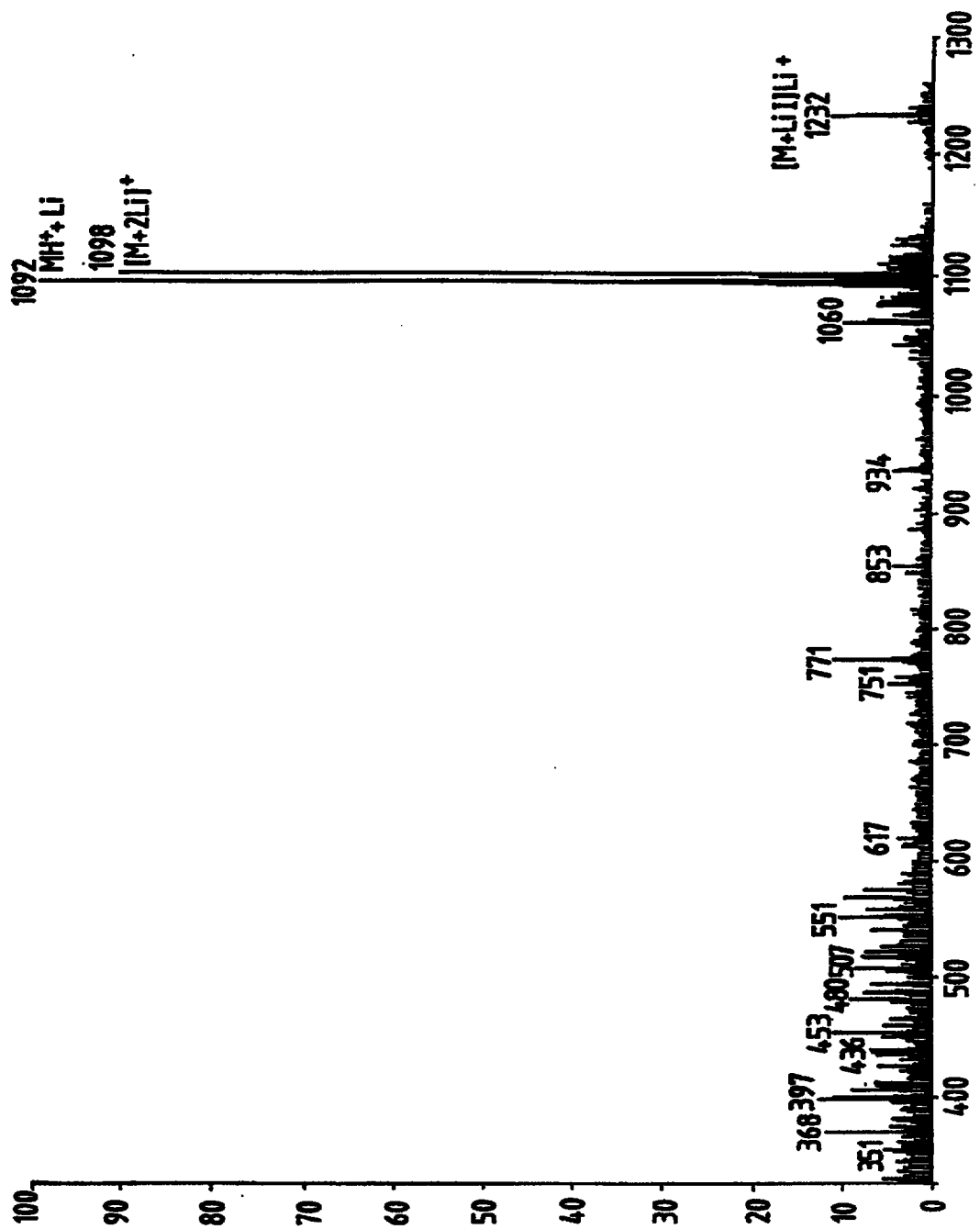


FIG. 3

4 / 12

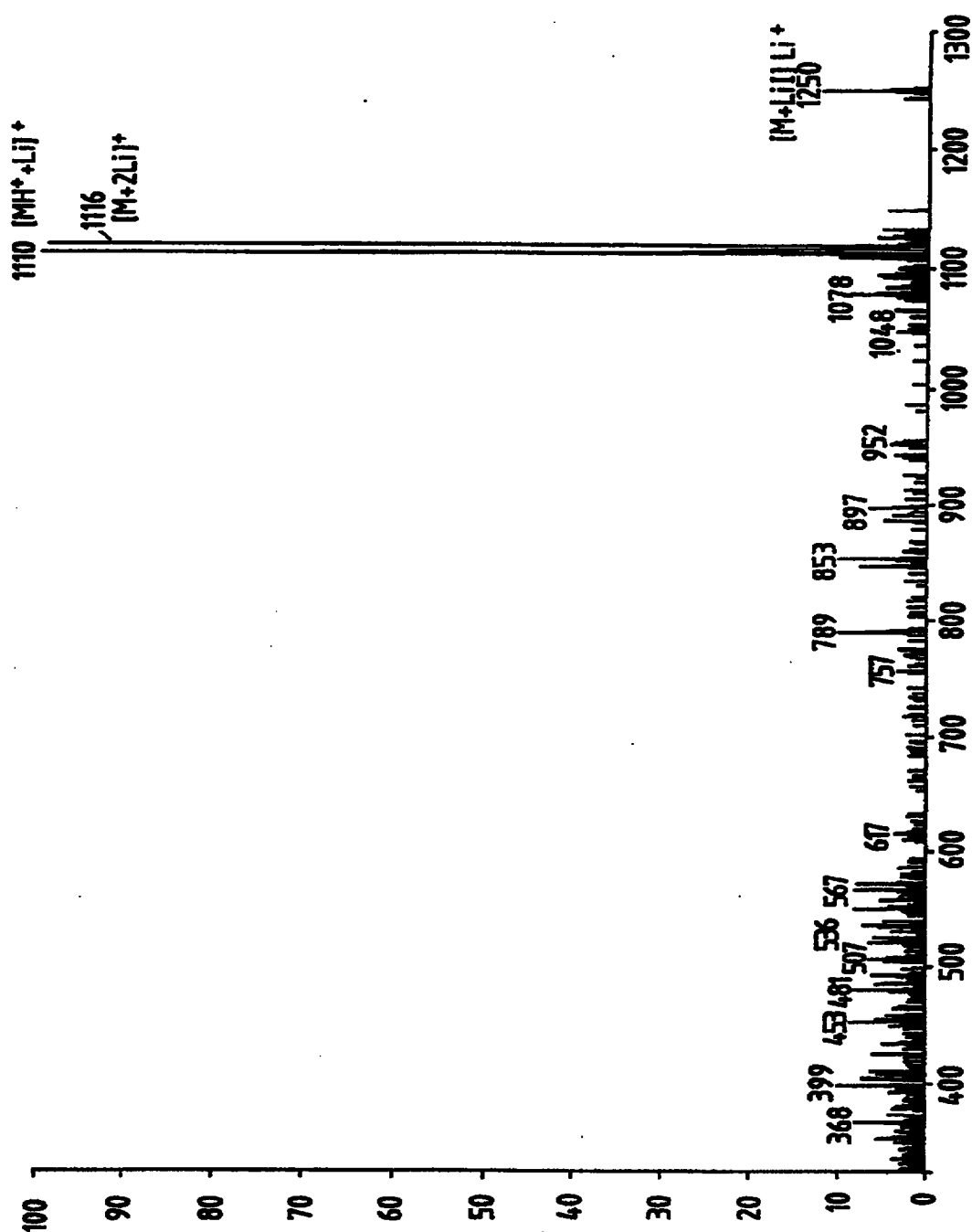


FIG. 4

5 / 12

FIG. 5

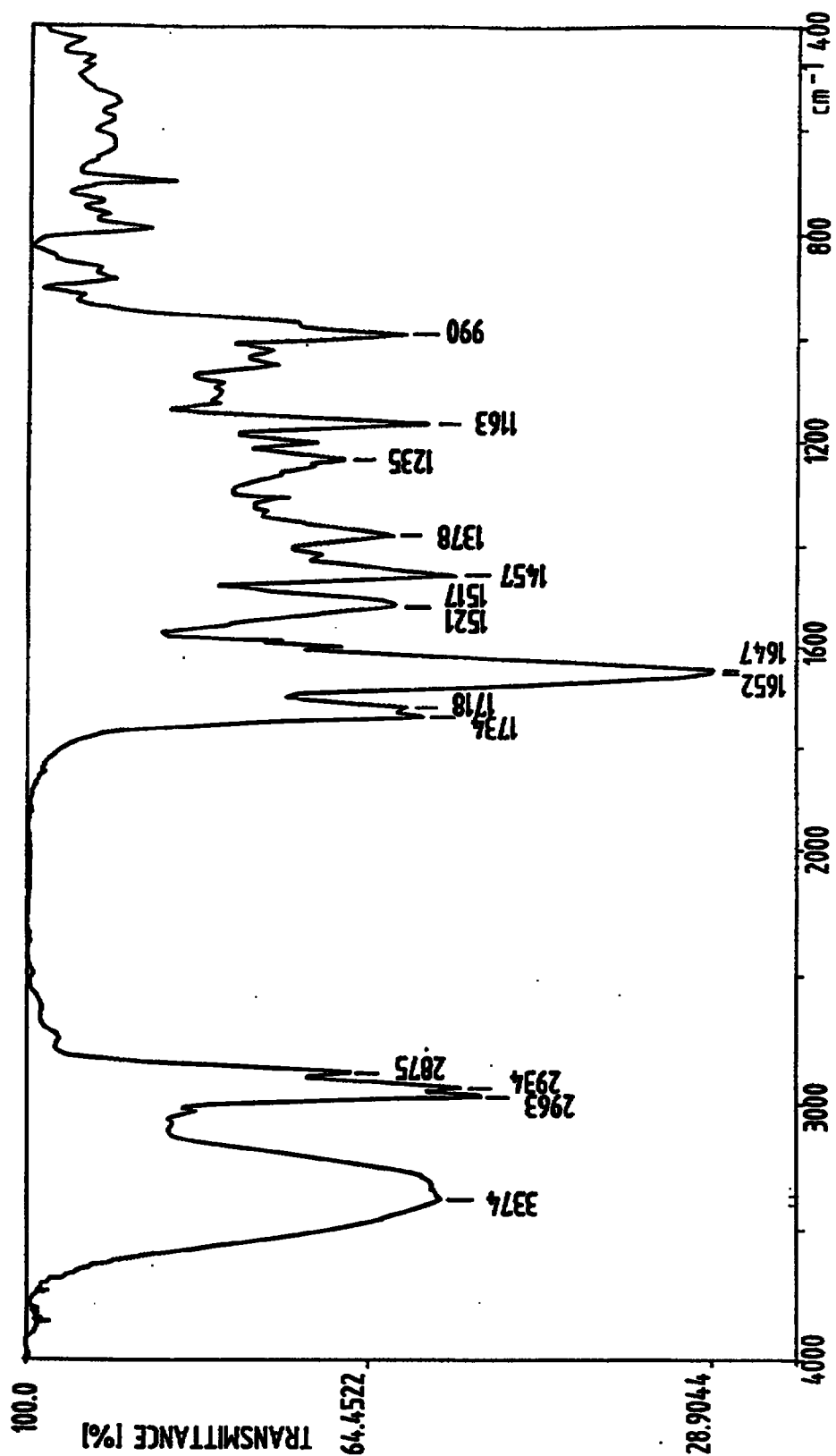


FIG. 6

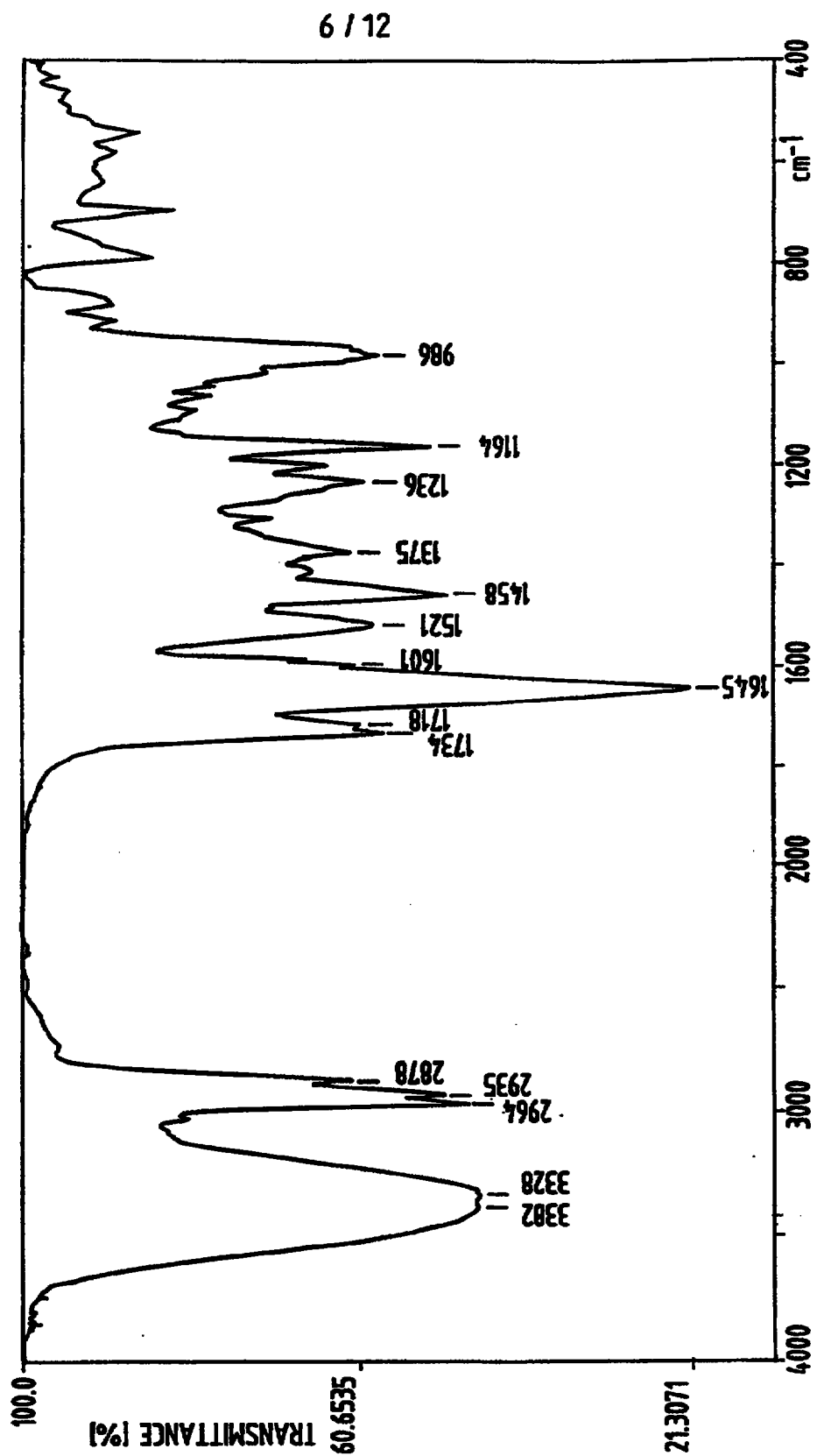


FIG. 7

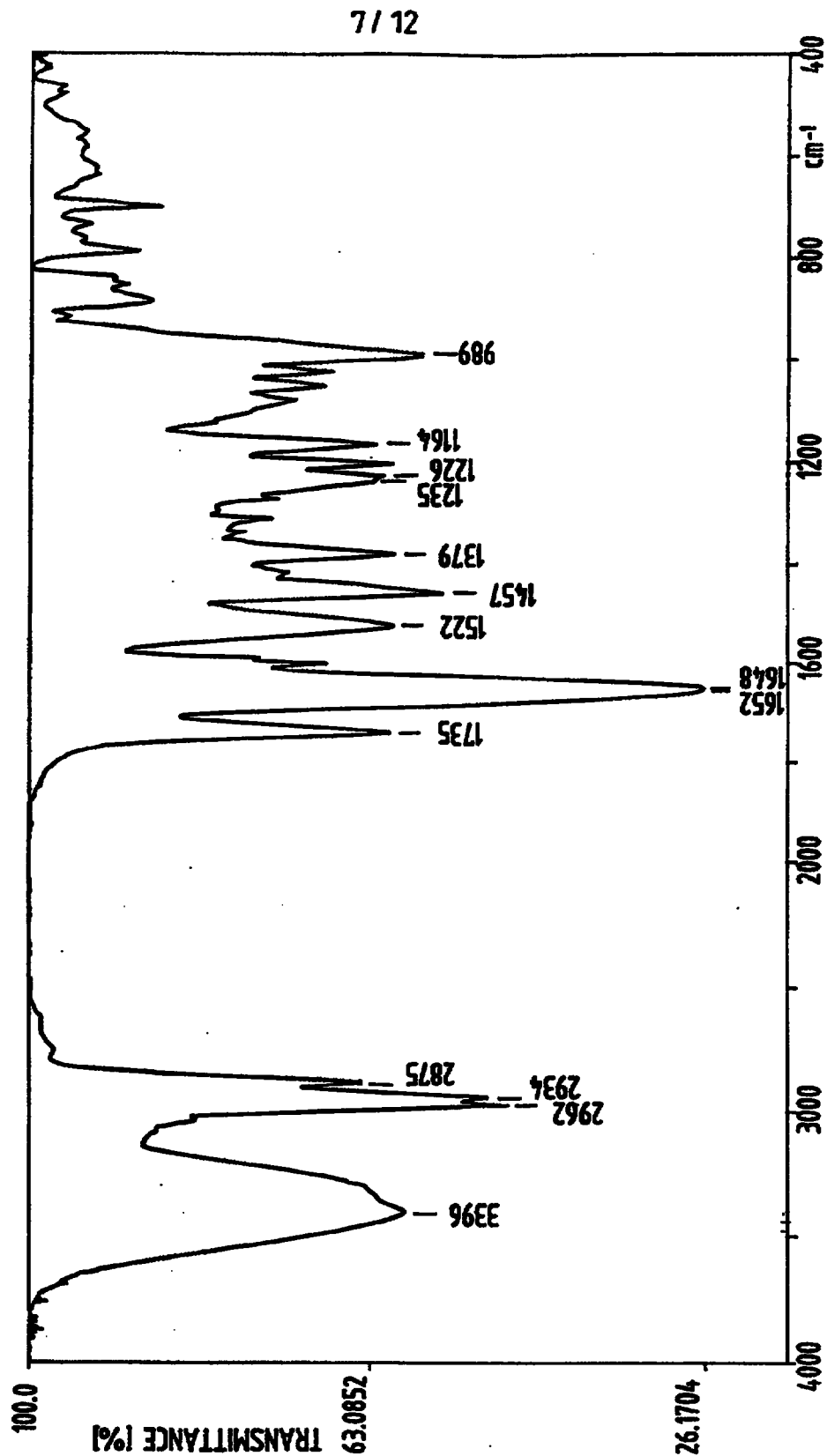
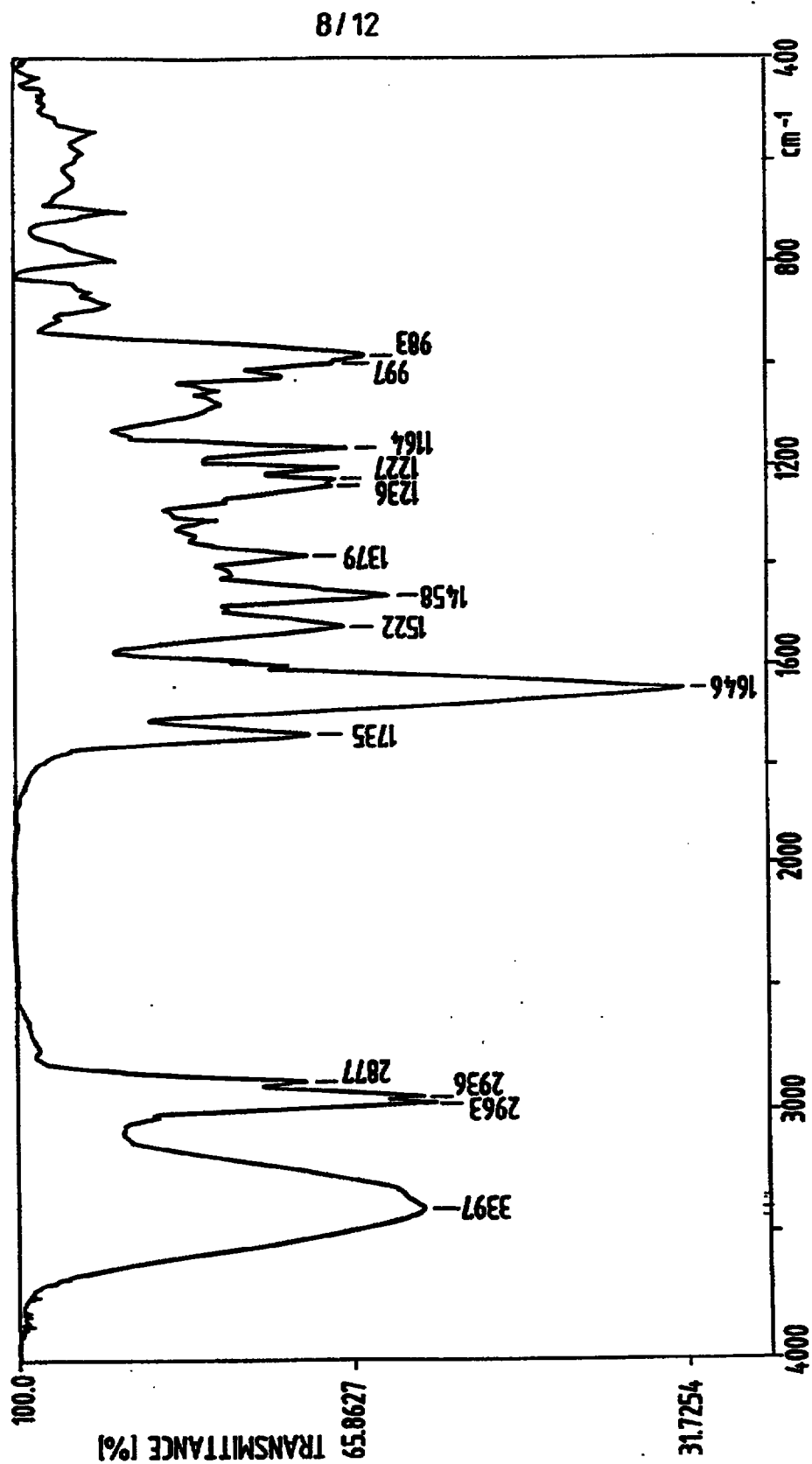
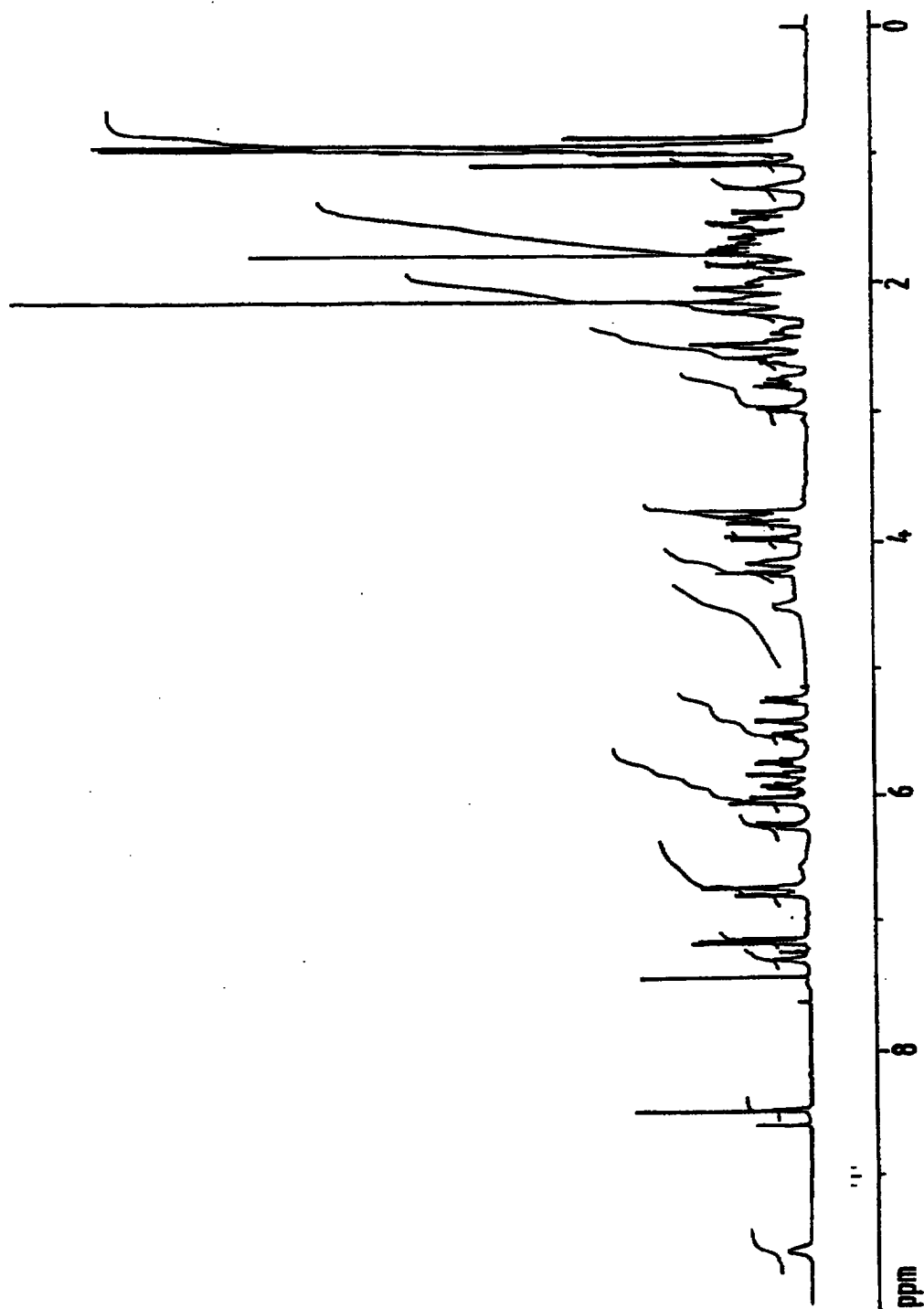


FIG. 8



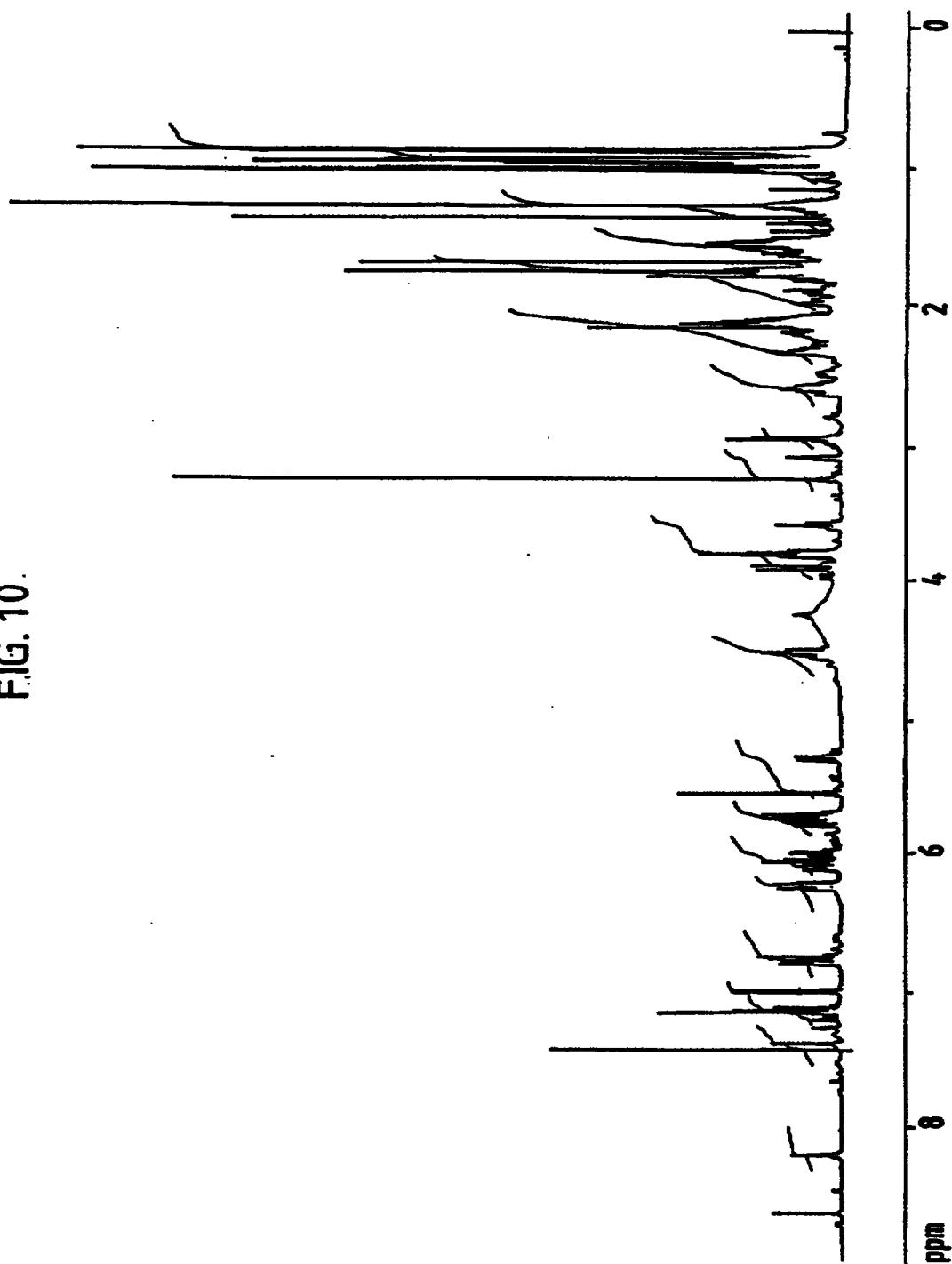
9/12

FIG. 9



10 / 12

FIG. 10.



11 / 12

FIG. 11

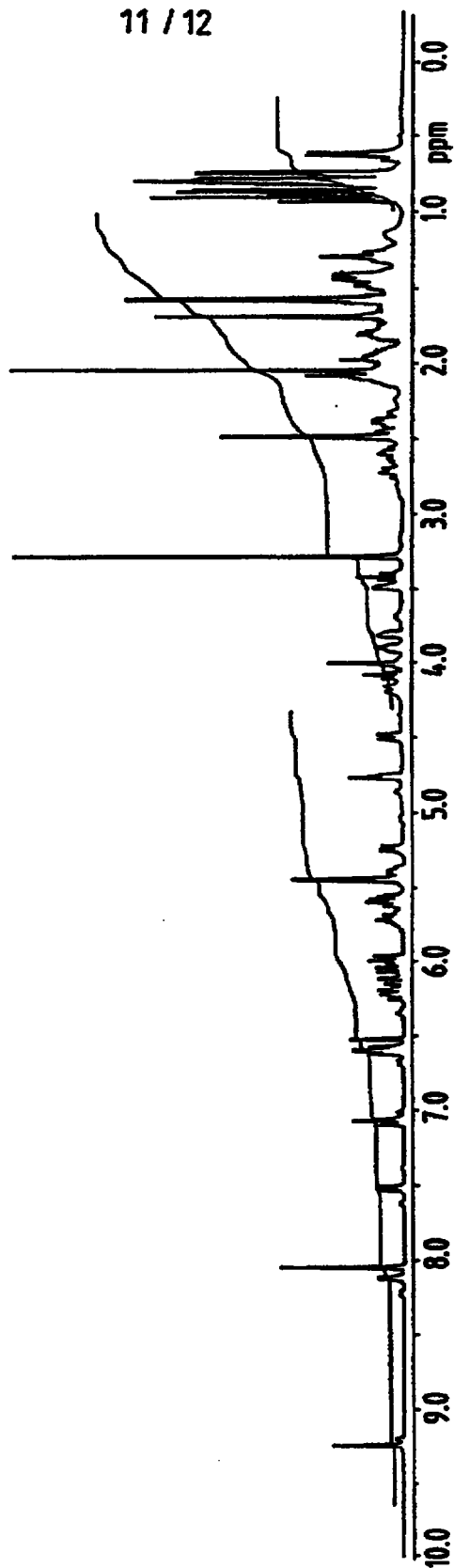
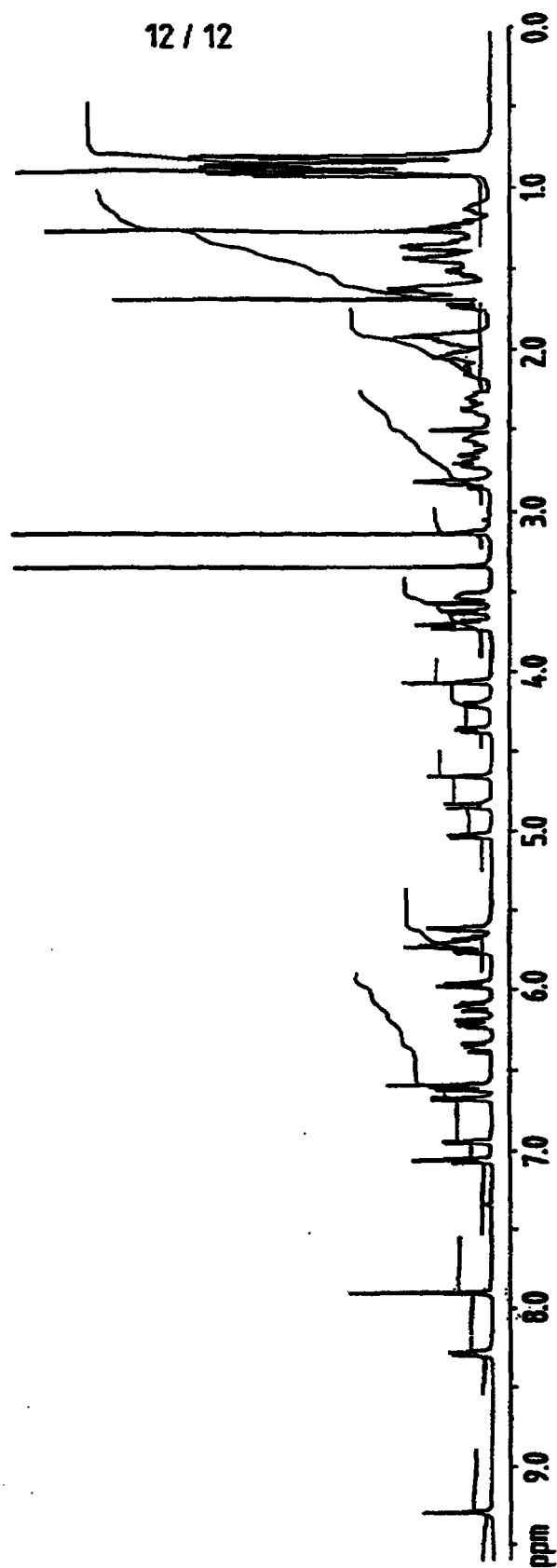


FIG. 12



- ☐ Description : paged ..... to paged .....  
☒ Claim(s) : paged 62 to paged 74  
☐ Drawing(s) : paged ..... to paged .....  
☐ Abstract : paged ..... to paged .....

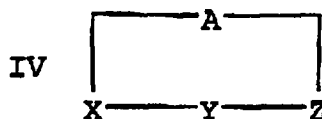
DATE OF RECEIPT 23-12-98

CLAIMS

## REPLACEMENT SHEET

23 DEC 1998

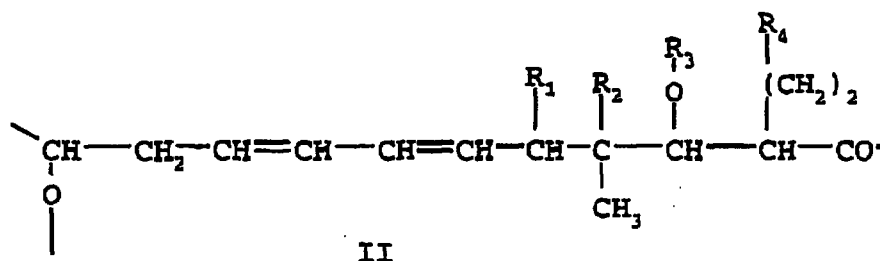
1. A macrolide comprising a macrocyclic ring of formula IV



wherein X, Y and Z are

- i) positions 2 to 6 inclusive of the macrocyclic ring and are provided by a piperidaziny carboxylic acid residue; and/or
- ii) positions 7 to 9 inclusive of the macrocyclic ring and are provided by a aromatic  $\alpha$ -amino acid residue; and/or
- iii) positions 10 to 12 inclusive of the macrocyclic ring and are provided by a aliphatic  $\alpha$ -amino acid residue,

and A is a residue comprising a hydroxy carboxylic acid residue which has a chain length of from 6 to 20 carbon atoms, preferably a hydroxy carboxylic acid residue of formula II.



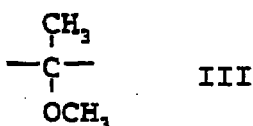
wherein

$R_1$  and  $R_2$  are both H or represent an extra bond;

$R_3$  is H;

$R_4$  is  $-\text{CO}-\text{CH}_3$  or  $-\text{CH}(\text{OH})-\text{CH}_3$  or

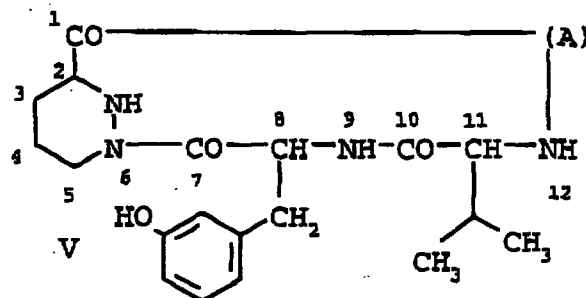
$R_3$  and  $R_4$  together represent a structure of formula III



23 DEC 1998

in free or protected form, or salt thereof.

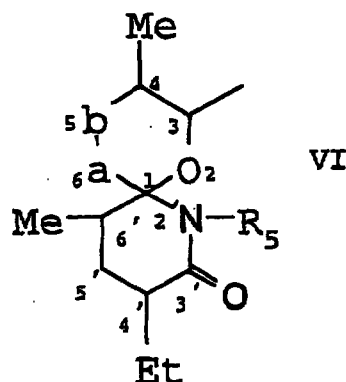
2. A macrolide according to claim 1 comprising a macrocyclic ring of formula V



wherein A is as defined in claim 1,

in free or protected form, or salt thereof.

3. A macrolide according to any one of the previous claims, which is substituted at the carbon atom adjacent the oxy moiety of the lactone bridge by a 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue.
4. A macrolide according to claim 3 in which the spirobicyclohexanyl residue is of the formula VI



wherein

-a-b- is  $-(\text{Me})\text{C}=\text{CH}-$  or  $-(\text{Me})\text{CH}-\text{CH}(\text{OH})-$  and

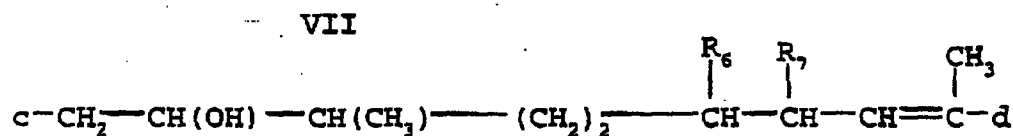
R<sub>5</sub> is H or Me

in free or protected form, or salt thereof, linked to the macrolide ring via a linker comprising a linear sequence of from 6 to 11, typically 9, carbon atoms between the spiro residue and the macrolide ring.



23 DEC 1998

5. A macrolide according to claim 4, in which the linker between the spiro residue and the macrolide ring is a group of formula VII



wherein

c represents linkage to the spiro residue;

d represents linkage to the macrocyclic ring and

$R_6$  and  $R_7$  are each OH or together represent an additional bond, in free or protected form.

6. A compound of formula VIII



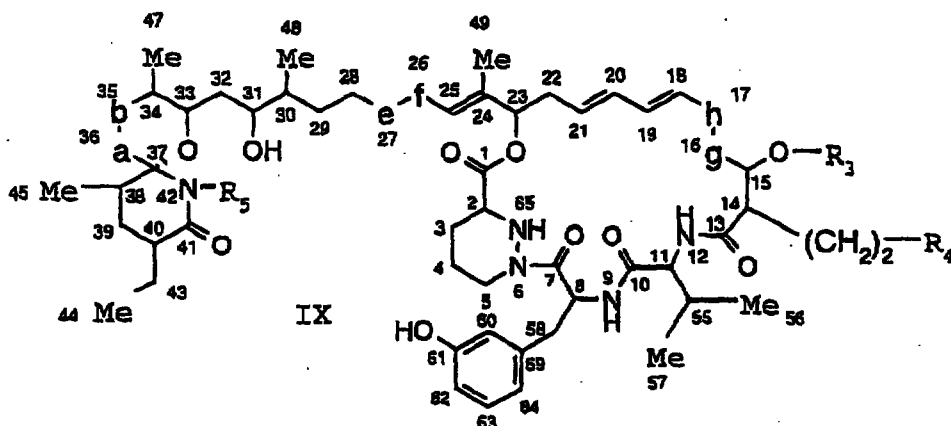
wherein

S represents a 2-oxy-2'-aza-3'-oxo-spirobicyclohexan-3-yl residue;

L represents a linker comprising a linear sequence of from 6 to 11, typically 9, carbon atoms, and

M represents a macrolide ring as defined in claim 1 or 2, in free or protected form, or salt thereof.

7. A compound according to claim 6 of formula IX



wherein

-a-b- is as defined above;

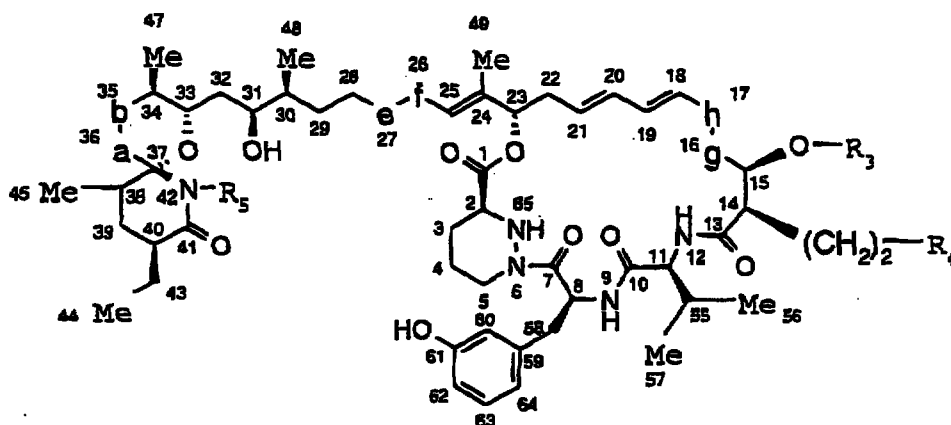
-e-f- is -CH(OH)-CH(OH)- or -CH=CH-;

-g-h- is as defined above for -a-b-, and

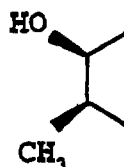
R<sub>3</sub>, R<sub>4</sub> and R<sub>5</sub> are as defined above,

in free or protected form or salt thereof.

8. A compound according to claim 7 the following conformation

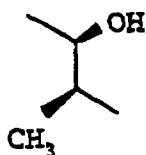


wherein when -a-b- is -(Me)CH-CH(OH)-, it preferably has the configuration:

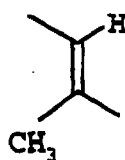


when -e-f- is -CH(OH)-CH(OH)-, it preferably has the (S),(S) configuration or the (R),(R) configuration;

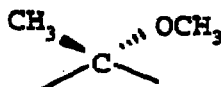
when -g-h- is -(Me)CH-CH(OH)-, it preferably has the configuration:



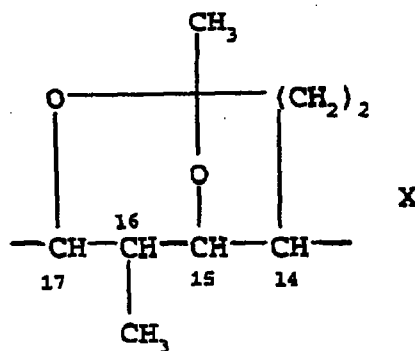
when -g-h- is -(Me)C=CH-, it preferably has the configuration:



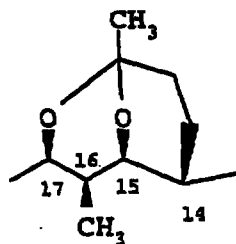
and when  $R_3$  and  $R_4$  are fused together they are preferably is of configuration



9. A Sanglifehrin selected from the group consisting of Sanglifehrin A, B, C and D.
10. A macrolide according to any one of claims 1 to 5 or a compound according to any one of claims 6 to 8 wherein the 14 to 17 positions of the macrocyclic ring comprise a residue of formula X:



e.g. of configuration

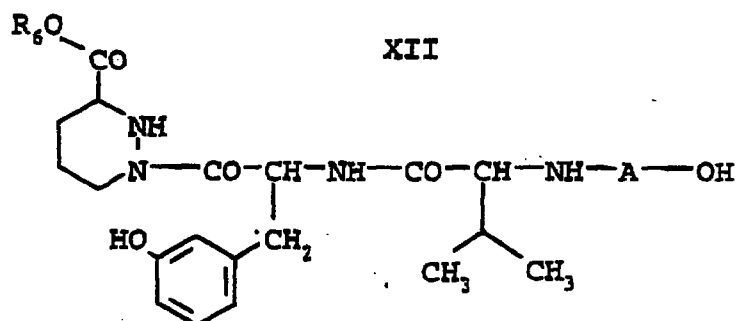


11. A compound of formula XI:

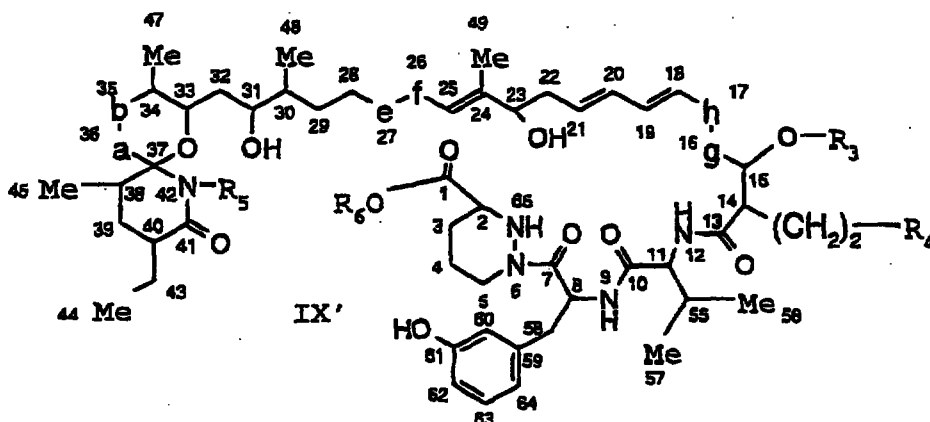


e.g. of formula XII





e.g. a compound of formula IX'



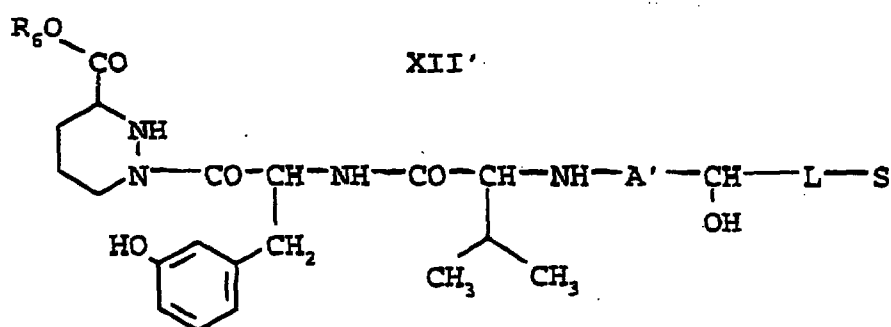
wherein X, Y, Z, A, R<sub>3</sub>, R<sub>4</sub> and R<sub>5</sub> are as defined above and R<sub>6</sub> is H or C<sub>1-4</sub> alkyl, e.g. methyl

in free or protected form, or a salt thereof.

12. A macrolide as defined in any one of claims 1 to 5 in ring-opened form, said ring-opened macrocycle being in free or protected form, or salt thereof;
13. A compound R<sub>6</sub>O - X - Y - Z - A - Oh wherein X, Y, Z and A are as defined in claim 1 and R<sub>6</sub> is H or C<sub>1-4</sub> alkyl, in free or protected form, or salt thereof.
14. A compound R<sub>6</sub>O - X - Y - Z - A' - CH(OH) - L - S wherein - A' - CH(OH) - is a hydroxy carboxylic acid residue as defined in claim 1 and the other symbols are as defined in claim 13, in free or protected form, or a salt thereof.
15. A compound of formula XII'

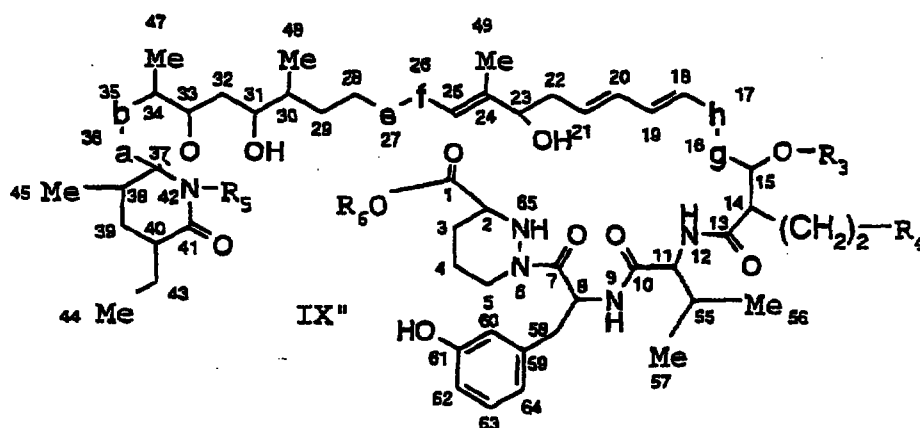


23 DEC 1998



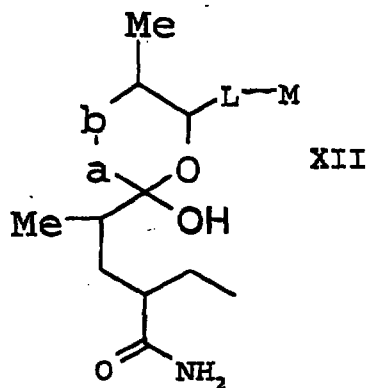
in free or protected form, or salt thereof;

16. A compound of formula IX''



in free or protected form or a salt thereof.

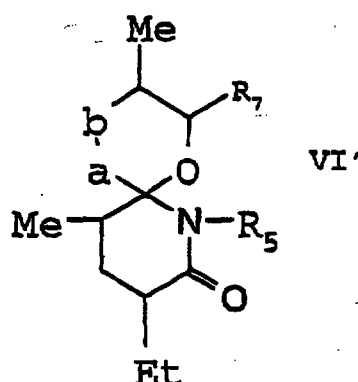
17. A compound in which the 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexane ring system is in ring-opened form, e.g. a compound of formula XII



23 DEC 1998

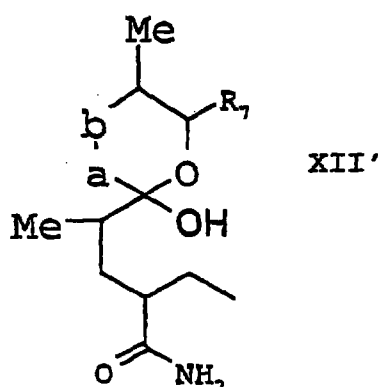
wherein a, b, L and M are as defined above,  
in free or protected form, or a salt thereof.

18. A 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexan, in free or protected form, or a salt thereof, in particular a compound of formula VI'



wherein  $R_7$  is H, an optionally protected OH group, a reactive functional group, or a  $-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CHO}$  group or the delta lactol equivalent thereof, in free or protected form or salt thereof.

19. A ring-open 2-oxy-2'-aza-3'-oxo-3'yl-spirobicyclo hexan, in free or protected form, or a salt thereof, in particular a compound of formula XII'

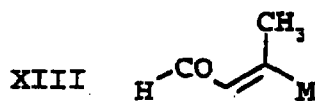


wherein a, b and  $R_7$  are as previously defined, in free or protected form, or a salt thereof.

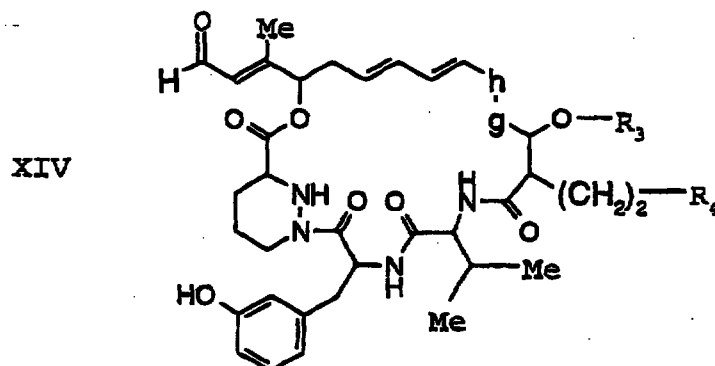
20. A macrolide of formula XIII



23 DEC 1998



wherein M is a macolide ring as defined in claim 1 or 2, in particular a macolide of formula XIV



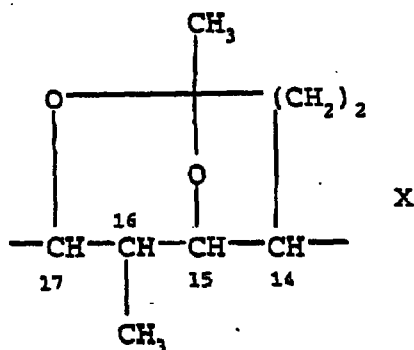
in free or protected form, or salt thereof.

21. A process for the production of any compound of the invention as hereinbefore defined, which process comprises:
  - i). for the production of any one of Sanglifehrins A, B, C or D, cultivating a Sanglifehrin A, B, C or D producing actinomycete strain in a culture medium and isolating the desired Sanglifehrin A, B, C or D from the obtained culture broth;
  - ii). for the production of Sanglifehrins C and D subjecting Sanglifehrins A and B to cyclisation at positions 15 and 16;
  - iii). for the production of Sanglifehrins A and B and subjecting Sanglifehrins C and D to ring opening of the lactol ring at positions 15 and 16;
  - iv). for the production of a macolide of formula IX or IX', wherein -g-h- is -C(CH<sub>3</sub>)=CH-, dehydrating a compound of formula IX or IX' wherein -g-h- is -CH(CH<sub>3</sub>)-CH(OH)- or a protected form thereof;



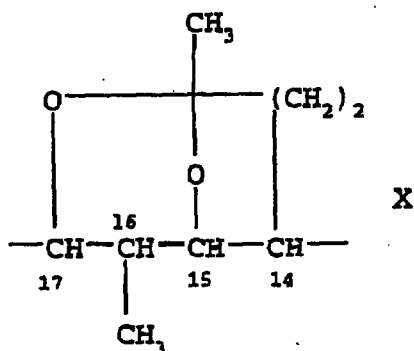
v). for the production of a macrolide of formula IX or IX' wherein  $R_4$  is -  
CH(OH)-CH<sub>3</sub>, hydrogenating a compound of formula IX or IX' wherein  $R_4$  is -  
C(O)-CH<sub>3</sub>;

vi). for the production of a macrolide of formula IX or IX' wherein the 14 to  
16 positions of the macrolide ring comprise a residue of formula X



causing a compound of formula IX or IX' to undergo internal protection;

vii). for the production of a macrolide of formula IX or IX', causing a  
compound of formula IX or IX' wherein the 14 to 16 positions of the  
macrolide ring comprise a residue of formula X



to undergo reversal of internal protection.

viii). for the production of a macrolide of formula IX or IX', in which  $R_5$  is  
methyl, subjecting a macrolide of formula IX or IX', in which  $R_5$  is H to  
methylation;



ix). for the production of a macrolide of formula IX or IX', in which  $R_4$  is in O-protected form, subjecting a macrolide of formula IX or IX', in which  $R_5$  is in O-unprotected form to O-protection;

x). for the production of a macrolide of formula IX or IX', in which  $R_4$  is in O-unprotected form, subjecting a macrolide of formula IX or IX', in which  $R_5$  is in O-protected form to O-deprotection;

xi). for the production of a macrolide of formula IX or IX', which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX', in which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-protection;

xii). for the production of a macrolide of formula IX or IX', which comprises an O-unprotected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring, subjecting a macrolide of formula IX or IX' which comprises an O-protected hydroxyphenylalanine residue at positions 7 to 10 of the macrocyclic ring to O-deprotection;

xiii). for the production of a macrolide of formula IX or IX', in which -e-f- is -CH(OH)-CH(OH)-, subjecting a macrolide of formula IX or IX' in which -e-f- is -CH=CH- to oxidative hydrolysis;

xiv). for the production of a compound of formula V' or a compound of formula XII, subjecting a compound of formula IX or IX' to cleavage of the linker group between the spiro bicyclo group and the macrocyclic ring.

xv). for the production of a compound of formula  $R_6O - X - Y - Z - A - OH$  or of formula  $R_6O - X - Y - Z - A' - CH(OH) - L - S$ , subjecting a macrocycle of formula IV or the macrocyclic ring of a compound of formula VIII to ring-opening;



23 DEC 1998

- xvi). for the production of a macrolide of formula IX or XII in ring-closed form, subjecting a compound of formula  $R_6O - X - Y - Z - A - OH$  or of formula  $R_6O - X - Y - Z - A' - CH(OH) - L - S$  to closure of the macrocyclic ring.
- xvii). for the production of a compound of formula XII or XII', subjecting a compound of formula IX or VI' to ring-opening within the spiro bicyclic ring system, and
- xix). for the production of a compound of formula IX or VI', subjecting a compound of formula XII or XII' to ring-closure within the spiro bicyclic ring system.
22. A biologically pure isolate of strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof which is capable of producing a macrolide of the invention.
23. A process for the production of a macrolide of the invention, which comprises cultivating strain Streptomyces sp. A92-308110 (DSM 9954) or a mutant, recombinant or modified form thereof in an appropriate culture medium and optionally recovering the sanglifehrin.
24. An agent of the invention for use as a pharmaceutical, e.g. for use as an immunosuppressant or for use
- i) for the prevention of acute and/or chronic organ allo- or xenotransplant rejection; or
  - ii) for the prevention of graft-versus-host disease; or
  - iii) for the treatment of autoimmune diseases; or



- iv) for the treatment of asthma.
25. A pharmaceutical composition comprising an agent of the invention in association with a pharmaceutically acceptable diluent or carrier.
26. Use of an agent of the invention for the preparation of a medicament for use as an immunosuppressant or for use
- i) for the prevention of acute and/or chronic organ allo- or xenotransplant rejection; or
  - ii) for the prevention of graft-versus-host disease; or
  - iii) for the treatment of autoimmune diseases; or
  - iv) for the treatment of asthma.
27. Use of a compound according to any one of claims 1, 2, 4-6, 11 or 12 as a reagent in a displacement immunoassay for a cyclosporins or other cyclophilin binding compounds.

