



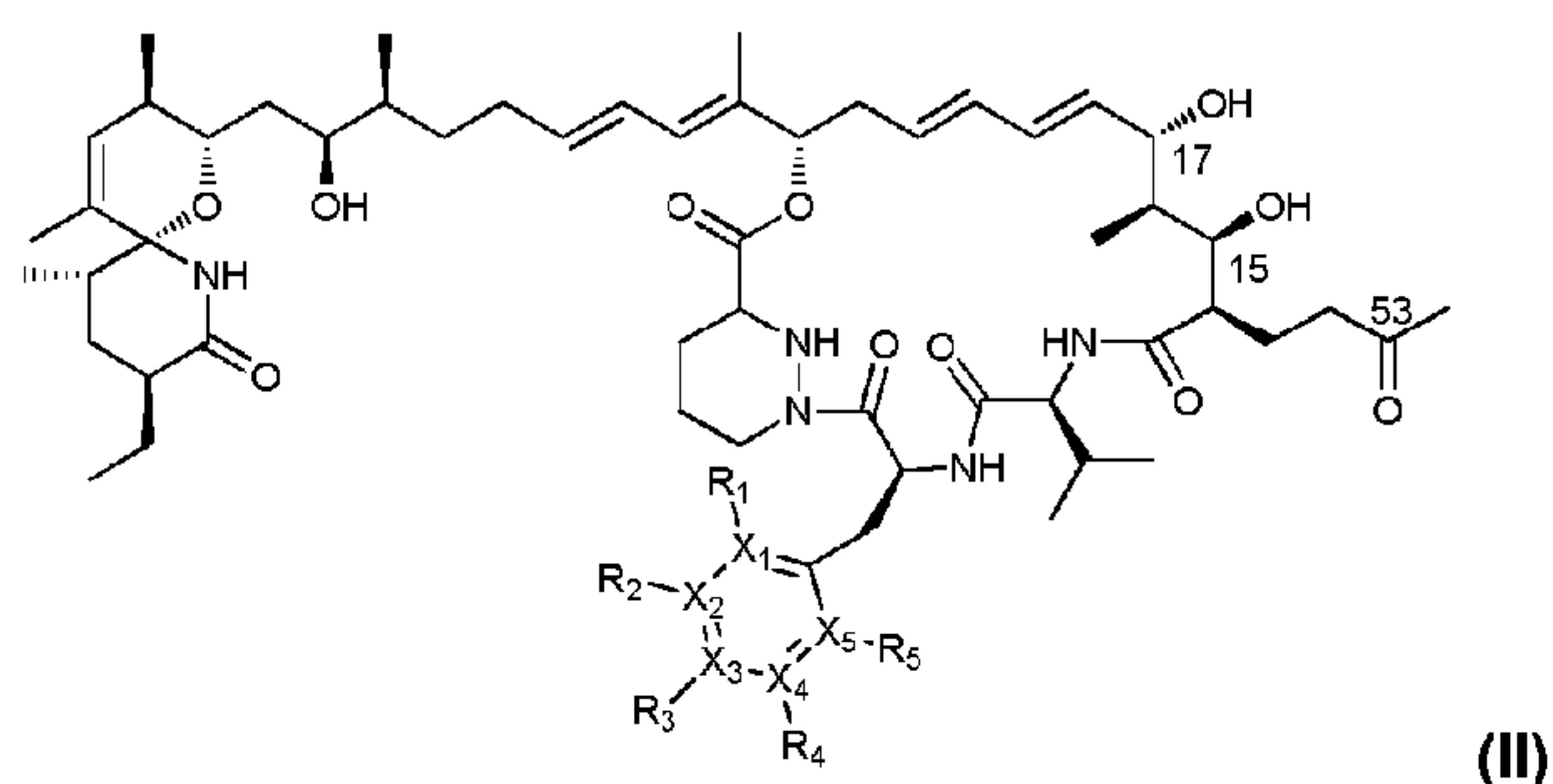
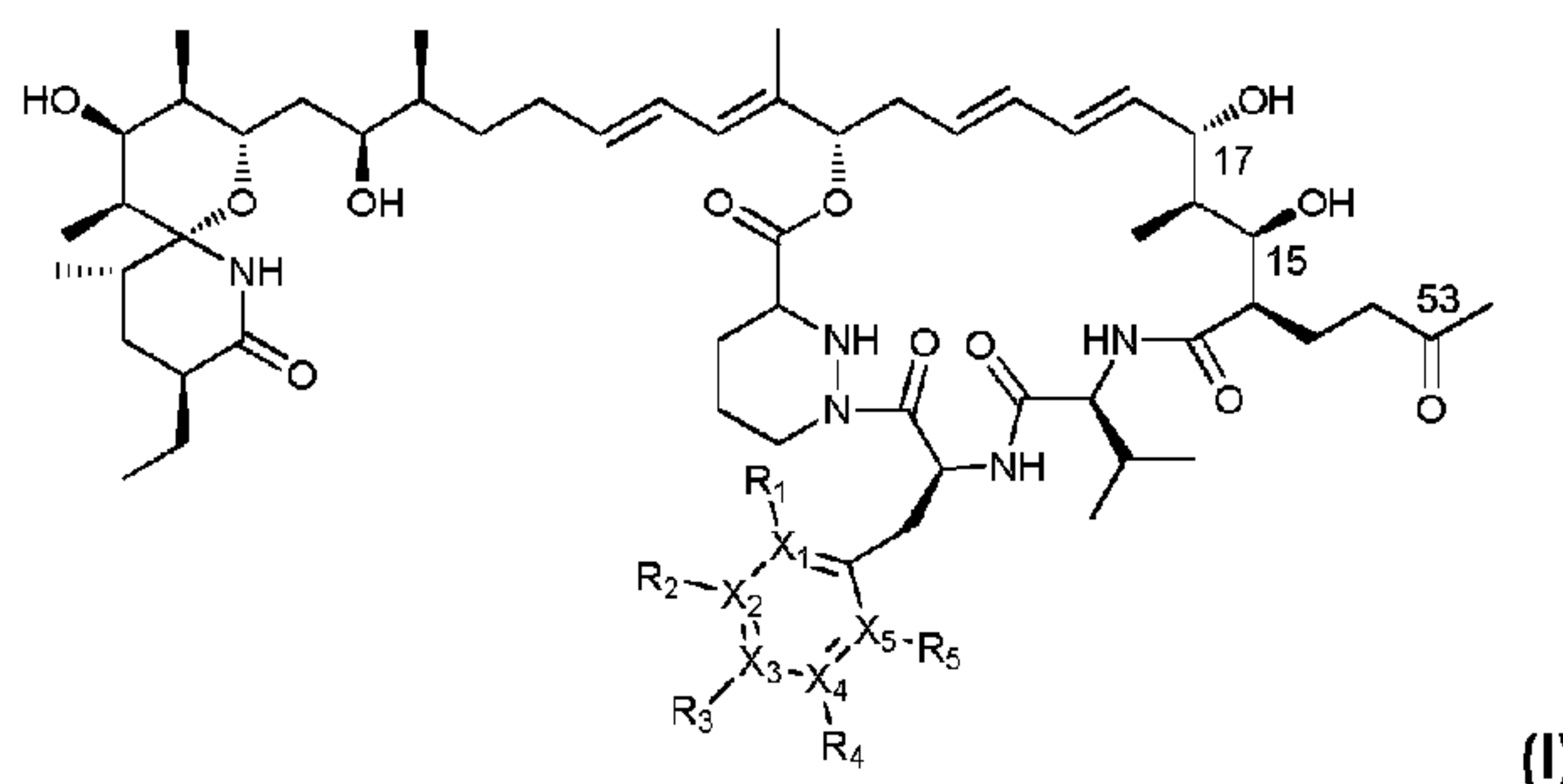
(86) Date de dépôt PCT/PCT Filing Date: 2011/12/20
(87) Date publication PCT/PCT Publication Date: 2012/06/28
(45) Date de délivrance/Issue Date: 2019/11/05
(85) Entrée phase nationale/National Entry: 2013/06/19
(86) N° demande PCT/PCT Application No.: GB 2011/052524
(87) N° publication PCT/PCT Publication No.: 2012/085553
(30) Priorités/Priorities: 2010/12/20 (GB1021522.6);
2011/08/08 (GB1113626.4)

(51) Cl.Int./Int.Cl. *A61K 31/5025* (2006.01),
A61K 38/05 (2006.01), *A61P 29/00* (2006.01),
A61P 31/12 (2006.01), *A61P 31/14* (2006.01),
A61P 31/18 (2006.01), *C07K 5/062* (2006.01),
C12N 1/20 (2006.01), *C12P 17/18* (2006.01),
C12P 21/02 (2006.01)

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(54) Titre : DERIVES DE SANGLIFEHRINE ET PROCEDES POUR LEUR PRODUCTION
(54) Title: SANGLIFEHRIN DERIVATIVES AND METHODS FOR THEIR PRODUCTION



(57) Abrégé/Abstract:

There are provided inter alia compounds of formula (I) and (II) and their use in therapy, particularly for the treatment of viral infection.

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

(19) World Intellectual Property
Organization
International Bureau

(10) International Publication Number

WO 2012/085553 A1(43) International Publication Date
28 June 2012 (28.06.2012)

(51) International Patent Classification:

A61K 31/5025 (2006.01) *A61P 31/12* (2006.01)

(21) International Application Number:

PCT/GB2011/052524

(22) International Filing Date:

20 December 2011 (20.12.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1021522.6 20 December 2010 (20.12.2010) GB

1113626.4 8 August 2011 (08.08.2011) GB

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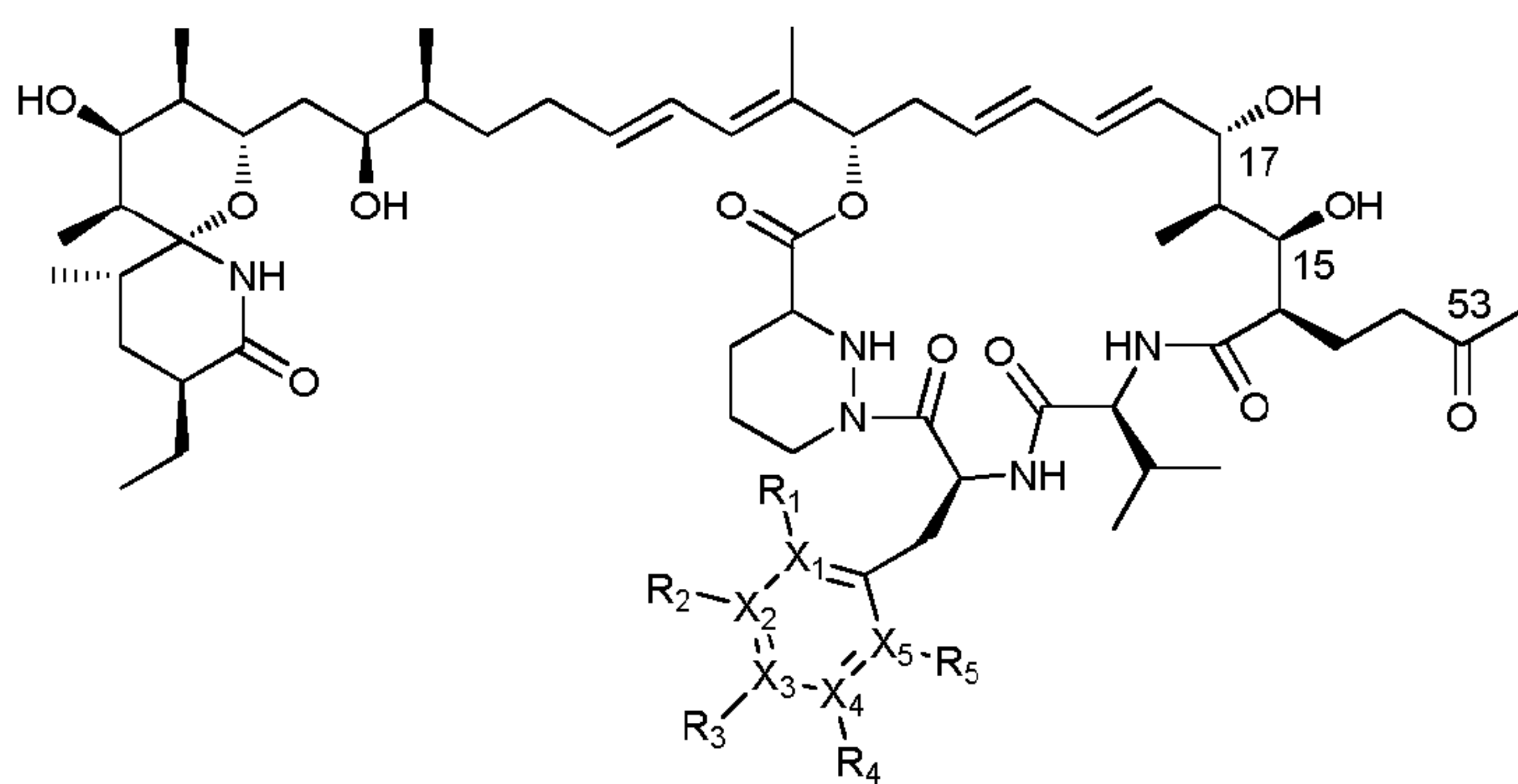
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,

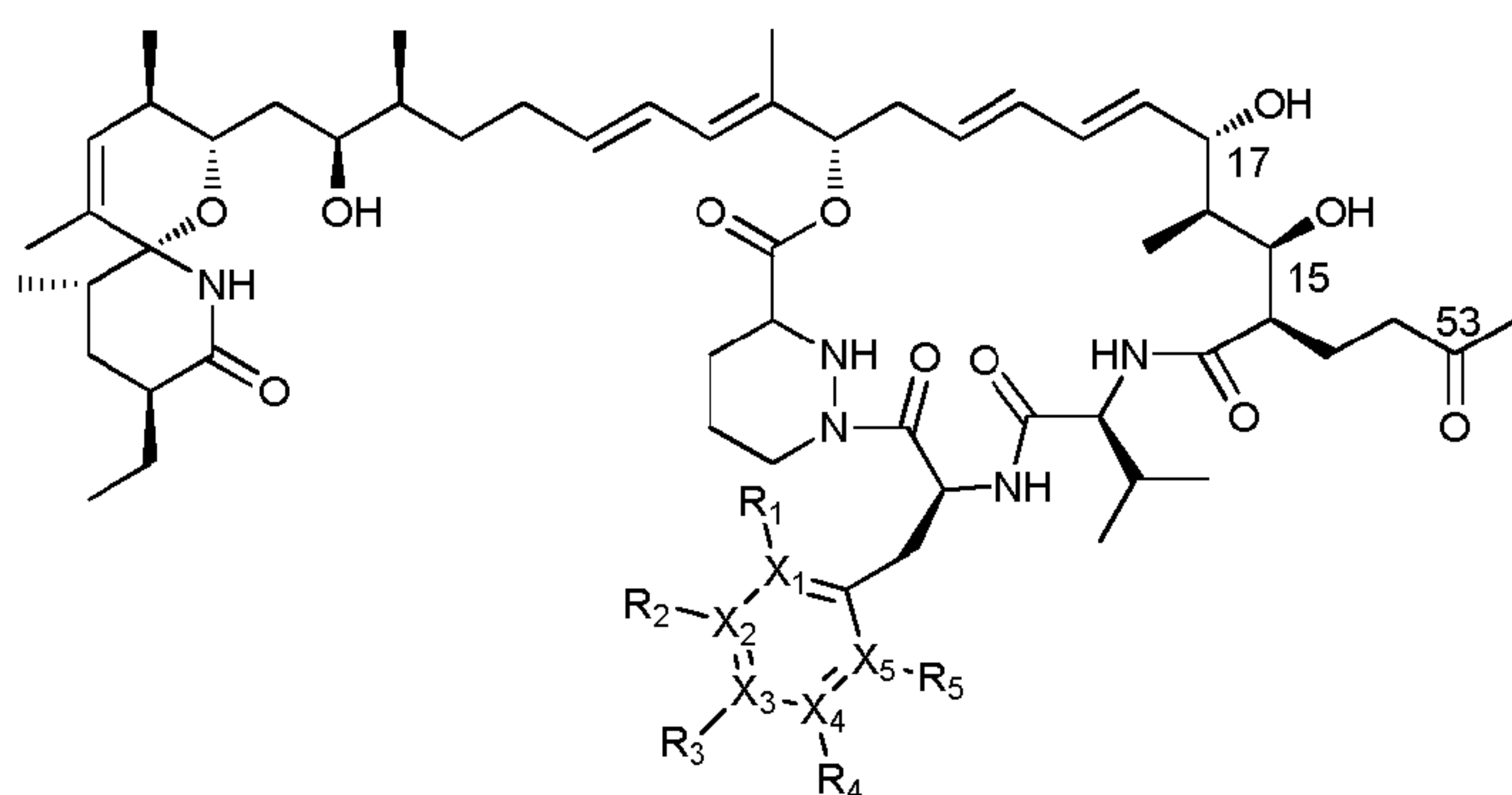
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(54) Title: SANGLIFEHRIN DERIVATIVES AND METHODS FOR THEIR PRODUCTION



(I)

(57) Abstract: There are provided inter alia compounds of formula (I) and (II) and their use in therapy, particularly for the treatment of viral infection.



(II)

WO 2012/085553 A1



MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT,
LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS,
SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(84) **Designated States** *(unless otherwise indicated, for every
kind of regional protection available):* ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD,
RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ,

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

SANGLIFEHRIN DERIVATIVES AND METHODS FOR THEIR PRODUCTION

Introduction

The present invention relates to sanglifehrin analogues, that are useful as cyclophilin inhibitors, e.g. in the treatment of viral infection, especially infection by RNA viruses such as Hepatitis C virus (HCV) and HIV and/or as immunosuppressants e.g. for use in prophylaxis of transplant rejection and as anti-inflammatory agents, e.g. for use in inflammatory disorders. The present invention also provides methods for their use in medicine, in particular for the treatment of HCV infection and for use as an immunosuppressant or anti-inflammatory agent, or as intermediates in the generation of further medicinally useful compounds.

Background of the invention**Hepatitis C**

Hepatitis C virus (HCV) is a positive strand RNA virus, and infection is a leading cause of post-transfusional hepatitis. HCV is the most common chronic blood borne infection, and the leading cause of death from liver disease in United States. The World Health Organization estimates that there are more than 170 million chronic carriers of HCV infection, which is about 3% of the world population. Among the un-treated HCV-infected patients, about 70%–85% develop chronic HCV infection, and are therefore at high risk to develop liver cirrhosis and hepatocellular carcinoma. In developed countries, 50-76% of all cases of liver cancer and two-thirds of all liver transplants are due to chronic HCV infection (Manns et al, 2007).

In addition to liver diseases, chronically infected patients may also develop other chronic HCV-related diseases, and serve as a source of transmission to others. HCV infection causes non-liver complications such as arthralgias (joint pain), skin rash, and internal organ damage predominantly to the kidney. HCV infection represents an important global health-care burden, and currently there is no vaccine available for hepatitis C (Strader et al., 2004; Jacobson et al. 2007; Manns et al., 2007 Pawlotsky, 2005; Zeuzem & Hermann, 2002).

Treatment of HCV

The current standard of care (SoC) is subcutaneous injections of pegylated interferon- α (pIFN α) and oral dosing of the antiviral drug ribavirin for a period of 24-48 weeks. Success in treatment is defined by *sustained virologic response* (SVR), which is defined by absence of HCV RNA in serum at the end of treatment period and 6 months later. Overall response rates to SoC depend mainly on genotype and pretreatment HCV RNA levels. Patients with genotype 2 and 3 are more likely to respond to SoC than patients infected with genotype 1 (Melnikova, 2008; Jacobson et al., 2007).

A significant number of HCV patients do not respond adequately to the SoC treatment, or cannot tolerate the therapy due to side effects, leading to frequent issues with completion of the full course. The overall clinical SVR rate of SoC is only around 50% (Melnikova, 2008).

Development of resistance is another underlying factor for failure of treatment (Jacobson et al.

5 et al. 2007). SoC is also contraindicated in some patients who are not considered candidates for treatment, such as patients with past significant episodes of depression or cardiac disease. Side effects of the SoC, which frequently lead to discontinuation of treatment include a flu-like illness, fever, fatigue, haematological disease, anaemia, leucopaenia, thrombocytopaenia, alopecia and depression (Manns et al., 2007).

10 Considering the side effects associated with the lengthy treatments using SoC, development of resistance, and suboptimum overall rate of success, more efficacious and safer new treatments are urgently needed for treatment of HCV infection. The objectives of new treatments include improved potency, improved toxicity profile, improved resistance profile, improved quality of life and the resulting improvement in patient compliance. HCV has a short life cycle and therefore
15 development of drug resistance during drug therapy is common.

Novel, specifically targeted antiviral therapy for hepatitis C (STAT-C), also known as direct acting antiviral (DAA) drugs are being developed that target viral proteins such as viral RNA polymerase NS5B or viral protease NS3 (Jacobson et al, 2007; Parfieniuk et al., 2007). In addition, novel compounds also are being developed that target human proteins (e.g.

20 cyclophilins) rather than viral targets, which might be expected to lead to a reduction in incidence of resistance during drug therapy (Manns et al., 2007; Pockros, 2008; Pawlotsky J-M, 2005).

Cyclophilin inhibitors

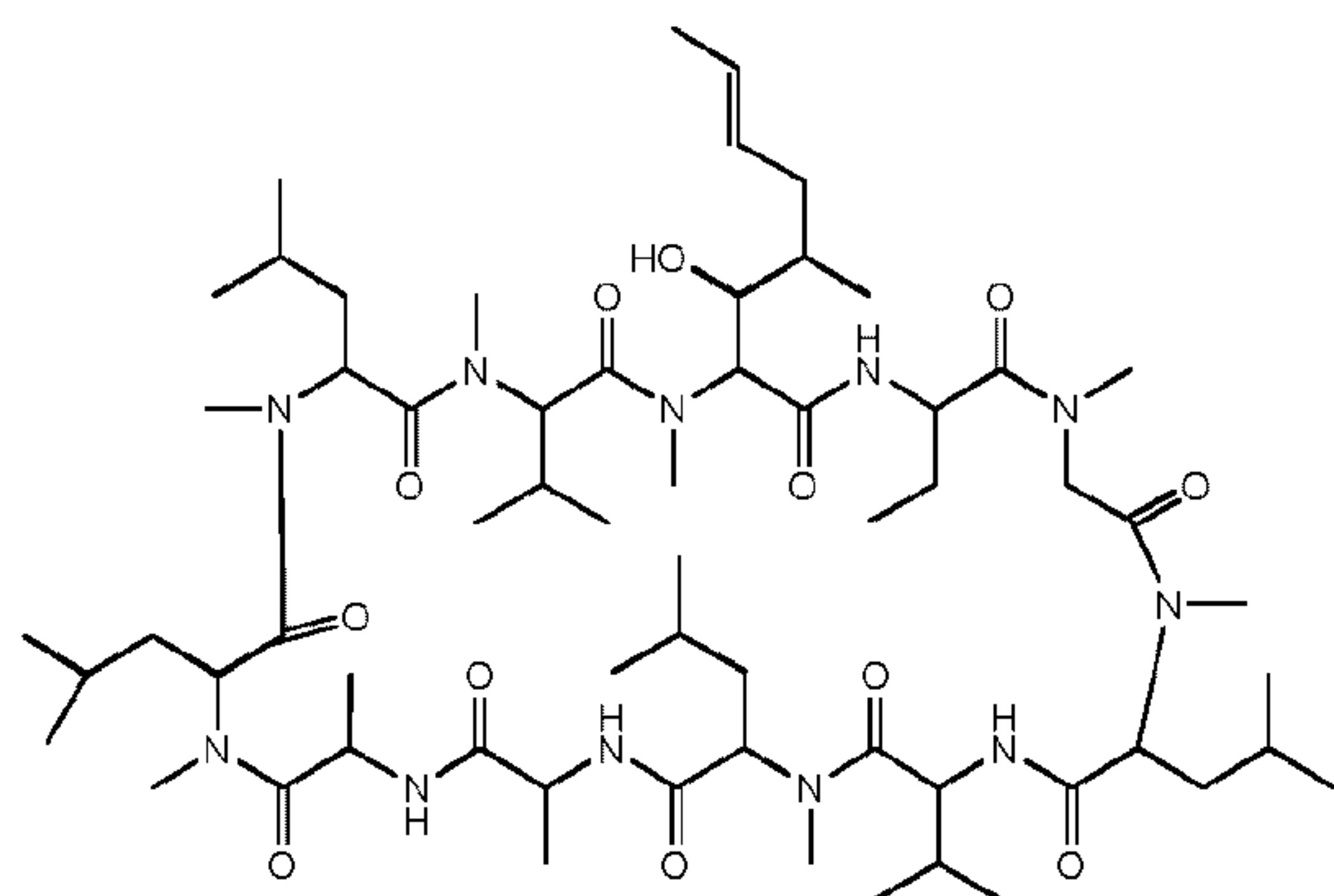
Cyclophilins (CyP) are a family of cellular proteins that display peptidyl-prolyl *cis-trans*

25 isomerase activity facilitating protein conformation changes and folding. CyPs are involved in cellular processes such as transcriptional regulation, immune response, protein secretion, and mitochondrial function. HCV virus recruits CyPs for its life cycle during human infection.

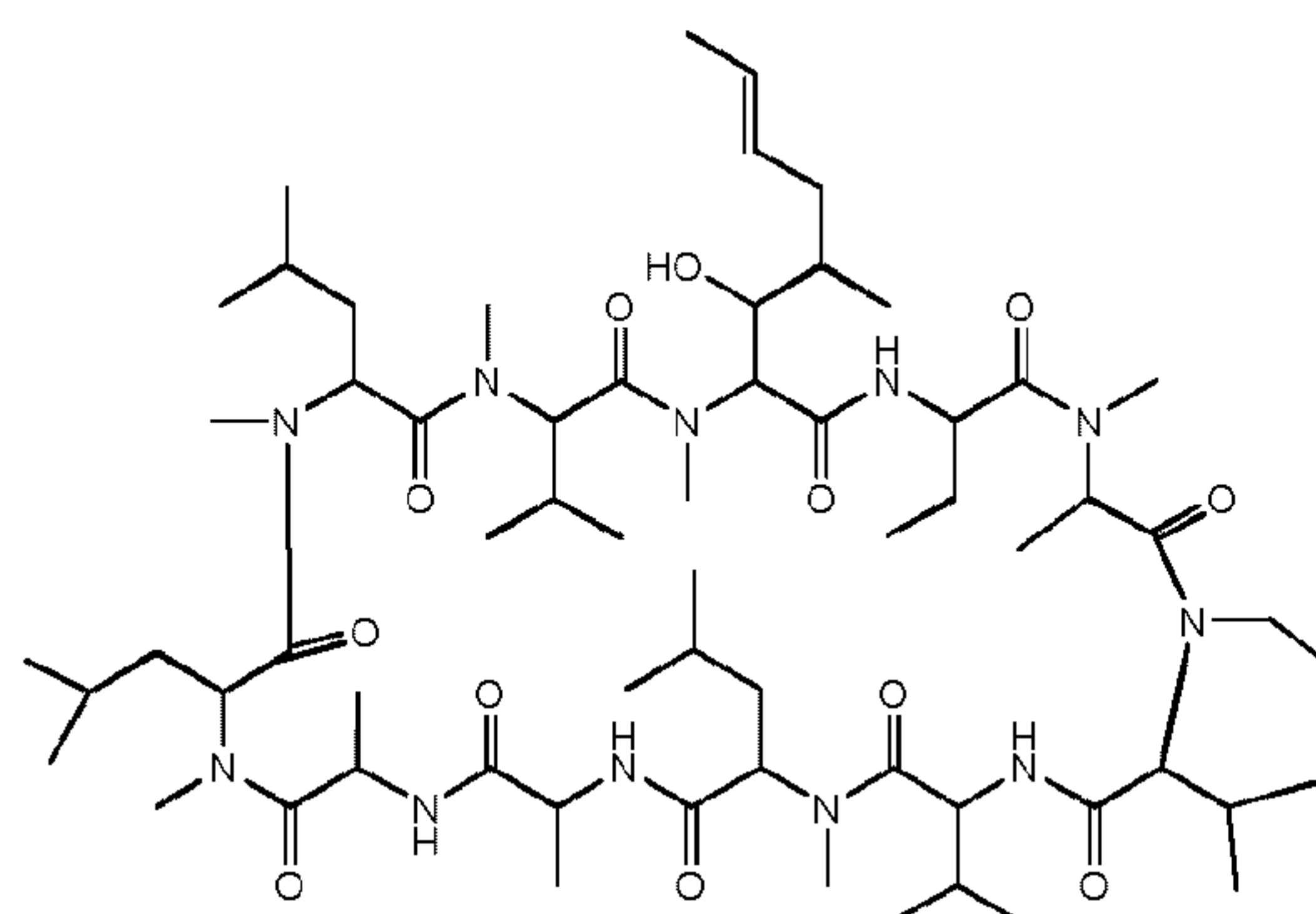
Originally, it was thought that CyPs stimulate the RNA binding activity of the HCV non-structural protein NS5B RNA polymerase that promotes RNA replication, although several alternative

30 hypotheses have been proposed including a requirement for CyP PPlase activity. Various isoforms of CyPs, including A and B, are believed to be involved in the HCV life cycle (Yang et al., 2008; Appel et al., 2006; Chatterji et al., 2009; Gaither et al., 2010). The ability to generate knockouts in mice (Colgan et al., 2000) and human T cells (Braaten and Luban, 2001) indicates that CyPA is optional for cell growth and survival. Similar results have been observed with
35 disruption of CyPA homologues in bacteria (Herrler et al., 1994), *Neurospora* (Tropschug et al.,

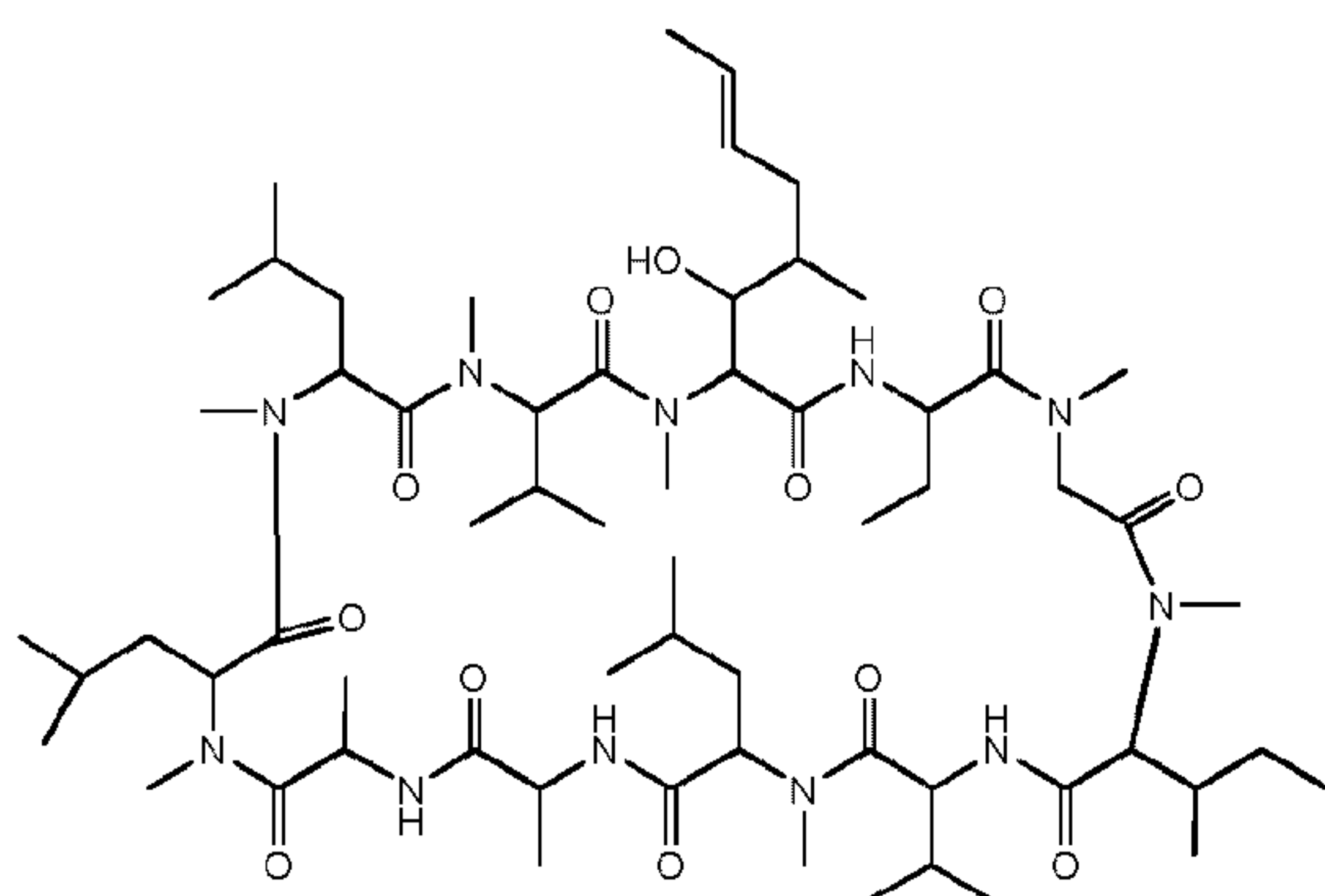
1989) and *Saccharomyces cerevisiae* (Dolinski et al. 1997). Therefore, inhibiting CyPs represents a novel and attractive host target for treating HCV infection, and a new potential addition to current SoC or STAT-C/DAA drugs, with the aim of increasing SVR, preventing emergence of resistance and lowering treatment side effects.



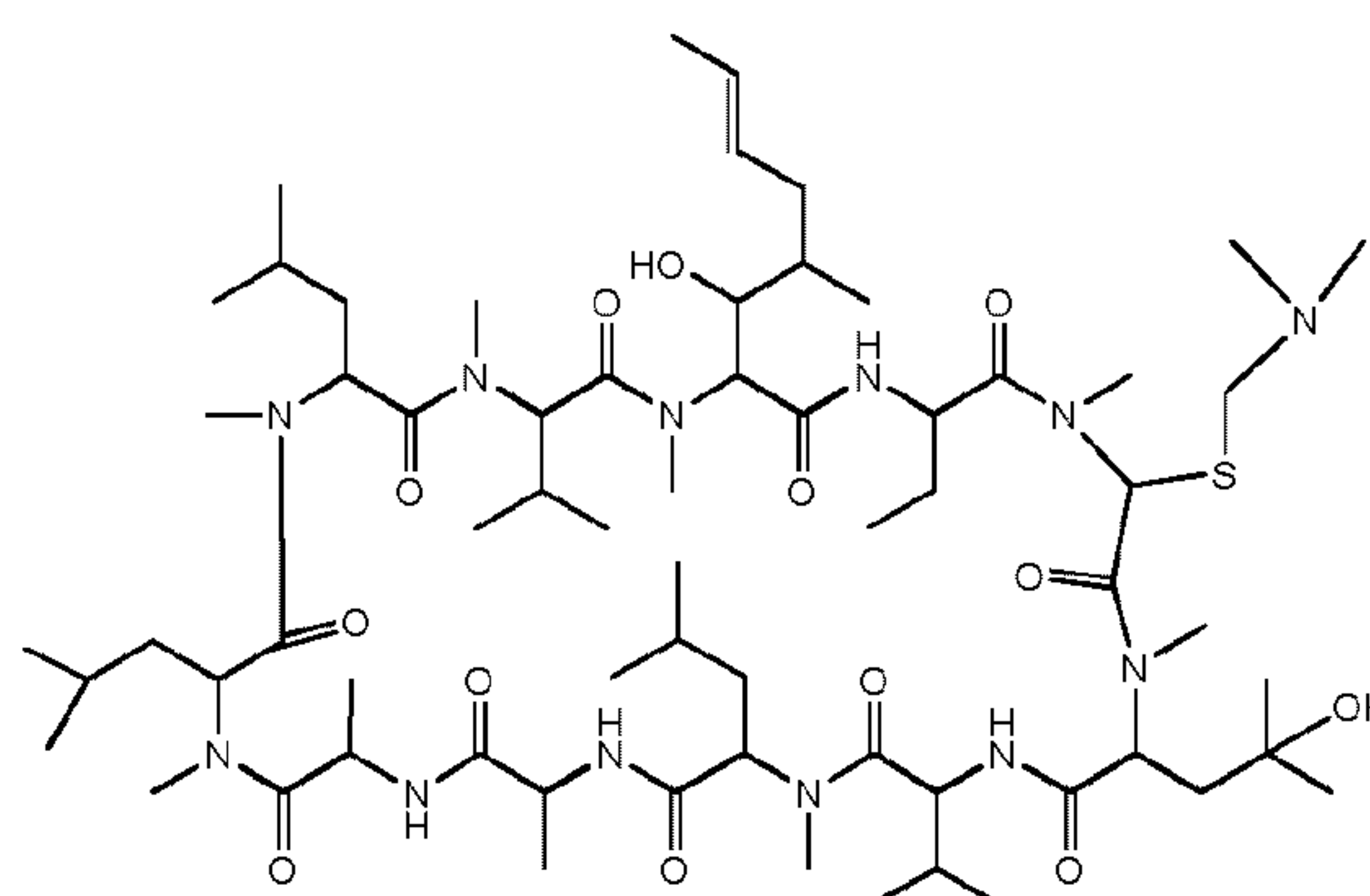
Cyclosporine A, 1



DEBIO-025, 2



NIM-811, 3



SCY-635, 4

5

Cyclosporine A (Inoue et al. 2003) ("CsA") and its closely structurally related non-immunosuppressive clinical analogues DEBIO-025 (Paeshuyse et al. 2006; Flisiak et al. 2008), NIM811 (Mathy et al. 2008) and SCY-635 (Hopkins et al., 2009) are known to bind to cyclophilins, and as cyclophilin inhibitors have shown *in vitro* and clinical efficacy in the treatment of HCV infection (Crabbe et al., 2009; Flisiak et al. 2008; Mathy et al. 2008; Inoue et al., 2007; Ishii et al., 2006; Paeshuyse et al., 2006). Although earlier resistance studies on CsA showed mutations in HCV NS5B RNA polymerase and suggested that only cyclophilin B would be involved in the HCV replication process (Robida et al., 2007), recent studies have suggested an essential role for cyclophilin A in HCV replication (Chatterji et al. 2009; Yang et al., 2008). Considering that mutations in NS5A viral protein are also associated with CsA resistance and that NS5A interacts with both CyPA and CypB for their specific peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, a role for both cyclophilins in viral life cycle is further suggested (Hanouille et al., 2009).

15

The anti-HCV effect of cyclosporine analogues is independent of the immunosuppressive property, which is dependent on calcineurin. This indicated that the essential requirement for HCV activity is CyP binding and calcineurin binding is not needed. DEBIO-025, the most clinically advanced cyclophilin inhibitor for the treatment of HCV, has shown *in vitro* and *in vivo* potency against the four most prevalent HCV genotypes (genotypes 1, 2, 3, and 4). Resistance studies showed that mutations conferring resistance to DEBIO-025 were different from those reported for polymerase and protease inhibitors, and that there was no cross resistance with STAT-C/DAA resistant viral replicons. More importantly, DEBIO-025 also prevented the development of escape mutations that confer resistance to both protease and polymerase inhibitors (Crabbe et al., 2009).

However, the CsA-based cyclophilin inhibitors in clinical development have a number of issues, which are thought to be related to their shared structural class, including: certain adverse events that can lead to a withdrawal of therapy and have limited the clinical dose levels; variable pharmacokinetics that can lead to variable efficacy; and an increased risk of drug-drug interactions that can lead to dosing issues.

The most frequently occurring adverse events (AEs) in patients who received DEBIO-025 included jaundice, abdominal pain, vomiting, fatigue, and pyrexia. The most clinically important AEs were hyperbilirubinemia and reduction in platelet count (thrombocytopaenia). Peg-IFN can cause profound thrombocytopaenia and combination with DEBIO-025 could represent a significant clinical problem. Both an increase in bilirubin and decrease in platelets have also been described in early clinical studies with NIM-811 (Ke et al., 2009). Although the hyperbilirubinemia observed during DEBIO-025 clinical studies was reversed after treatment cessation, it was the cause for discontinuation of treatment in 4 out of 16 patients, and a reduction in dose levels for future trials. As the anti-viral effect of cyclophilin inhibitors in HCV is dose related, a reduction in dose has led to a reduction in anti-viral effect, and a number of later trials with CsA-based cyclophilin inhibitors have shown no or poor reductions in HCV viral load when dosed as a monotherapy (Lawitz et al., 2009; Hopkins et al., 2009; Nelson et al., 2009). DEBIO-025 and cyclosporine A are known to be inhibitors of biliary transporters such as bile salt export pumps and other hepatic transporters (especially MRP2/cMOAT/ABCC2) (Crabbe et al., 2009). It has been suggested that the interaction with biliary transporters, in particular MRP2, may be the cause of the hyperbilirubinaemia seen at high dose levels of DEBIO-025 (Nelson et al., 2009, Wring et al., 2010). CsA class-related drug-drug interactions (DDIs) via inhibition of other drug transporters such as OAT1B1 and OAT1B3 (Konig et al., 2010) may also be a concern, potentially limiting certain combinations and use in some patients undergoing treatment for co-infections such as HIV (Seden et al., 2010).

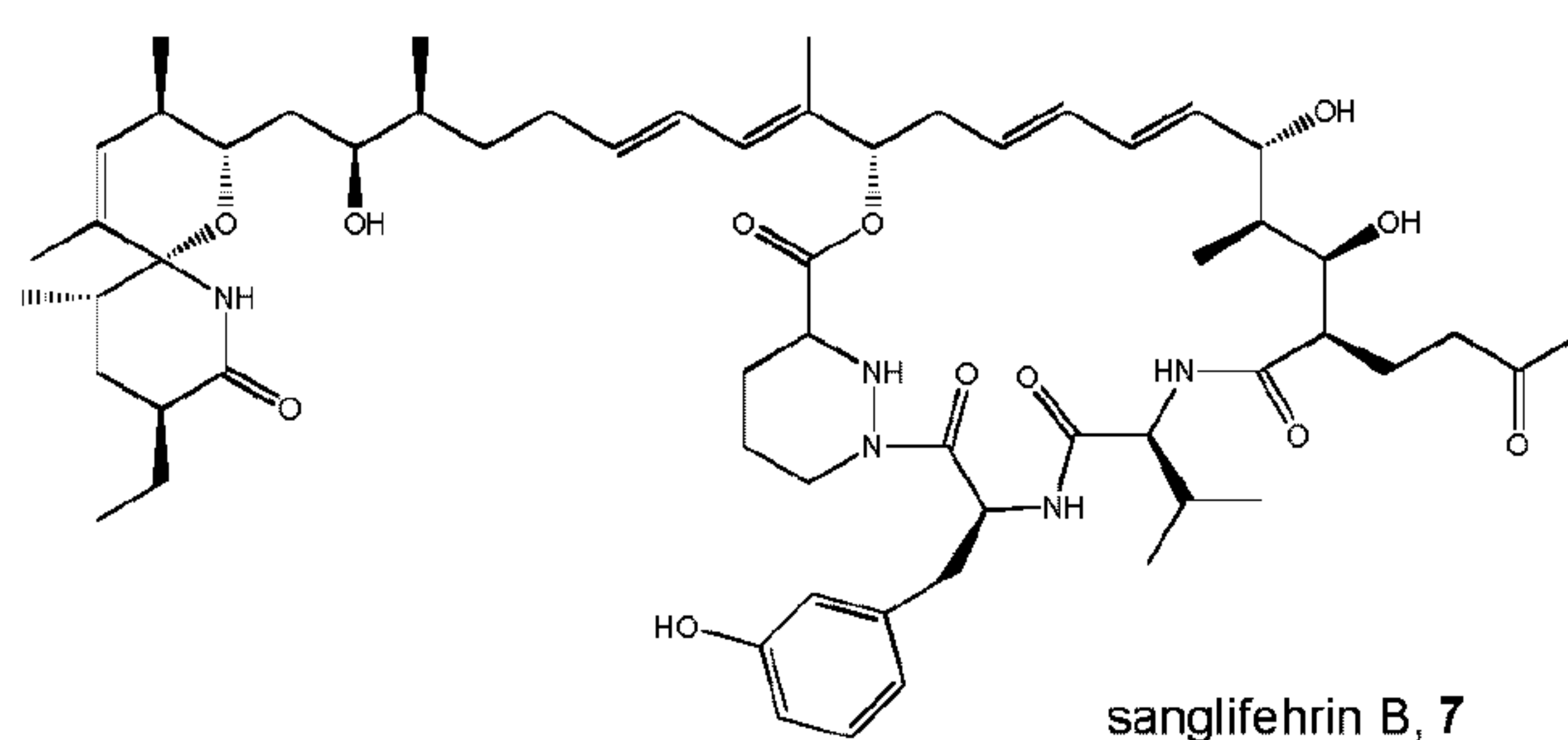
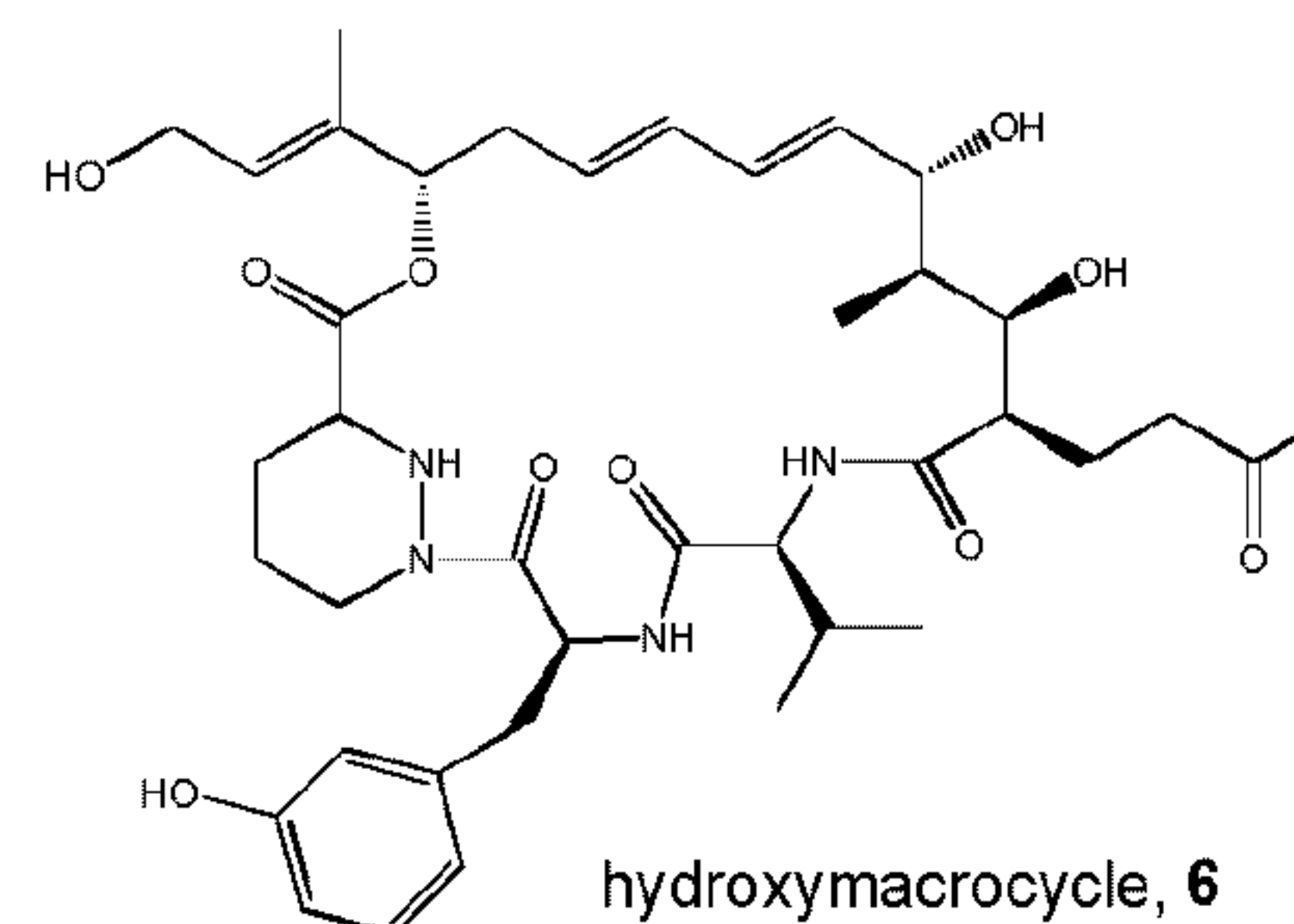
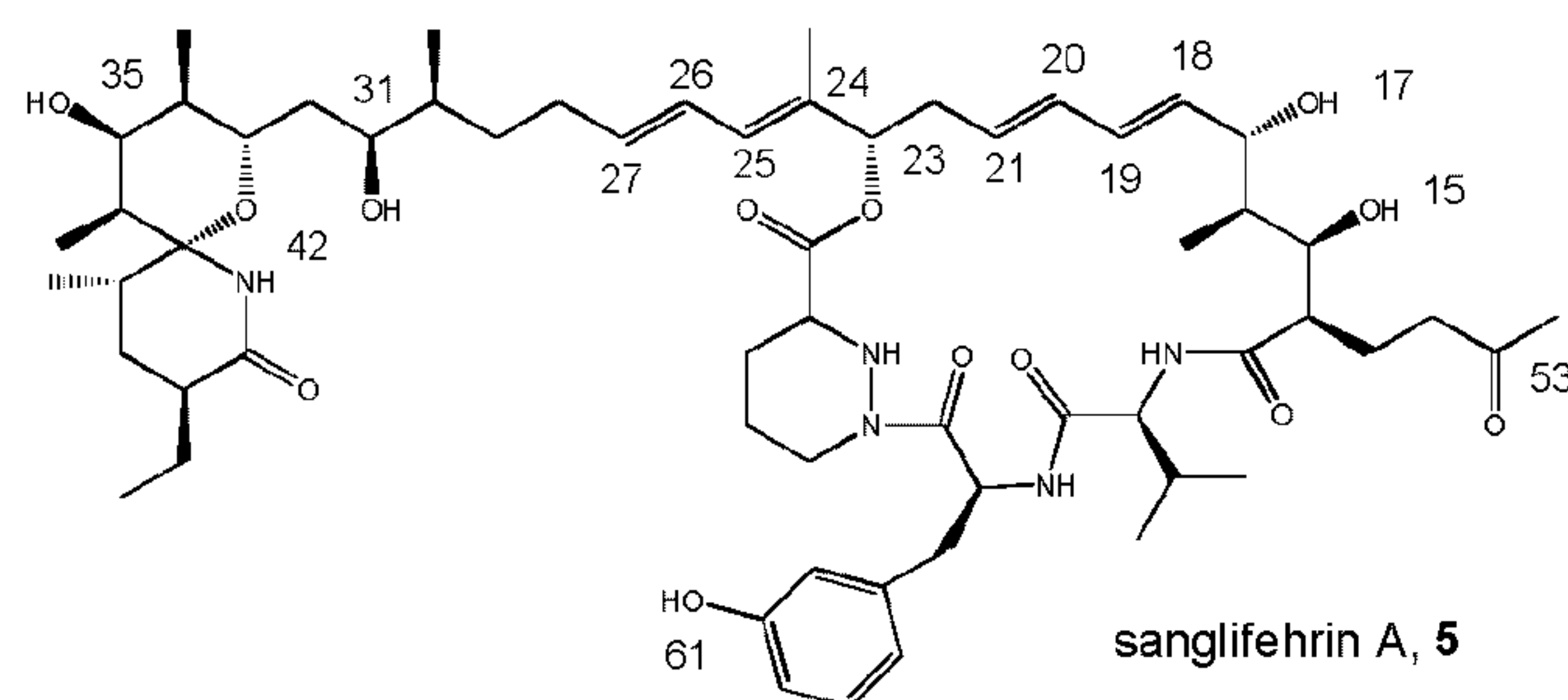
Moreover, DEBIO-025 and cyclosporine A are substrates for metabolism by cytochrome P450 (especially CYP3A4), and are known to be substrates and inhibitors of human P-glycoprotein (MDR1) (Crabbe et al., 2009). Cyclosporine A has also been shown to be an inhibitor of CYP3A4 *in vitro* (Niwa et al., 2007). This indicates that there could be an increased risk of drug-
5 drug interactions with other drugs that are CYP3A4 substrates, inducers or inhibitors such as for example ketoconazole, cimetidine and rifampicin. In addition, interactions are also expected with drugs that are subject to transport by P-glycoprotein (e.g. digoxin), which could cause severe drug-drug interactions in HCV patients receiving medical treatments for other concomitant diseases (Crabbe et al. 2009). CsA is also known to have highly variable
10 pharmacokinetics, with early formulations showing oral bioavailability from 1-89% (Kapurtzak et al., 2004). Without expensive monitoring of patient blood levels, this can lead to increased prevalence of side effects due to increased plasma levels, or reduced clinical response due to lowered plasma levels.

Considering that inhibition of cyclophilins represent a promising new approach for treatment of
15 HCV, there is a need for discovery and development of more potent and safer CyP inhibitors for use in combination therapy against HCV infection.

Sanglifehrins

Sanglifehrin A (SfA) and its natural congeners belong to a class of mixed non-ribosomal peptide/polyketides, produced by *Streptomyces* sp. A92-308110 (also known as DSM 9954)
20 (see WO 97/02285), which were originally discovered on the basis of their high affinity to cyclophilin A (CyPA). SfA is the most abundant component in fermentation broths and exhibits approximately 20-fold higher affinity for CyPA compared to CsA. This has led to the suggestion that sanglifehrins could be useful for the treatment of HCV (WO2006/138507). Sanglifehrins have also been shown to exhibit a lower immunosuppressive activity than CsA when tested *in*
25 *vitro* (Sanglier et al., 1999; Fehr et al., 1999). SfA binds with high affinity to the CsA binding site of CyPA (Kallen et al., 2005).

6



Biosynthesis of sanglifehrins

Sanglifehrins are biosynthesised by a mixed polyketide synthase (PKS)/Non-ribosomal peptide synthetase (NRPS) (see WO2010/034243, Qu et al., 2011). The 22-membered macrolide backbone consists of a polyketide carbon chain and a tripeptide chain. The peptide chain consists of one natural amino acid, valine, and two non-natural amino acids: (S)-meta-tyrosine and (S)-piperazic acid, linked by an amide bond. Hydroxylation of phenylalanine (either *in situ* on the NRPS or prior to biosynthesis) to generate (S)-meta-tyrosine is thought to occur via the gene product of *sfaA*.

Immunosuppressive action of sanglifehrins

The immunosuppressive mechanism of action of SfA is different to that of other known immunophilin-binding immunosuppressive drugs such as CsA, FK506 and rapamycin. SfA does not inhibit the phosphatase activity of calcineurin, the target of CsA (Zenke et al. 2001), instead its immunosuppressive activity has been attributed to the inhibition of interleukin-6 (Hartel et al., 2005), interleukin-12 (Steinschulte et al., 2003) and inhibition of interleukin-2-dependent T cell proliferation (Zhang & Liu, 2001). However, the molecular target and mechanism through which SfA exerts its immunosuppressive effect is hitherto unknown.

The molecular structure of SfA is complex and its interaction with CyPA is thought to be mediated largely by the macrocyclic portion of the molecule. In fact, a macrocyclic compound (hydroxymacrocycle) derived from oxidative cleavage of SfA has shown strong affinity for CyPA (Sedrani et al., 2003). X-ray crystal structure data has shown that the hydroxymacrocycle binds

to the same active site of CyPA as CsA. Analogues based on the macrocycle moiety of SfA have also previously been shown to be devoid of immunosuppressive properties (Sedrani et al., 2003), providing opportunity for design of non-immunosuppressive CyP inhibitors for potential use in HCV therapy.

- 5 Converse to this, there is also an opportunity to develop immunosuppressive agents with low toxicity for use in such areas as prophylaxis of transplant rejection, autoimmune, inflammatory and respiratory disorders, including, but not limited to, Crohn's disease, Behcet syndrome, uveitis, psoriasis, atopic dermatitis, rheumatoid arthritis, nephritic syndrome, aplastic anaemia, biliary cirrhosis, asthma, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and
10 celiac disease. Sanglifehrins have been shown to have a novel mechanism of immunosuppressive activity (Zenke et al., 2001), potentially acting through dendritic cell chemokines (Immecke et al., 2011) and there is therefore an opportunity to develop agents with a mechanism of action different to current clinical agents, such as cyclosporine A, rapamycin and FK506.

15 **Other therapeutic uses of cyclophilin inhibitors**

Human Immunodeficiency Virus (HIV)

- Cyclophilin inhibitors, such as CsA and DEBIO-025 have also shown potential utility in inhibition of HIV replication. The cyclophilin inhibitors are thought to interfere with function of CyPA during progression/completion of HIV reverse transcription (Ptak et al., 2008). However, when tested
20 clinically, DEBIO-025 only reduced HIV-1 RNA levels ≥ 0.5 and >1 log₁₀ copies/mL in nine and two patients respectively, whilst 27 of the treated patients showed no reduction in HIV-1 RNA levels (Steyn et al., 2006). Following this, DEBIO-025 was trialed in HCV/HIV coinfecting patients, and showed better efficacy against HCV, and the HIV clinical trials were discontinued (see Watashi et al., 2010).

25 **Treatment of HIV**

- More than 30 million people are infected by HIV-1 worldwide, with 3 million new cases each year. Treatment options have improved dramatically with the introduction of highly active antiretroviral therapy (HAART) (Schopman et al., 2010). By 2008, nearly 25 antiretroviral drugs had been licensed for treatment of HIV-1, including nine nucleoside reverse transcriptase
30 inhibitors (NRTI), four non-nucleoside reverse transcriptase inhibitors (NNRTI), nine protease inhibitors (PI), one fusion inhibitor, one CCR5 inhibitor and one integrase inhibitor (Shafer and Schapiro, 2008). However, none of these current regimens leads to complete viral clearance, they can lead to severe side effects and antiviral resistance is still a major concern. Therefore, there still remains a need for new antiviral therapies, especially in mechanism of action classes
35 where there are no approved drugs, such as is the case for cyclophilin inhibitors.

Hepatitis B Virus

Hepatitis B is a DNA virus of the family hepadnaviridae, and is the causative agent of Hepatitis B. As opposed to the cases with HCV and HIV, there have been very few published accounts of activity of cyclophilin inhibitors against Hepatitis B virus. Ptak et al. 2008 have described weak
5 activity of Debio-025 against HBV (IC₅₀ of 4.1 μM), whilst Xie et al., 2007 described some activity of CsA against HBV (IC₅₀ >1.3 μg/mL). This is in contrast to HIV and HCV, where there are numerous reports of nanomolar antiviral activity of cyclophilin inhibitors.

Treatment of HBV

HBV infects up to 400 million people worldwide and is a major cause of chronic viral hepatitis
10 and hepatocellular carcinoma. As of 2008, there were six drugs licensed for the treatment of HBV; interferon alpha and pegylated interferon alpha, three nucleoside analogues (lamivudine, entecavir and telbivudine) and one nucleotide analogue (adefovir dipivoxil). However, due to high rates of resistance, poor tolerability and possible side effects, new therapeutic options are needed (Ferir et al., 2008).

15 Inhibition of the Mitochondrial Permeability Transition Pore (mPTP)

Opening of the high conductance permeability transition pores in mitochondria initiates onset of the mitochondrial permeability transition (MPT). This is a causative event, leading to necrosis and apoptosis in hepatocytes after oxidative stress, Ca²⁺ toxicity, and ischaemia/reperfusion. Inhibition of Cyclophilin D (also known as Cyclophilin F) by cyclophilin inhibitors has been
20 shown to block opening of permeability transition pores and protects cell death after these stresses. Cyclophilin D inhibitors may therefore be useful in indications where the mPTP opening has been implicated, such as muscular dystrophy, in particular Ullrich congenital muscular dystrophy and Bethlem myopathy (Millay et al., 2008, WO2008/084368, Palma et al., 2009), multiple sclerosis (Forte et al., 2009), diabetes (Fujimoto et al., 2010), amyotrophic
25 lateral sclerosis (Martin 2009), bipolar disorder (Kubota et al., 2010), Alzheimer's disease (Du and Yan, 2010), Huntington's disease (Perry et al., 2010), recovery after myocardial infarction (Gomez et al., 2007) and chronic alcohol consumption (King et al., 2010).

Further therapeutic uses

Cyclophilin inhibitors have potential activity against and therefore in the treatment of infections
30 of other viruses, such as Varicella-zoster virus (Ptak et al., 2008), Influenza A virus (Liu et al., 2009), Severe acute respiratory syndrome coronavirus and other human and feline coronaviruses (Chen et al., 2005, Ptak et al., 2008), Dengue virus (Kaul et al., 2009), Yellow fever virus (Qing et al., 2009), West Nile virus (Qing et al., 2009), Western equine encephalitis virus (Qing et al., 2009), Cytomegalovirus (Kawasaki et al., 2007) and Vaccinia virus (Castro et
35 al., 2003).

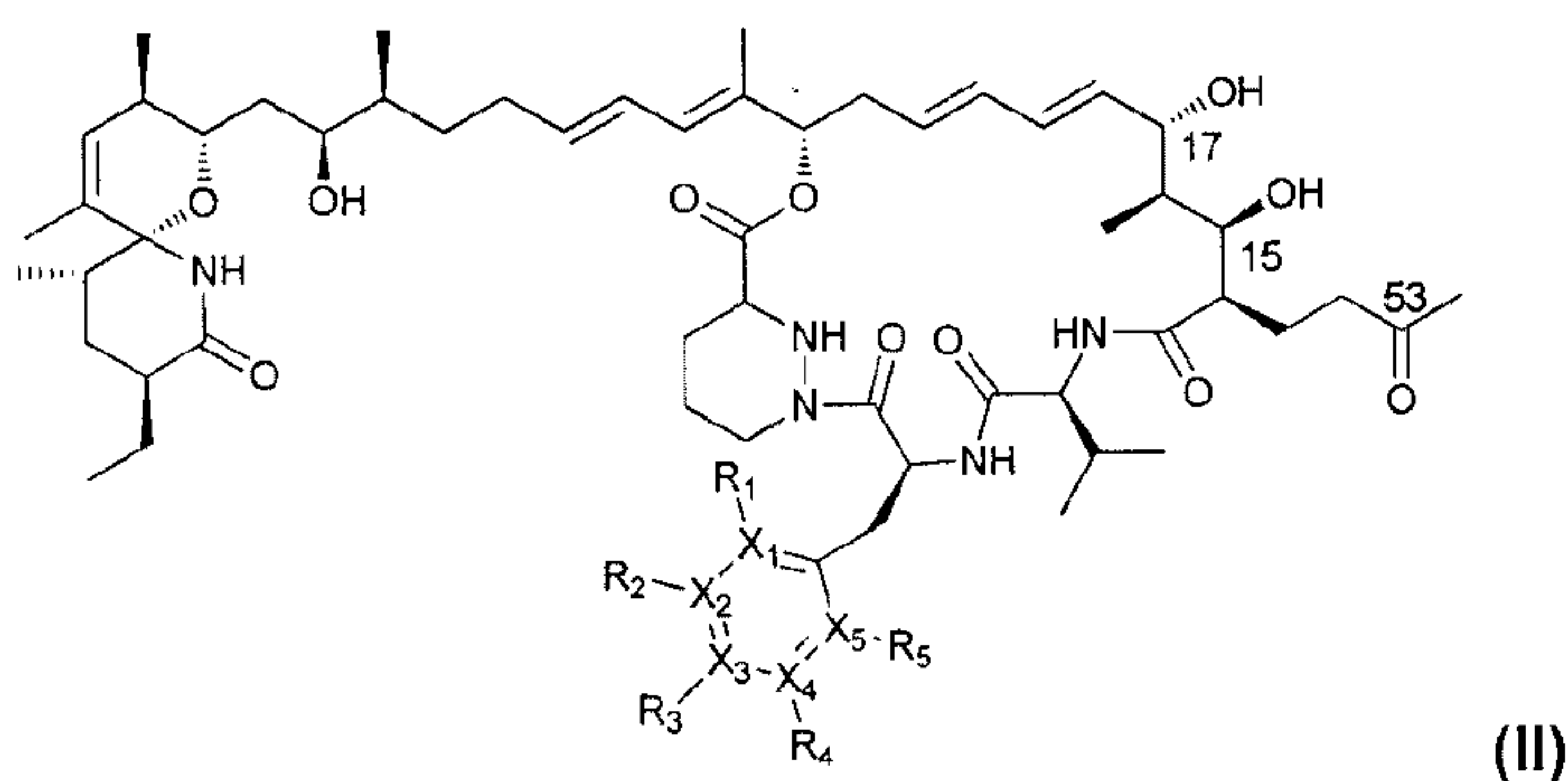
There are also reports of utility of cyclophilin inhibitors and cyclophilin inhibition in other therapeutic areas, such as in cancer (Han et al., 2009).

General comments on sanglifehrins

One of the issues in drug development of compounds such as sanglifehrins is rapid metabolism
5 and glucuronidation, leading to low oral bioavailability. This can lead to an increased chance of food effect, more frequent incomplete release from the dosage form and higher interpatient variability.

Therefore there remains a need to identify novel cyclophilin inhibitors and anti-inflammatory
agents, which may have utility, particularly in the treatment of HCV infection and anti-
10 inflammatory conditions, but also in the treatment of other disease areas where inhibition of cyclophilins may be useful, such as HIV infection, Muscular Dystrophy or aiding recovery after myocardial infarction or where immunosuppression is useful. Preferably, such cyclophilin inhibitors have improved properties over the currently available cyclophilin inhibitors, including one or more of the following properties: longer half-life or increased oral bioavailability, possibly
15 via reduced P450 metabolism and/or reduced glucuronidation, improved water solubility, improved potency against HCV, reduced toxicity (including hepatotoxicity), improved pharmacological profile, such as high exposure to target organ (e.g. liver in the case of HCV) and/or long half life (enabling less frequent dosing), reduced drug-drug interactions, such as via reduced levels of CYP3A4 metabolism and inhibition and reduced (Pgp) inhibition (enabling
20 easier multi-drug combinations) and improved side-effect profile, such as low binding to MRP2, leading to a reduced chance of hyperbilirubinaemia, lower immunosuppressive effect, improved activity against resistant virus species, in particular CsA and CsA analogue (e.g. DEBIO-025) resistant virus species and higher therapeutic (and/or selectivity) index. The present invention discloses novel sanglifehrin analogues which may have one or more of the above properties. In
25 particular, the present invention discloses novel mutasynthetic sanglifehrin analogues which, in at least some embodiments, have reduced metabolism via P450 or glucuronidation, for example as shown by increased microsome half-life and/or improved potency against HCV, for example as shown by a low replicon EC₅₀ and/or increased selectivity index.

There is also a need to develop novel immunosuppressive agents, which may have utility in the
30 prophylaxis of transplant rejection, or in the treatment of autoimmune, inflammatory and respiratory disorders. Preferably, such immunosuppressants have improved properties over the known natural sanglifehrins, including one or more of the following properties: longer half-life or increased oral bioavailability, possibly via reduced P450 metabolism and/or reduced glucuronidation, improved water solubility, improved potency in immunosuppressive activity,
35 such as might be seen in t-cell proliferation assays, reduced toxicity (including hepatotoxicity),



wherein:

R_1 , R_2 , R_3 , R_4 and R_5 independently represent H, F, Cl, Br, C_{2-6} alkenyl or C_{1-10} alkyl wherein one or more carbon atoms of said alkyl group are optionally replaced by a heteroatom selected from O, N and $S(O)_p$ in which p represents 0, 1 or 2 and wherein one or more carbon atoms of said alkyl group are optionally replaced by carbonyl and which alkyl group may optionally be substituted by one or more halogen atoms;

X_1 , X_2 , X_3 , X_4 and X_5 independently represent C or N, and in the case of any of these groups representing N the attached substituent is absent;

with the proviso that where R_1 , R_3 , R_4 and R_5 all represent H and X_1 , X_2 , X_3 , X_4 and X_5 all represent C, then R_2 cannot represent OH;

including any tautomer thereof; or an isomer thereof in which the C=C bond at the C26, 27 position C=C (by reference to the structure of sanglifehrin A) shown as trans is cis; and including a methanol adduct thereof in which a ketal is formed by the combination of the C-53 keto and the C-15 hydroxyl group and methanol.

The above structure shows a representative tautomer and the invention embraces all tautomers of the compounds of formula (I) for example keto compounds where enol compounds are illustrated and *vice versa*.

According to another aspect, the present disclosure provides a compound as described herein for use as a pharmaceutical for the treatment of a viral infection.

According to still a further aspect, the present disclosure provides a pharmaceutical composition comprising a compound as defined herein together with a pharmaceutically acceptable diluent or carrier.

According to a further aspect, the present disclosure provides use of a therapeutically effective amount of the compound defined herein for the treatment of a viral infection.

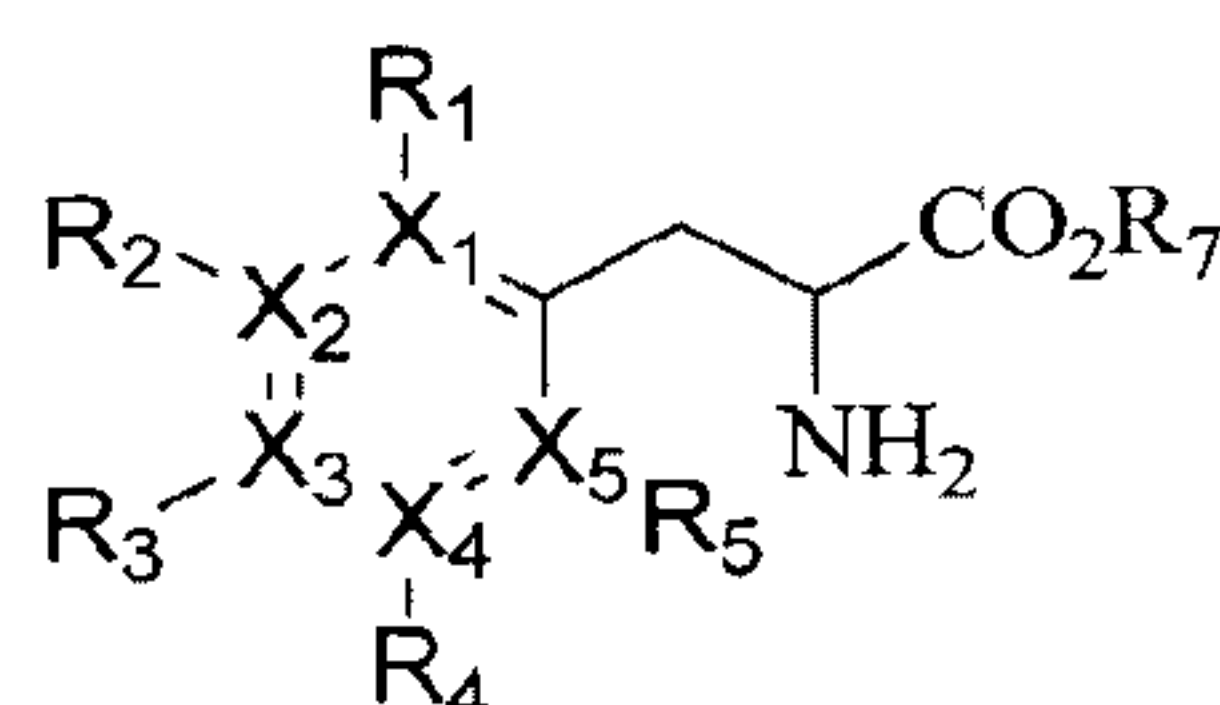
According to another aspect, the present disclosure provides use of a therapeutically effective amount of the compound as defined herein for the manufacture of a medicament for the treatment of a viral infection.

According to still another aspect, the present disclosure provides use of a therapeutically effective amount of the compound as defined herein for the treatment of an inflammatory disorder.

According to another aspect, the present disclosure provides use of a therapeutically effective amount of the compound described herein for the manufacture of a medicament for the treatment of an inflammatory disorder.

According to a further aspect, the present disclosure provides a compound described herein for use as a pharmaceutical for the treatment of an inflammatory disorder.

According to another aspect, the present disclosure provides a process for producing a mutasynthetic sanglifehrin which comprises feeding a sanglifehrin producing bacterium a compound of formula (III)



Formula (III)

wherein X₁, X₂, X₃, X₄, X₅, R₁, R₂, R₃, R₄ and R₅ are as defined herein and R₇ represents H or an ester forming group, or a salt thereof, and culturing the bacterium such that a mutasynthetic sanglifehrin is produced.

Specific tautomers that are included within the definition of formula (I) are those in which (i) the C-53 keto group forms a hemiketal with the C-15 hydroxyl, or (ii) the C-15 and C-17 hydroxyl can combine with the C-53 keto to form a ketal. All numberings use the system for the parent sanglifehrin A structure.

Definitions

The articles “a” and “an” are used herein to refer to one or to more than one (i.e. at least one) of the grammatical objects of the article. By way of example “an analogue” means one analogue or more than one analogue.

As used herein the term “**analogue(s)**” refers to chemical compounds that are structurally similar to another but which differ slightly in composition (as in the replacement of one atom by another or in the presence or absence of a particular functional group).

As used herein the term “**inflammatory disorders**” refers to the list of disorders caused by inflammation, including, but not limited to Acne vulgaris, Atherosclerosis, Asthma, Automimmune diseases (such as Acute Disseminated Encephalomyelitis (ADEM), Addison's Disease, Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Antibody Syndrome (APS), Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Autoimmune Inner Ear Disease, Bullous Pemphigoid, Coeliac Disease, Chagas Disease, Chronic Obstructive Pulmonary Disease (COPD), Crohn's Disease, Dermatomyositis, Diabetes Mellitus Type 1, Endometriosis, Goodpasture's Syndrome, Graves' Disease, Guillain-Barré Syndrome, Hashimoto's Disease, Hidradenitis Suppurativa, Kawasaki Disease, IgA Nephropathy, Idiopathic Thrombocytopenic Purpura, Interstitial Cystitis, Lupus Erythematosus, Mixed Connective Tissue Disease, Morphea, Multiple sclerosis (MS), Myasthenia Gravis, Narcolepsy, Neuromyotonia, Pemphigus Vulgaris, Pernicious Anaemia, Psoriasis, Psoriatic Arthritis, Polymyositis, Primary Biliary Cirrhosis, Rheumatoid Arthritis, Schizophrenia, Scleroderma, Sjögren's Syndrome, Stiff Person Syndrome, Temporal Arteritis, Ulcerative Colitis, Vasculitis, Vitiligo, Wegener's Granulomatosis), Inflammatory Bowel disease, Pelvic inflammatory disease, Rheumatoid arthritis and transplant rejection.

As used herein the term “**sanglifehrin(s)**” refers to chemical compounds that are structurally similar to sanglifehrin A but which differ slightly in composition (as in the replacement of one atom by another or in the presence or absence of a particular functional group), in particular those generated by fermentation of *Streptomyces* sp. A92-308110. Examples include the sanglifehrin-like compounds discussed in WO97/02285 and WO98/07743, such as sanglifehrin B.

As used herein the term “**mutasynthetic sanglifehrin(s)**” or “**mutasynthetic sanglifehrin analogue(s)**” refers to chemical compounds that are structurally similar to sanglifehrin A, B, C or D but which differ slightly in composition (as in the replacement of one or more atom by another or in the presence or absence of a particular functional group), in particular, those generated by fermentation of *Streptomyces* sp. A92-308110 or a mutant thereof, where the culture is fed with a meta-tyrosine analogue.

As used herein the term “**meta-tyrosine analogue(s)**” refers to chemical compounds that are structurally similar to meta-tyrosine but which differ slightly in composition (as in the replacement of one or more atom by another or in the presence or absence of a particular functional group), in particular, those described in formula (III).

As used herein, the term “**HCV**” refers to Hepatitis C Virus, a single stranded, RNA, enveloped virus in the viral family *Flaviviridae*.

As used herein, the term “**HIV**” refers to Human Immunodeficiency Virus, the causative agent of Human Acquired Immune Deficiency Syndrome.

5 As used herein, the term “**bioavailability**” refers to the degree to which or rate at which a drug or other substance is absorbed or becomes available at the site of biological activity after administration. This property is dependent upon a number of factors including the solubility of the compound, rate of absorption in the gut, the extent of protein binding and metabolism etc. Various tests for bioavailability that would be familiar to a person of skill in the art are described
10 herein (see also Egorin *et al.* 2002).

The term “**water solubility**” as used in this application refers to solubility in aqueous media, e.g. phosphate buffered saline (PBS) at pH 7.4, or in 5% glucose solution. Tests for water solubility are given below in the Examples as “water solubility assay”.

The pharmaceutically acceptable salts of compounds of the invention such as the compounds
15 of formula (I) include conventional salts formed from pharmaceutically acceptable inorganic or organic acids or bases as well as quaternary ammonium acid addition salts. More specific examples of suitable acid salts include hydrochloric, hydrobromic, sulfuric, phosphoric, nitric, perchloric, fumaric, acetic, propionic, succinic, glycolic, formic, lactic, maleic, tartaric, citric, palmoic, malonic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, fumaric,
20 toluenesulfonic, methanesulfonic, naphthalene-2-sulfonic, benzenesulfonic hydroxynaphthoic, hydroiodic, malic, steroic, tannic and the like. Hydrochloric acid salts are of particular interest. Other acids such as oxalic, while not in themselves pharmaceutically acceptable, may be useful in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable salts. More specific examples of suitable basic salts
25 include sodium, lithium, potassium, magnesium, aluminium, calcium, zinc, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, N-methylglucamine and procaine salts. References hereinafter to a compound according to the invention include both compounds of formula (I) and their pharmaceutically acceptable salts.

As used herein, the term “**alkyl**” represents a straight chain or branched alkyl group. Exemplary
30 alkyl is C₁₋₆ alkyl eg C₁₋₄alkyl.

“**Alkenyl**” refers to an alkyl group containing two or more carbons which is unsaturated with one or more double bonds.

Examples of alkyl groups include C₁₋₄ alkyl groups such as methyl, ethyl, n-propyl, i-propyl, and n-butyl. Examples of alkenyl groups include C₂₋₄ alkenyl groups such as –CH=CH₂ and –CH₂CH=CH₂.

The term “**treatment**” includes prophylactic as well as therapeutic treatment.

5 **Figure Legend**

Figure 1: compound structures and sanglifehrin A numbering system

Figure 2: ¹H NMR of compound **14**

Figure 3: ¹H NMR of compound **15**

Figure 4: ¹H NMR of compound **16**

10 **Figure 5:** ¹H NMR of compound **17**

Figure 6: ¹H NMR of compound **18**

Figure 7: ¹H NMR of compound **19**

Figure 8: ¹H NMR of compound **20**

15 **Description of the Invention**

The present invention provides mutasynthetic sanglifehrin analogues, as set out above, methods for preparation of these compounds and methods for the use of these compounds in medicine.

20 In one embodiment, the compound is a methanol adduct thereof in which a ketal is formed by the combination of the C-53 keto and the C-15 hydroxyl groups and methanol. In another embodiment it is not.

In certain embodiments a carbon atom of the C₁₋₁₀alkyl group (e.g. C₁₋₆alkyl group) that one or more of R₁, R₂, R₃, R₄ and R₅ may represent is replaced by a heteroatom.

25 If –CH₃ is replaced by N, the group formed is –NH₂. If –CH₂– is replaced by N, the group formed is –NH–. If –CHR– is replaced by N the group formed is –NR–. Hence nitrogen atoms within R₁, R₂, R₃, R₄ and R₅ may be primary, secondary or tertiary nitrogen atoms.

If –CH₃ is replaced by O, the group formed is –OH.

30 When a carbon atom of the C₁₋₁₀alkyl group (e.g. C₁₋₆alkyl group) that one or more of R₁, R₂, R₃, R₄ and R₅ may represent is replaced by a heteroatom, it is suitably replaced by O, S or N, especially N or O particularly O.

When any one of R_1 , R_2 , R_3 , R_4 and R_5 contains a group $S(O)_p$, variable p suitably represents 0 or 1. In one embodiment p represents 0. In another embodiment p represents 1. In another embodiment p represents 2.

When a C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) that one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent contains more than one heteroatom, these should typically be separated by two or more carbon atoms.

Suitably, a carbon atom of a C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) that one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent is not replaced by any heteroatom or else represents OH or NH_2 .

When a carbon atom of the C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) that one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent is replaced by a carbonyl, the carbonyl is suitably located adjacent to another carbon atom or a nitrogen atom. Suitably carbonyl groups are not located adjacent to sulfur or oxygen atoms.

For example one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent $-COC_{1-3}$ alkyl e.g. $-COMe$.

Suitably a carbon atom of the C_{1-10} alkyl (e.g. C_{1-6} alkyl) group that one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent is not replaced by a carbonyl.

The C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) that one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent may be substituted by one or more halogen atoms. For example one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent $-CF_3$. Alternatively one or more of R_1 , R_2 , R_3 , R_4 and R_5 may represent C_{1-10} alkyl (e.g. C_{1-6} alkyl) substituted by one or more (eg one) Cl or F atom (eg $-CH_2CH_2Cl$).

Suitably a C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) of R_1 , R_2 , R_3 , R_4 or R_5 is not substituted by halogen.

When one or more of R_1 , R_2 , R_3 , R_4 and R_5 group(s) represent a C_{1-10} alkyl group (e.g. C_{1-6} alkyl group) suitably the group(s) represent C_{1-4} alkyl (e.g. C_{1-2} alkyl such as methyl).

In an embodiment, one or more of R_1 , R_2 , R_3 , R_4 and R_5 represent C_{1-6} alkyl (such as C_{1-2} alkyl) or C_{2-3} alkenyl e.g. one or more of R_1 , R_2 , R_3 , R_4 and R_5 represent methyl.

Suitably R_1 represents H, F, Cl, CF_3 , OH or C_{1-6} alkyl (e.g. methyl). Most suitably, R_1 represents H or F, especially H.

Suitably R_2 represents H, F, Cl, CF_3 , OH, NH_2 or C_{1-6} alkyl (e.g. methyl). More suitably, R_2 represents H, F, OH or NH_2 , especially OH.

Suitably R_3 represents H, F, Cl, CF_3 , OH or C_{1-6} alkyl (e.g. methyl). More suitably, R_3 represents H, Me or F. R_3 may also represent ethyl.

Suitably R_4 represents H, F, Cl, CF_3 , OH or C_{1-6} alkyl (e.g. methyl). More suitably, R_4 represents H or F.

Suitably R_5 represents H, F, Cl, CF_3 , OH or C_{1-6} alkyl (e.g. methyl). More suitably, R_5 represents H or F

- 5 Suitably one or more, more suitably two or more (for example three or more) of R_1 , R_2 , R_3 , R_4 or R_5 do not represent H.

Suitably one or more, for example two or more of R_1 , R_2 , R_3 , R_4 or R_5 represent F.

Suitably R_3 or R_4 or R_3 and R_4 represent F. In another embodiment R_1 and R_3 represent F.

- 10 In one embodiment X_1 represents N (therefore R_1 is absent). In another more preferable embodiment X_1 represents C.

Suitably X_2 represents C.

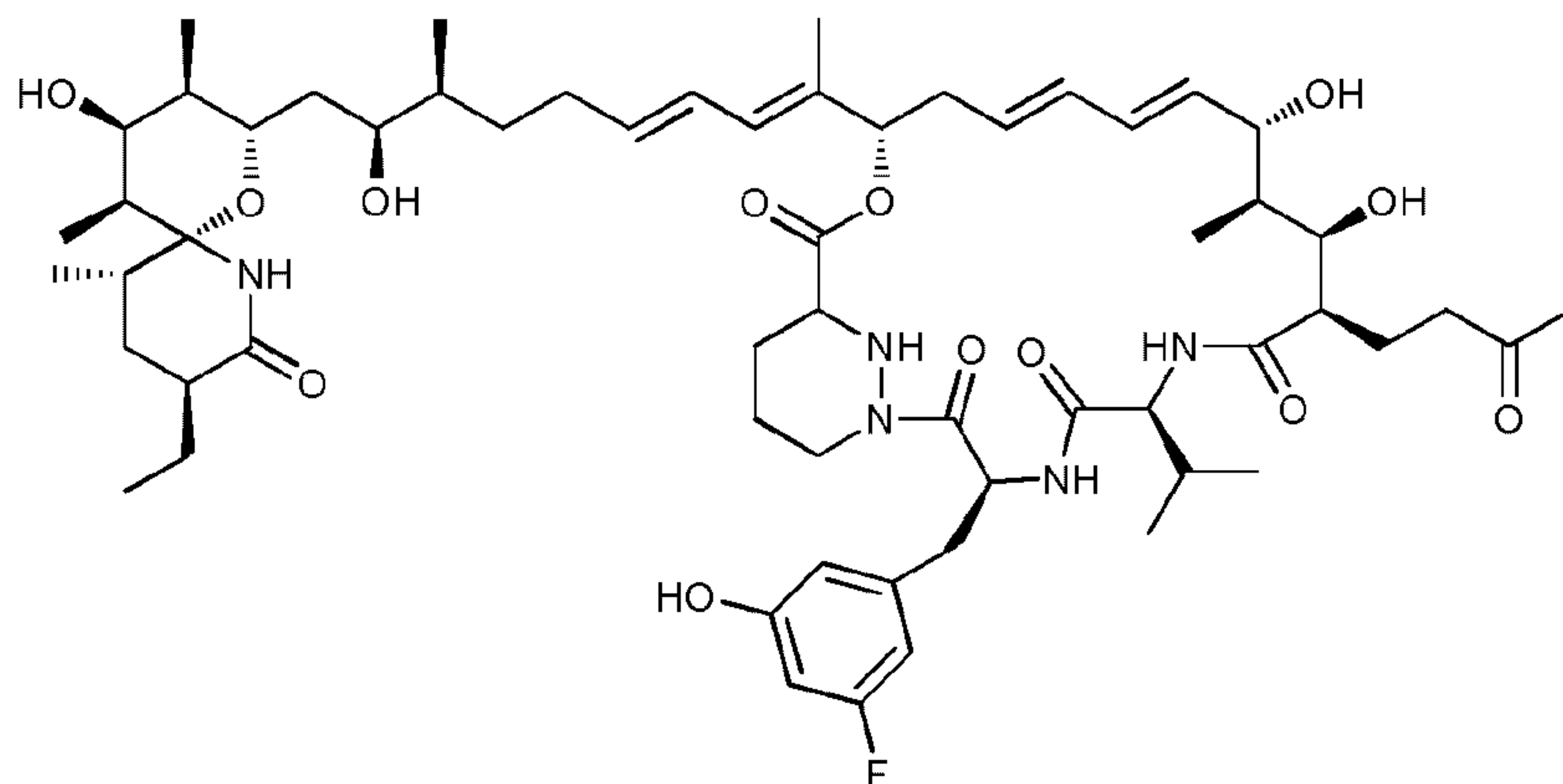
Suitably X_3 represents C.

Suitably X_4 represents C.

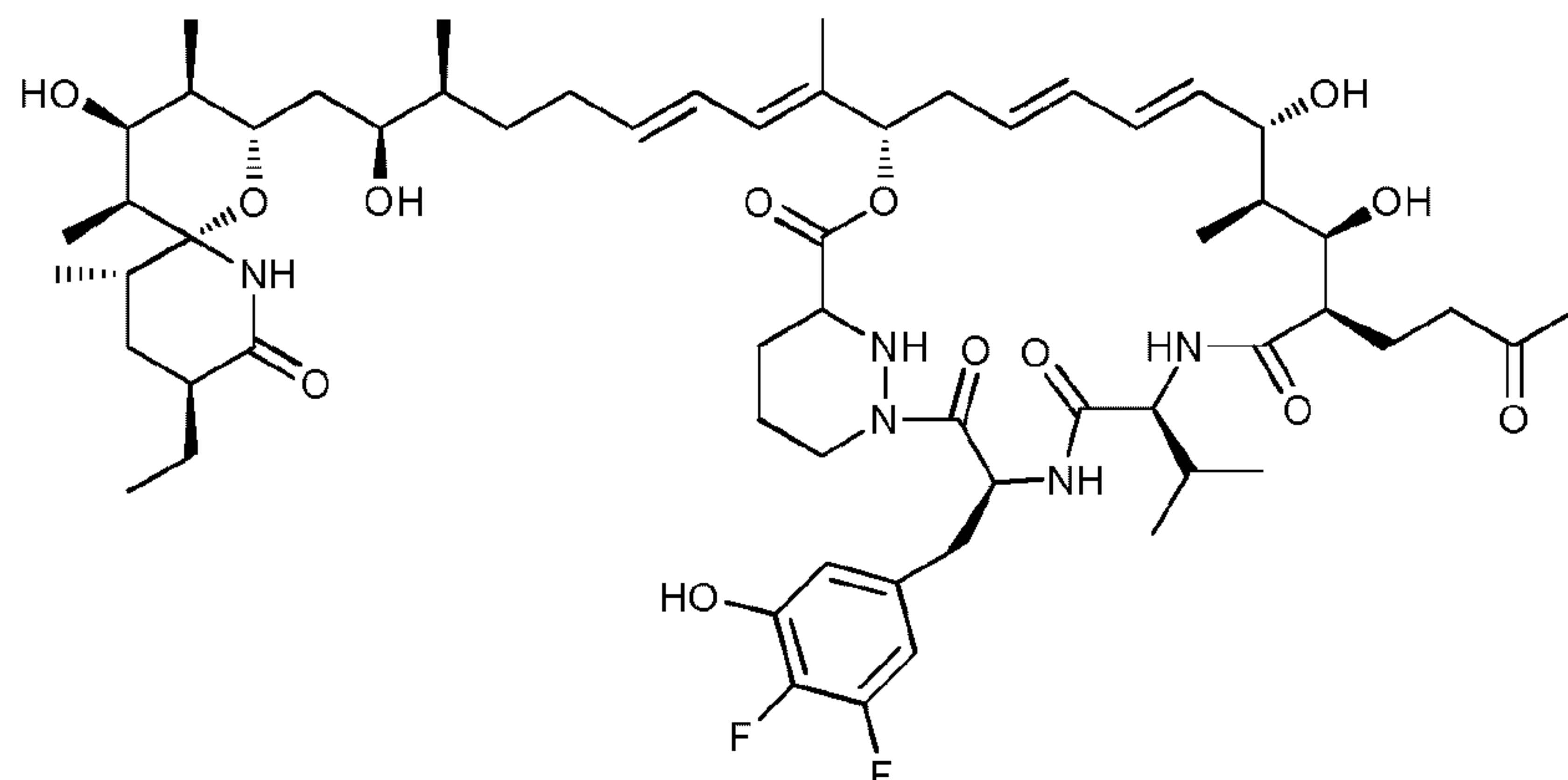
Suitably X_5 represents C.

- 15 In one embodiment the compound is a compound of formula (I). In another embodiment the compound is a compound of formula (II).

- 20 In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents H, R_4 represents F, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

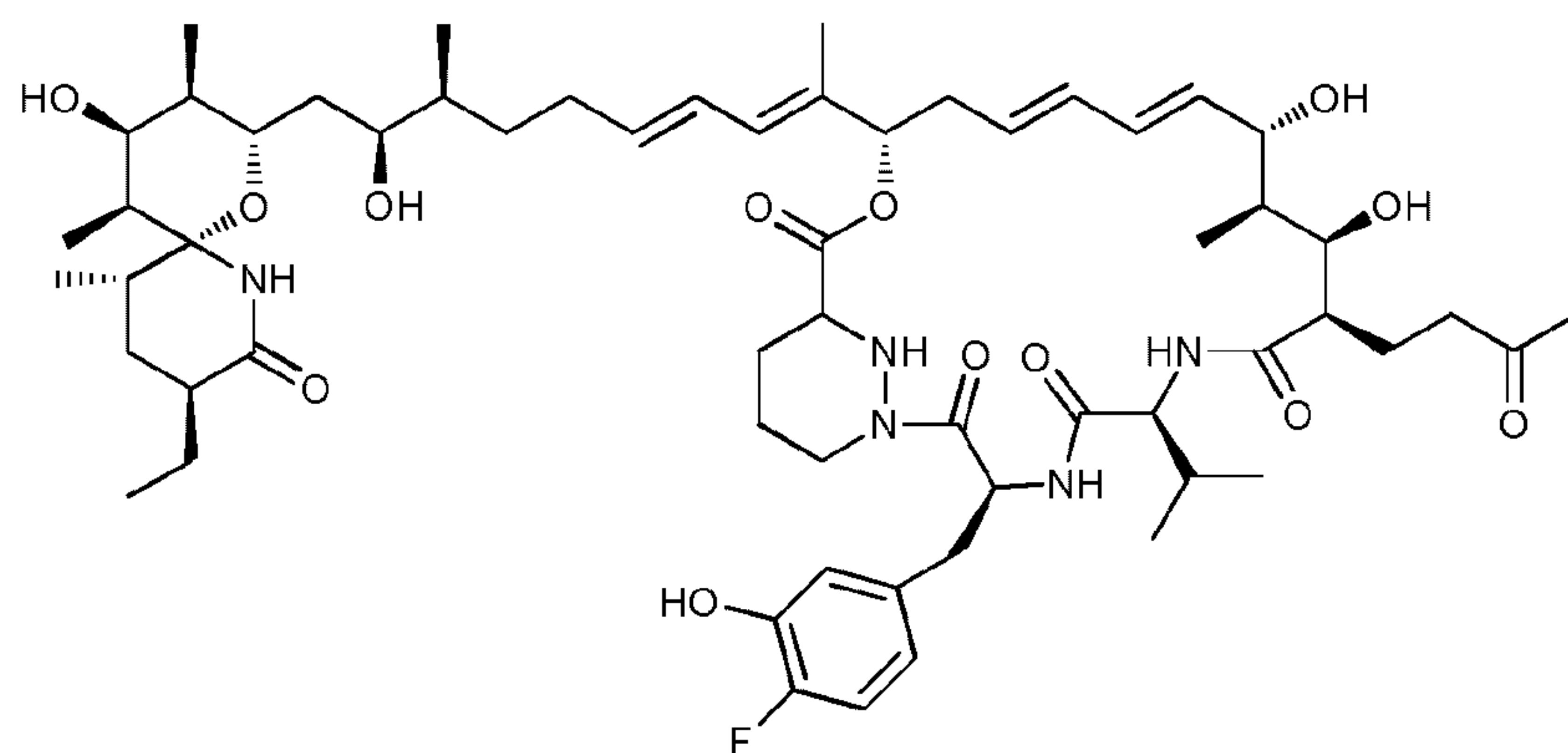


In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents F, R_4 represents F, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



5

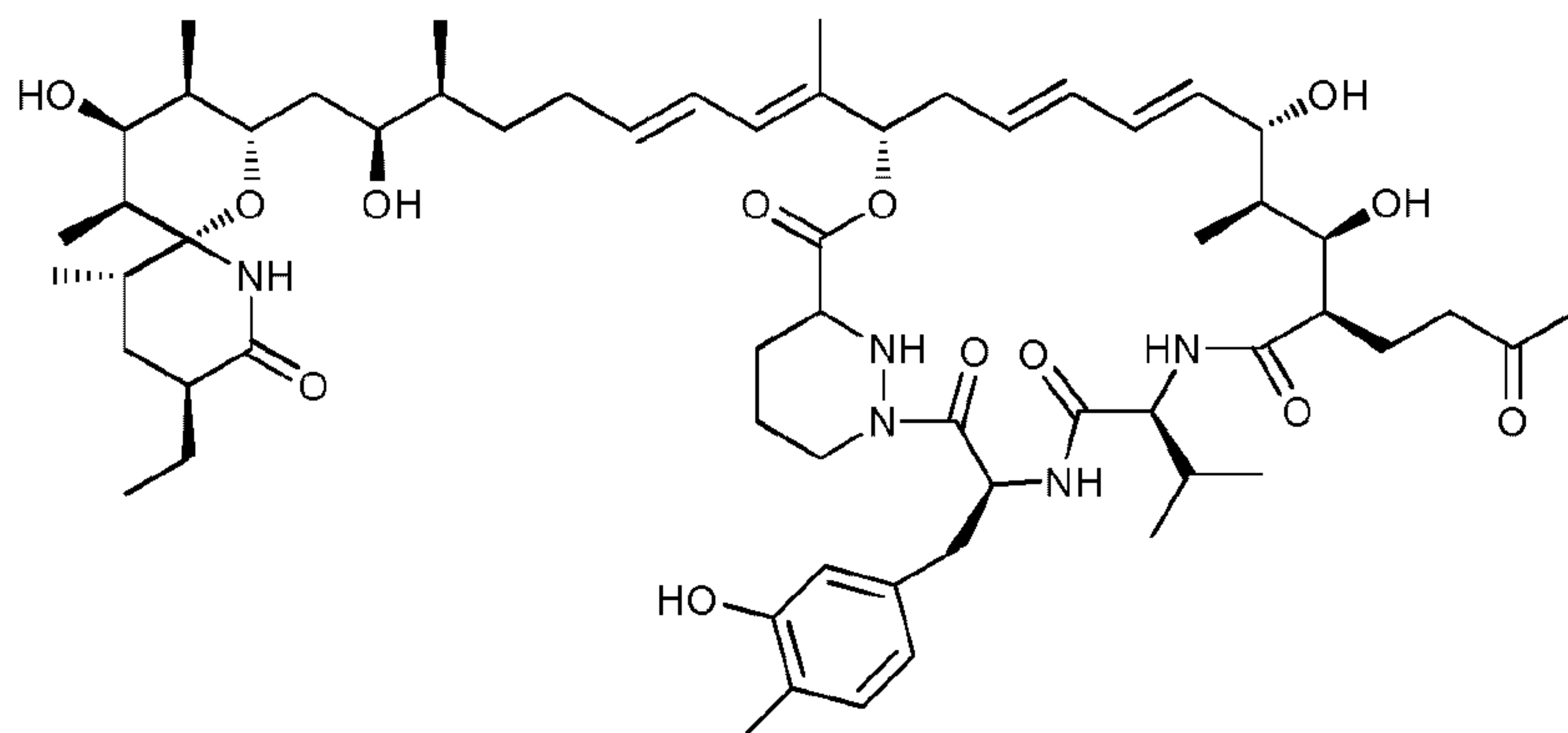
In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents F, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



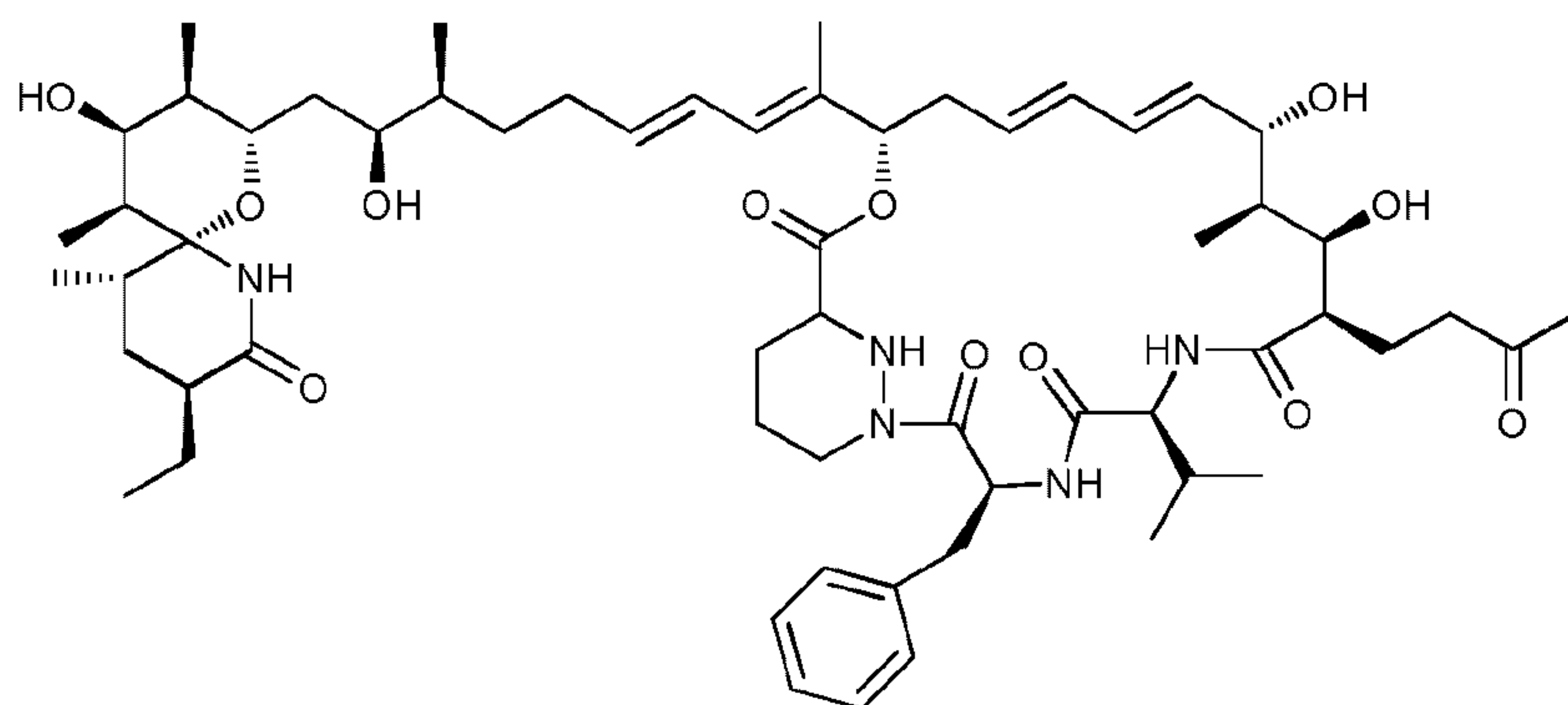
10

In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents Me, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

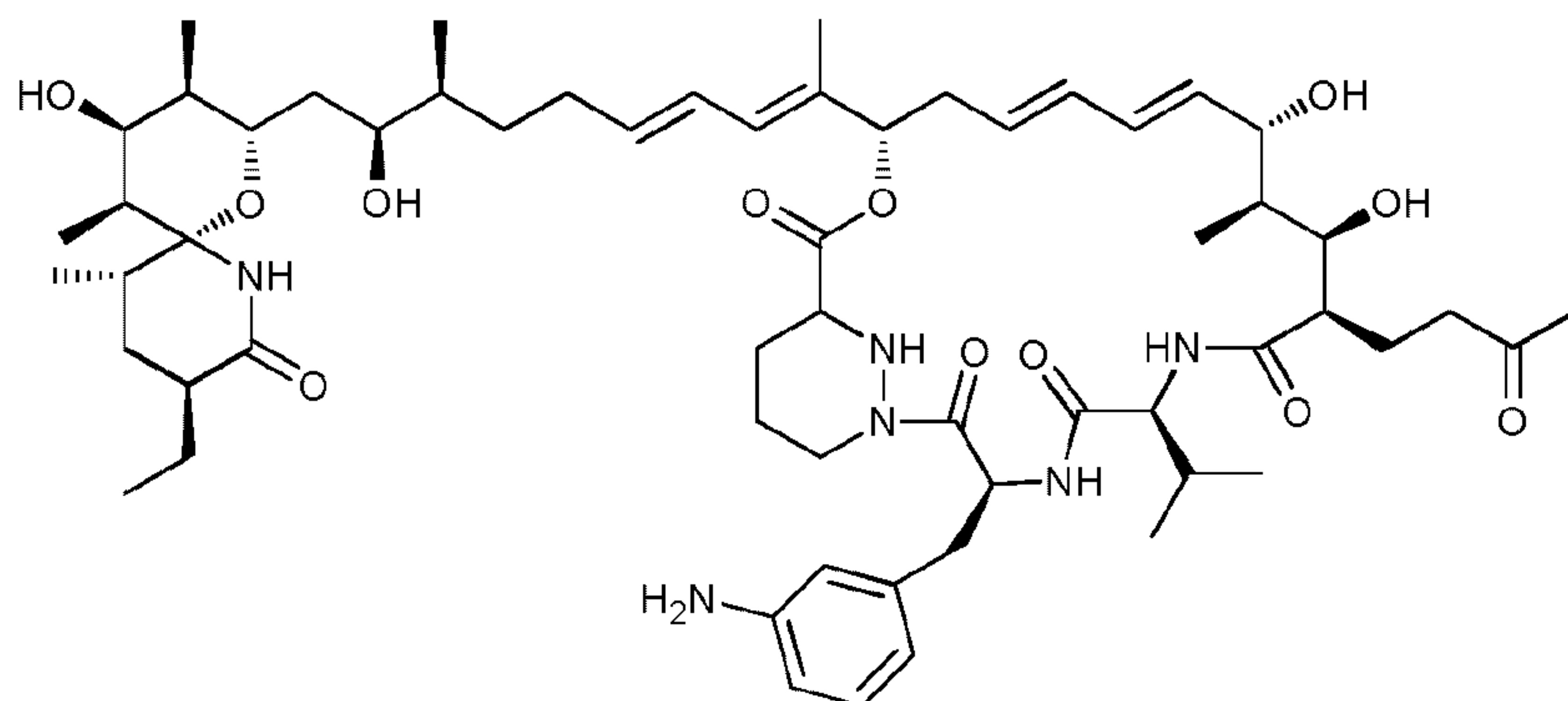
18



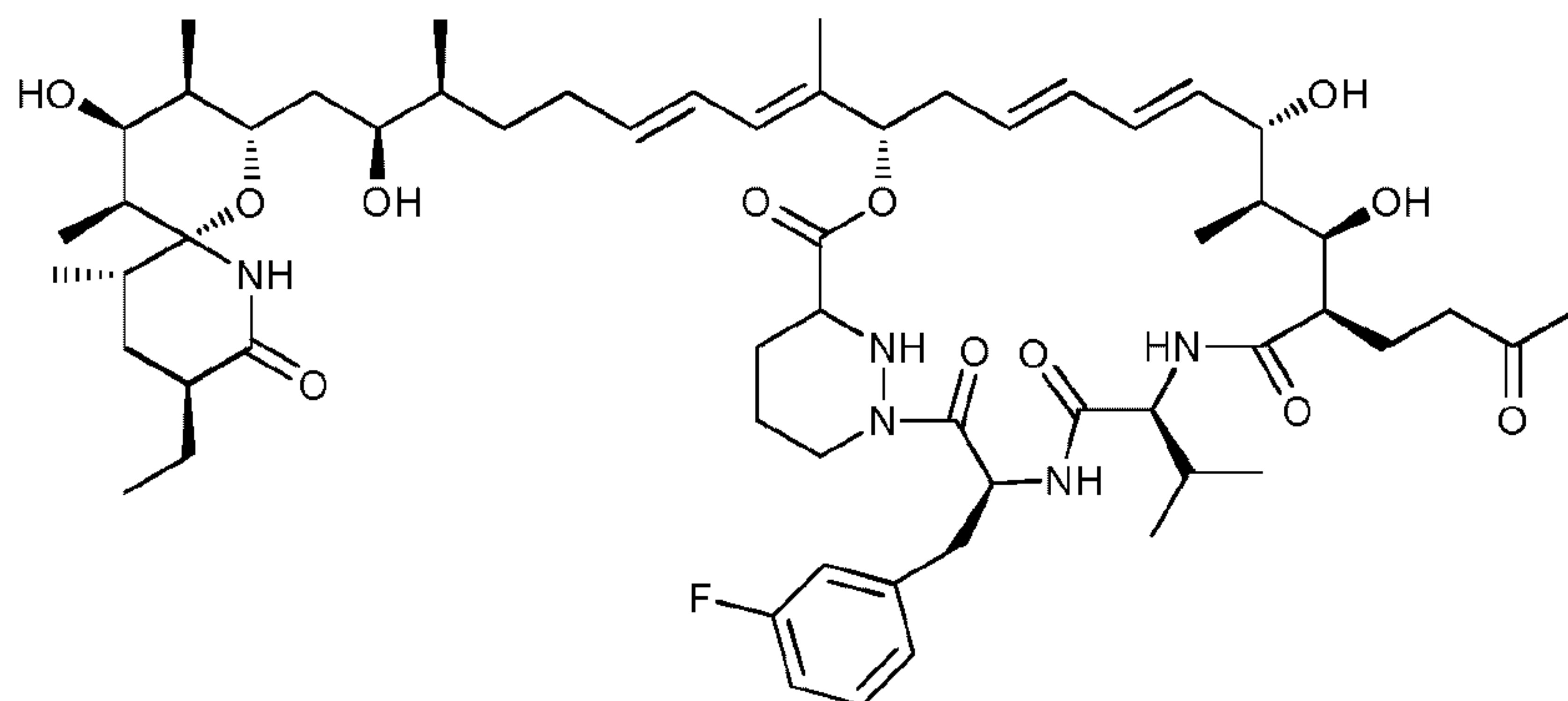
In a suitable embodiment of the invention, R_1 represents H, R_2 represents H, R_3 represents H, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



In a suitable embodiment of the invention, R_1 represents H, R_2 represents NH_2 , R_3 represents H, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

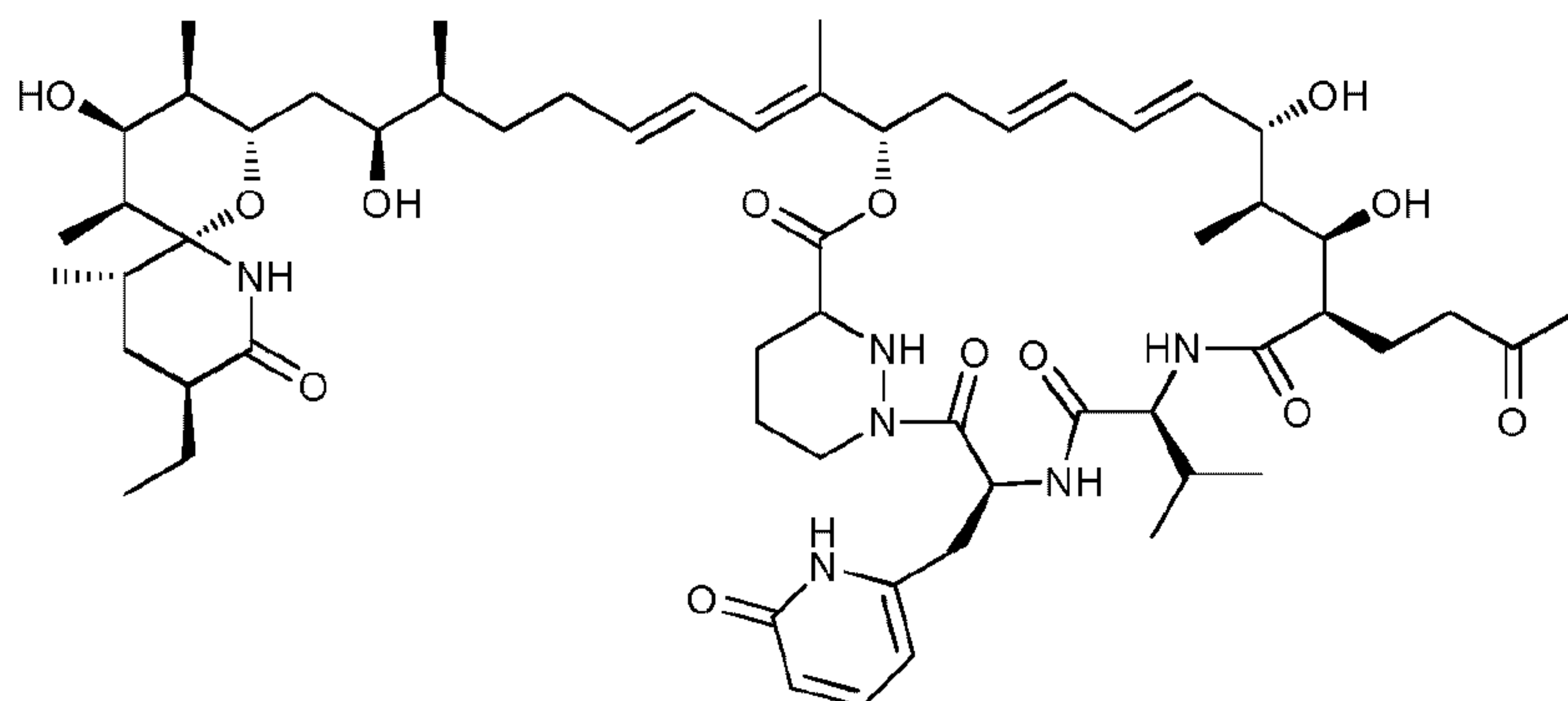


In a suitable embodiment of the invention, R_1 represents H, R_2 represents F, R_3 represents H, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

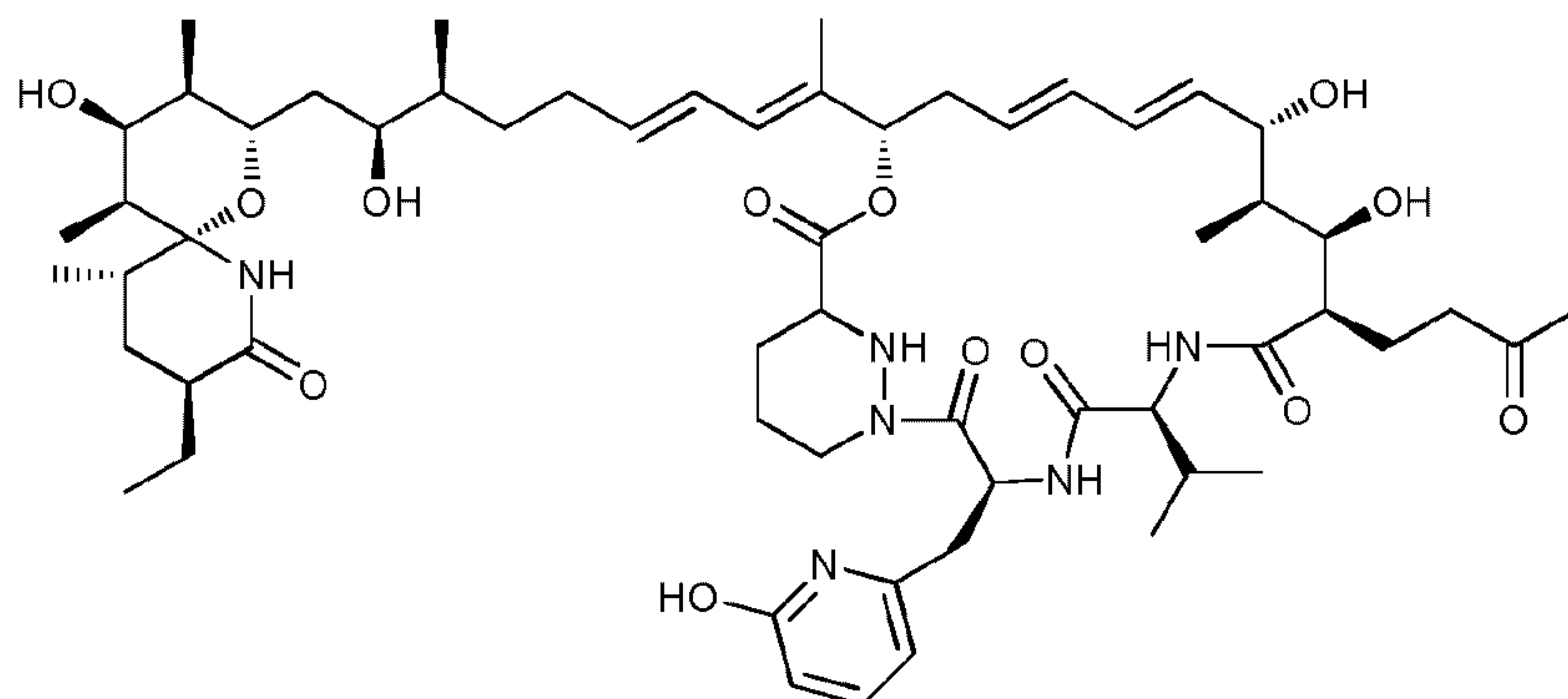


5

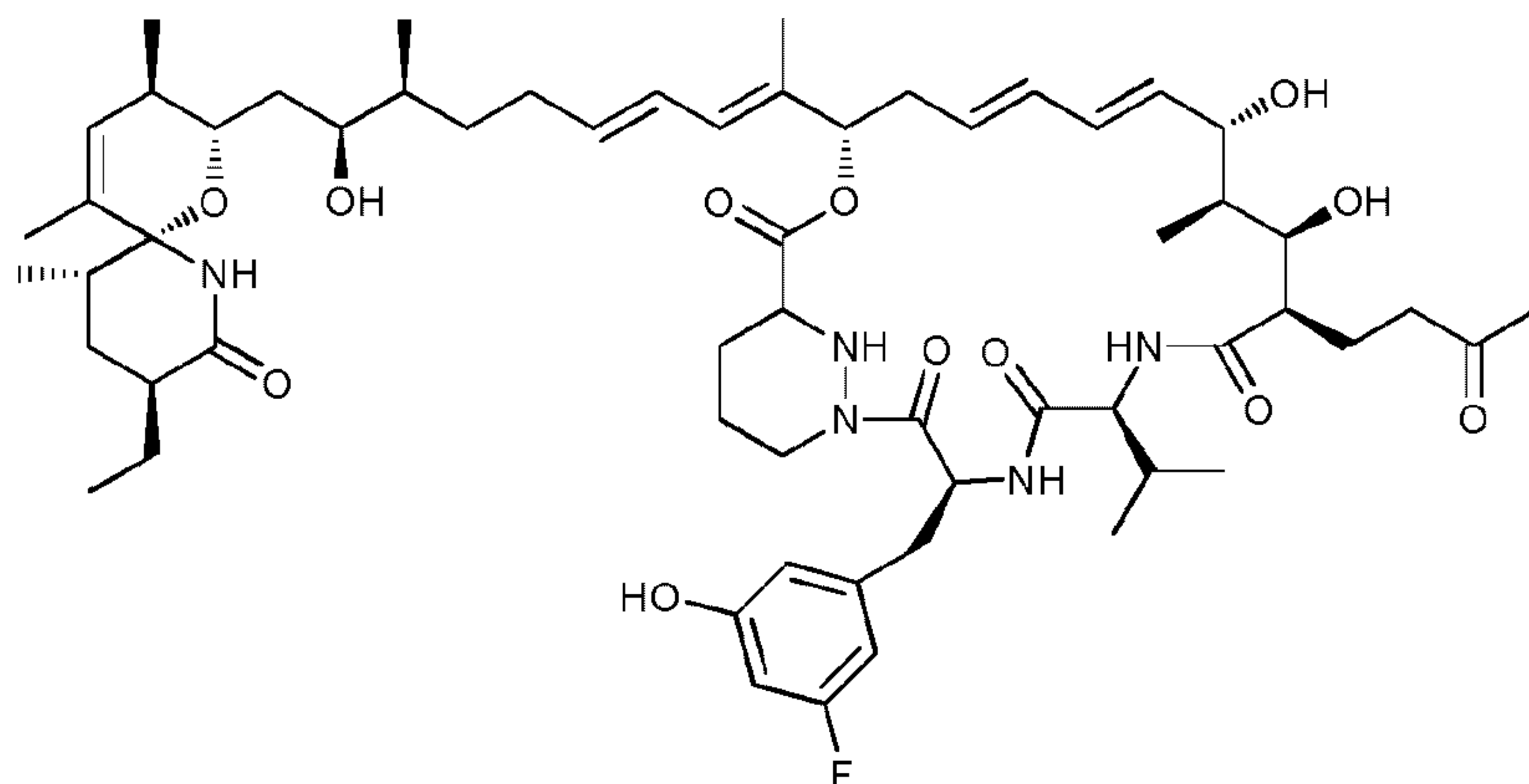
In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents H, R_4 represents F, R_5 represents H, X_1 represents N and X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



10 which can also be represented as:

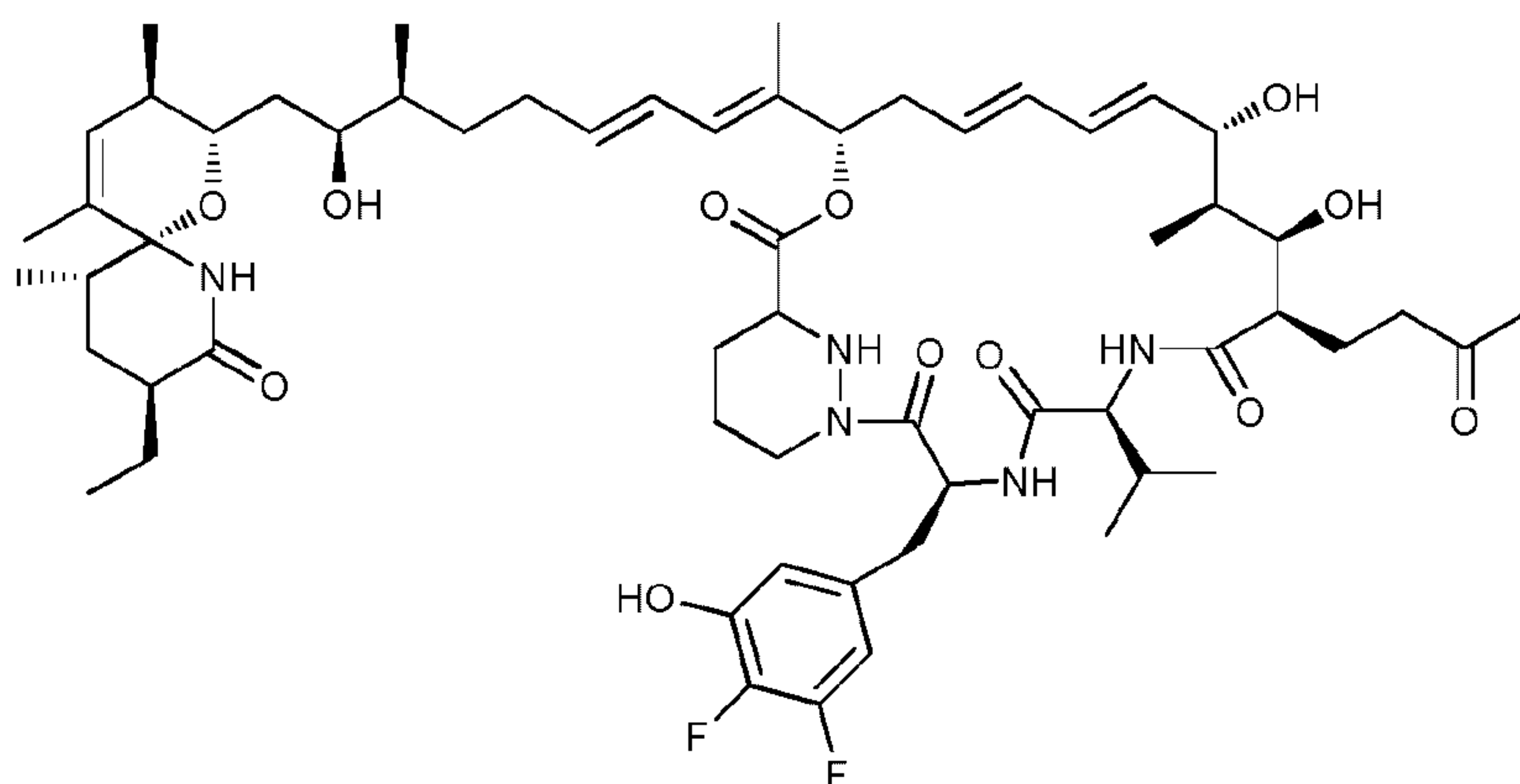


In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents H, R_4 represents F, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



5

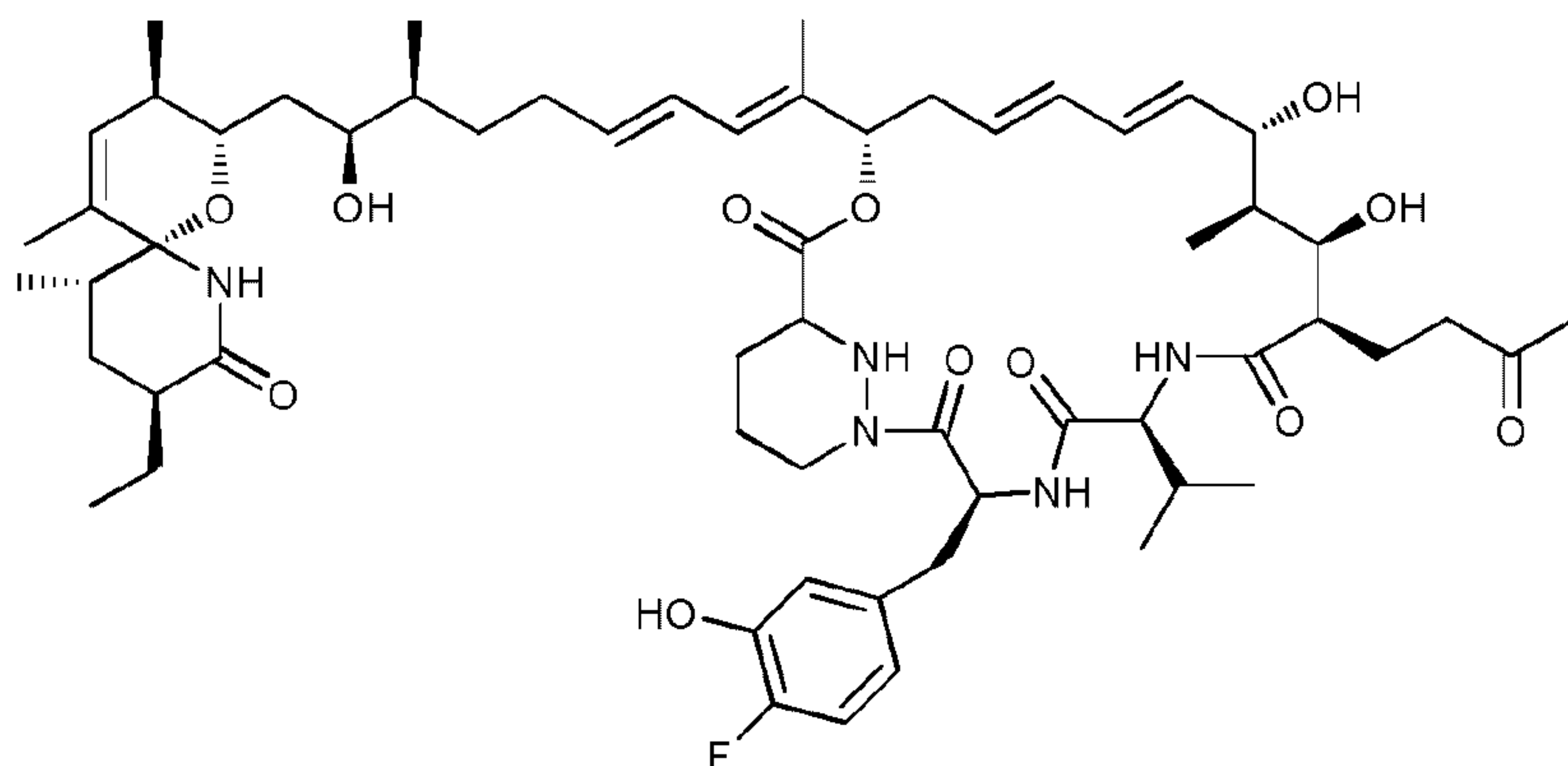
In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents F, R_4 represents F, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



10

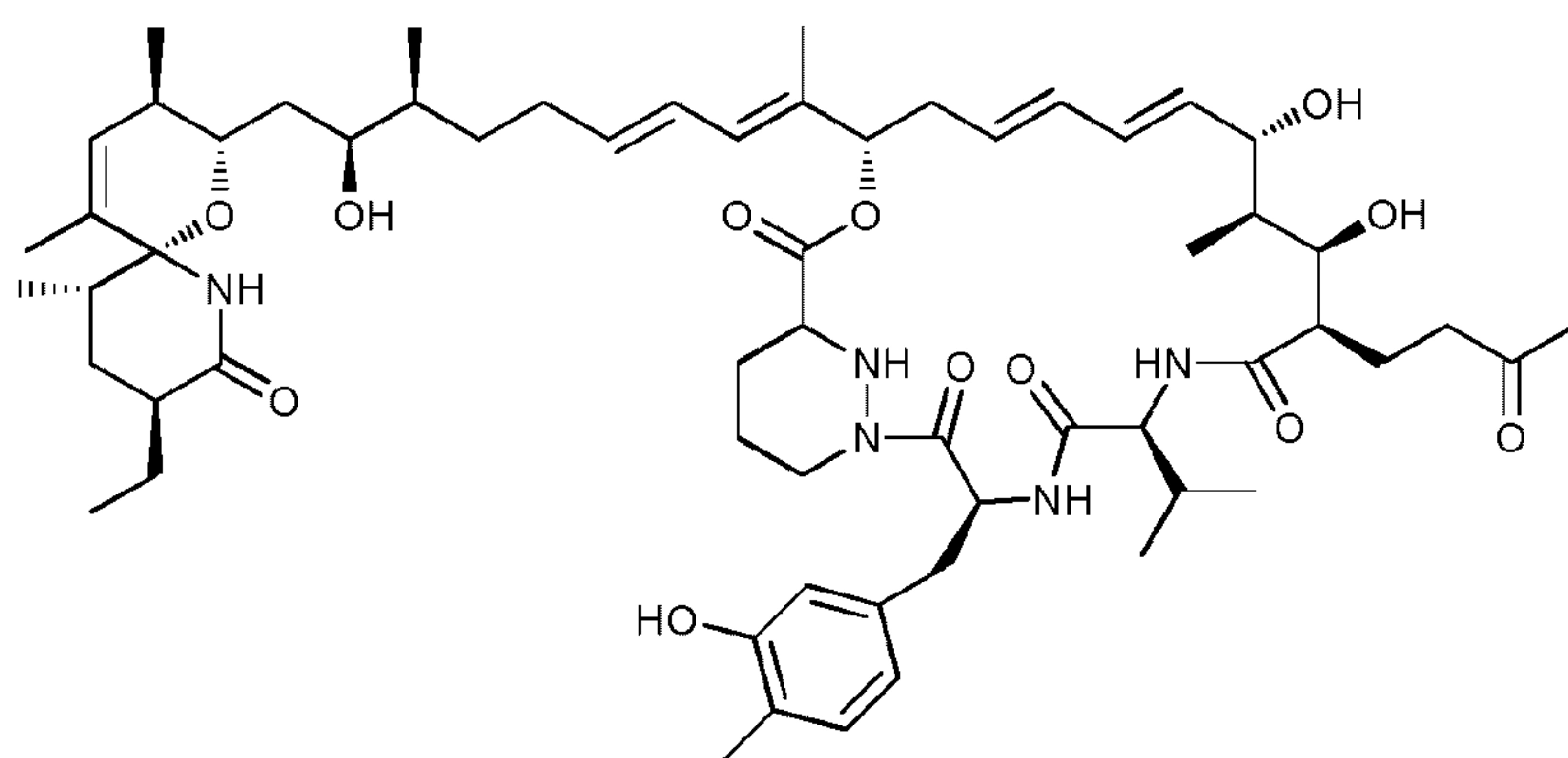
In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents F, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

21



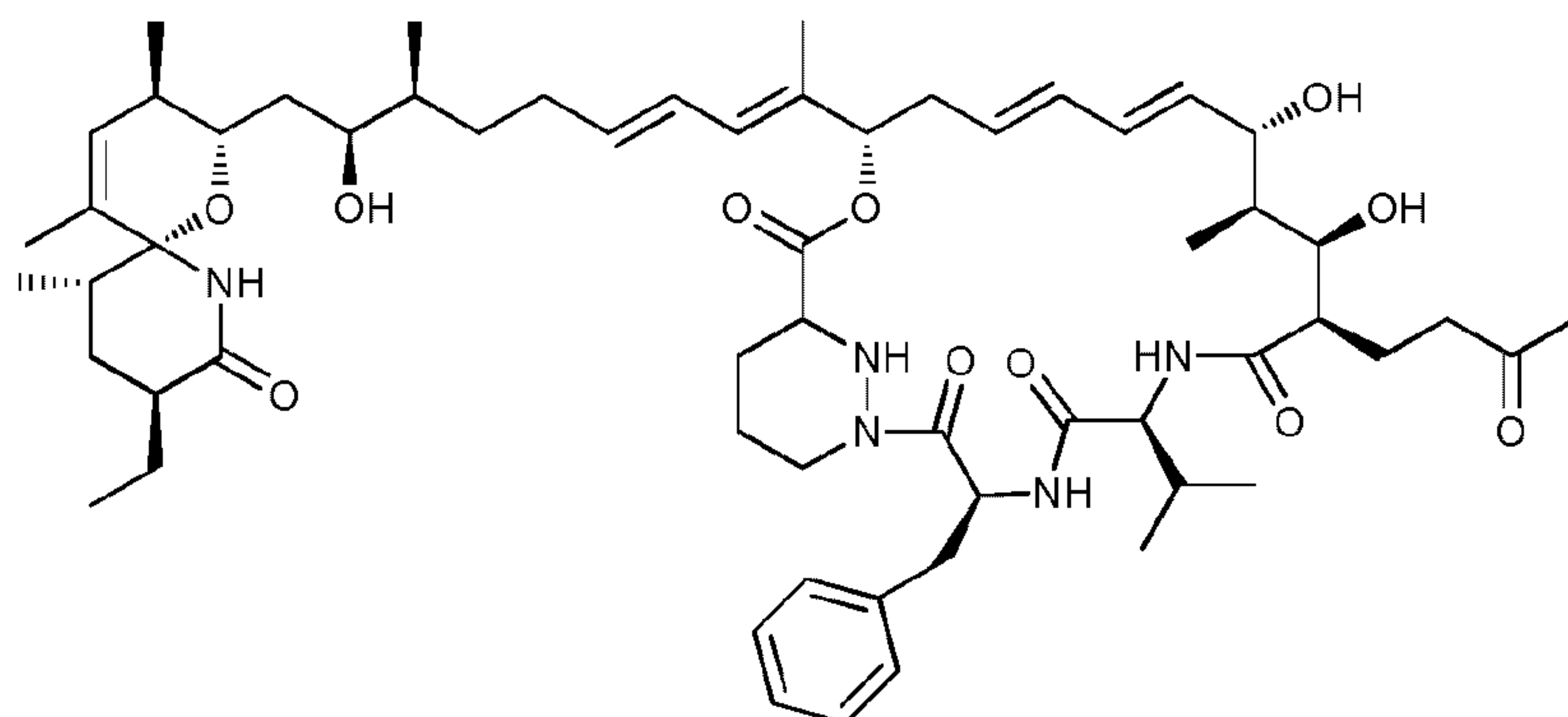
In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents Me, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by

5 the following structure:

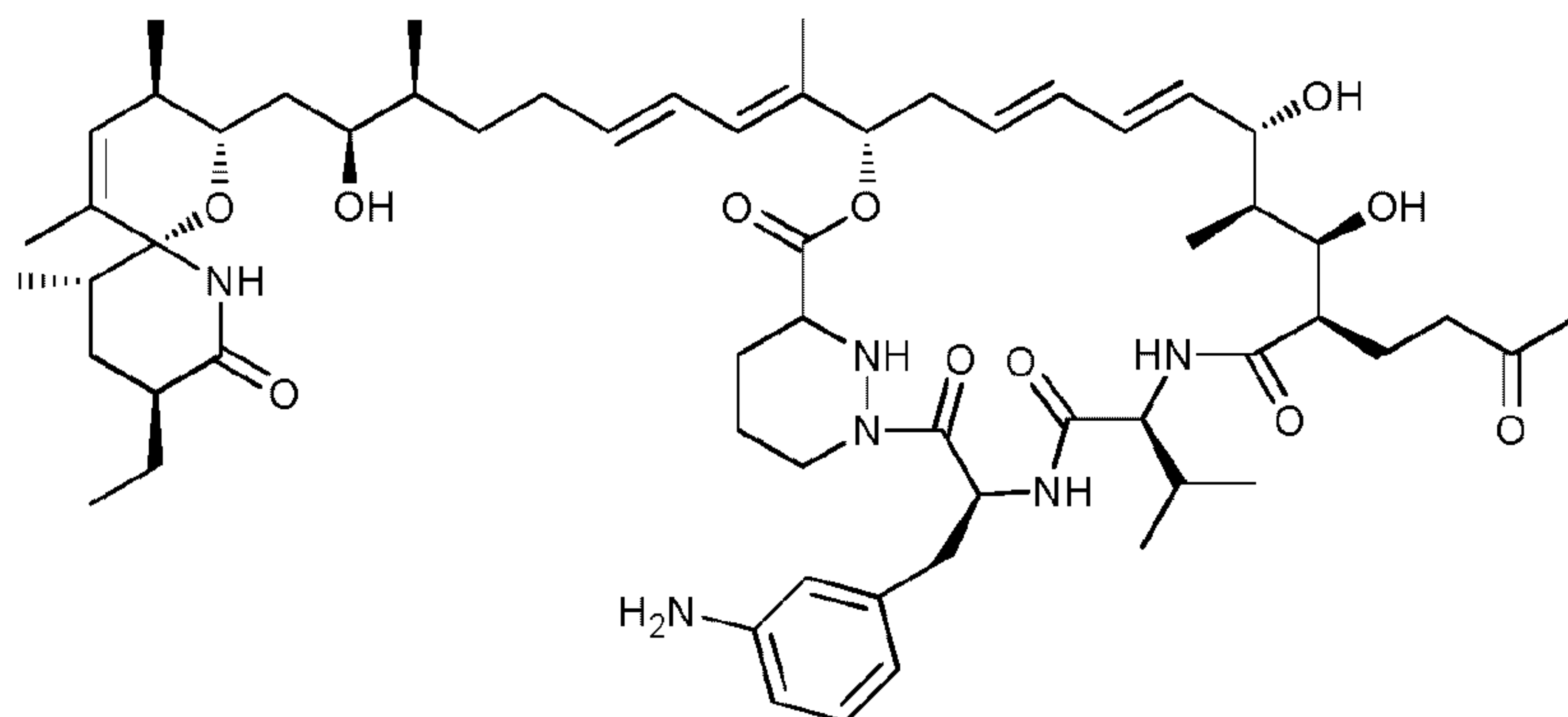


In a suitable embodiment of the invention, R_1 represents H, R_2 represents H, R_3 represents H, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the

10 following structure:

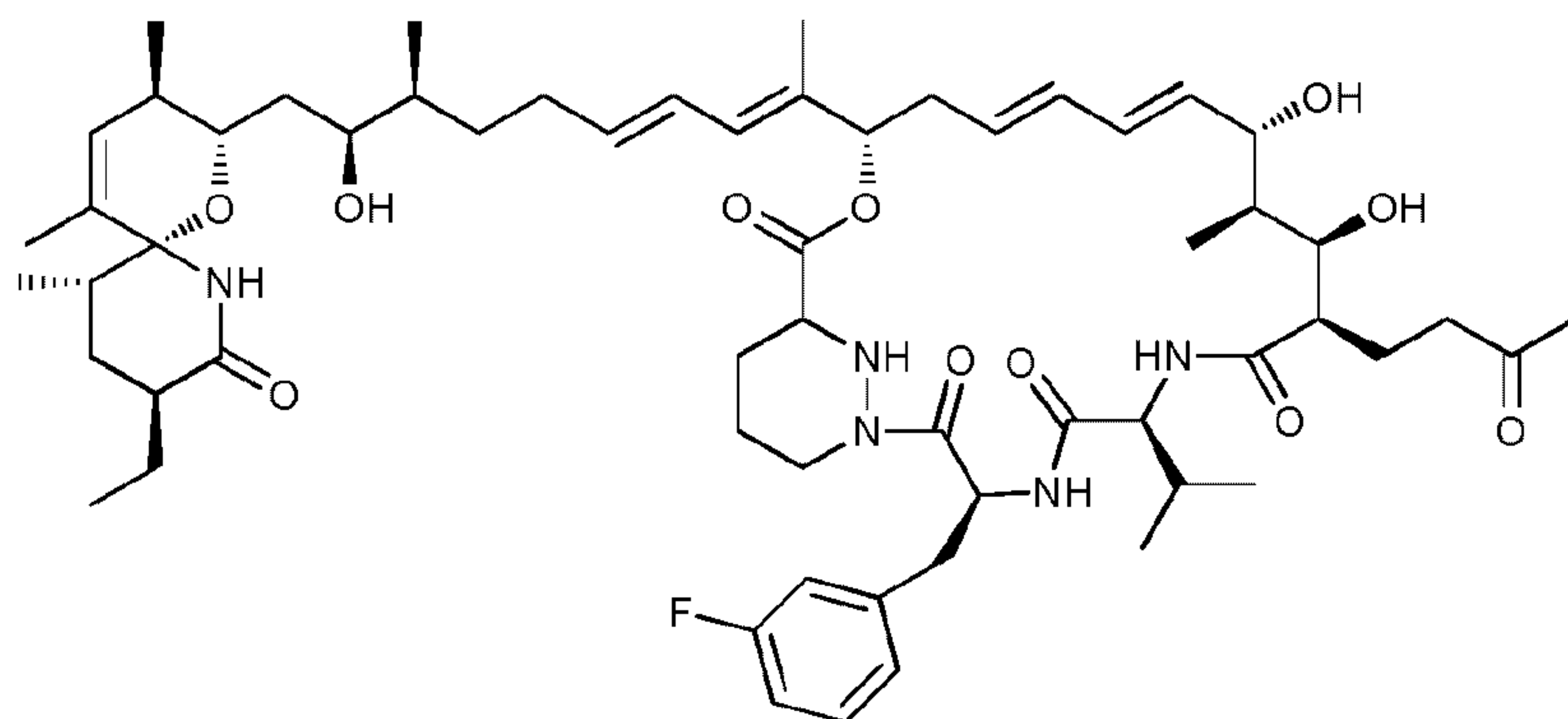


In a suitable embodiment of the invention, R_1 represents H, R_2 represents NH_2 , R_3 represents H, R_4 represents H, R_5 represents H and X_1, X_2, X_3, X_4 and X_5 represent C as represented by the following structure:

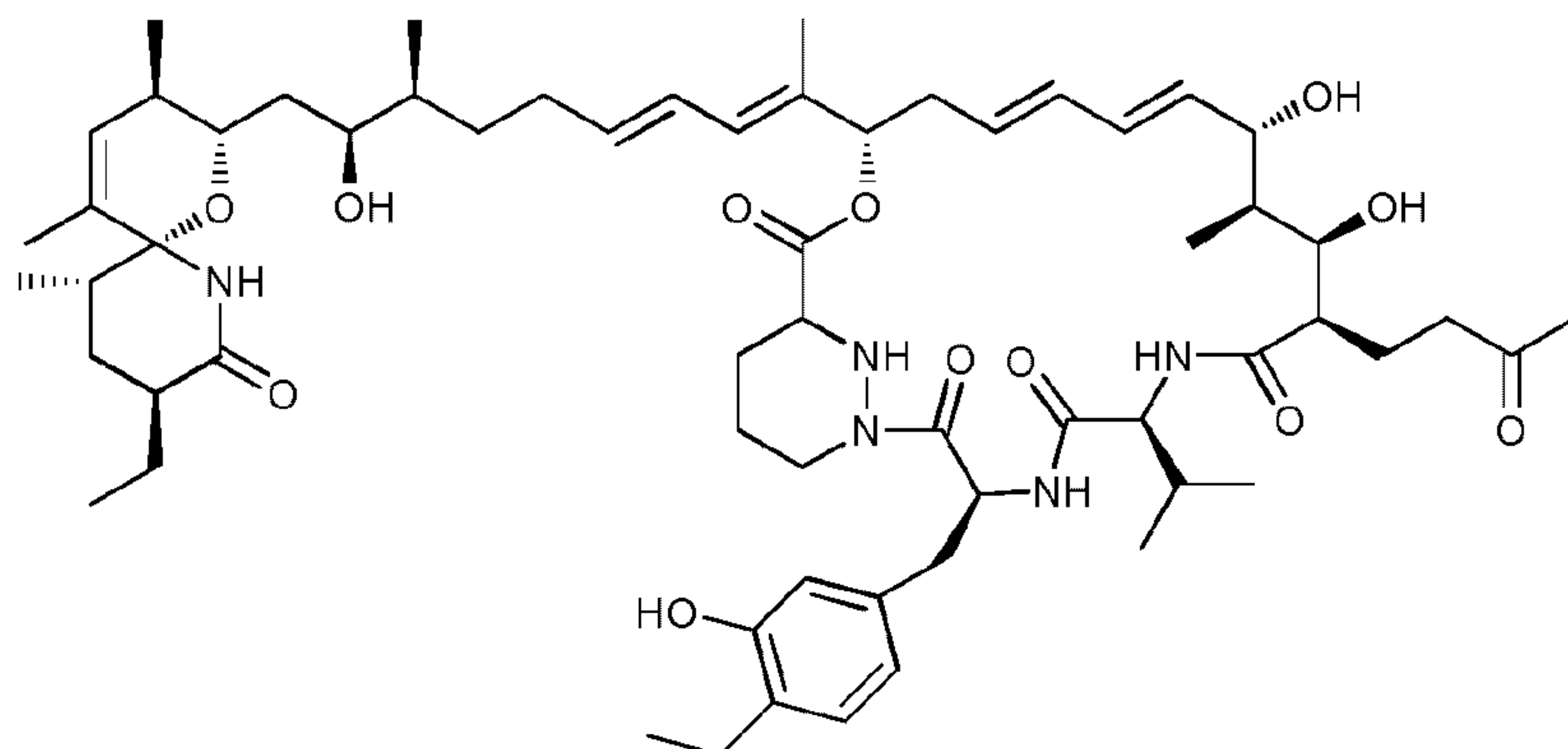


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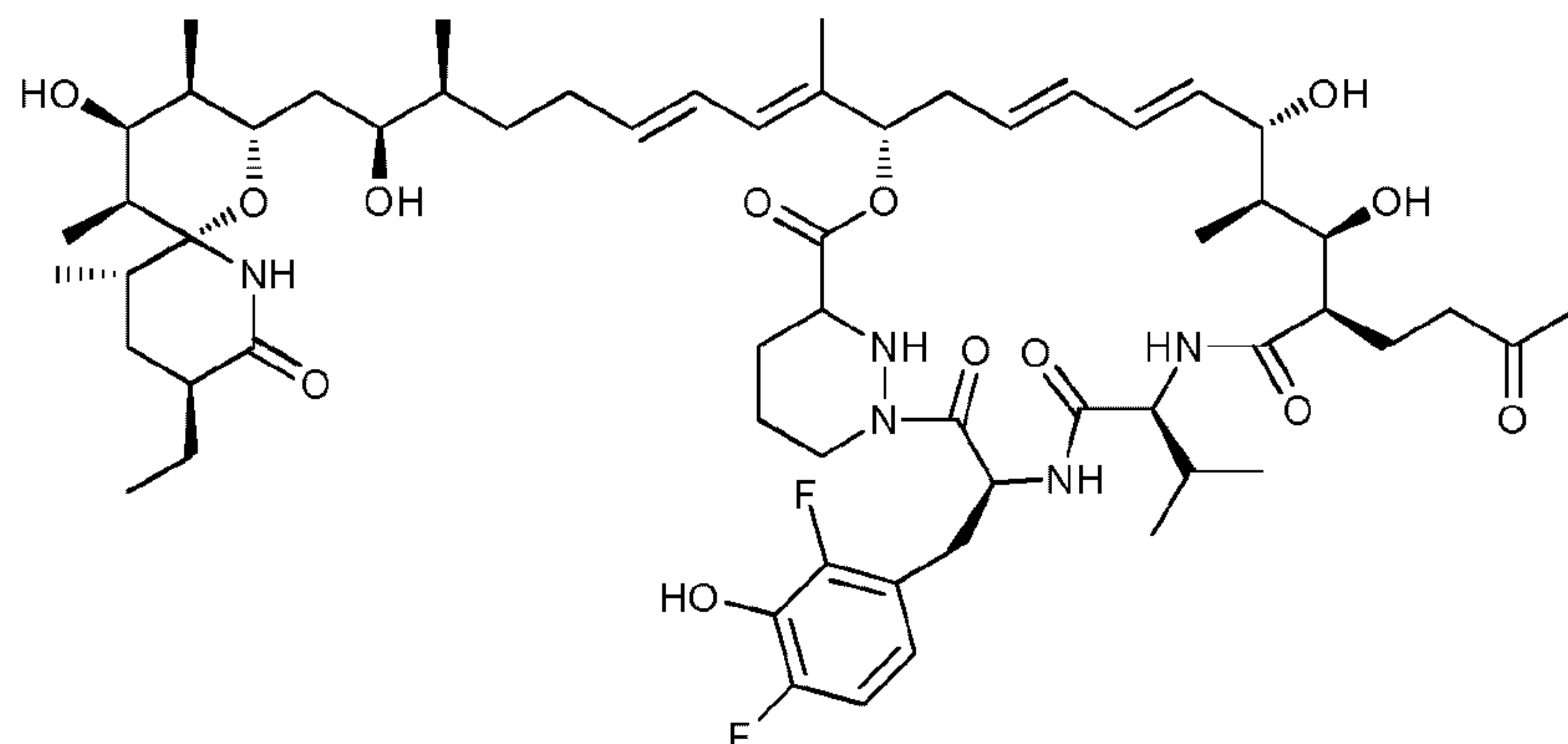
In a suitable embodiment of the invention, R_1 represents H, R_2 represents F, R_3 represents H, R_4 represents H, R_5 represents H and X_1, X_2, X_3, X_4 and X_5 represent C as represented by the following structure:



10 In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents Et, R_4 represents H, R_5 represents H and X_1, X_2, X_3, X_4 and X_5 represent C as represented by the following structure:

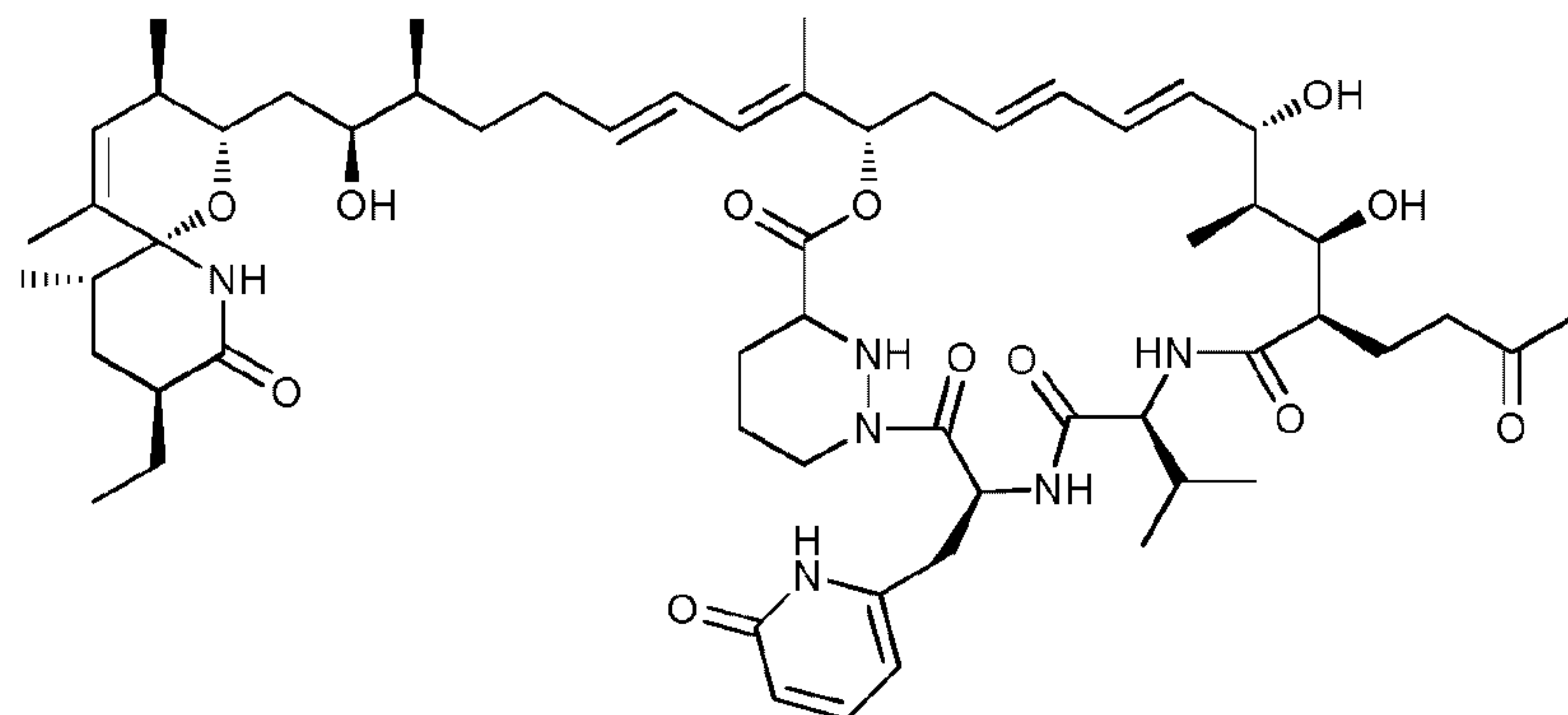


In a suitable embodiment of the invention, R_1 represents F, R_2 represents OH, R_3 represents F, R_4 represents H, R_5 represents H and X_1 , X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:

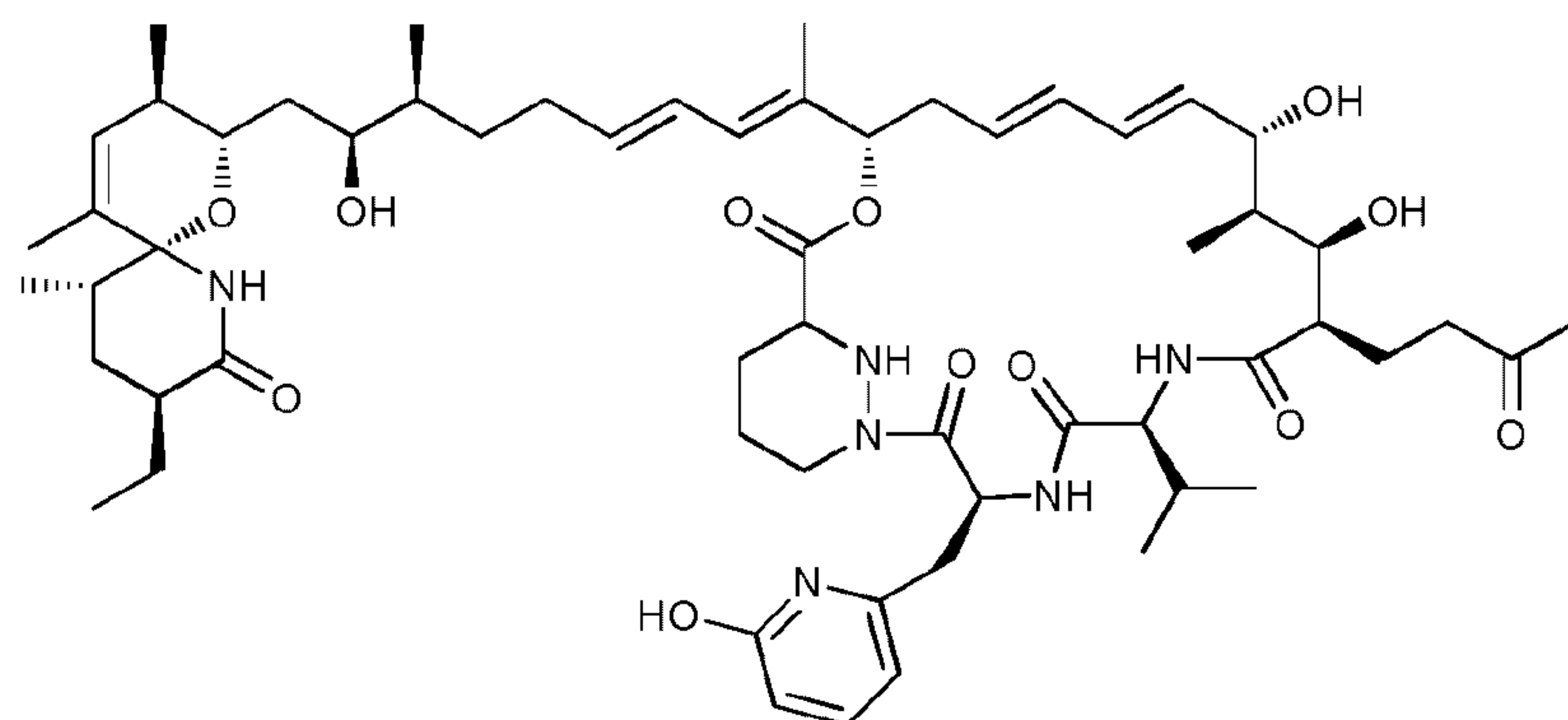


5

In a suitable embodiment of the invention, R_1 represents H, R_2 represents OH, R_3 represents H, R_4 represents F, R_5 represents H, X_1 represents N and X_2 , X_3 , X_4 and X_5 represent C as represented by the following structure:



10 which can also be represented as:

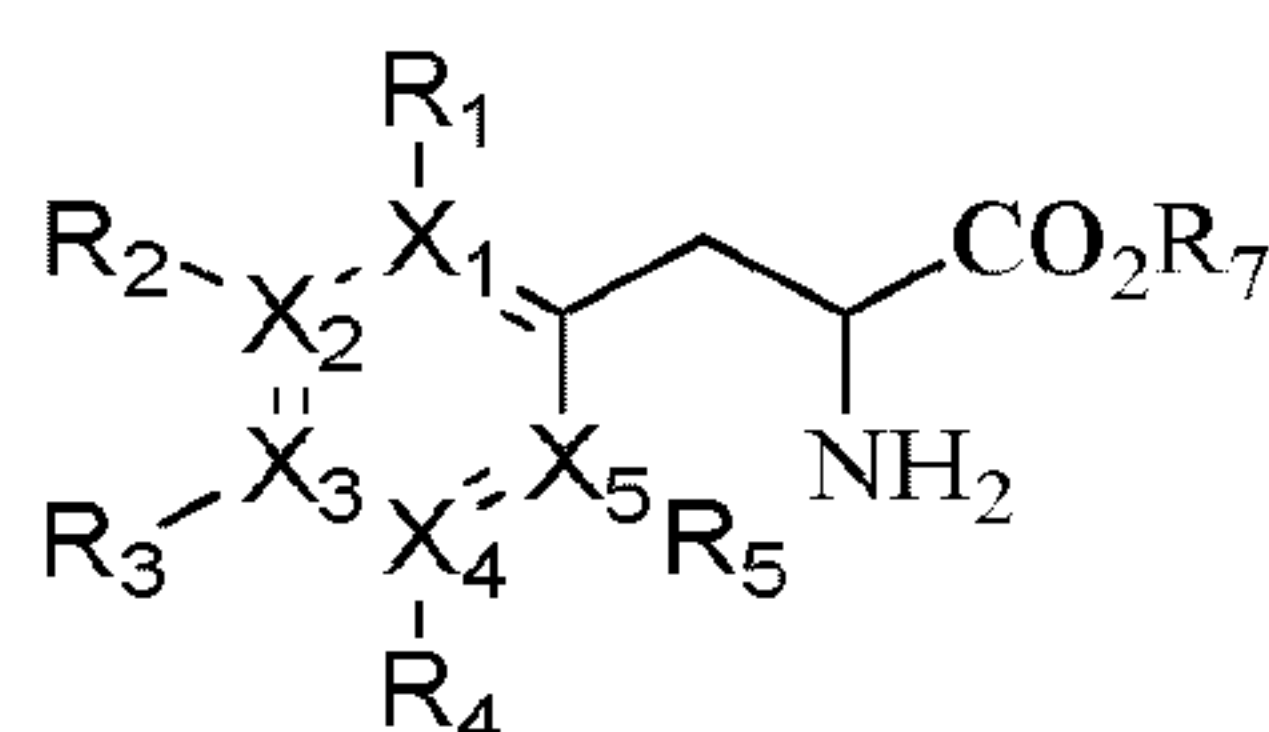


In some embodiments the double bond at the C26,27 position (by reference to the structure of sanglifehrin A) may be in the cis form instead of the trans form.

In general, the compounds of the invention are prepared by mutasynthesis.

In general, a process for preparing certain compounds of formula (I) or (II) or a pharmaceutically acceptable salt thereof comprises:

- Inoculating a fermentation broth with a culture of a sanglifehrin producer (such as *Streptomyces* sp. A92-308110 (also known as DSM 9954) or more preferably, a sanglifehrin producer with the *sfaA* gene or *sfaA* gene homologue inactivated or deleted;
- Feeding the fermentation broth with an meta-tyrosine analogue (as shown in formula (III))
- Allowing fermentation to continue until sanglifehrin analogues are produced
- Extracting and isolating the sanglifehrin analogue



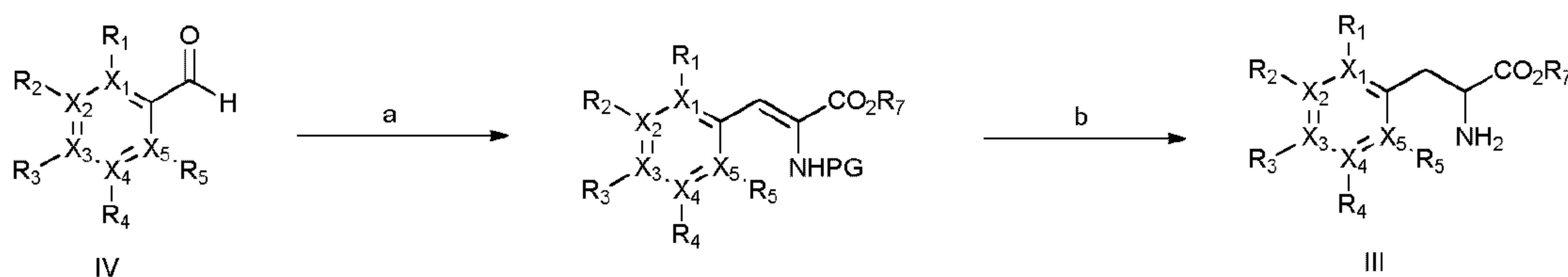
Formula (III)

- where R_7 represents H or an ester forming group such as an alkyl group, e.g. C_{1-6} alkyl such as Me.

Suitable X_1 , X_2 , X_3 , X_4 , X_5 , R_1 , R_2 , R_3 , R_4 and R_5 groups in Formula (III) are as defined for compounds of formula (I) and (II).

The feed may be racemic or the L-form of a compound of formula (III).

- Compounds of formula (III) are either commercially available or prepared by standard organic synthetic chemistry techniques. One generic route to compounds of formula (III) is as shown in the following scheme 1:.



Scheme 1: a) coupling aldehyde of formula (IV) with suitable fragment, e.g.

- $(R_7O)_2P(O)CH(NHPG)CO_2R_7$, b) hydrogenation and deprotection as necessary. PG = protecting group.

Aldehydes of formula (IV) may be commercially available or readily synthesised by one skilled in the art. Protection and deprotection chemistry may need to be employed in generating compounds of formula (III) from compounds of formula (IV). These techniques are known to one skilled in the art and suitable protecting groups are described in Greene's Protective
5 Groups in Organic Synthesis (Wuts and Greene, 4th Edition, 2007)

In addition to the specific methods and references provided herein a person of skill in the art may also consult standard textbook references for synthetic methods, including, but not limited to Vogel's Textbook of Practical Organic Chemistry (Furniss *et al.*, 1989) and March's Advanced Organic Chemistry (Smith and March, 2001).

10 A mutasynthetic sanglifehrin analogue according to the invention may be administered alone or in combination with other therapeutic agents. Co-administration of two (or more) agents may allow for lower doses of each to be used, thereby reducing side effect, can lead to improved potency and therefore higher SVR, and a reduction in resistance.

Therefore in one embodiment, the mutasynthetic sanglifehrin analogue is co-administered with
15 one or more therapeutic agent/s for the treatment of HCV infection, taken from the standard of care treatments. This could be an interferon (e.g. pIFN α and/or ribavirin).

In an alternative embodiment, a mutasynthetic sanglifehrin analogue is co-administered with one or more other anti-viral agents, such as a STAT-C/DAA (specifically targeted agent for treatment of HCV), which could be one or more of the following: Non-nucleoside Polymerase
20 inhibitors (e.g. IDX375, VCH-222, BI 207127, ANA598, VCH-916), Nucleoside or nucleotide polymerase inhibitors (e.g. 2'-C-methylcytidine, 2'-C-methyladenosine, R1479, PSI-6130, R7128, R1626), Protease inhibitors (e.g. BILN-2061, VX-950(Telaprevir), SCH503034(Boceprevir), TMC435350, MK-7009, R7227/ITMN-191, EA-058, EA-063) or viral entry inhibitors (e.g. PRO 206).

25 The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or
30 finely divided solid carriers or both, and then, if necessary, shaping the product.

The compounds of the invention will normally be administered orally in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration,
35 the compositions may be administered at varying doses.

For example, the compounds of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications.

- 5 Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycolate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and
10 acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds
15 of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the
20 active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g. sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with
25 an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a
30 predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

It should be understood that in addition to the ingredients particularly mentioned above the
35 formulations of this invention may include other agents conventional in the art having regard to

the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

Advantageously, agents such as preservatives and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the
5 water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use.

The dosage to be administered of a compound of the invention will vary according to the particular compound, the disease involved, the subject, and the nature and severity of the disease and the physical condition of the subject, and the selected route of administration. The
10 appropriate dosage can be readily determined by a person skilled in the art.

The compositions may contain from 0.1% by weight, preferably from 5-60%, more preferably from 10-30% by weight, of a compound of invention, depending on the method of administration.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual
15 dosages of a compound of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage may be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance
20 with normal clinical practice.

Further aspects of the invention include:

-A compound according to the invention for use as a pharmaceutical;

-A compound according to the invention for use as a pharmaceutical for the treatment of viral infections (especially RNA virus infections) such as HCV or HIV infection, for use as an anti-
25 inflammatory or for prophylaxis of organ transplant rejection;

-A pharmaceutical composition comprising a compound according to the invention together with a pharmaceutically acceptable diluent or carrier;

-A pharmaceutical composition comprising a compound according to the invention together with a pharmaceutically acceptable diluent or carrier further comprising a second or subsequent
30 active ingredient, especially an active ingredient indicated for the treatment of viral infections such as HCV or HIV infection, for use as an anti-inflammatory or for prophylaxis of organ transplant rejection;

-A method of treatment of viral infections (especially RNA virus infections) such as HCV or HIV infection, for use as an anti-inflammatory or for prophylaxis of organ transplant rejection which comprises administering to a subject a therapeutically effective amount of a compound according to the invention;

- 5 -Use of a compound according to the invention for the manufacture of a medicament for the treatment of viral infections such as HCV or HIV infection, for use as an anti-inflammatory or for prophylaxis of organ transplant rejection.

-A process for producing a mutasynthetic sanglifehrin (such as a compound of formula (I) or (II)) which comprises feeding a sanglifehrin producing bacterium, such as a *Streptomyces* sp (eg
10 A92-308110), a compound of formula (III) or a salt thereof, and culturing the bacterium such that a mutasynthetic sanglifehrin is produced.

-A process according to the previous paragraph wherein the sanglifehrin producing bacterium is a *Streptomyces* sp in which the *sfaA* gene or *sfaA* gene homologue is inactivated or deleted.

-A process according to the previous two paragraphs further comprising the step of isolating the
15 mutasynthetic sanglifehrin.

Novel compounds of formula (III) (such as those listed in Table 1 and the acids and esters of any of the compounds of formula (III) listed in Table 1) and (IV) including their salts and esters also form an aspect of the invention.

General Methods

20 Materials and Methods

Bacterial strains and growth conditions

The sanglifehrin producer *Streptomyces* sp. A92-308110 (DSM no 9954, purchased from DSMZ, Braunschweig, Germany) also termed BIOT-4253 and BIOT-4370 or its derivatives, such as BIOT-4585 are maintained on medium oatmeal agar, MAM, ISP4 or ISP2 (see below)
25 at 28 °C.

pKC1139, a standard *Streptomyces*-*Ecoli* shuttle plasmid, was obtained from the John Innes Centre, UK, and is described in Bierman et al., 1992 and Kieser et al., 2000.

BIOT-4585 was grown on oatmeal agar at 28 °C for 7-10 days. Spores from the surface of the agar plate were collected into 20% w/v sterile glycerol in distilled water and stored in 0.5 ml
30 aliquots at -80 °C. Frozen spore stock was used for inoculating seed media SGS or SM25-3. The inoculated seed medium was incubated with shaking between 200 and 300 rpm at 5.0 or 2.5 cm throw at 27 °C for 24 hours. The fermentation medium SGP-2 or BT6 were inoculated

with 2.5%-10% of the seed culture and incubated with shaking between 200 and 300 rpm with a 5 or 2.5 cm throw at 24 °C for 4-5 days. The culture was then harvested for extraction.

Meta-tyrosine analogues

Methyl (2*S*)-2-amino-3-(6-hydroxy(2-pyridyl))propanoate, L-3-aminophenylalanine methyl ester, L-4-methyl-*meta*-tyrosine methyl ester, L-4-fluoro-*meta*-tyrosine methyl ester and L-4,5-difluoro-*meta*-tyrosine methyl ester were purchased from Netchem (USA).

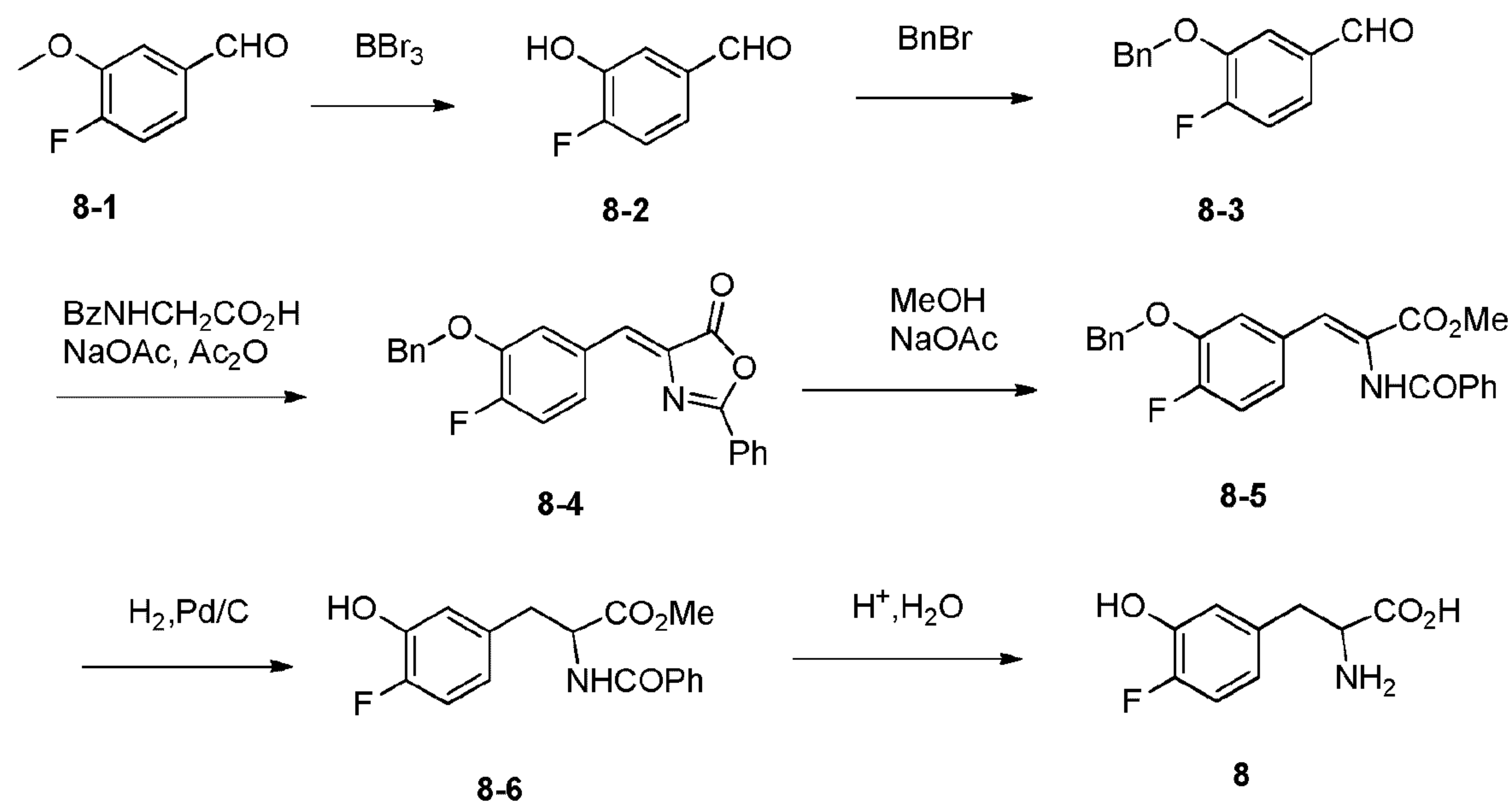
DL-3-fluorophenylalanine and L-phenylalanine were purchased from Sigma (UK).

DL-*meta*-tyrosine was purchased from Fluorochem (UK).

L-*meta*-tyrosine was purchased from Alfa Aesar (UK).

DL-4-fluorometa-tyrosine (**8**), DL-5-fluorometa-tyrosine (**9**), methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl)propanoate (**10**), methyl 2-amino-3-(2-fluoro-5-hydroxyphenyl)propanoate (**11**), methyl 2-amino-3-(2-fluoro-3-hydroxyphenyl)propanoate (**12**) and methyl 2-amino-3-(2,6-difluoro-3-hydroxyphenyl)propanoate (**13**) were synthesised as follows:

DL-4-fluoro meta-tyrosine (**8**)



To a solution of **8-1** (3 g, 19.5 mmol) in dry DCM (150 mL) was added dropwise BBr₃ (4 M in DCM, 14.6 ml, 58.5 mmol) at -70°C. After the addition, the reaction mixture was stirred at -20°C for 3 h, ice-water was added carefully, and extracted with DCM. The organic layers were washed with water and brine, dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatographed on silica to give the desired compound **8-2**.

To a solution of **8-2** (0.9 g, 6.4 mmol) in acetone (40 mL) was added K_2CO_3 (2.2 g, 16 mmol) at room temperature. The reaction mixture was stirred at room temperature overnight. Water was added and acetone was removed under vacuum, and then extracted with EtOAc, the organic layers were washed with water and brine, dried over Na_2SO_4 , filtered and concentrated. The residue was purified by flash chromatographed on silica to give the desired compound **8-3**.

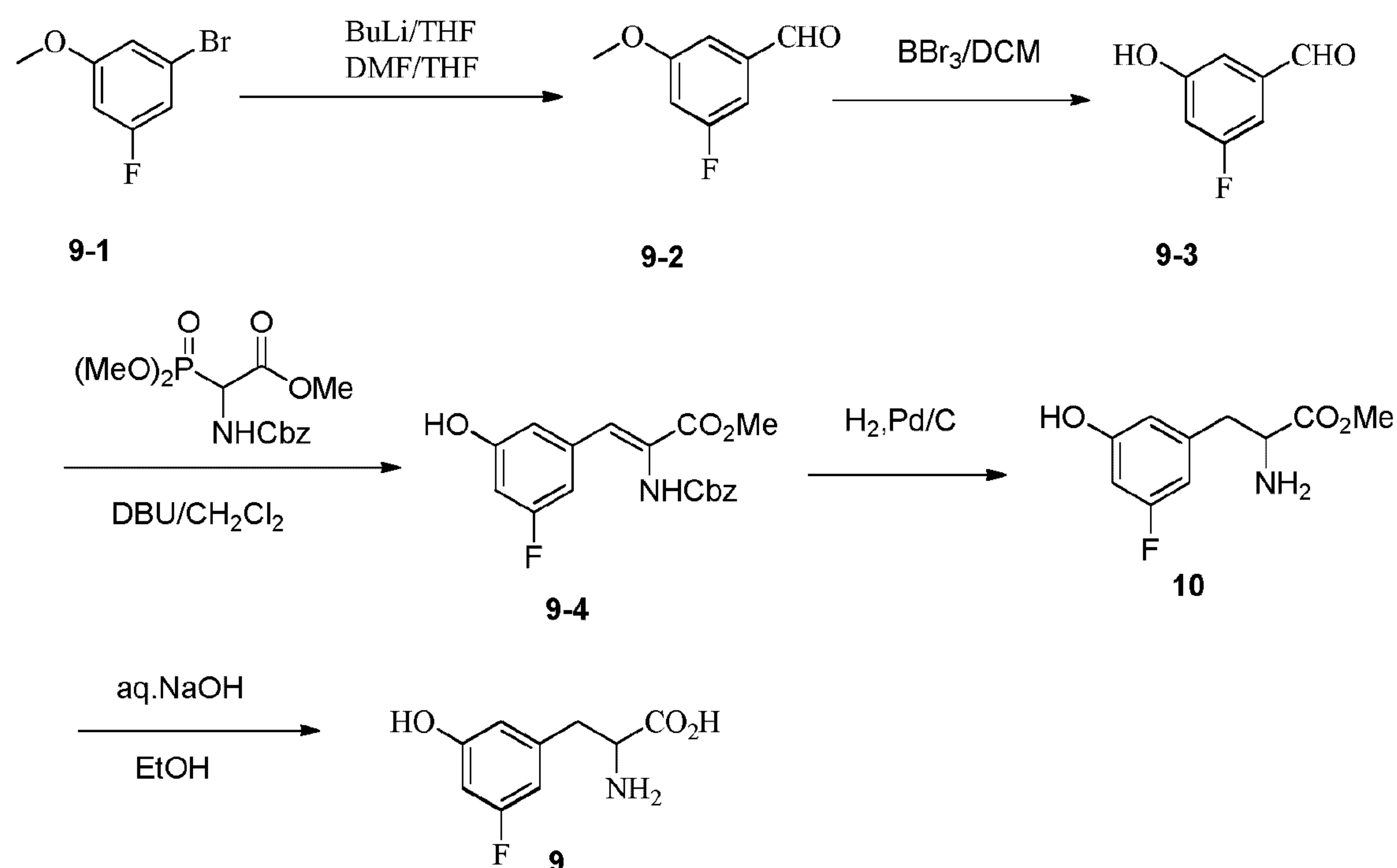
A mixture of **8-3** (1 g, 4.34 mmol), hippuric acid (860 mg, 4.80 mmol), NaOAc (400 mg) and Ac_2O (2.2 mL) was stirred at 80 °C for 2 h. The yellow reaction mixture was cooled and cold EtOH (10 mL) was added, the mixture was cooled in an ice bath for 15 min and then was poured into 30 mL of ice water, chilled and the product was collected by filtration. The solid was dried in vacuo to yield **8-4**.

A solution of **8-4** (300 mg, 0.8 mmol) and NaOAc (71 mg, 0.87 mmol) in MeOH (50 mL) was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the residue was dissolved in 50 mL of EtOAc, the EtOAc solution was washed two times with water and concentrated to give **8-5**.

A solution of **8-5** (360 mg, 0.89 mmol) in MeOH (50 mL) was hydrogenated over 10% Pd/C (77 mg) at normal pressure for 20 h. After removal of the catalyst by filtration, the solvent was evaporated to give the product **8-6**.

A solution of **8-6** (210 mg) in 3 N HCl (10 mL) was refluxed for 24 h. the solution was concentrated to dryness and the residue was purified by reverse-combiflash to give the target product **8**.

DL-5-fluoro meta-tyrosine (9) and methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl)propanoate (10)



To a solution of **9-1** (20 g, 97.55 mmol) in tetrahydrofuran (100 mL) was added dropwise n-butyl lithium (43 mL, 2.5 M, 107.3 mmol) at -78 °C. It was stirred for 30 minutes and N,N-dimethylformamide (15.1 mL, 195.1 mmol) was added at this temperature. It was stirred for another 30 minutes and the cold bath was removed. After 1 hour, the reaction was quenched with saturated aqueous ammonium chloride. The organic layer was washed with water and saturated aqueous sodium chloride, dried (sodium sulfate), filtered and concentrated. The residue was purified by chromatography on silica to give **9-2**.

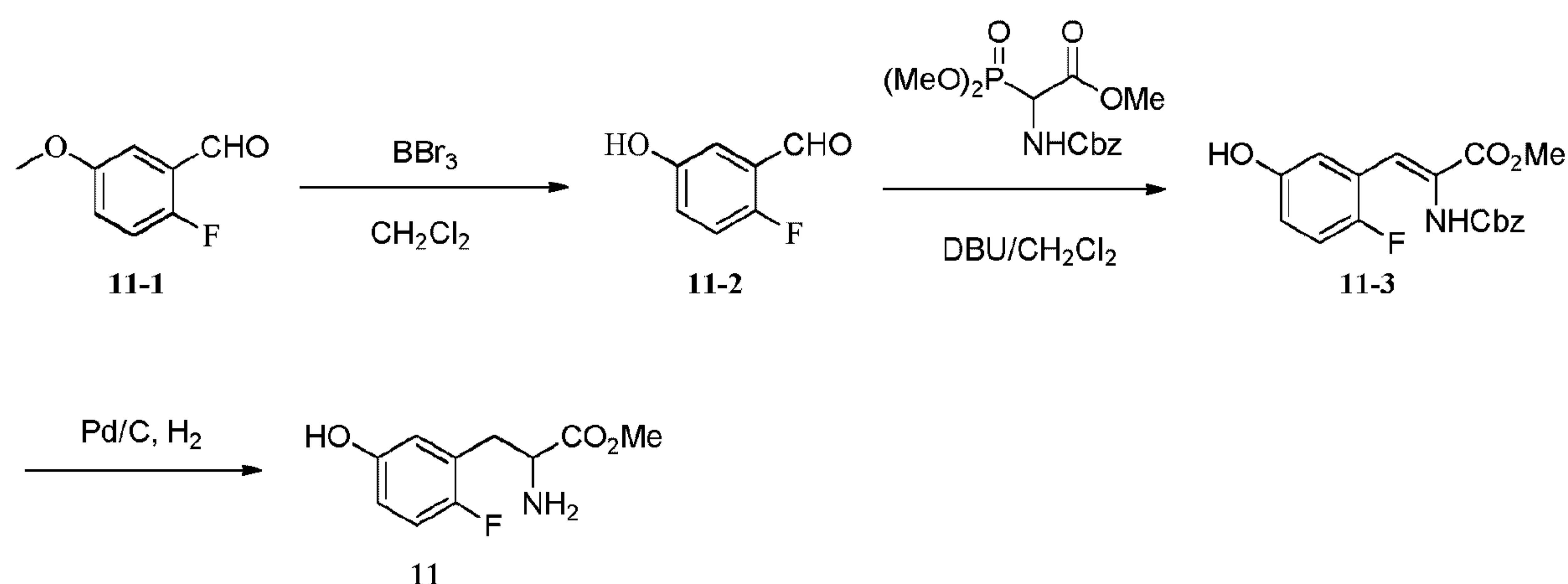
To a solution of **9-2** (6 g, 38.9 mmol) in dry DCM (200 mL) was added dropwise BBr₃ (4 M in DCM, 30 mL, 116.8 mmol) at -70 °C. After the addition, the reaction mixture was stirred at -20 °C for 3 hours, ice-water was added carefully, and extracted with DCM. The organic layers were washed with water and brine, dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatography on silica to give the desired compound **9-3**.

To a solution of methyl 2-(benzyloxycarbonylamino)-2-(dimethoxyphosphoryl)acetate (4.64 g, 14 mmol) in DCM (150 mL) was added DBU (4.26 g, 28 mmol) at room temperature. After 10 min, **9-3** (1.95 g, 14 mmol) was added and the resulting mixture was stirred at room temperature overnight. The solution was diluted with EtOAc (150 mL), separated and the organic layer was washed with 1 N HCl, dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatography on silica to give **9-4**.

A solution of **9-4** (1 g) in MeOH (20 mL) was hydrogenated over 200 mg of 10% Pd/C at normal pressure overnight. After removal of the catalyst by filtration, the solvent was evaporated to give **10**.

To a solution of **10** (300 mg, 1.4 mmol) in EtOH (30 mL) was added aq. NaOH (2 N, 4 mL), the reaction was stirred at room temperature for 30 minutes. The solvent was removed and the residue was neutralized to pH=6 with 2 N HCl and the white crystals that formed were collected by filtration to give the target compound **9**.

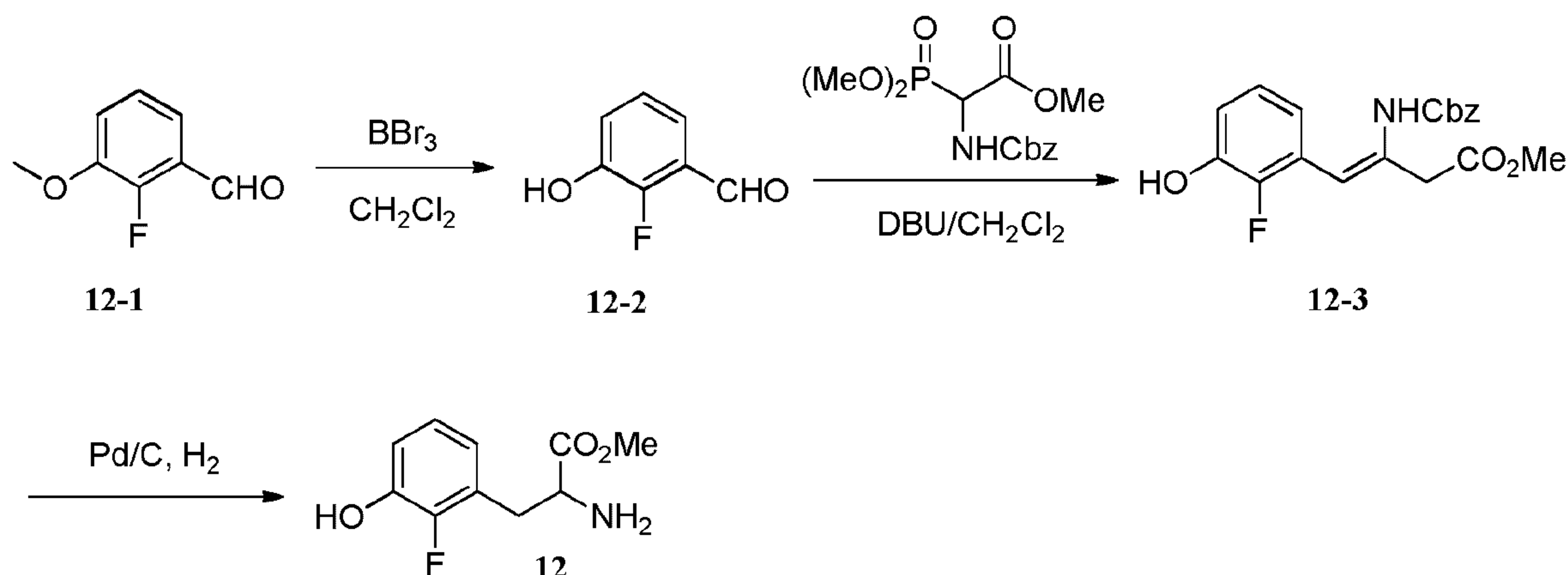
methyl 2-amino-3-(2-fluoro-5-hydroxyphenyl)propanoate (11)



To a solution of the compound **11-1** (1.4 g, 9 mmol) in 50 mL DCM was added dropwise BBr_3 (4M in DCM, 3.6 mL, 13.5 mmol) at -78°C . After the addition, the reaction was stirred at -20°C for 4 hours. Then slow addition of ice/water, the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated to dryness. The residue was used to next step without further purification.

To a solution of methyl 2-(benzyloxycarbonylamino)-2-(dimethoxyphosphoryl)acetate (3 g, 9 mmol) in 100 mL DCM was added DBU (2.8 g, 18 mmol) at room temperature, after 10 mins, the compound **11-2** (crude compound from last step) was added, stirred at room temperature for 2 hours. The solution was then diluted with DCM (50 mL), washed with 1N HCl (20 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate=5/1) to give **11-3**.

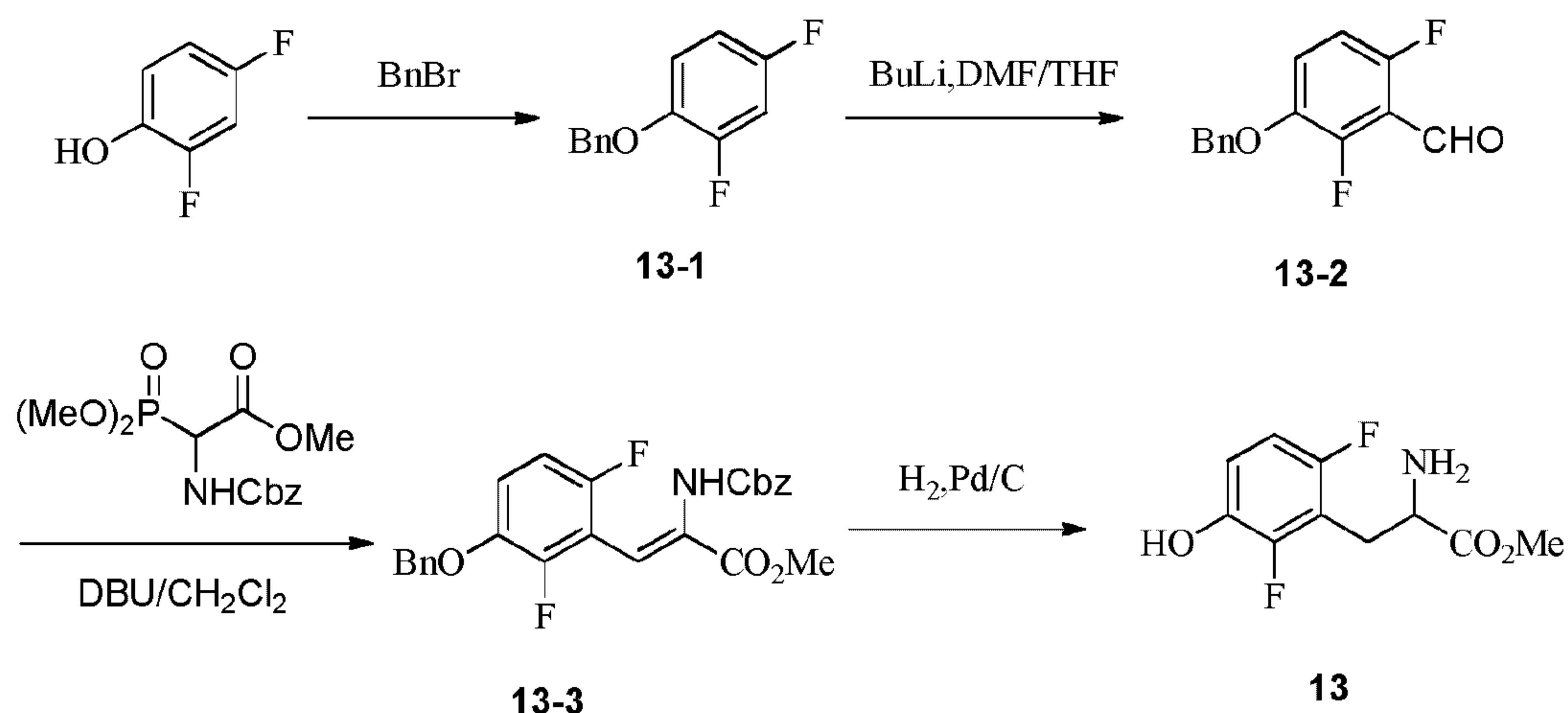
A mixture of the compound **11-3** (500 mg, 1.5 mmol) in MeOH (20 mL) was hydrogenated over 50 mg of 10% Pd/C at normal pressure overnight. After removal of the catalyst by filtration, the solvent was evaporated to get the crude product, which was purified by reverse-combiflash to get **11** as a white solid.

methyl 2-amino-3-(2-fluoro-3-hydroxyphenyl)propanoate (12)

To a solution of the compound **12-1** (1.4 g, 9 mmol) in 50 mL DCM was added dropwise BBr_3 (4M in DCM, 3.6 mL, 13.5 mmol) at -78°C . After the addition, the reaction was stirred at -20°C for 4 hours. After slow addition of ice/water, the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated to dryness. The residue was used to next step without further purification.

To a solution of methyl 2-(benzyloxycarbonylamino)-2-(dimethoxyphosphoryl)acetate (3 g, 9 mmol) in 100 mL DCM was added DBU (2.7 mL, 18 mmol) at room temperature, after 10mins, the compound **12-2** (crude compound from last step) was added, stirred at room temperature for 2 hours. The solution was then diluted with DCM (100 mL), washed with 1N HCl (30 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate =5/1) to give **12-3**.

A mixture of the compound **12-3** (500 mg, 1.44 mmol) in MeOH (10 mL) was hydrogenated over 100 mg of 10% Pd/C at normal pressure overnight. After removal of the catalyst by filtration, the solvent was evaporated to get the crude product, which was purified by reverse-combiflash to get the desired compound **12** as a white solid.

methyl 2-amino-3-(2,6-difluoro-3-hydroxyphenyl)propanoate (13)

To a solution of 2,4-difluorophenol (2 g, 15.4 mmol) in 50 mL DMF was added K_2CO_3 (3.2 g, 23.1 mmol) and BnBr (2.2 mL, 18.5 mmol) at 0 °C. The reaction was stirred at room temperature for 2 hours. Water (100 mL) and EA (200 mL) was added, the organic layers was washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate =10/1) to give the crude **13-1**.

To a solution of the compound **13-1** (2 g, 9 mmol) in 10 mL THF was added dropwise n-BuLi (4 mL, 2.5 M) at -78 °C and stirred for 30 mins. DMF (1.3 g, 0.018 mmol) was added and stirred for 30 mins again. The cold bath was then removed and the reaction mixture was stirred at room temperature for 1 hour before being quenched with water. It was extracted with ethyl acetate (20 mL x3), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate =10/1) to give **13-2** as a yellow solid.

To a solution of methyl 2-(benzyloxycarbonylamino)-2-(dimethoxyphosphoryl)acetate (728 mg, 2.2 mmol) in 20 mL DCM was added DBU (319 mg, 2.1 mmol) at room temperature. After 10 mins, the compound **13-2** (500 mg, 2 mmol) was added and stirred at room temperature for 2 hours. The solution was then diluted with DCM (50 mL), washed with 1N HCl (20 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (petroleum ether/ethyl acetate =5/1) to give **13-3** as a yellow oil.

The compound **13-3** (600 mg, 1.32 mmol) in MeOH (20mL) was hydrogenated over 60 mg of 10% Pd/C at normal pressure overnight. After removal of the catalyst by filtration, the solvent was evaporated to get the crude product, which was purified by reverse-combiflash to get the desired compound **13** as a white solid.

Media Recipes

Water used for preparing media was prepared using Millipore™ Elix Analytical Grade Water Purification System

SGS Seed Medium

Ingredient (and supplier)	Recipe	
Glucose (Sigma, G7021)	7.50	g
Glycerol (Fisher scientific, G/0650/25)	7.50	g
yeast extract (Becton Dickinson, 212770)	1.35	g
malt extract (Becton Dickinson, 218630)	3.75	g
potato starch (soluble) (Sigma, S2004)	7.50	g
NZ-amine A (Sigma, C0626)	2.50	g
toasted soy flour, Nutrisoy™ (ADM, 063-160)	2.50	g
L-asparagine (Sigma, A0884)	1.00	g
CaCO ₃ (Calcitec, V/40S)	0.05	g
NaCl (Fisher scientific, S/3160/65)	0.05	g
KH ₂ PO ₄ (Sigma, P3786)	0.25	g
K ₂ HPO ₄ (Sigma, P5379)	0.50	g
MgSO ₄ ·7H ₂ O (Sigma, M7774)	0.10	g
trace element solution B	1.00	mL
agar	1.00	g
SAG471 Antifoam (GE Silicones, SAG471)	* 0.20	mL
RO H ₂ O	to final vol. of	** 1.00 L

pre-sterilisation pH was adjusted to pH 7.0 with 10M NaOH/10M H₂SO₄

sterilised by heating 121°C, 20-30 min (autoclaving)

*Notes***antifoam only used in seed fermenters, NOT seed flasks****final volume adjusted accordingly to account for seed volume***Trace Element Solution B**

Ingredient	Recipe	
FeSO ₄ ·7H ₂ O (Sigma, F8633)	5.00	g
ZnSO ₄ ·7H ₂ O (Sigma, Z0251)	4.00	g
MnCl ₂ ·4H ₂ O (Sigma, M8530)	2.00	g
CuSO ₄ ·5H ₂ O (Aldrich, 20,919-8)	0.20	g
(NH ₄) ₆ Mo ₇ O ₂₄ (Fisher scientific, A/5720/48)	0.20	g
CoCl ₂ ·6H ₂ O (Sigma, C2644)	0.10	g
H ₃ BO ₃ (Sigma, B6768)	0.10	g
KI (Alfa Aesar, A12704)	0.05	g
H ₂ SO ₄ (95%) (Fluka, 84720)	1.00	mL
RO H ₂ O <i>to final vol. of</i>	1.00	L

SGP2 Production Medium

Ingredient	Recipe	
toasted soy flour (Nutrisoy™) (ADM, 063-160)	20.00	g
Glycerol (Fisher scientific, G/0650/25)	40.00	g
MES buffer (Acros, 172595000)	19.52	g
SAG471 Antifoam (GE Silicones, SAG471)	*0.20	mL
RO H ₂ O <i>to final vol. of</i>	**1.00	L

pre-sterilisation pH adjusted to pH 6.8 with 10M NaOH

sterilised by heating 121°C, 20-30 min (autoclaving)

Notes

**final volume adjusted accordingly to account for seed volume*

*** antifoam was used only in fermentors not flasks*

SM25-3 Medium

Ingredient

Glycerol (Fisher scientific, G/0650/25)	40g
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Soy Peptone A3 SC (Organotechnie)	10g
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Malt extract (Difco)	21g
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to final vol. of	1L
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pre-sterilisation pH not adjusted (i.e. pH 7.0)

ISP4 Medium

Ingredient

Soluble Starch (Difco)	10g
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K ₂ HPO ₄	1g
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MgSO ₄ ·7H ₂ O	1g
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NaCl	1g
------	----

(NH ₄) ₂ SO ₄	2g
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CaCO ₃	2g
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ISP Trace Salts Solution	1mL
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Agar	20g
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to final vol. of	1L
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Make a paste with the starch in a small volume of cold

water and bring to volume of 500ml

Add other ingredients to solution II in 500mls water pH should be between pH 7.0 and pH 7.4 (pH 7.3) Mix two solutions together and add agar

ISP Trace Salts

Ingredient

FeSO ₄ ·7H ₂ O	1g
MnCl ₂ ·4H ₂ O	1g
ZnSO ₄ ·7H ₂ O	1g
to final vol. of	1L

Store at 4 degrees C

General Fermentation Method

Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 4.0mL of spore stock into 400mL medium SM25 in 2L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 27°C and 250rpm (5.0cm throw). From the seed culture 25mL was transferred into 250mL production medium SGP2+5%HP20 in 2L Erlenmeyer flasks with foam plug. After 24 hours cultivation at 24°C and 250rpm (2.5cm throw), 2mL of a 250mM racemic or 125mM enantiomerically pure solution of the desired precursor in 1M hydrochloric acid and 2mL of a 250mM methanolic solution of DL-piperazic acid was added to each production flask to give a final 1mM concentration of the individual enantiomers of the precursors. Cultivation was continued for further four days at 24°C and 250rpm (2.5cm throw).

Analysis of culture broths by LC-UV and LC-UV-MS

Culture broth (1 mL) and ethyl acetate (1 mL) is added and mixed for 15-30 min followed by centrifugation for 10 min. 0.4 mL of the organic layer is collected, evaporated to dryness and then re-dissolved in 0.20 mL of acetonitrile.

HPLC conditions:

C18 Hyperclone™ BDS C18 Column 3u, 4.6 mm x 150 mm

Fitted with a Phenomenex™ Analytical C18 Security Guard Cartridge (KJ0-4282)

Column temp at 50 °C

Flow rate 1 mL/min

Monitor UV at 240 nm

Inject 20 uL aliquot

5 Solvent gradient:

0 min: 55% B

1.0 min: 55% B

6.5 min: 100% B

10.0 min: 100% B

10 10.05 min: 55% B

13.0 min: 55% B

Solvent A is Water + 0.1% Formic Acid

Solvent B is Acetonitrile + 0.1% Formic Acid

Under these conditions SfA elutes at 5.5 min

15 Under these conditions SfB elutes at 6.5 min

LCMS is performed on an integrated Agilent™ HP1100 HPLC system in combination with a Bruker™ Daltonics Esquire 3000+ electrospray mass spectrometer operating in positive ion mode using the chromatography and solvents described above.

QC LC-MS method

20 HPLC conditions:

C18 Hyperclone™ BDS C18 Column 3u, 4.6 mm x 150 mm

Fitted with a Phenomenex™ Analytical C18 Security Guard Cartridge (KJ0-4282)

Column temp at 50 °C

Flow rate 1 mL/min

25 Monitor UV at 210, 240 and 254 nm

Solvent gradient:

0 min: 10% B

2.0 min: 10% B

15 min: 100% B

17 min: 100% B

17.05 min: 10% B

20 min: 10% B

5 Solvent A is Water + 0.1% Formic Acid

Solvent B is Acetonitrile + 0.1% Formic Acid

MS conditions:

MS operates in switching mode (switching between positive and negative), scanning from 150 to 1500 amu.

10 *In vitro* analysis LC-MS method (e.g. for microsome stability assessment)

Using an API-2000, API-4000 or UPLC instrument

HPLC conditions:

For **15**: ACQUITY UPLC BEH C18 (2.1×50 mm, 1.7 µm)

For cpds **5, 14, 16, 17, 18, 19** Ultimate XB-C18 (2.1×50 mm, 5 µm) Column temp at 50 °C

15 Flow rate 0.6 mL/min

Solvent gradient A1 (e.g. for cpd **15**):

0.2 min: 20% B

0.6 min: 95% B

1.1 min: 95% B

20 1.15 min: 20% B

1.5 min: stop

Solvent A is H₂O-0.025% FA- 1 mM NH₄OAC

Solvent B is ACN-0.025% FA- 1 mM NH₄OAC

Solvent gradient A2 (e.g. for cpds **5, 14, 16, 17, 18, 19**):

25 0.3 min: 10% B

0.8 min: 95% B

2.3 min: 95% B

2.31 min: 10% B

3.5 min: stop

Solvent A is H₂O-0.1% FA

Solvent B is MeOH-0.1% FA

negative scan mode:

5 *MRM setup:*

transitions [Da]

21 1089.7 → 504.2

positive scan mode:

10 *MRM setup:*

transitions [Da]

5 1090.6 → 1054.6

14 1108.9 → 1072.5

15 1126.7 → 1090.0

15 **16** 1108.8 → 1072.3

17 1104.5 → 1068.5

18 1074.6 → 1038.8

In vitro replicon assay for assessment of HCV antiviral activity

20 Antiviral efficacy against genotype 1 HCV may be tested as follows: One day before addition of the test article, Huh5.2 cells, containing the HCV genotype 1b I389luc-ubi-neo/NS3-3'/5.1 replicon (Vrolijk et al., 2003) and subcultured in cell growth medium [DMEM (Cat No. 41965039) supplemented with 10% FCS, 1% non-essential amino acids (11140035), 1% penicillin/streptomycin (15140148) and 2% Geneticin (10131027); Invitrogen] at a ratio of 1.3-
25 1.4 and grown for 3-4 days in 75mL tissue culture flasks (Techno Plastic Products), were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6500 cells/well (100uL/well) in 96-well tissue culture microtitre plates (Falcon, Beckton Dickinson for evaluation of the anti-metabolic effect and CulturPlate, Perkin Elmer for evaluation of antiviral effect). The microtitre plates are incubated
30 overnight (37°C, 5% CO₂, 95-99% relative humidity), yielding a non-confluent cell monolayer.

Dilution series are prepared; each dilution series is performed in at least duplicate. Following assay setup, the microtitre plates are incubated for 72 hours (37°C, 5% CO₂, 95-99% relative humidity).

For the evaluation of anti-metabolic effects, the assay medium is aspirated, replaced with 75uL of a 5% MTS (Promega) solution in phenol red-free medium and incubated for 1.5 hours (37°C, 5% CO₂, 95-99% relative humidity). Absorbance is measured at a wavelength of 498nm (Safire², Tecan) and optical densities (OD values) are converted to percentage of untreated controls.

For the evaluation of antiviral effects, assay medium is aspirated and the cell monolayers are washed with PBS. The wash buffer is aspirated, 25uL of Glo Lysis Buffer (Cat. N°. E2661, Promega) is added after which lysis is allowed to proceed for 5min at room temperature. Subsequently, 50uL of Luciferase Assay System (Cat. N°. E1501, Promega) is added and the luciferase luminescence signal is quantified immediately (1000ms integration time/well, Safire², Tecan). Relative luminescence units are converted to percentage of untreated controls.

The EC₅₀ and EC₉₀ (values derived from the dose-response curve) represent the concentrations at which respectively 50% and 90% inhibition of viral replication would be observed. The CC₅₀ (value derived from the dose-response curve) represents the concentration at which the metabolic activity of the cells would be reduced to 50 % of the metabolic activity of untreated cells. The selectivity index (SI), indicative of the therapeutic window of the compound, is calculated as CC₅₀/EC₅₀.

A concentration of compound is considered to elicit a genuine antiviral effect in the HCV replicon system when, at that particular concentration, the anti-replicon effect is above the 70% threshold and no more than 30% reduction in metabolic activity is observed.

For results see Example 12.

In vitro replicon assay for assessment of HCV antiviral activity in genotypes 1a and 2a

The replicon cells (subgenomic replicons of genotype 1a (H77) and 2a (JFH-1)) are grown in Dulbecco's modified essential media (DMEM), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (pen-strep), 1% glutamine, 1% non-essential amino acids, 250 µg/ml G418 in a 5% CO₂ incubator at 37°C. All cell culture reagents may be purchased from Mediatech (Herndon, VA).

The replicon cells are trypsinized and seeded at 5 x 10³ cells per well in 96-well plates with the above media without G418. On the following day, the culture medium is replaced with DMEM containing compounds serially diluted in the presence of 5% FBS. The HCV replicon antiviral assay examines the effects of compounds in a serial of compound dilutions. Briefly, the cells

containing the HCV replicon are seeded into 96-well plates. Test article is serially diluted with DMEM plus 5% FBS. The diluted compound is applied to appropriate wells in the plate. After 72 hr incubation at 37°C, the cells are processed. The intracellular RNA from each well is extracted with an RNeasy 96 kit (Qiagen). The level of HCV RNA is determined by a reverse transcriptase-real time PCR assay using TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) and an ABI Prism 7900 sequence detection system (Applied Biosystems) as described previously (Vrolijk et al., 2003). The cytotoxic effects are measured with TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems) as an indication of cell numbers. The amount of the HCV RNA and ribosomal RNA is then used to derive applicable IC₅₀ values (concentration inhibiting on replicon replication by 50%).

Assessment of microsome metabolism (microsome stability assay)

Rate of metabolism in microsomes may be tested as follows:

Mouse or human liver microsomes were diluted with buffer C (0.1 M Potassium Phosphate buffer, 1.0 mM EDTA, pH 7.4) to a concentration of 2.5 mg/mL. Microsomal stability samples were then prepared by adding 50 uL of 5 uM compound spiking solution (0.5 uL 10 mM DMSO stock solution in 9.5 uL ACN, added to 990 uL Buffer C) to 50 uL of microsomal solution (2.5 mg/mL), 110 uL Buffer C and mixed well. All samples were pre-incubated for approximately 15 minutes at 37°C. Following this, the reaction was initiated by adding 40 uL of the NADPH solution (12.5 mM) with gentle mixing. Aliquots (40 uL) were removed at 0, 15, 30, 45 and 60 minutes and quenched with ACN containing internal standard (120 uL). Protein was removed by centrifugation (4000 rpm, 15 min) and the sample plate analysed for compound concentration by LC-MS/MS. Half-lives were then calculated by standard methods, comparing the concentration of analyte with the amount originally present.

For results see Example 13.

Assessment of hepatocyte stability

Cryopreserved hepatocytes, previously stored in liquid nitrogen are placed in a 37 ± 1°C shaking water bath for 2 min ± 15 sec. The hepatocytes are then added to 10X volume of pre-warmed Krebs-Henseleit bicarbonate (KHB) buffer (2000mg/L glucose, no calcium carbonate and sodium bicarbonate, Sigma), mixed gently and centrifuged at 500 rpm for 3 minutes. After centrifugation, the supernatant is carefully removed and a 10X volume of pre-warmed KHB buffer added to resuspend the cell pellet. This is mixed gently and centrifuged at 500 rpm for 3 minutes. The supernatant is then removed and discarded. The cell viability and yield are then determined by cell counts, and these values used to generate human hepatocyte suspensions to the appropriate seeding density (viable cell density = 2 × 10⁶ cells/mL). A 2X dosing solution is prepared in pre-warmed KHB (1% DMSO) (200 uM spiking solution: 20 uL of substrate stock

solution (10 mM) in 980 uL of DMSO, 2X dosing solution: 10 uL of 200 uM spiking solution in 990 uL of KHB (2uM after dilution).

50 uL of pre-warmed 2X dosing solution is added to the wells and 50 uL of pre-warmed hepatocyte solution (2 × 10⁶ cells/mL) added and timing started. The plate is then incubated at 37°C. 100 uL of acetonitrile containing internal standard is added to each the wells after completion of incubation time (0, 15, 30, 60 and 120 minutes) mixed gently, and 50 uL of pre-warmed hepatocyte solution added (2 × 10⁶ cells/mL). At the end of the incubation, cell viability is determined. Samples are centrifuged at 4000 rpm for 15 minutes at 4°C, supernatants diluted 2-fold with ultrapure water and compound levels analysed by LC-MS/MS.

Assessment of water solubility

Water solubility may be tested as follows: A 10 mM stock solution of the sanglifehrin analogue is prepared in 100% DMSO at room temperature. Triplicate 0.01 mL aliquots are made up to 0.5 mL with either 0.1 M PBS, pH 7.3 solution or 100% DMSO in amber vials. The resulting 0.2 mM solutions are shaken, at room temperature on an IKA® vibrax VXR shaker for 6 h, followed by transfer of the resulting solutions or suspensions into 2 mL Eppendorf tubes and centrifugation for 30 min at 13200 rpm. Aliquots of the supernatant fluid are then analysed by the LCMS method as described above.

Alternatively, solubility in PBS at pH7.4 may be tested as follows: A calibration curve is generated by diluting the test compounds and control compounds to 40uM, 16uM, 4uM, 1.6uM, 0.4uM, 0.16uM, 0.04uM and 0.002uM, with 50% MeOH in H₂O. The standard points are then further diluted 1:20 in MeOH:PBS 1:1. The final concentrations after 1:20 dilution are 2000nM, 800nM, 200nM, 80nM, 20nM, 8nM, 2nM and 1nM. Standards are then mixed with the same volume (1:1) of ACN containing internal standard (hydroxymacrocycle, **6**). The samples are centrifuged (5min, 12000rpm), then analysed by LC/MS.

	Solution(uL)	MeOH/ H ₂ O(1:1) (uL)		Working solution (uM)	Solution(uL)	MeOH/buffer (1:1) (uL)		Final solution(nM)
10mM	10	240	→	400				
400uM	50	450	→	40	20	380	→	2000
	20	480	→	16	20	380	→	800
40uM	50	450	→	4	20	380	→	200
16uM	50	450	→	1.6	20	380	→	80

4uM	50	450	→	0.4	20	380	→	20
1.6uM	50	450	→	0.16	20	380	→	8
0.4uM	50	450	→	0.04	20	380	→	2
0.04uM	50	950	→	0.002	20	380	→	1

Test compounds are prepared as stock solutions in DMSO at 10mM concentration. The stock solutions are diluted in duplicate into PBS, pH7.4 in 1.5mL Eppendorf tubes to a target concentration of 100uM with a final DMSO concentration of 1% (e.g. 4uL of 10mM DMSO stock solution into 396uL 100mM phosphate buffer). Sample tubes are then gently shaken for 4 hours at room temperature. Samples are centrifuged (10min, 15000rpm) to precipitate undissolved particles. Supernatants are transferred into new tubes and diluted (the dilution factor for the individual test article is confirmed by the signal level of the compound on the applied analytical instrument) with PBS. Diluted samples are then mixed with the same volume (1:1) of MeOH. Samples are finally mixed with the same volume (1:1) of ACN containing internal standard (hydroxymacrocycle, **6**) for LC-MS/MS analysis.

Assessment of cell permeability

Cell permeability may be tested as follows: The test compound is dissolved to 10mM in DMSO and then diluted further in buffer to produce a final 10uM dosing concentration. The fluorescence marker lucifer yellow is also included to monitor membrane integrity. Test compound is then applied to the apical surface of Caco-2 cell monolayers and compound permeation into the basolateral compartment is measured. This is performed in the reverse direction (basolateral to apical) to investigate active transport. LC-MS/MS is used to quantify levels of both the test and standard control compounds (such as Propanolol and Acebutolol).

In vivo assessment of pharmacokinetics

In vivo assays may also be used to measure the bioavailability of a compound. Generally, a compound is administered to a test animal (e.g. mouse or rat) both intravenously (i.v.) and orally (p.o.) and blood samples are taken at regular intervals to examine how the plasma concentration of the drug varies over time. The time course of plasma concentration over time can be used to calculate the absolute bioavailability of the compound as a percentage using standard models. An example of a typical protocol is described below.

Mice are dosed with 1, 10, or 100 mg/kg of the compound of the invention or the parent compound i.v. or p.o.. Blood samples are taken at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 420 and 2880 minutes and the concentration of the compound of the invention or parent compound in the sample is determined via HPLC. The time-course of plasma concentrations

can then be used to derive key parameters such as the area under the plasma concentration-time curve (AUC – which is directly proportional to the total amount of unchanged drug that reaches the systemic circulation), the maximum (peak) plasma drug concentration, the time at which maximum plasma drug concentration occurs (peak time), additional factors which are used in the accurate determination of bioavailability include: the compound's terminal half life, total body clearance, steady-state volume of distribution and F%. These parameters are then analysed by non-compartmental or compartmental methods to give a calculated percentage bioavailability, for an example of this type of method see Egorin et al. 2002, and references therein.

10 In vivo assessment of oral and intravenous pharmacokinetics (specific method)

For sanglifehrin analogues, whole blood is analysed. Compounds are formulated in 5% ethanol / 5% cremophor EL / 90% saline for both p.o. and i.v. administration. Groups of 3 male CD1 mice are dosed with either 1 mg/kg i.v. or 10mg/kg p.o. Blood samples (40uL) are taken via saphenous vein, pre-dose and at 0.25, 0.5, 2, 8, and 24 hours, and diluted with an equal amount of dH₂O and put on dry ice immediately. Samples are stored at -70°C until analysis. The concentration of the compound of the invention or parent compound in the sample is determined via LCMS as follows: 20 uL of blood:H₂O (1:1, v/v)/PK sample is added with 20 uL Internal standard (hydroxyl macrocycle, **6**) at 100 ng/mL, 20 uL working solution/MeOH and 150 uL of ACN, vortexed for 1 minute at 1500 rpm, and centrifuged at 12000 rpm for 5 min. The supernatant is then injected into LC-MS/MS. The time-course of blood concentrations is plotted and used to derive area under the whole blood concentration-time curve (AUC – which is directly proportional to the total amount of unchanged drug that reaches the systemic circulation). These values are used to generate the oral bioavailability (F%) and other PK parameters where possible.

25 In vitro assessment of cytotoxicity

Huh-7 and HepG2 cells obtained from ATCC are grown in Dulbecco's modified essential media (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (pen-strep) and 1% glutamine; whereas CEM cells (human T-cell leukemia cells obtained from ATCC) are grown in RPMI 1640 medium with 10% FBS, 1% pen-strep and 1% glutamine. Fresh human PBMCs are isolated from whole blood obtained from at least two normal screened donors. Briefly, peripheral blood cells are pelleted/washed 2-3 times by low speed centrifugation and resuspension in PBS to remove contaminating platelets. The washed blood cells are then diluted 1:1 with Dulbecco's phosphate buffered saline (D-PBS) and layered over 14 mL of Lymphocyte Separation Medium (LSM; cellgrow by Mediatech, Inc.; density 1.078+/-0.002 g/ml; Cat.# 85-072-CL) in a 50 mL centrifuge tube and centrifuged for 30 minutes at 600 X g. Banded

PBMCs are gently aspirated from the resulting interface and subsequently washed 2X with PBS by low speed centrifugation. After the final wash, cells are counted by trypan blue exclusion and resuspended at 1×10^7 cells/mL in RPMI 1640 supplemented with 15 % Fetal Bovine Serum (FBS), 2 mM L-glutamine, 4 μ g/mL PHA-P. The cells are allowed to incubate for 48-72 hours at 37°C. After incubation, PBMCs are centrifuged and resuspended in RPMI 1640 with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 μ g/mL gentamycin, and 20 U/mL recombinant human IL-2.

Compound cytotoxicity is evaluated by testing half-log concentrations of each compound in triplicate against the cells described above. Cell containing medium alone served as the cell control (CC). Huh-7 and HepG2 cells are seeded in 96-well plates at a concentration of 5×10^3 cells per well. On the following day, the media is aspirated, and 100 μ L of corresponding media containing 5% FBS is added. Test drug dilutions are prepared at a 2X concentration in microtiter tubes and 100 μ L of each concentration is placed in appropriate wells in a standard format. After 72 hours, the cells are processed for cytotoxicity assessment.

PBMCs are diluted in fresh medium and plated in the interior wells of a 96 well round bottom microplate at 5×10^4 cells/well in a volume of 100 μ L. Similarly, CEM cells are plated at 1×10^4 cells/well. Then, 100 μ L of 2X preparations of the test drugs are added in appropriate wells in a standard format. The cultures are maintained for six to seven days and then processed for cytotoxicity determination.

Cytotoxicity is determined using CytoTox-ONE™ homogeneous membrane integrity assay kit (Promega). The assay measures the release of lactate dehydrogenase (LDH) from cells with damaged membranes in a fluorometric, homogeneous format. LDH released into the culture medium is measured with a coupled enzymatic assay that results in the conversion of resazurin into a fluorescent resorufin product. The amount of fluorescence produced is proportional to the number of lysed cells. Six serially diluted concentrations of each compound are applied to the cells to derive where applicable TC50 (toxic concentration of the drug decreasing cell viability by 50%) and TC90 (toxic concentration of the drug decreasing cell viability by 90%) values.

In vitro assessment of inhibition of MDR1 and MRP2 transporters

To assess the inhibition and activation of the MDR1 (P-glycoprotein 1) and MRP2 transporters, an in vitro ATPase assay from Solvo Biotechnology Inc. can be used (Glavinas et al., 2003). The compounds (at 0.1, 1, 10 and 100 μ M) are incubated with MDR1 or MRP2 membrane vesicles both in the absence and presence of vanadate to study the potential ATPase activation. In addition, similar incubations are conducted in the presence of verapamil/sulfasalazine in order to detect possible inhibition of the transporter ATPase activity.

ATPase activity is measured by quantifying inorganic phosphate spectrophotometrically.

Activation is calculated from the vanadate sensitive increase in ATPase activity. Inhibition is determined by decrease in verapamil/sulfasalazine mediated ATPase activity.

EXAMPLES

Example 1 – Construction of an *sfaA* deletion mutant of *Streptomyces* sp. A92-308110 (DSM9954)

1.1 Construction of the *sfaA* deletion construct

The ~7kb *EcoRV*-*StuI* fragment of cosmid TL3006 (SEQ ID NO. 3) encompassing *sfaA* (nucleotide position 14396-21362, NCBI sequence accession number FJ809786) was excised by digestion with *EcoRV* and *StuI* and the resulting isolated fragment ligated directly into pKC1139 that had previously been digested with *EcoRV* and treated with shrimp alkaline phosphatase (Roche). This plasmid was designated pSGK268.

An in frame deletion of the *sfaA* gene contained within this clone was performed using the Red/ET recombination kit supplied by Gene Bridges (catalog number K006).

(SEQ ID NO. 1) SfaA17161f 5'-

CGCTCTGTGGCGCCTGGTTTCCAAGCGGCTCGCGGACCGGCACCGGCACATGCATAATTA
ACCCTCACTAAAGGGCG-3'

(SEQ ID NO. 2) SfaA17825r 5'-

TGGATGTATCGTCGCAGGACGCCCAGAATTCACCTGCGACGTCCTCCAGATGCATTAATAC
GACTCACTATAGGGCTC-3'

Two oligonucleotides, SfaA17161f and SfaA17825r were used to amplify the neomycin marker from the FRT-PGK-gb2-neo-FRT template DNA supplied in the kit using KOD DNA polymerase. The resulting ~1.7kb amplified product was isolated by gel electrophoresis and purified from the gel with QiaEXTM resin.

Plasmid pSGK268 was transformed into *E. coli* DH10B using standard techniques and selected on plates containing apramycin (50 µg/ml). Introduction of the deletion construct was performed essentially following the Gene Bridges kit protocol. A single colony was grown overnight in 2TY apramycin (50 µg/ml) and transformed with the pRedET (tet) plasmid and selected on apramycin (50 µg/ml) and tetracycline (3 µg/ml) at 30 °C. A single colony was used to prepare an overnight culture of this strain in 3ml 2TY apramycin (50 µg/ml) and tetracycline (3 µg/ml) at 30 °C. 0.5 ml of this culture was used to inoculate 10 ml 2TY apramycin (50 µg/ml) and tetracycline (3 µg/ml) at 30 °C and grown to an OD_{600nm} ~0.5. 1.4 ml of this culture was transferred to each of 2 eppendorf tubes and 50 µl 10 % arabinose added to one tube to induce expression of the Red/ET recombination proteins. Tubes were shaken for ~1 hour at 37 °C. Induced and non-induced cells were pelleted in a bench top centrifuge and washed twice with

chilled sterile water; resuspending and centrifuging to pellet the cells each time. The resulting pellets were suspended in about 30-40 μ l of water and kept on ice. The 1.7kb disruption fragment isolated previously was added to the induced and non-induced tubes and transferred to 1mm BioradTM electrocuvettes on ice. The samples were electroporated (BioradTM Micropulser at 1.8kV, resulting time constant $\sim$ 4ms) and 1 ml 2TY (no antibiotics) added and mixed to remove the cells from the cuvette. Cells were incubated for $\sim$ 3hours at 37 °C with shaking (1100rpm, eppendorf thermomixer compact) before plating onto 2TY plates containing apramycin (50 μ g/ml and kanamycin 25 μ g/ml and incubating over night at 37 °C. Colonies from the induced sample plates were streaked onto 2TY plates containing kanamycin at 50 μ g/ml to purify and confirm introduction of the kanamycin resistance cassette. PCR on individual bacterial colonies was used to confirm the introduction of the cassette. Plasmids were prepared from these cultures and digested to confirm the expected plasmid pSGK270. Plasmids were then digested with *Nsi*I to remove the marker fragment, and the remainder religated to produce the *sfaA* in-frame deletion construct pSGK271.

1.2 Conjugation of *Streptomyces* sp. A92-308110 (DSM9954) and introduction of an *sfaA* deletion

Plasmid pSGK271 was transformed into *E. coli* ET12567 pUZ8002 using standard techniques and selected on 2TY plates containing apramycin (50 μ g/ml), kanamycin (25 μ g/ml) and chloroamphenicol (10 μ g/ml). The resulting strain was inoculated into 3ml liquid 2TY containing apramycin (50 μ g/ml), kanamycin (25 μ g/ml) and chloroamphenicol (10 μ g/ml) and incubated overnight at 37°C, 250rpm. 0.8 ml of this culture was used to inoculate 10 ml liquid 2TY containing apramycin (50 μ g/ml), kanamycin (25 μ g/ml) and chloroamphenicol (10 μ g/ml) in a 50 ml Falcon tube and incubated at 37°C 250 rpm until OD_{600nm} $\sim$ 0.5 was reached. The resulting culture was centrifuged at 3500 rpm for 10 minutes at 4°C, washed twice with 10 ml 2TY media using centrifugation to pellet the cells after each wash. The resulting pellet was resuspended in 0.5ml 2TY and kept on ice before use. This process was timed to coincide with the complete preparation of *Streptomyces* spores described below.

Spores of *Streptomyces* sp. A92-308110 (DSM9954) (Biot-4370) were harvested from a 1-2 week old confluent plate by resuspending in $\sim$ 3 ml 20 % glycerol. Spores were centrifuged (5000rpm, 10 minutes room temperature) and washed twice with 50 mM TES buffer before resuspending in 1ml 50 mM TES buffer and splitting between 2 eppendorf tubes. These tubes were heat shocked at 50°C for 10 minutes in a water bath before adding 0.5 ml 2TY and incubating in an Eppendorf Thermomixer compact at 37°C for 4-5 hours.

The prepared *E. coli* ET12567 pUZ8002 pSGK271 and Biot-4370 were mixed at ratios 1:1 (250 μ L each strain) and 1:3 (100 μ L *E. coli*) and immediately spread on R6 plates and transferred to

a 37°C incubator. After approximately 2 hours incubation these plates were overlaid with 2ml of sterile water containing nalidixic acid to give a final in-plate concentration of 25 ug/L. Plates were returned to the 37°C incubator overnight before overlaying with 2ml of sterile water containing apramycin to give a final in-plate concentration of 20-25 ug/L. Ex-conjugant colonies appearing after ~4-7 days were patched to ISP4 media containing apramycin (25 ug/L) and nalidixic acid (25 ug/L) and incubated at 37°C. Once adequate mycelial growth was observed strains were repatched to ISP4 media containing apramycin (25 ug/L) at 37°C and allowed to sporulate. Strains were then subcultured three times (to promote removal of the temperature sensitive plasmid) by patching to ISP4 (without antibiotic) and incubating at 37°C for 3-4 days. Strains were finally patched to ISP4 and incubated at 28°C to allow full sporulation (5-7 days). Spores were harvested and serially diluted onto ISP4 plates at 28°C to allow selection of single colonies. Sporulated single colonies were doubly patched to ISP4 plates with or without apramycin (25 ug/L) to confirm loss of plasmid and allowed to grow ~ 7 days before testing for production of sanglifehrins.

1.3 Screening strains for production of sanglifehrins in falcon tubes

A single ~7mm agar plug of a well sporulated strain was used to inoculate 7 ml of sterile SM25-3 media and incubated at 27°C 200rpm in a 2" throw shaker. After 48 hours of growth 0.7 ml of this culture was transferred to a sterilised falcon tube containing 7 ml of SGP2 media with 5 % HP20 resin. Cultures were grown at 24°C 300rpm on a 1 inch throw shaking incubator for 5 days before harvest. 0.8 ml bacterial culture was removed and aliquoted into a 2ml eppendorf tube ensuring adequate dispersal of the resin in throughout the culture prior to aliquoting. 0.8 ml acetonitrile and 15 ul of formic acid were added and the tube mixed for about 30 minutes. The mixture was cleared by centrifugation and 170 ul of the extract removed into a HPLC vial and analysed by HPLC.

1.4 Analysis of strains for reversion to wild type or *sfaA* phenotype.

Extracts of strains were analysed by HPLC. Strains that produced sanglifehrin A and B were not analysed further as these had reverted to wild type. Strains lacking sanglifehrin A and B production showed small levels (~1-2 mg/L) of a peak retention time 6.5 minutes that displayed a sanglifehrin like chromophore. Analysis by LCMS indicated this peak had a m/z 1073, -16 units from the expected m/z of sanglifehrin. It was postulated this peak was due to incorporation of phenylalanine in absence of meta-hydroxytyrosine.

Eight strains showing loss of sanglifeherin production were subsequently regrown to assess whether the potential *sfaA* mutation could be complemented chemically allowing a mutasynthetic process to novel sanglifehrins. Strains were grown in SM25-3 seed media for 48

hours before transferring to SGP2 production media with 5 % resin. After a further 24 hours growth strains were fed in triplicate with 2mM DL meta-hydroxytyrosine (addition of 100 ul of a 0.16M solution in 1M HCL) or 2mM L-phenylalanine with an unfed strain used as control. Strains were also fed pipecolic acid (2mM) in methanol) to enhance product yields. Strains were harvested after a further 4 days
 5 growth and extracted and analysed by HPLC. Meta-hydroxy tyrosine was shown to completely complement the *sfaA* mutation and addition of L-phenylalanine increased levels of the -16 amu compound. Strain Biot-4585 was chosen for further study as the *sfaA* deletion mutant.

Example 2 – Other methods for construction of the *sfaA* deletion construct

Other methods can be used to generate *sfaA* deletion mutants. Examples include *sfaA*
 10 insertional inactivation mutants (such as example 12 from WO2010/034243). This strain was generated as described in WO2010/034243, and given the strain designation BIOT-4452.

In an alternative procedure to generate the deletion of *sfaA* two oligonucleotides 15209F 5'-CAGAGAATTCGCGGTACGGGGCGGACGACAAGGTGTC-3'

(SEQ ID NO. 4) and 17219R 5'-

15 GCGCATGCATGTGCCGGTGCCGGTCCGCGAGCCGCTTGG-3'

(SEQ ID NO. 5) are used to amplify an upstream region of homology using cosmid TL3006 (SEQ ID NO. 3) as template and KOD DNA polymerase. The amplified product is treated with T4 polynucleotide kinase (NEB) and cloned into pUC18 that has been dephosphorylated by treating with shrimp alkaline phosphatase (Roche). The resulting construct is checked by
 20 restriction digestion and thoroughly sequenced to ensure the desired sequence is generated and that errors have not been introduced during polymerase amplification. This construct is digested with *EcoRI* and *NsiI* and the products analysed by gel electrophoresis. The desired sequence-containing band (i.e. upstream homology ~2 kb) is excised from the gel and purified using standard procedures (QiaEXTM resin). A second series of oligonucleotides (SEQ ID NO.

25 6) 17766F 5'-CCTCATGCATCTGGAGGACGTCGCAGGTGAATTCTGGGCG-3'

and 19763R 5'-GGGCAAGCTTCTCCTGGCTGAGCTTGAACATCG-3'

(SEQ ID NO. 7) are used to amplify a downstream region of homology using cosmid TL3006 (SEQ ID NO. 3) as template and KOD DNA polymerase. The amplified product is treated with T4 polynucleotide kinase (NEB) and cloned into pUC18 that has been dephosphorylated by
 30 treating with shrimp alkaline phosphatase (Roche). The resulting construct is analysed by restriction digestion and thoroughly sequenced to ensure the desired sequence is generated and that errors have not been introduced during polymerase amplification. This construct is

digested with *Hind*III and *Nsi*I and the products analysed by gel electrophoresis. The desired sequence-containing band (i.e. downstream homology ~2 kb) is excised from the gel and purified using standard procedures (QiaEXTM resin). Vector pKC1139 is digested with *Eco*RI and *Hind*III and the large vector fragment isolated by gel electrophoresis and purified by standard methods (QiaEXTM resin). The isolated upstream and downstream homology fragments are then cloned into this fragment of pKC1139 in a three-way ligation to generate the desired *sfaA* deletion construct.

In a further alternative procedure for generation of a *sfaA* deletion construct commercial gene synthesis (i.e. Genscript or other vendor) is used to generate a construct containing the desired sequence (SEQ ID NO. 8). This purchased construct is digested using *Bam*HI and *Xba*I to excise the sequence of interest and the products analysed by gel electrophoresis. The desired sequence-containing band (~4kb) is excised from the gel and purified using standard procedures. Vector pKC1139 is digested with *Bam*HI and *Xba*I and the large fragment isolated by gel electrophoresis and purified by standard methods. The two isolated fragments are then ligated together to generate the desired *sfaA* deletion construct.

These alternative *sfaA* deletion constructs are introduced into *Streptomyces* sp. A92-308110 (DSM9954) by conjugation using the methods in Example section 1.2.

20 **Example 3 - Array feed of the *sfaA* deletion mutant**

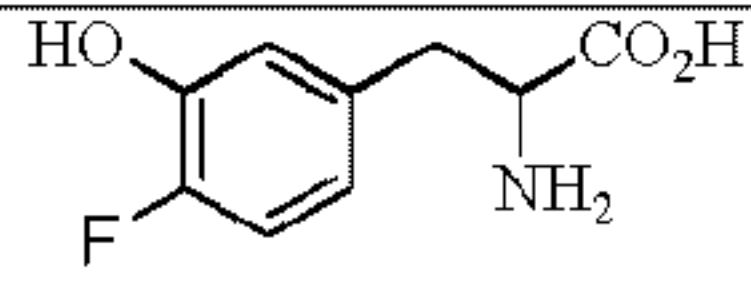
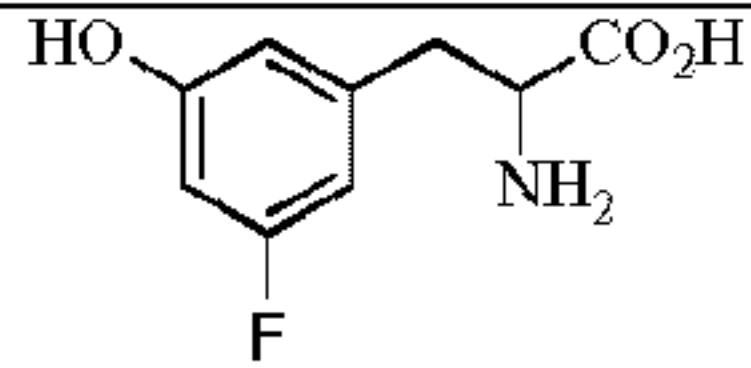
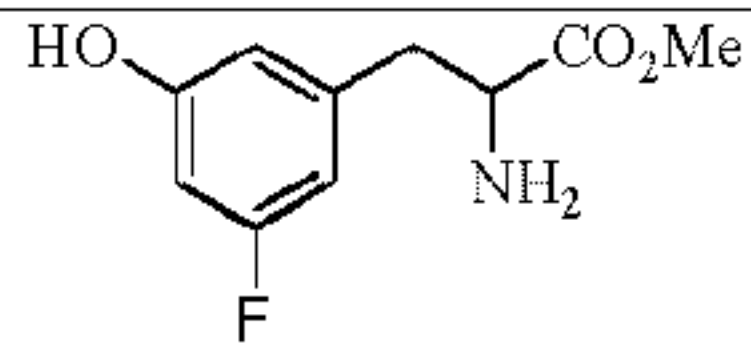
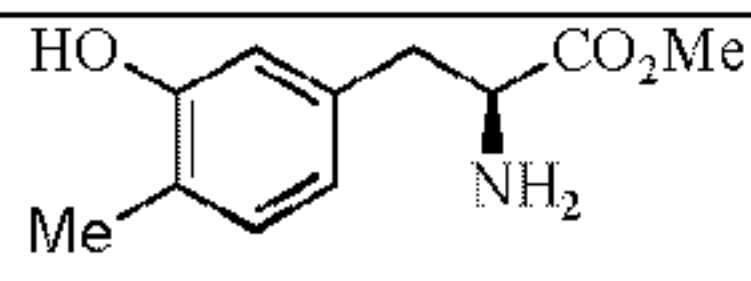
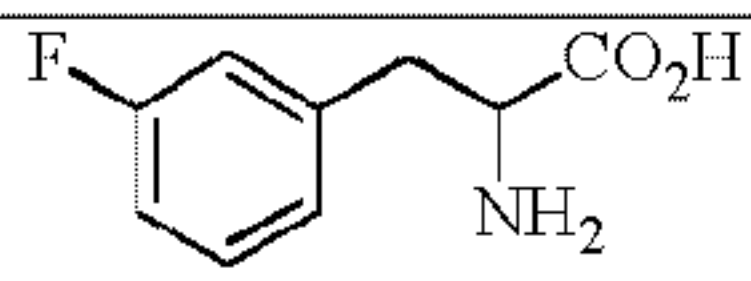
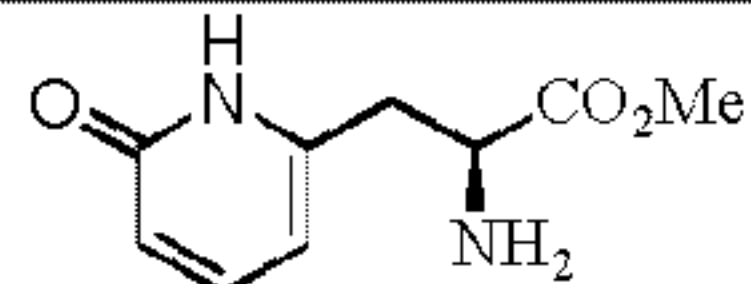
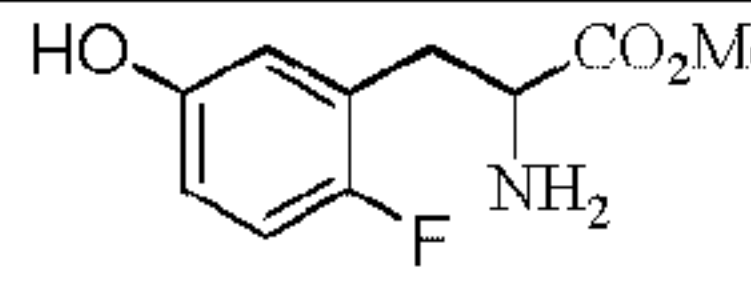
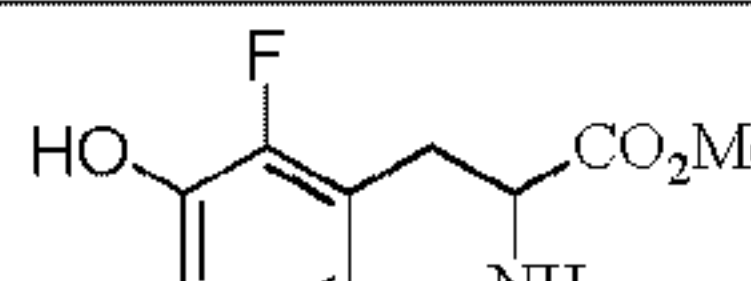
Spore stocks of a mutant disrupted in *sfaA* (BIOT-4452 or BIOT-4585) were prepared after growth on MAM, ISP4, ISP3 or ISP2 medium, and preserved in 20% w/v glycerol in distilled water and stored at -80 °C. Vegetative cultures (seed cultures) were prepared by inoculating spore stock (1% v/v) into 7 mL seed medium (SM25 medium) in 50 mL centrifuge tubes with foam plugs. The culture tubes were incubated at 27 °C, 250 rpm (5 cm throw) for 48 h. From the seed culture 10 % (v/v) was transferred into 7 mL production medium SGP-2 in 50 mL centrifuge tubes with foam plugs. Cultivation was carried out at 24 °C and 300 rpm (2.5 cm throw). For production of sanglifehrin mutasynthetic analogues, 0.05 mL of a 0.32 M solution (in 1N HCl) of the feed compound (mutasynthon) was added to each tube at 24 hours post inoculation to give a final concentration of 2 mM. Additionally, 0.05 ml of a 0.32 M solution of piperazic acid (in methanol) was added to each tube at 24 hours to give a final concentration of 2 mM. Cultivation was continued for an additional four days post feeding.

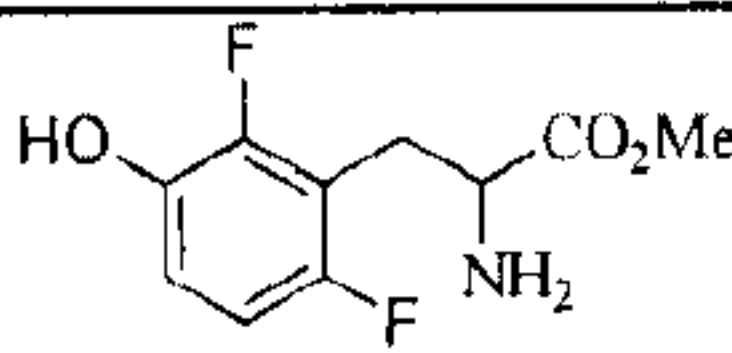
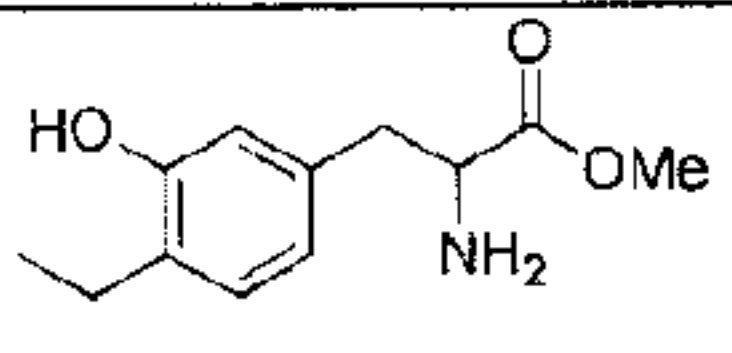
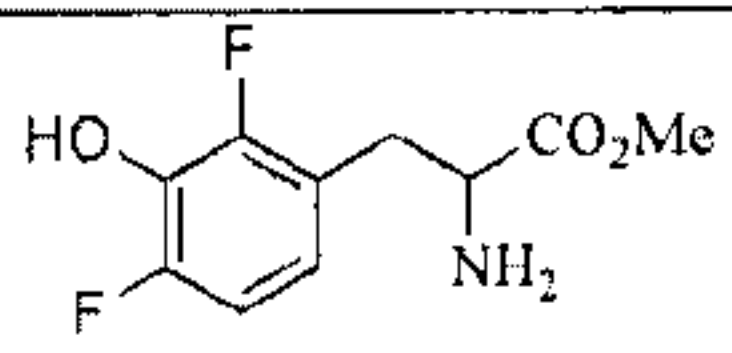
Samples were extracted by transferring 0.8 ml of the whole broth into a 2 ml capped eppendorf tube. 0.8 ml of acetonitrile was added, along with 0.015 ml of formic acid. The mixture was then

shaken for 30 minutes on a vibrax. The tube was then centrifuged at 13000 rpm for 10 minutes and 0.15 ml of the supernatant was removed for analysis. Extracts were analysed as described in general methods.

5 Table 1 shows the mutasynthons that were fed in this way, along with the LCMS H⁺ and Na⁺ adducts, anticipated molecular mass and retention time of the sanglifehrin mutasynthetic products observed. The major peaks, relating to the sanglifehrin A analogues, are shown. In all cases, LCMS peaks were also seen for the sanglifehrin B analogues (Mass – 18).

Table 1

mutasynthon fed	mutasynthon name	[M-H] ⁻ observed (m/z)	[M+Na] ⁺ observed (m/z)	molecular mass (amu)	retention time (minutes)
	2-amino-3-(4-fluoro-3-hydroxyphenyl)propanoic acid	1106.4	1130.4	1107.4	5.5
	2-amino-3-(3-fluoro-5-hydroxyphenyl)propanoic acid	1106.4	1130.4	1107.4	5.7
	methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl)proprionate	1106.4	1130.4	1107.4	5.7
	(S)-methyl 2-amino-3-(3-hydroxy-4-methylphenyl)propanoate	1102.5	1126.7	1103.5	6.0
	2-amino-3-(3-fluorophenyl)propanoic acid	1090.4	1114.5	1091	6.1
	methyl (2S)-2-amino-3-(3-hydroxy(2-pyridyl))propanoate	1089.5	1113.7	1090.5	4.4
	methyl 2-amino-3-(2-fluoro-5-hydroxyphenyl)propanoate	1106.5	1130.6	1107.5	5.5
	methyl 2-amino-3-(2-fluoro-5-hydroxyphenyl)propanoate	1106.5	1130.6	1107.5	5.1

	3-hydroxyphenyl)propanoate				
	methyl 2-amino-3-(2,6-difluoro-3-hydroxyphenyl)propanoate	1124.4	1148.5	1125.5	5.1
	methyl 2-amino-3-(4-ethyl-3-hydroxyphenyl)propanoate	1116.7	1141.0	1117.7	7.2
	methyl 2-amino-3-(2,4-difluoro-3-hydroxyphenyl)propanoate	1124.7	1148.8	1125.7	6.0

Example 4 – Isolation of 63-fluoro sanglifehrin A, compound 14

Fermentation carried out as described in general methods utilising methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl)propanoate and DL-piperazic acid as precursors, both were added at 26
5 hours.

After harvesting the culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using a magnetic
10 stirrer. The acetonitrile extract was recovered either by centrifugation or by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give
15 the final crude (1.3g).

The crude extract (1.3 g) was dissolved in ethyl acetate (2 ml) and loaded onto a silica gel column (10 x 2 cm) conditioned with ethyl acetate (500 ml). The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC,
20 combined and taken to dryness. This material (278 mg) was dissolved in methanol (1.8 ml) and purified by preparative HPLC. A Waters Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection

followed by a gradient to 100 % B at 30 minutes. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (20 mg).

Example 5 - Isolation of 62,63-fluoro sanglifehrin A, compound 15

5 Fermentation carried out as described in general methods utilising methyl (S)-2-amino-3-(3,4-difluoro-5-hydroxyphenyl)propanoate and DL-piperazic acid as precursors, both were added at 26 hours.

After harvesting the culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The
10 clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using a magnetic stirrer. The acetonitrile extract was recovered either by centrifugation or by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions. The combined acetonitrile extracts were concentrated to a residual
15 aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude (1.6g).

The crude extract (1.6 g) was dissolved in 2 ml ethyl acetate and loaded onto a silica gel column (10 x 2 cm) conditioned with 500 ml ethyl acetate. The column was eluted with ethyl
20 acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (188 mg) was dissolved in 1.8 ml methanol and purified by preparative HPLC. A Waters Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was
25 acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection followed by a gradient to 100 % B at 30 minutes. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (15 mg).

Example 6 - Isolation of 62-fluoro sanglifehrin A, compound 16

Employed methyl (S)-2-amino-3-(4-fluoro-3-hydroxyphenyl)propanoate and DL-piperazic acid
30 precursors. Carried out in accordance with general method with exception that precursors were added at 27 hours.

After harvesting the culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound

present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using magnetic stirrer. The acetonitrile extract was recovered either by centrifugation or by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions.

- 5 The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final oily crude (4.2g).

10 The crude extract (4.2 g) was dissolved in 4 ml ethyl acetate and loaded onto a silica gel column (15 x 2 cm) conditioned with 500 ml ethyl acetate. The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (390 mg) was dissolved in 2.4 ml methanol and purified by preparative HPLC. A Waters XterraTM MSC18 column (10 micron, 19 cm x 250 mm)
15 was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection followed by a gradient to 100 % B at 30 minutes. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (38 mg).

20 ***Example 7 - Isolation of 62-methyl sanglifehrin A, compound 17***

Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 0.4mL of spore stock into 400mL medium SM25 in 2L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 27 °C and 250rpm (2.5cm throw). From the seed culture 20mL was transferred into 400mL production
25 medium SGP2+5%HP20 in 2L Erlenmeyer flasks with foam plug. After 24 hours cultivation at 24°C and 250rpm (2.5cm throw), 2mL of a 200mM solution of methyl (S)-2-amino-3-(3-hydroxy-4-methylphenyl)propanoate in 1M hydrochloric acid and 2mL of a 400mM methanolic solution of DL-piperazic acid was added to each production flask to give a final 1mM concentration of the individual enantiomers of the precursors. Cultivation was continued for further four days at 24°C
30 and 250rpm (2.5cm throw).

The culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using an overhead paddle
35 stirrer. The acetonitrile extract was recovered by allowing it to settle under gravity. A second

acetonitrile extraction of the cells and resin was then performed under the same conditions. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude extract (7.6g).

The crude extract (7.6 g) was dissolved in 5 ml ethyl acetate and loaded onto a silica gel column (15 x 2 cm) conditioned with 500 ml ethyl acetate. The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (319 mg) was dissolved in 2.4 ml methanol and purified by preparative HPLC. A Waters Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection followed by a gradient to 100 % B at 30 minutes. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (14.9 mg).

Example 8 - Isolation of 61-deshydroxy sanglifehrin A, compound 18

Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 0.4mL of spore stock into 400mL medium SM25 in 2L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 27°C and 250rpm (2.5cm throw). From the seed culture 500mL was transferred into 4.5L production medium SGP2+5%HP20 in a 7L Applikon fermenter and cultivated at 24°C, 400rpm (cascade DOT control), 2.5L/min air flow and 30% DOT (cascade agitation control). After 24 hours cultivation, 7.5mL of a 667mM solution of (S)-2-amino-3-phenylpropanoic acid in 1M hydrochloric acid was added to the fermenter to give a final 1mM concentration of the precursor. Cultivation was continued for further four days at 24°C, 400rpm (cascade DOT control), 2.5L/min air flow and 30% DOT (cascade agitation control).

The culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using an overhead paddle stirrer. The acetonitrile extract was recovered by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions, but with the second extract being recovered by centrifugation. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to

pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude (55g).

The crude extract (55 g) was suspended in 80% methanol in water and extracted with 300 ml hexane twice. The target compound was found in methanol/water part which was taken to dryness. This dried extract (48 g) was dissolved in 30 ml ethyl acetate and loaded onto a silica gel column (20 x 5 cm) conditioned with 1 L ethyl acetate. The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (813 mg) was dissolved in methanol and purified by preparative HPLC. A Waters Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection followed by a gradient to 100 % B at 30 minutes. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (34 mg).

Example 9 - Isolation 58-des(3-hydroxyphenyl)-58-(3-hydroxy(2-pyridyl)-sanglifehrin A, compound 19

Employed methyl (2S)-2-amino-3-(3-hydroxy(2-pyridyl))propanoate and DL-piperazic acid precursors. Carried out in accordance with general method with exception that the incubator throw during vegetative (seed) cultivation was 2.5cm.

The culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using an overhead paddle stirrer. The acetonitrile extract was recovered by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude extract (7g).

The crude extract (7 g) was dissolved in 4 ml ethyl acetate and loaded onto a silica gel column (15 x 2 cm) conditioned with 500 ml ethyl acetate. The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate to 100% acetone then 1% methanol to stepwise 5% methanol in acetone). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (204 mg) was dissolved in methanol and purified by preparative HPLC. A Waters

Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was run isocratically at 50 % B for 6 minutes following the injection followed by a gradient to 100 % B at 30 minutes. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (4 mg).

Example 10 - Isolation of 61-deshydroxy-61-fluoro sanglifehrin A, compound 20

Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 0.4mL of spore stock into 400mL medium SM25 in 2L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 27°C and 250rpm (2.5cm throw). From the seed culture 20mL was transferred into 400mL production medium SGP2+5%HP20 in 2L Erlenmeyer flasks with foam plug. After 24 hours cultivation at 24°C and 250rpm (2.5cm throw), 2mL of a 400mM solution of 2-amino-3-(3-fluorophenyl)propanoic acid in 1M hydrochloric acid and 2mL of a 400mM methanolic solution of DL-piperazic acid was added to each production flask to give a final 1mM concentration of the individual enantiomers of the precursors. Cultivation was continued for further four days at 24°C and 250rpm (2.5cm throw).

The culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using an overhead paddle stirrer. The acetonitrile extract was recovered either by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions. A third extract was obtained by centrifugation of the residual cell and resin mix. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude extract (10.5g).

The crude extract (10.5 g) was dissolved in 7 ml ethyl acetate and loaded onto a silica gel column (15 x 2 cm) conditioned with 500 ml ethyl acetate. The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (342 mg) was dissolved in methanol and purified by preparative HPLC. A Waters Xterra™ MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The

column was run isocratically at 53 % B for 30 minutes following the injection. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid (6 mg).

Example 11 - Isolation of 61-deshydroxy-61-amino sanglifehrin A, compound 21

5 Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 0.4mL of spore stock into 400mL medium SM25 in 2L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 hours at 27°C and 250rpm (2.5cm throw). From the seed culture 20mL was transferred into 400mL production medium SGP2+5%HP20 in 2L Erlenmeyer flasks with foam plug. After 24 hours cultivation at
10 24°C and 250rpm (2.5cm throw), 2mL of a 200mM solution of methyl (S)-2-amino-3-(3-aminophenyl)propanoate in 1M hydrochloric acid was added to each production flask to give a final 1mM concentration of the precursors. Cultivation was continued for further four days at 24°C and 250rpm (2.5cm throw).

The culture broths were pooled and adjusted to approx. pH 3 with formic acid and centrifuged
15 (3300g) for 25mins to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1hr using an overhead paddle stirrer. The acetonitrile extract was recovered either by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same
20 conditions. A third extract was obtained by centrifugation of the residual cell and resin mix.

The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure and then adjusted to pH 6. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude.

The crude extract is dissolved in ethyl acetate and loaded onto a silica gel column (conditioned
25 with ethyl acetate. The column is eluted with organic solvent with increasing polarity. Approx. 250 mL fractions are collected and the target compound identified by analytical LC, combined and taken to dryness. This material is dissolved in methanol and purified by preparative HPLC. Pure fractions are identified by HPLC-UV and combined. These fractions are taken to dryness under reduced pressure to yield the target compound as an off-white amorphous solid.

30 ***Example 12 - Biological data – In vitro evaluation of HCV antiviral activity in the replicon system***

Compounds were analysed in the replicon assay using Huh5.2 cells as described in the General Methods. Cyclosporine A, **1**, sanglifehrin A, **5**, and the hydroxymacrocycle, **6** were included as a comparison.

Name	EC50 (μ M)	CC50 (μ M)	Selectivity index (CC50/EC50)
Cyclosporine A, 1	0.2	4.3	21.5
Sanglifehrin A, 5	0.318	9.1	28.7
Hydroxymacrocycle, 6	8.4	83.6	9.9
14	0.135	12.8	121
15	0.195	16.6	88
16	0.89	29.7	32
17	0.083	11.6	143
18	3.4	11.7	3.5
19	24.3	48.1	3.5

As can be seen, **14**, **15**, **16** and **17** are all very potent in the Huh5.2 replicon assay (as shown by the low EC50), with good selectivity against the cell line (as shown by a high selectivity index). The previously described sanglifehrin A, **5**, is less potent than **14**, **15** and **17** at HCV inhibition, and cyclosporine A, **1** is less potent and both **1** and **5** have poorer selectivity indices.

Example 13 – Microsome stability

Stability of the compounds in human and mouse liver microsomes was analysed as described in the General Methods. Sanglifehrin A, **5** was included as a comparison.

Name	Human liver microsome half life (mins)	Mouse liver microsome half life (mins)
Sanglifehrin A, 5	6.38	6.15
14	9.32	9.78

15	10.81	9.22
16	6.61	5.48
17	9.50	9.67
18	7.64	3.46
19	10.37	4.71

As can be seen, the compounds of the invention, **14**, **15**, **16**, **17**, **18** and **19** all have increased stability in human liver microsomes when compared to sanglifehrin A (**5**) and **14**, **15** and **17** are also all more stable in mouse liver microsomes.

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References

- Appel, N., T. Schaller, et al. (2006). "From structure to function: new insights into hepatitis C virus RNA replication." J Biol Chem **281**(15): 9833-6.
- Banteli, R., J. Wagner, et al. (2001). "Synthesis of derivatives of the novel cyclophilin-binding immunosuppressant sanglifehrin A with reduced numbers of polar functions." Bioorg Med Chem Lett **11**(12): 1609-12.
- Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Nagaraja Rao, R., and Schoner, B.E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**: 43-49.
- 15 Chatterji, U., M. Bobardt, et al. (2009). "The isomerase active site of cyclophilin a is critical for HCV replication." J Biol Chem.
- Colgan, J., M. Asmal, et al. (2000). "Isolation, characterization and targeted disruption of mouse ppia: cyclophilin A is not essential for mammalian cell viability." Genomics **68**(2): 167-78.
- Crabbe, R., G. Vuagniaux, et al. (2009). "An evaluation of the cyclophilin inhibitor Debio 025
20 and its potential as a treatment for chronic hepatitis C." Expert Opin Investig Drugs **18**(2): 211-20.
- Dolinski, K., S. Muir, et al. (1997). "All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*." Proc Natl Acad Sci U S A **94**(24): 13093-8.
- 25 E. Lawitz, R. R., T. Nguyen, M. Huang, J. Ke, J. Praestgaard, D. Serra, M. Koziel, T. Evans (2009). "Safety And Antiviral Efficacy Of 14 Days Of The Cycophilin Inhibitor Nim811 In

Combination With Pegylated Interferon .2a In Relapsed Genotype 1 Hcv Infected Patients." Journal of Hepatology **50**(S1): S379.

Egorin, M. J., T. F. Lagattuta, et al. (2002). "Pharmacokinetics, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC 707545) in
5 CD2F1 mice and Fischer 344 rats." Cancer Chemother Pharmacol **49**(1): 7-19.

Fehr, T., J. Kallen, et al. (1999). "Sanglifehrins A, B, C and D, novel cyclophilin-binding compounds isolated from *Streptomyces* sp. A92-308110. II. Structure elucidation, stereochemistry and physico-chemical properties." J Antibiot (Tokyo) **52**(5): 474-9.

Flisiak, R., A. Horban, et al. (2008). "The cyclophilin inhibitor Debio-025 shows potent anti-
10 hepatitis C effect in patients coinfectd with hepatitis C and human immunodeficiency virus." Hepatology **47**(3): 817-26.

Furniss, B. S., Furniss, A.I., Vogel, A.I., Ed. (1989). Vogel's Textbook of Practical Organic Chemistry, Prentice Hall.

Gaither, L. A., Borawski, J., Anderson, L. J., Balabanis, K. A. et al., (2010). "Multiple cyclophilins
15 involved in different cellular pathways mediate HCV replication" Virology 397: 43-55

Glavinas, H., Krajcsi, P., Cserepes, J., Sarkadi, B. (2004). "The role of ABC transporters in drug resistance, metabolism and toxicity." Curr. Drug. Deliv. **1**(1): 27-42.

Gomez, L., H. Thibault, et al. (2007). "Inhibition of mitochondrial permeability transition improves functional recovery and reduces mortality following acute myocardial infarction in mice." Am J
20 Physiol Heart Circ Physiol **293**(3): H1654-61.

Goto, K., Watashi, K., Inoue, D., Hijikata, M., Shimotohno, K. (2009) "Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor" Cancer Science **100**(10): 1943-1950

Hanoulle, X., Badillo A, Wieruszeski JM, Verdegem D, Landrieu I, Bartenschlager R, Penin F,
25 Lippens G (2009). "Hepatitis C virus NS5A protein is a substrate for the Peptidyl-Prolyl cis/trans isomerase activity of Cyclophilins A and B." J Biol Chem.

Hartel, C., P. Iblher, et al. (2006). "Immunosuppressive activity of the immunophilin-binding drug Sanglifehrin A in human whole blood: potent inhibition of interleukin-6 produced by lymphocytes and monocytes." Scand J Immunol **63**(1): 26-34.

Herrler, M., H. Bang, et al. (1994). "Cloning and characterization of ppiB, a *Bacillus subtilis* gene which encodes a cyclosporin A-sensitive peptidyl-prolyl cis-trans isomerase." Mol Microbiol **11**(6): 1073-83.

- Hite, M., Turner, S., Federici, C. (2003). "Part 1: Oral delivery of poorly soluble drugs". Pharmaceutical Manufacturing and Packing Sourcer. Summer 2003 issue.
- Immecke, S.N., Baal., N, et al. (2011). "The Cyclophilin-Binding Agent Sanglifehrin A Is a Dendritic Cell Chemokine and Migration Inhibitor." PLOS one **6**(3):e18406
- 5 Inoue, K., K. Sekiyama, et al. (2003). "Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial." J Gastroenterol **38**(6): 567-72.
- Inoue, K., T. Umehara, et al. (2007). "Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo." Hepatology **45**(4): 921-8.
- Ishii, N., K. Watashi, et al. (2006). "Diverse effects of cyclosporine on hepatitis C virus strain
10 replication." J Virol **80**(9): 4510-20.
- J. Ke, E. L., R. Rozier, T. Marbury, N. Nguyen, D. Serra, K. Dole, J. Praestgaard, M. Huang, T. Evans (2009). "Safety, And Tolerability Of Nim811, A Novel Cyclophilin Inhibitor For Hcv, Following Single And Multiple Ascending Doses In Healthy Volunteers And Hcv-Infected Patients." Journal of Hepatology **50**(S1): S229.
- 15 Jacobson, I., McHutchison, JG, Sulkowski, M. (2007). Gastroenterol & Hepatol **3**(S34): 1-10.
- Illing, G.T., Normansell, I.D. and Peberdy J.F. (1989) "Protoplast Isolation and Regeneration in *Streptomyces clavuligerus*." J. Gen. Microbiol **135**, 2289-2297
- Kallen, J., R. Sedrani, et al. (2005). "Structure of human cyclophilin A in complex with the novel immunosuppressant sanglifehrin A at 1.6 Å resolution." J Biol Chem **280**(23): 21965-71.
- 20 Kawasaki, H., E. S. Mocarski, et al. (2007). "Cyclosporine inhibits mouse cytomegalovirus infection via a cyclophilin-dependent pathway specifically in neural stem/progenitor cells." J Virol **81**(17): 9013-23.
- Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000) Practical Streptomyces Genetics, John Innes Foundation, Norwich.
- 25 Konig, J. H., Glaeser, M. Keiser, K. Mandery, U. Klotz and M. F. Fromm (2010), Drug Metab Dispos, 39, 1097-1102. Manns, M. P., G. R. Foster, et al. (2007). "The way forward in HCV treatment--finding the right path." Nat Rev Drug Discov **6**(12): 991-1000.
- Martin Cabrejas, L. M., S. Rohrbach, et al. (1999). "Macrolide Analogues of the Novel Immunosuppressant Sanglifehrin: New Application of the Ring-Closing Metathesis Reaction."
30 Angew Chem Int Ed Engl **38**(16): 2443-2446.

- Mathy, J. E., S. Ma, et al. (2008). "Combinations of cyclophilin inhibitor NIM811 with hepatitis C Virus NS3-4A Protease or NS5B polymerase inhibitors enhance antiviral activity and suppress the emergence of resistance." Antimicrob Agents Chemother **52**(9): 3267-75.
- Melnikova, I. (2008). "Hepatitis C therapies." Nature Rev Drug Disc **7**: 799-800.
- 5 Metternich, R., Denni, D., Thai, B, Sedrani, R. (1999). "Toward a Total Synthesis of the Immunosuppressant Sanglifehrin A. Preparation of Two Relay Compounds by Degradation and Their Use in the Reassembly of the Natural Product." J. Org. Chem. **64**: 9632-9639.
- Millay, D. P., M. A. Sargent, et al. (2008). "Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy." Nat Med **14**(4): 442-7.
- 10 Nelson, D. R., Ghalib, R.H., Sulkowski, M., Schiff, E., Rustgi, V., Pockros, P.J., Wang, C., Decosterd Kerhuel, D., and P. Groscurin, Porchet, H., Crabbe, R. (2009). "Efficacy And Safety Of The Cyclophilin Inhibitor Debio 025 In Combination With Pegylated Interferon Alpha-2a And Ribavirin In Previously Null-Responder Genotype 1 Hcv Patients." Journal of Hepatology **50**(S1): S40.
- 15 Niwa, T., Yamamoto, S, Saito, M, Shiraga, T, Takagi, A. (2007). "Effect of Cyclosporine and Tacrolimus on Cytochrome P450 Activities in Human Liver Microsomes." Yakugaku Zasshi **127**(1): 209--216.
- Paeshuyse, J., A. Kaul, et al. (2006). "The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro." Hepatology **43**(4): 761-70.
- 20 Parfieniuk, A., J. Jaroszewicz, et al. (2007). "Specifically targeted antiviral therapy for hepatitis C virus." World J Gastroenterol **13**(43): 5673-81.
- Pawlotsky, J. M. (2000). "Hepatitis C virus resistance to antiviral therapy." Hepatology **32**(5): 889-96.
- Pawlotsky, J. M. (2005). "Current and future concepts in hepatitis C therapy." Semin Liver Dis
25 **25**(1): 72-83.
- Pawlotsky, J. M. (2006). "Virology of hepatitis B and C viruses and antiviral targets." J Hepatol **44**(1 Suppl): S10-3.
- Pemberton, T. J. and J. E. Kay (2003). "Cyclophilin sensitivity to sanglifehrin A can be correlated to the same specific tryptophan residue as cyclosporin A." FEBS Lett **555**(2): 335-40.
- 30 Pockros, P. (2008). "Emerging Therapies for Chronic Hepatitis C Virus." Gastroenterol and Hepatology **4**(10): 729-734.

- Ptak, R. G., P. A. Gallay, et al. (2008). "Inhibition of human immunodeficiency virus type 1 replication in human cells by Debio-025, a novel cyclophilin binding agent." Antimicrob Agents Chemother **52**(4): 1302-17.
- 5 Qu, X., Jiang, N. et al., (2011). "Cloning, sequencing and characterization of the biosynthetic gene cluster of sanglifehrin A, a potent cyclophilin inhibitor." Mol. Biosyst. **7**:852-861
- Robida, J. M., H. B. Nelson, et al. (2007). "Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro." J Virol **81**(11): 5829-40.
- Hopkins, S. D. H., E. Gavis, J. Lalezari, E. Glutzer, B. DiMassimo, P. Rusnak, S. Wring, C. Smitley, Y. and Ribeill (2009). "Safety, plasma pharmacokinetics, and anti-viral activity of SCY-635 in adult patients with chronic hepatitis C virus infection." Journal of Hepatology **50**(S1): S36.
- 10 Sanglier, J. J., V. Quesniaux, et al. (1999). "Sanglifehrins A, B, C and D, novel cyclophilin-binding compounds isolated from *Streptomyces* sp. A92-308110. I. Taxonomy, fermentation, isolation and biological activity." J Antibiot (Tokyo) **52**(5): 466-73.
- Schneider, M. D. (2005). "Cyclophilin D: knocking on death's door." Sci STKE **2005**(287): pe26.
- 15 Sedrani, R., J. Kallen, et al. (2003). "Sanglifehrin-cyclophilin interaction: degradation work, synthetic macrocyclic analogues, X-ray crystal structure, and binding data." J Am Chem Soc **125**(13): 3849-59.
- Seden, K. D. Back and S. Khoo (2010), J Antimicrob Chemother, **65**, 1079-1085.
- Smith, M. B. a. M., J. , Ed. (2001). March's advanced organic chemistry, John Wiley and Sons Inc., UK.
- 20 Steinschulte, C., T. Taner, et al. (2003). "Cutting edge: sanglifehrin A, a novel cyclophilin-binding immunosuppressant blocks bioactive IL-12 production by human dendritic cells." J Immunol **171**(2): 542-6.
- Strader, D. B., T. Wright, et al. (2004). "Diagnosis, management, and treatment of hepatitis C." Hepatology **39**(4): 1147-71.
- 25 Tropschug, M., I. B. Barthelmess, et al. (1989). "Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*." Nature **342**(6252): 953-5.
- Vrolijk, J. M., A. Kaul, et al. (2003). "A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C." J Virol Methods **110**(2): 201-9.
- 30 Wring, S. , C. Wille, C. Rewerts, R. Randolph, A. Scribner and S. Hopkins (2010), Journal of Hepatology, **52**, S263.

Yang, F., J. M. Robotham, et al. (2008). "Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro." J Virol **82**(11): 5269-78.

5 Zenke, G., U. Strittmatter, et al. (2001). "Sanglifehrin A, a novel cyclophilin-binding compound showing immunosuppressive activity with a new mechanism of action." J Immunol **166**(12): 7165-71.

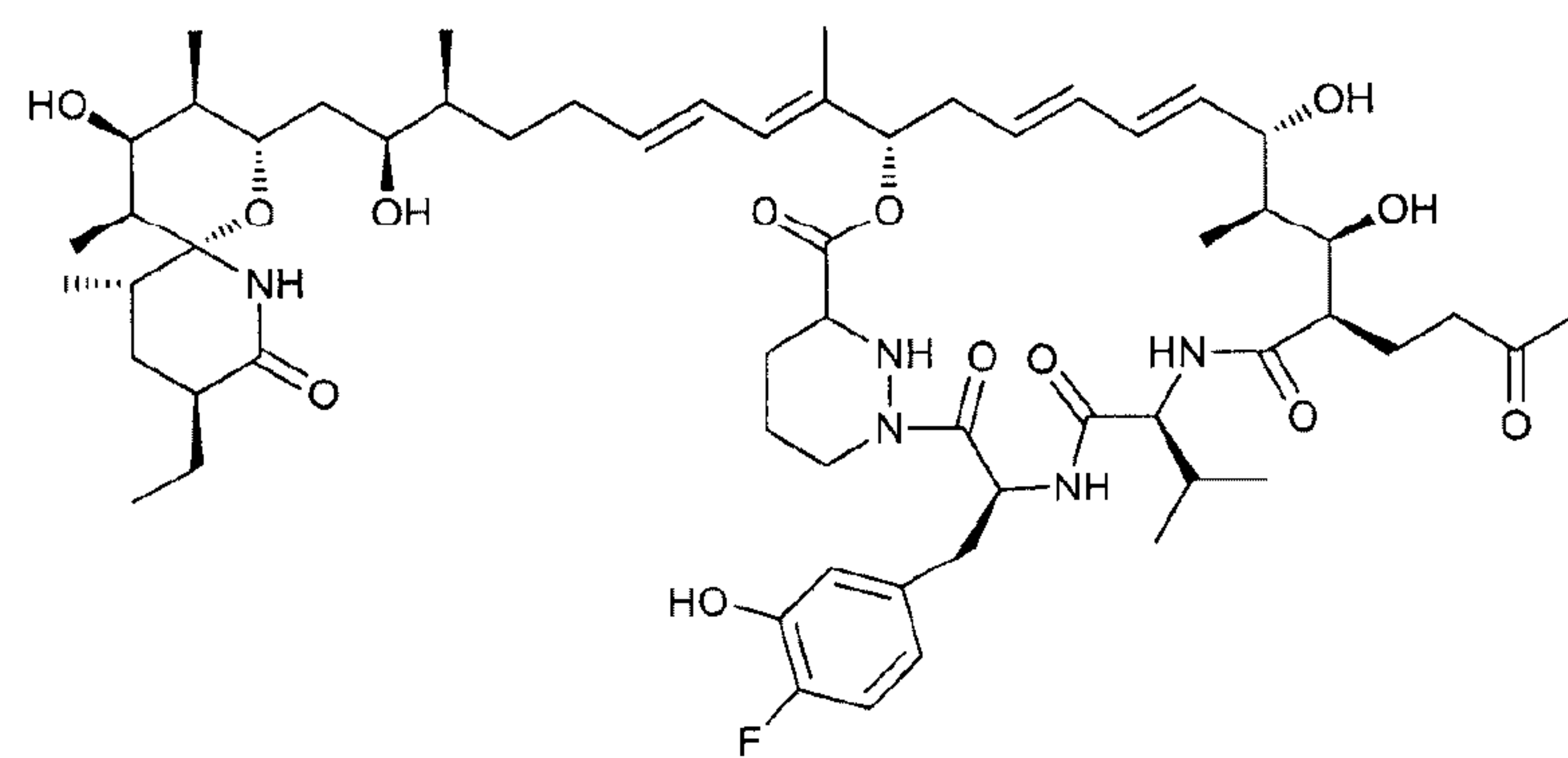
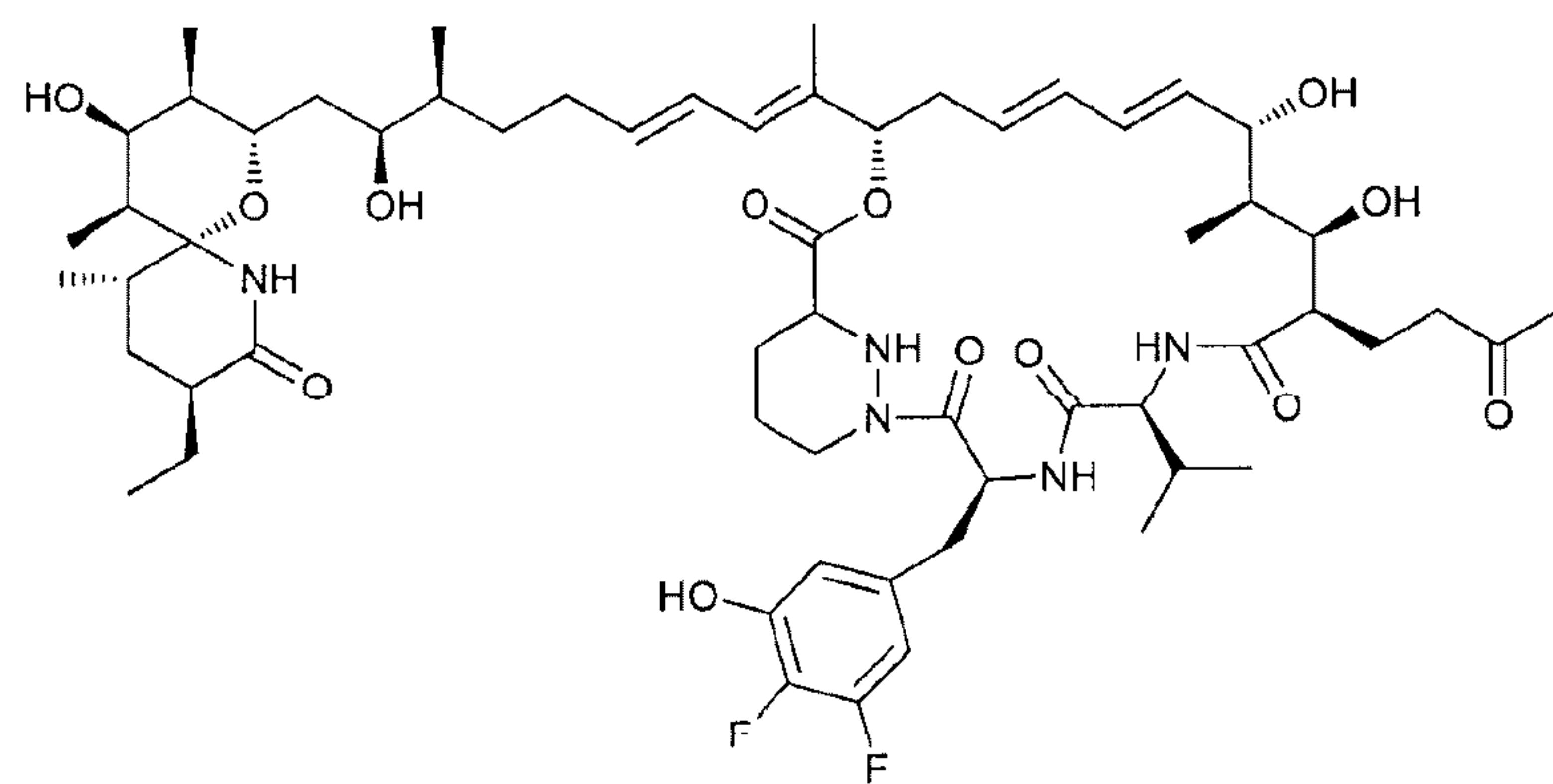
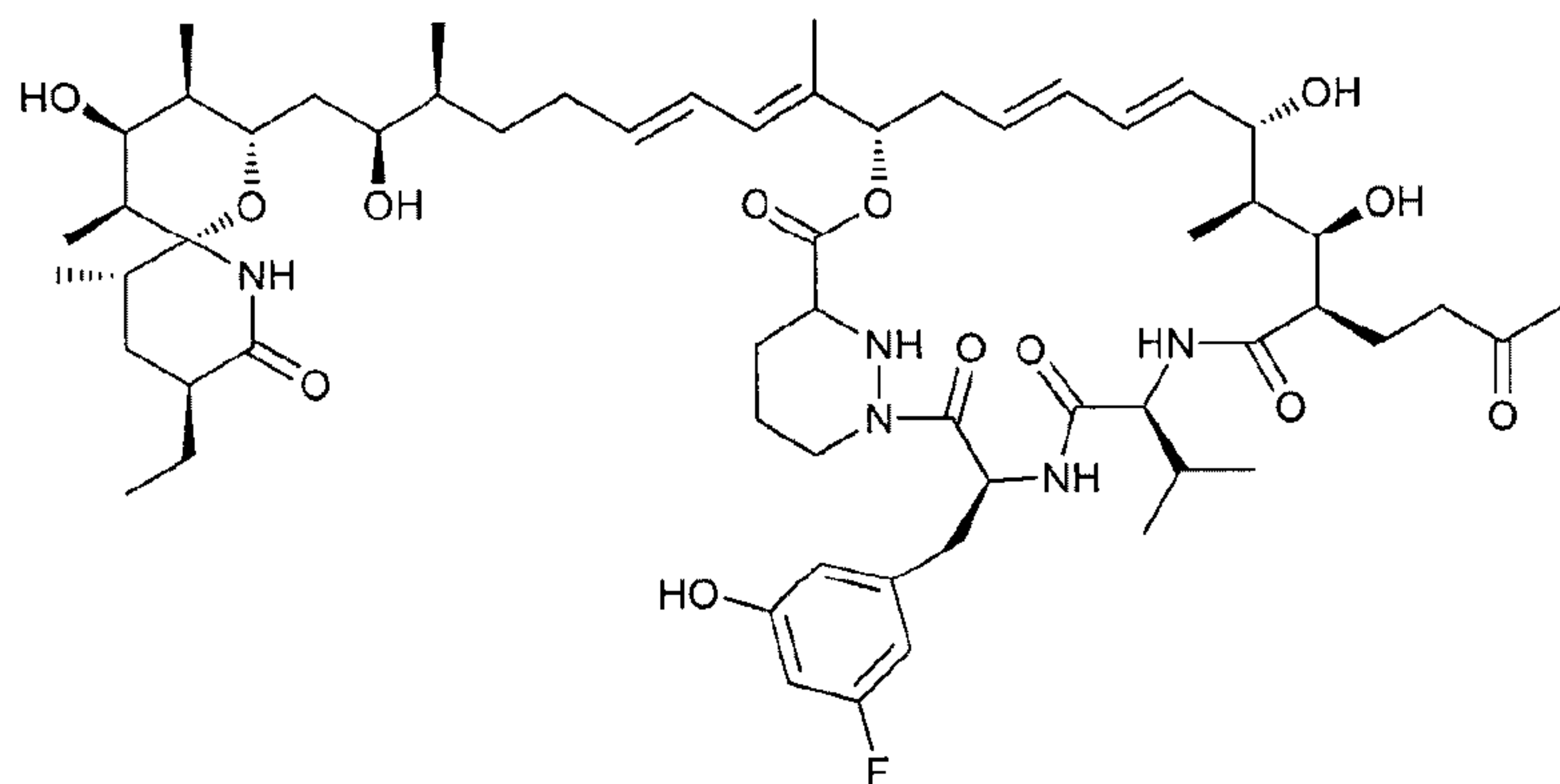
Zeuzem, S. and E. Herrmann (2002). "Dynamics of hepatitis C virus infection." Ann Hepatol **1**(2): 56-63.

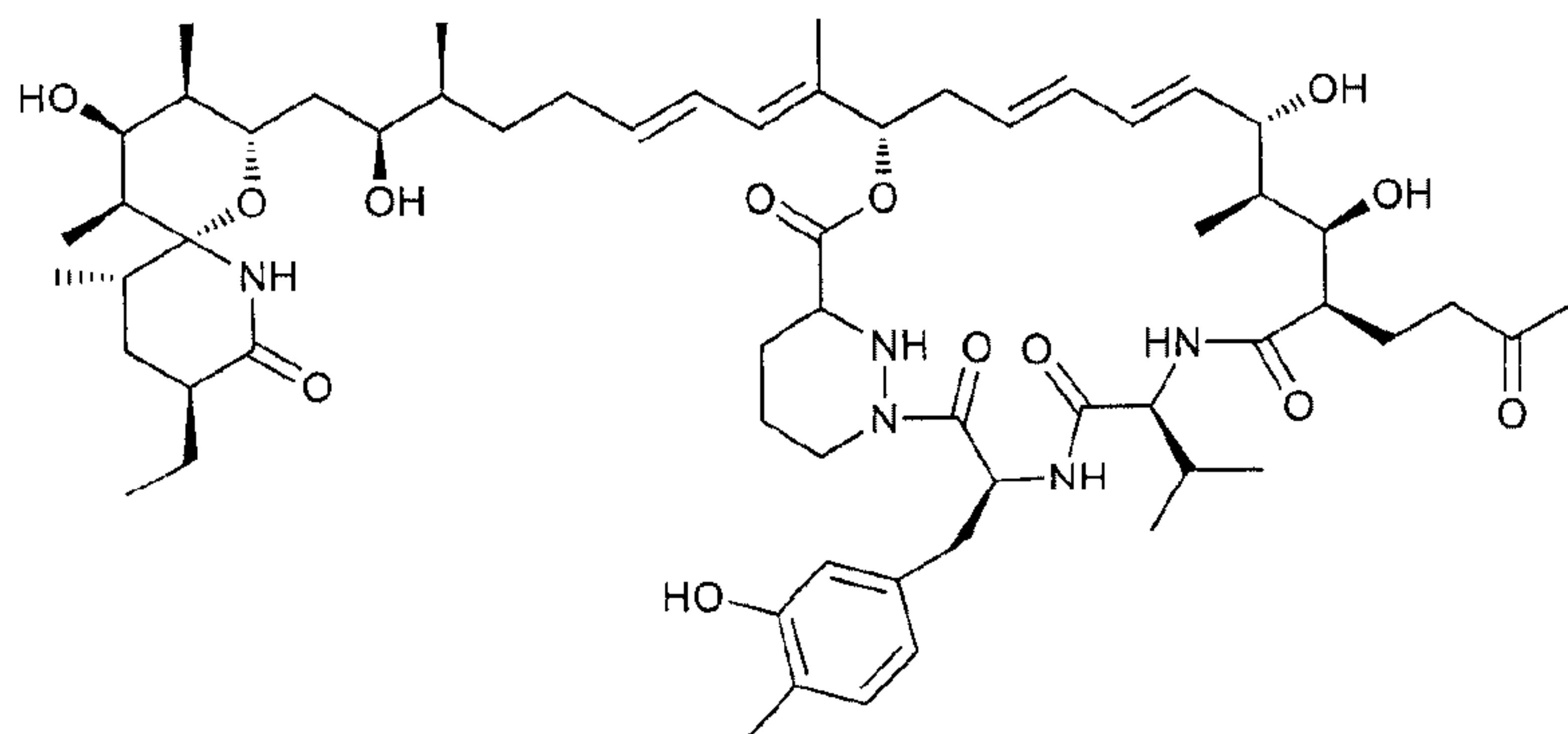
10 Zhang, L. H. and J. O. Liu (2001). "Sanglifehrin A, a novel cyclophilin-binding immunosuppressant, inhibits IL-2-dependent T cell proliferation at the G1 phase of the cell cycle." J Immunol **166**(9): 5611-8.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word 'comprise', and variations such as 'comprises' and 'comprising', will be understood to imply the inclusion of a stated integer or step or group of integers but not to the exclusion of any
15 other integer or step or group of integers or steps.

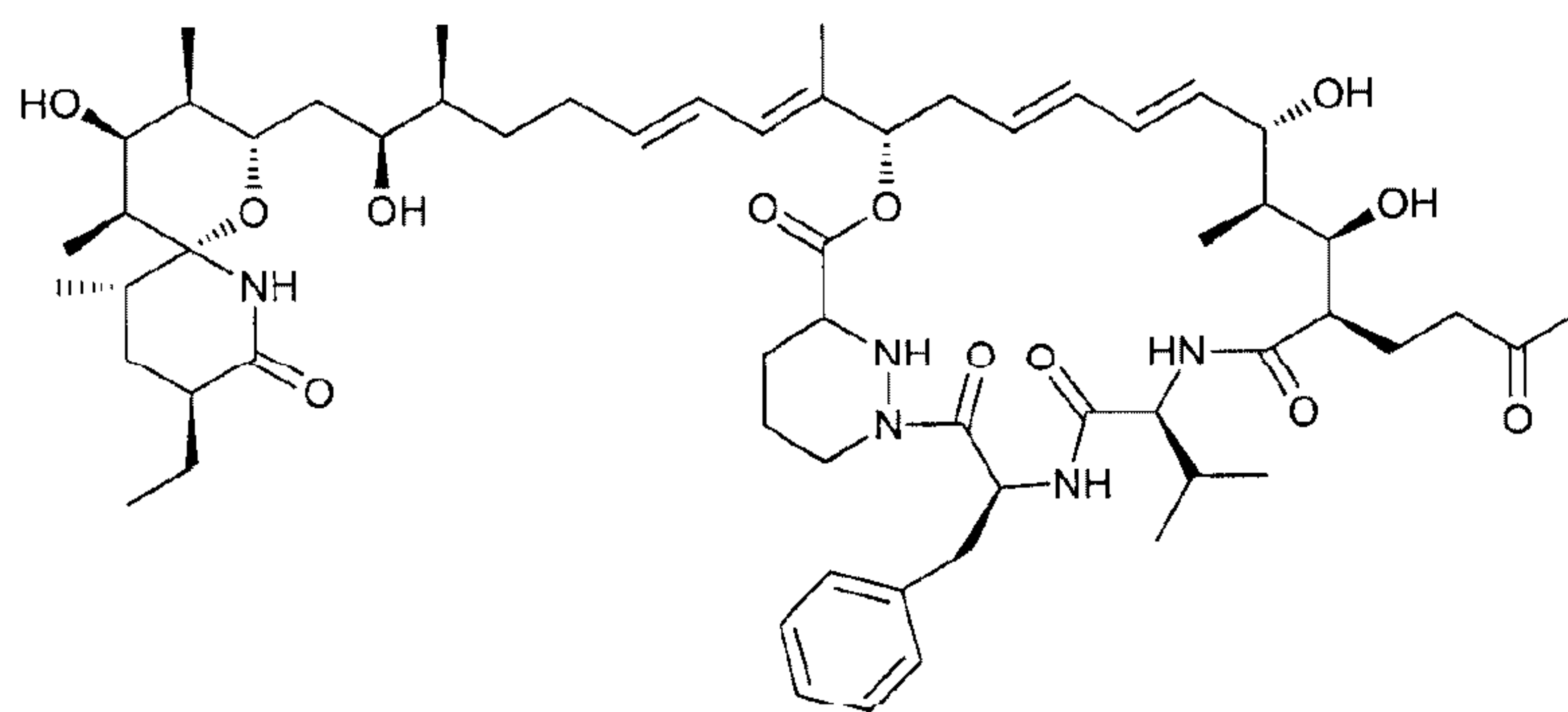
Claims

1. A compound selected from the group consisting of:

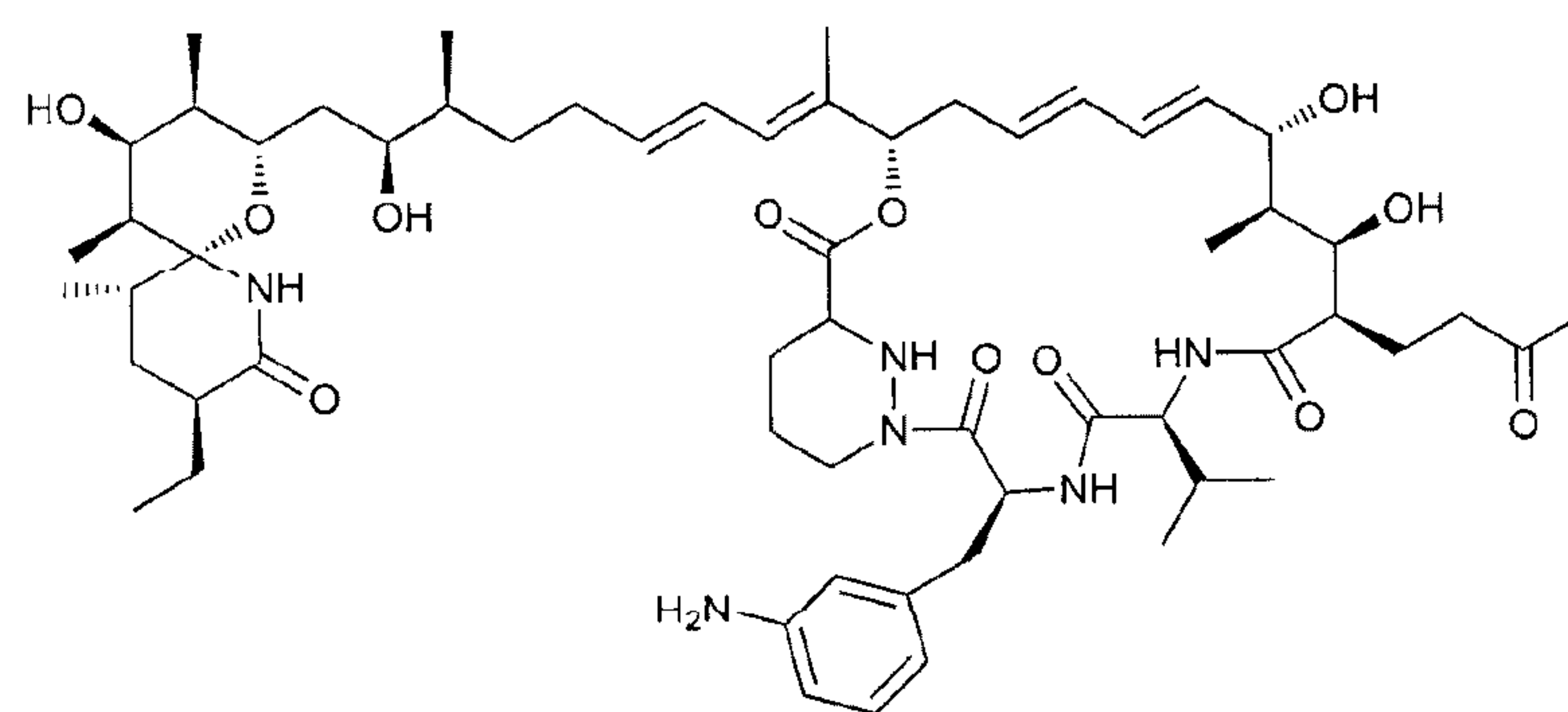




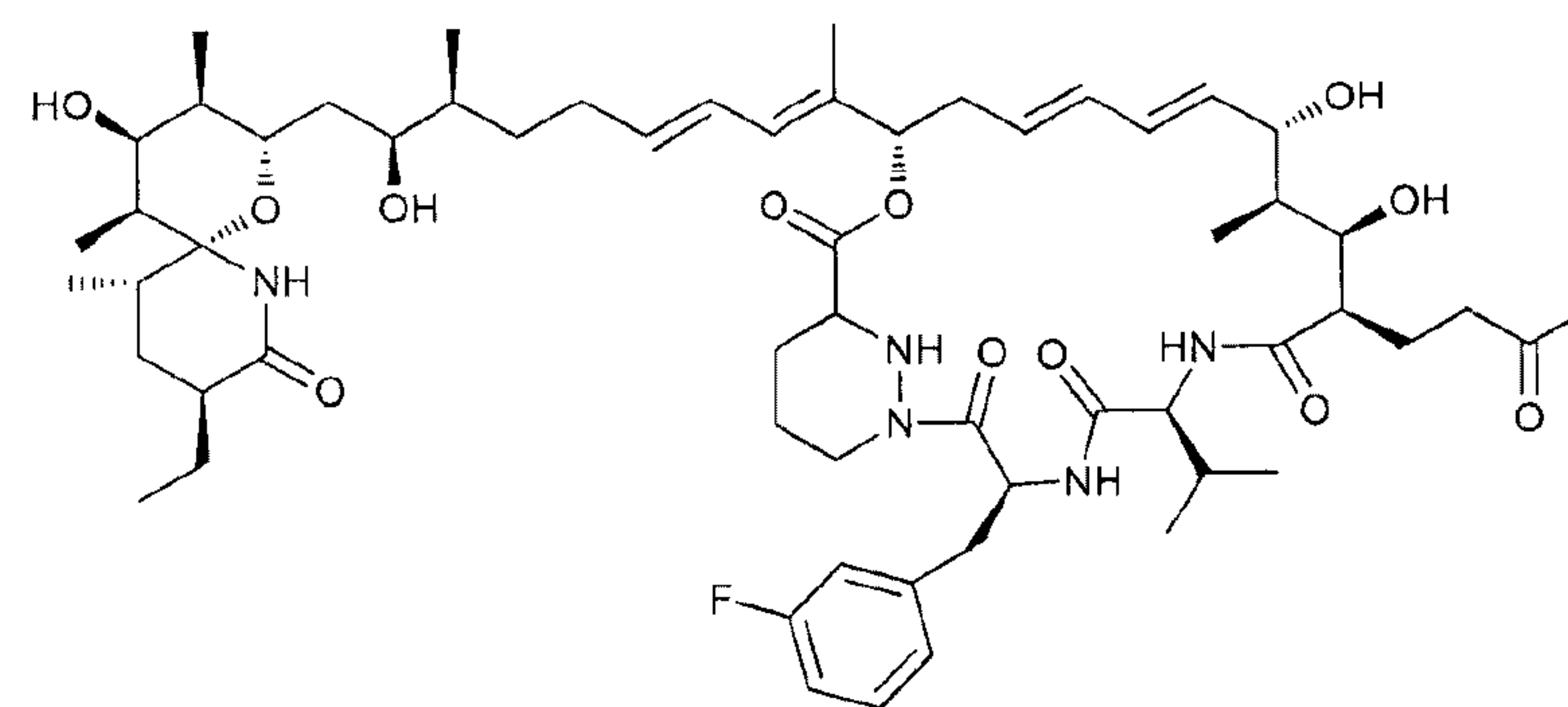
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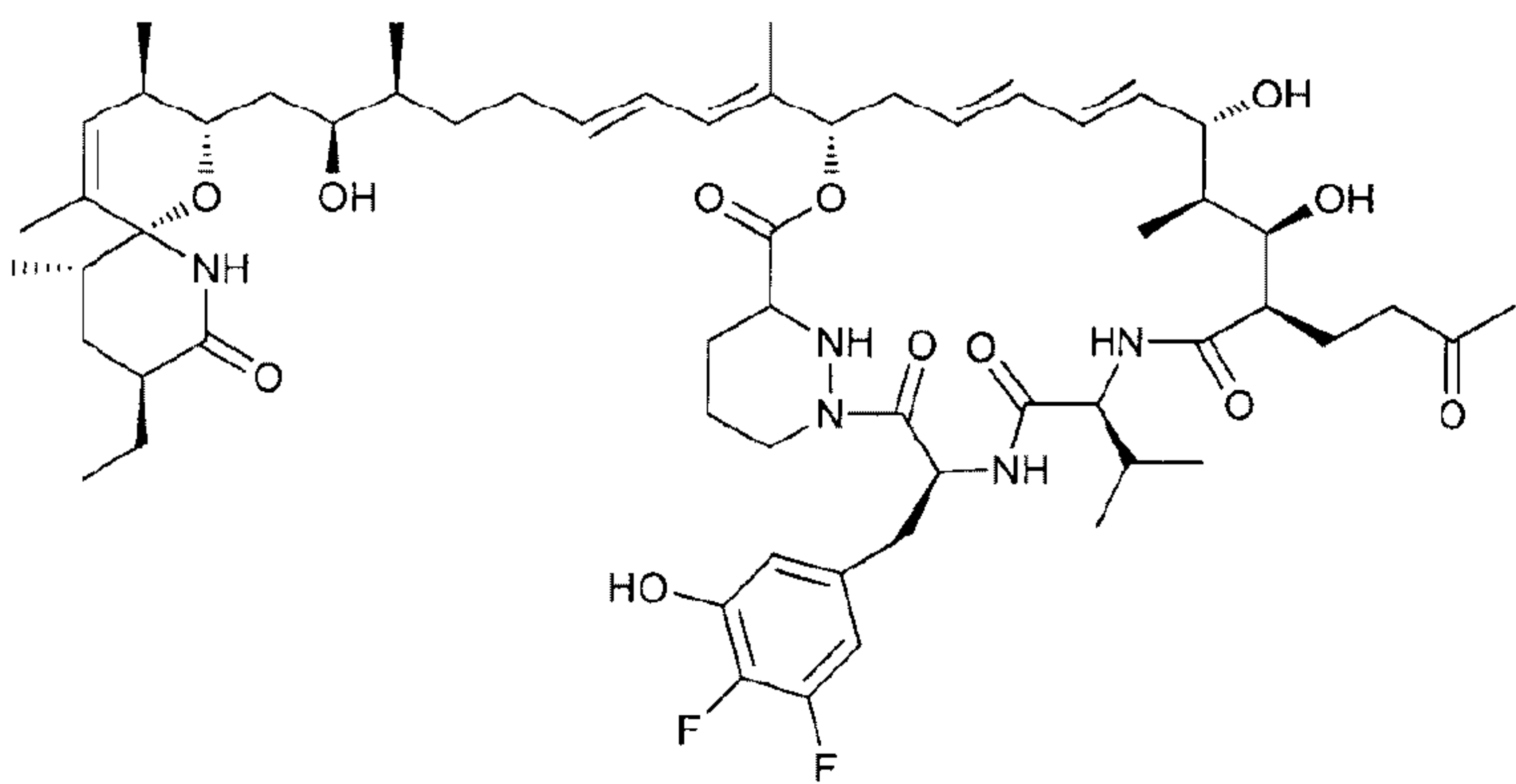
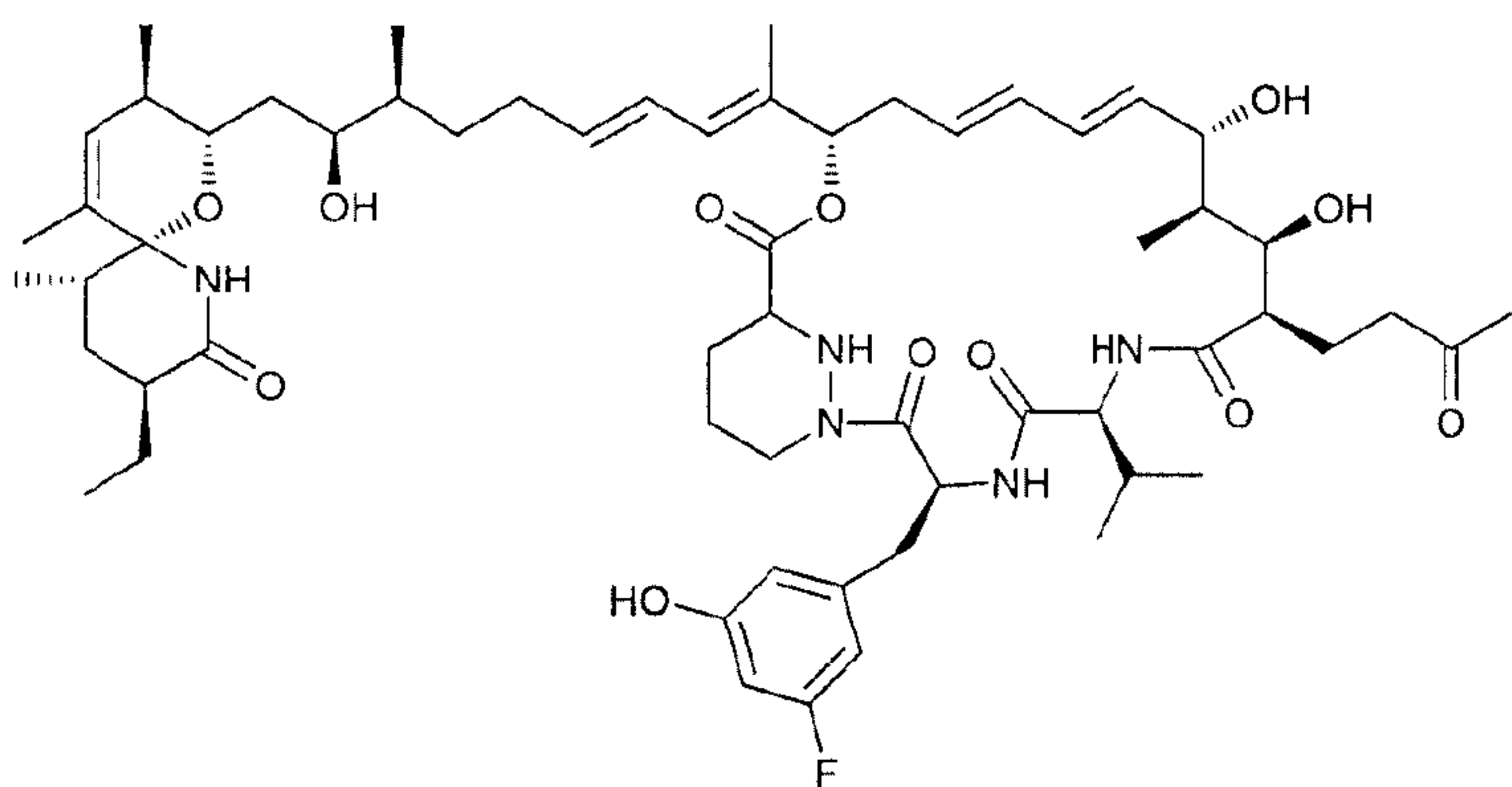
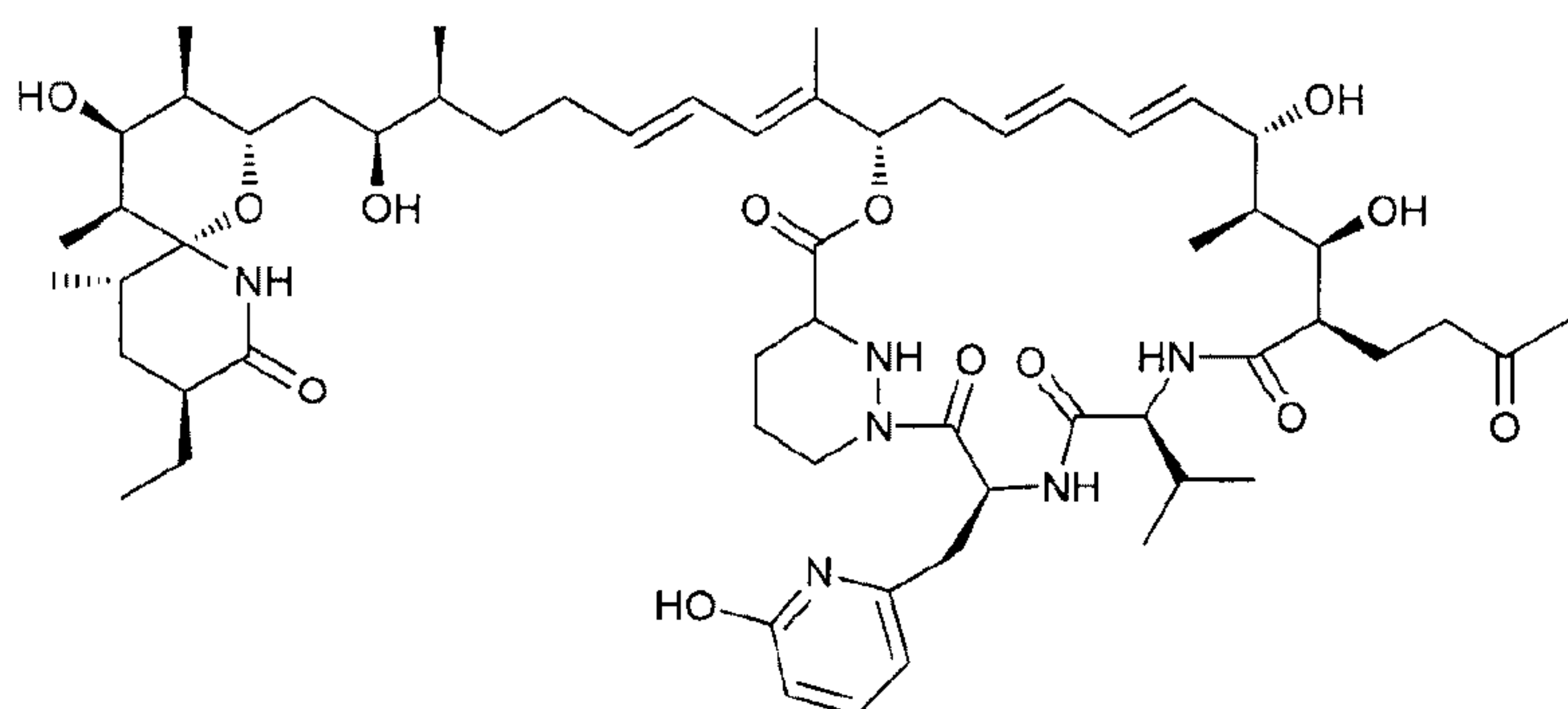
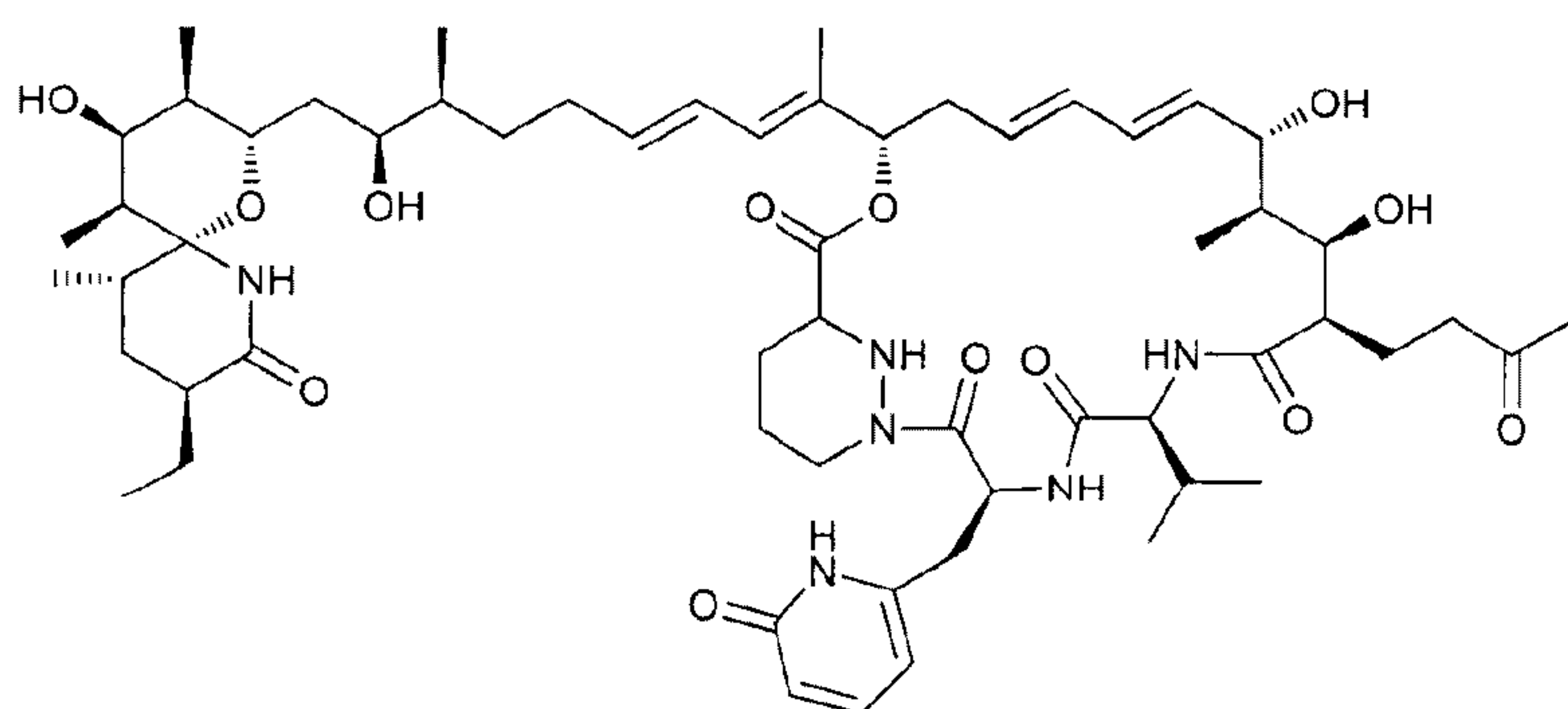
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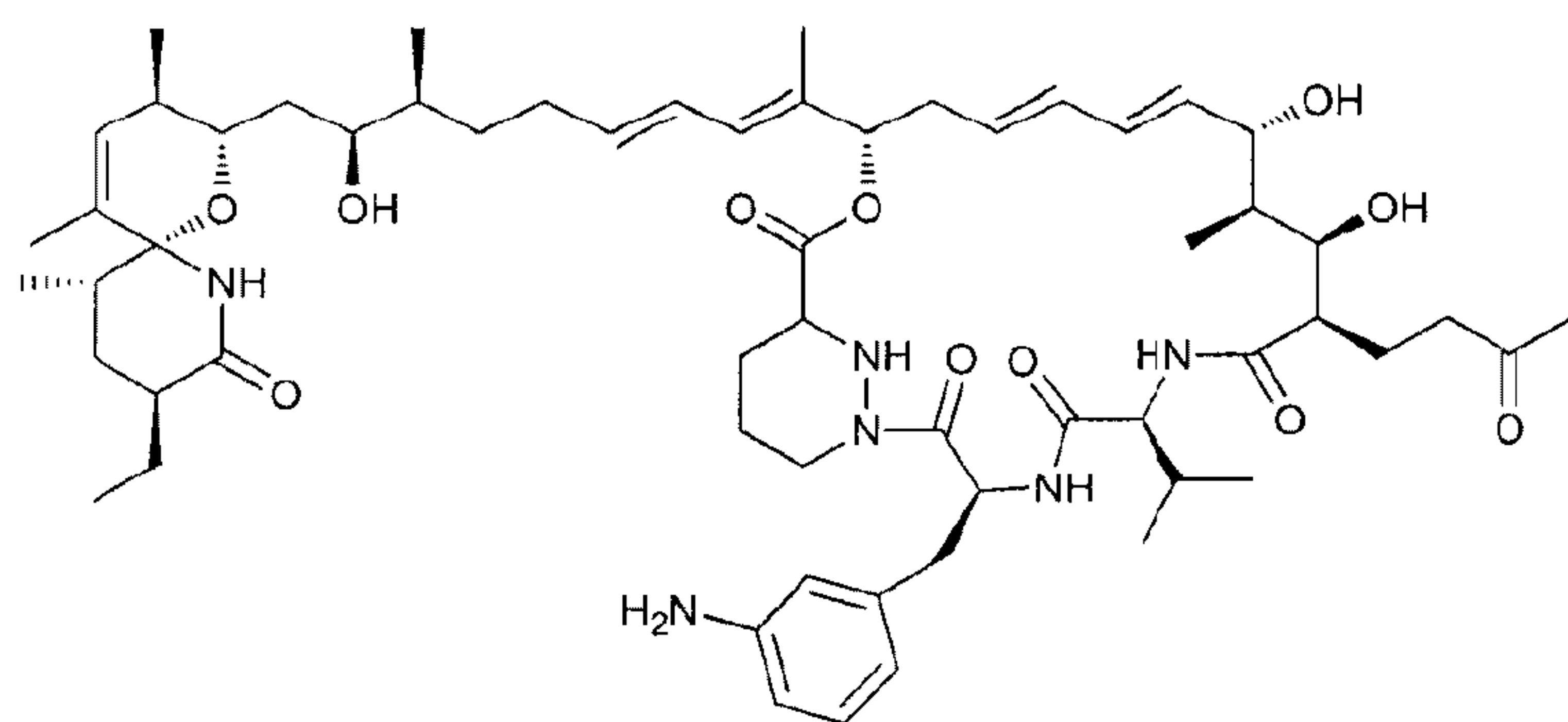
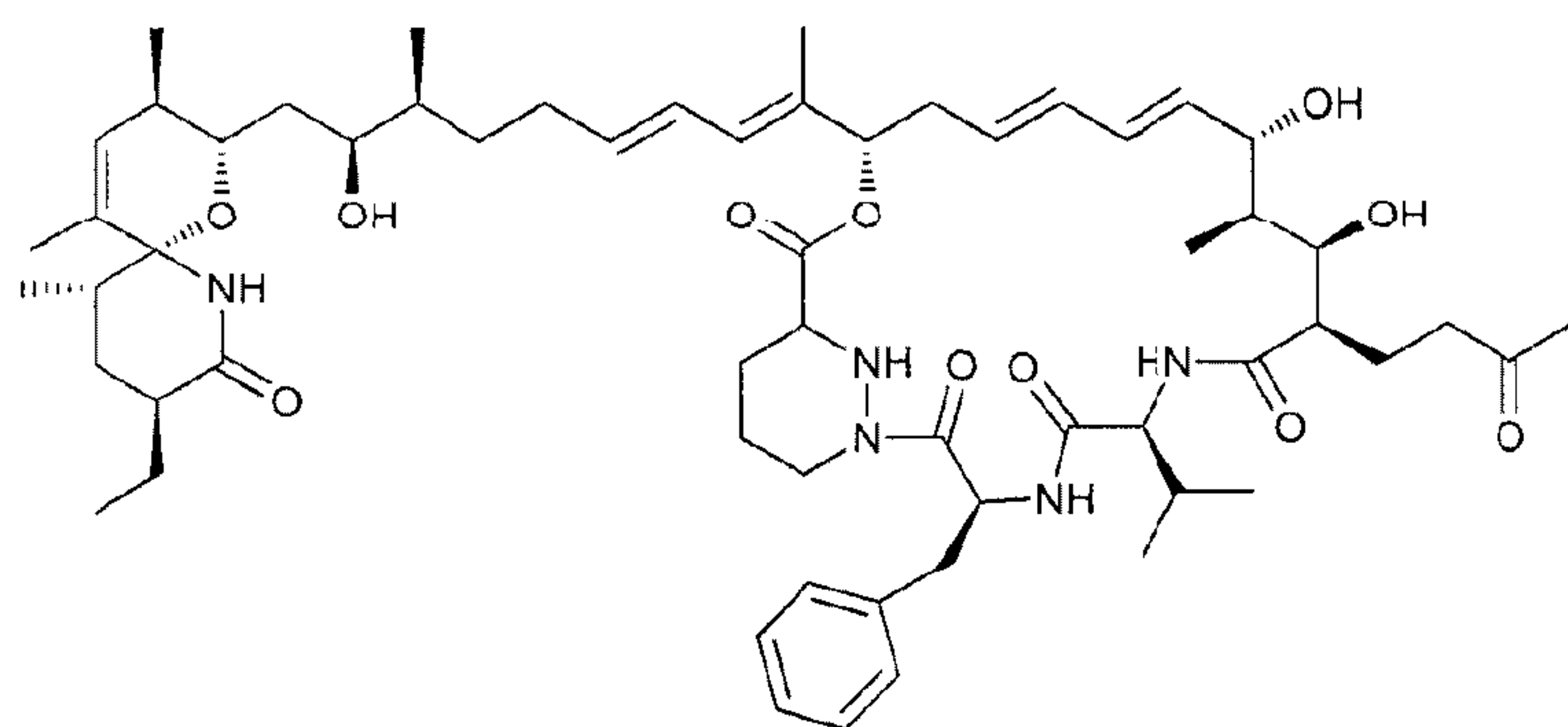
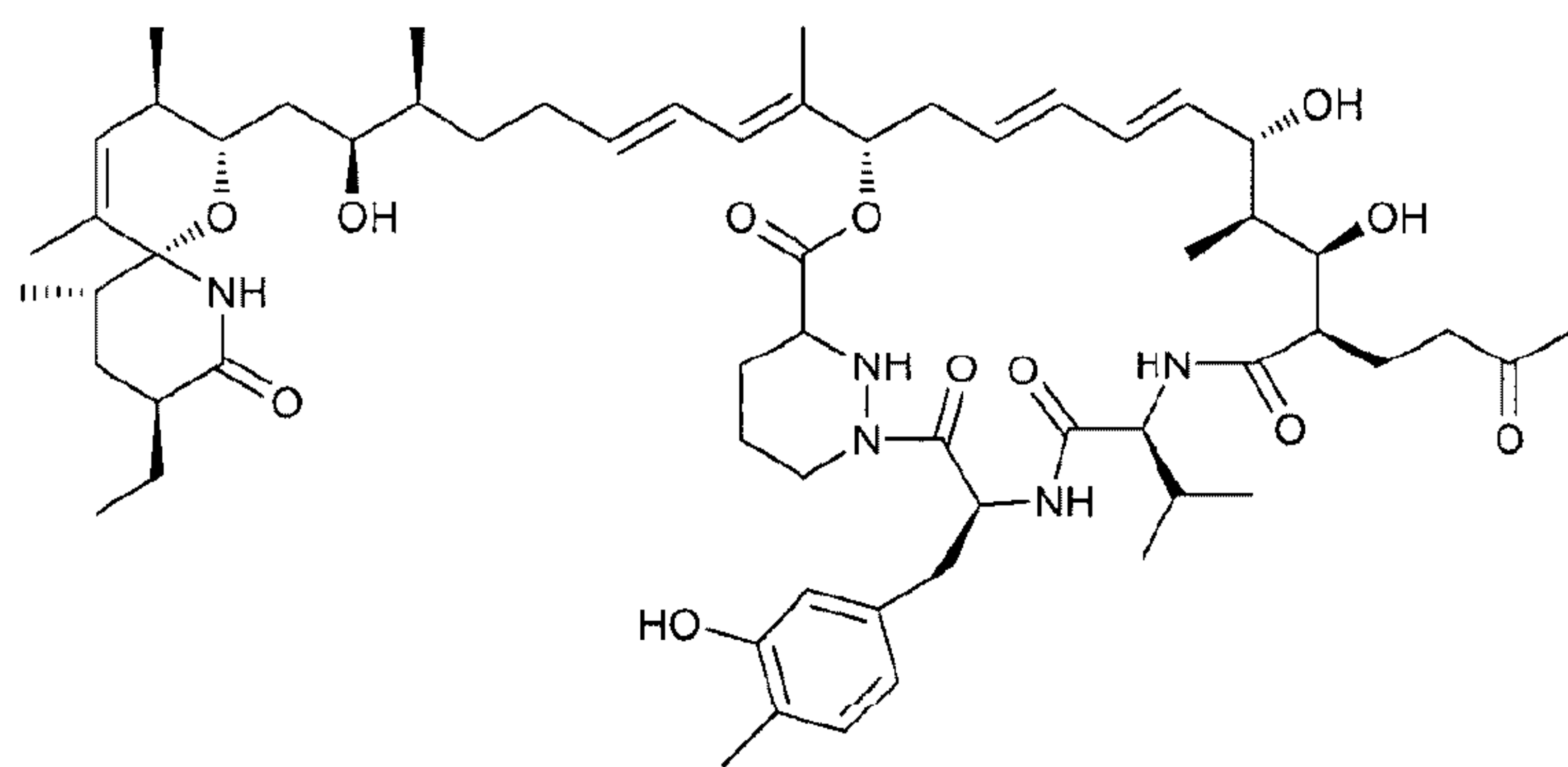
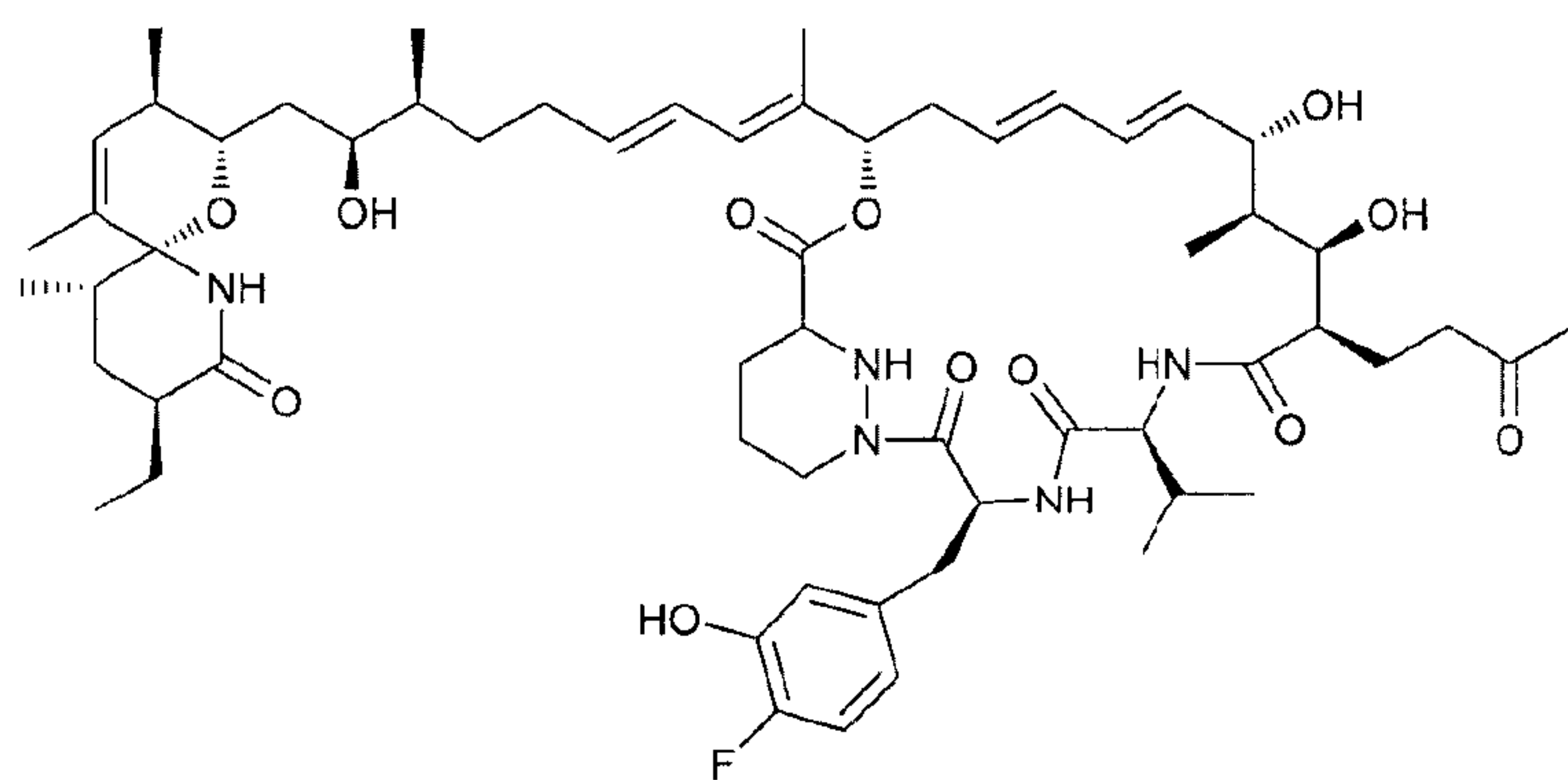


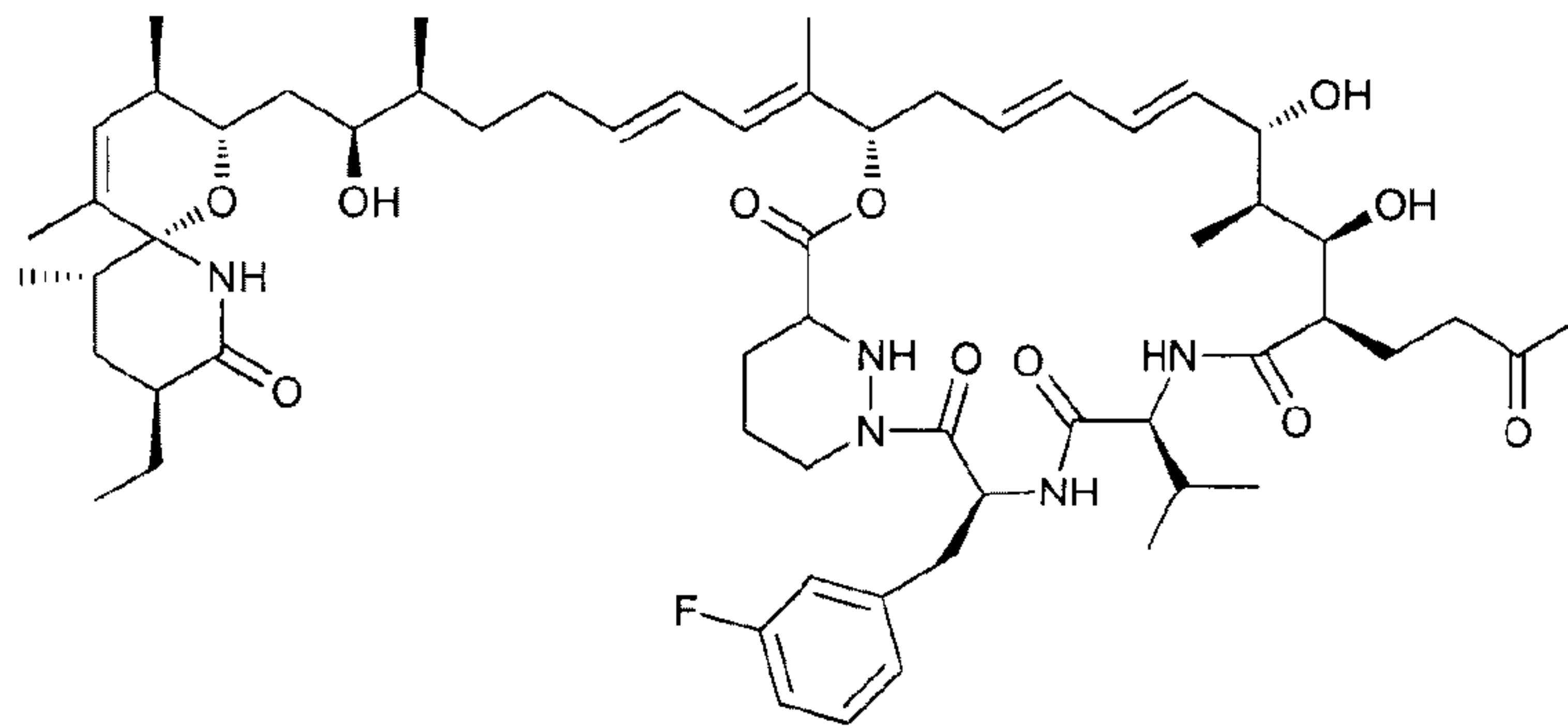
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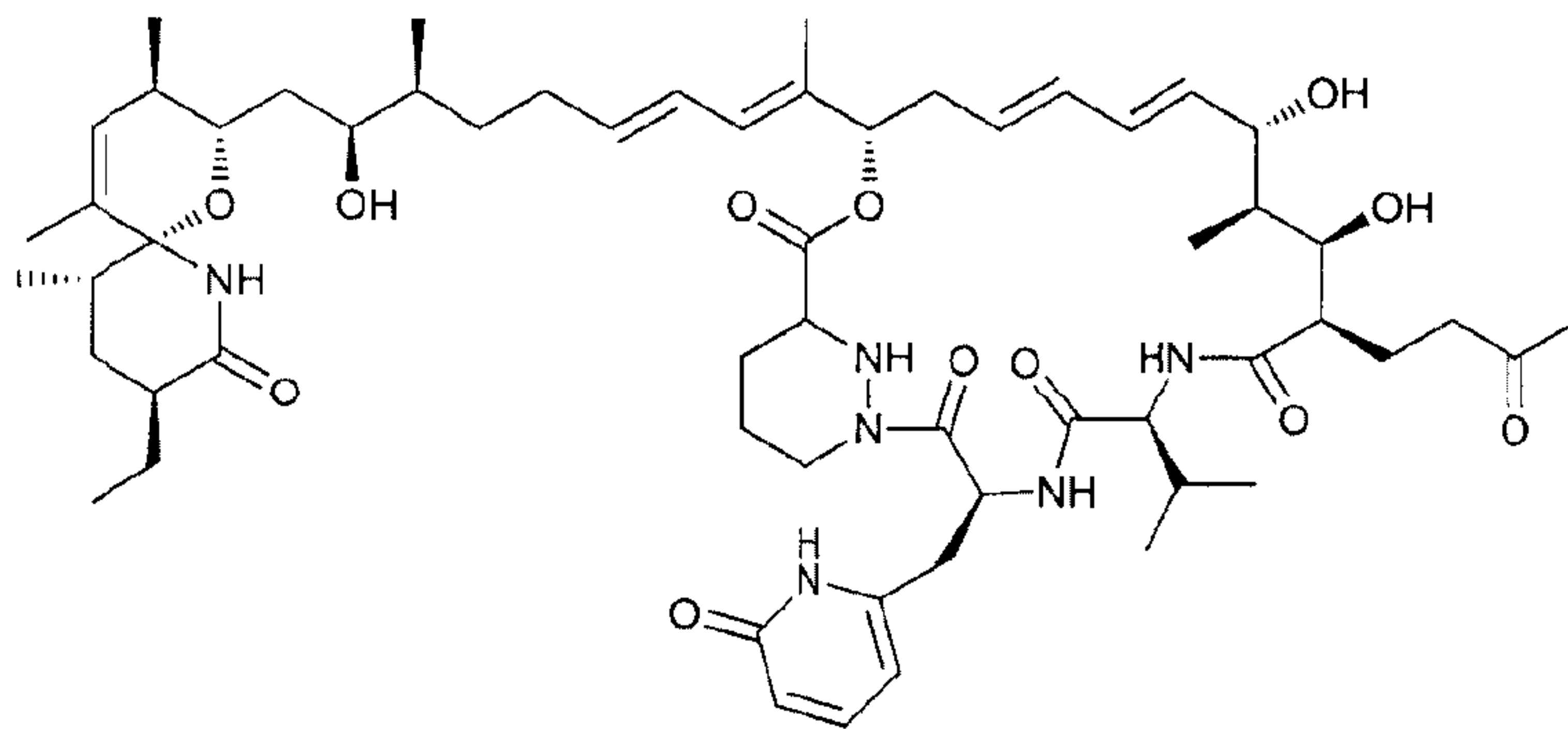
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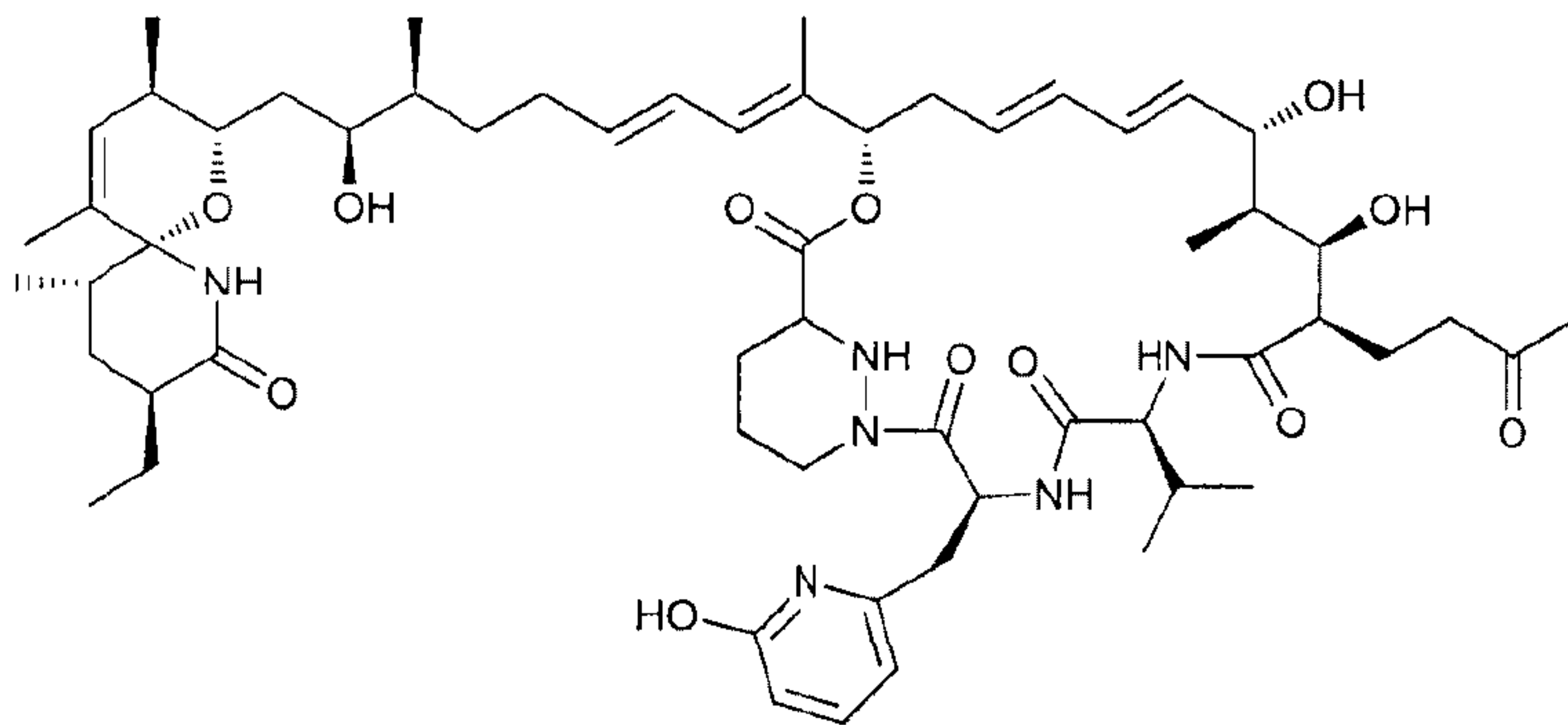




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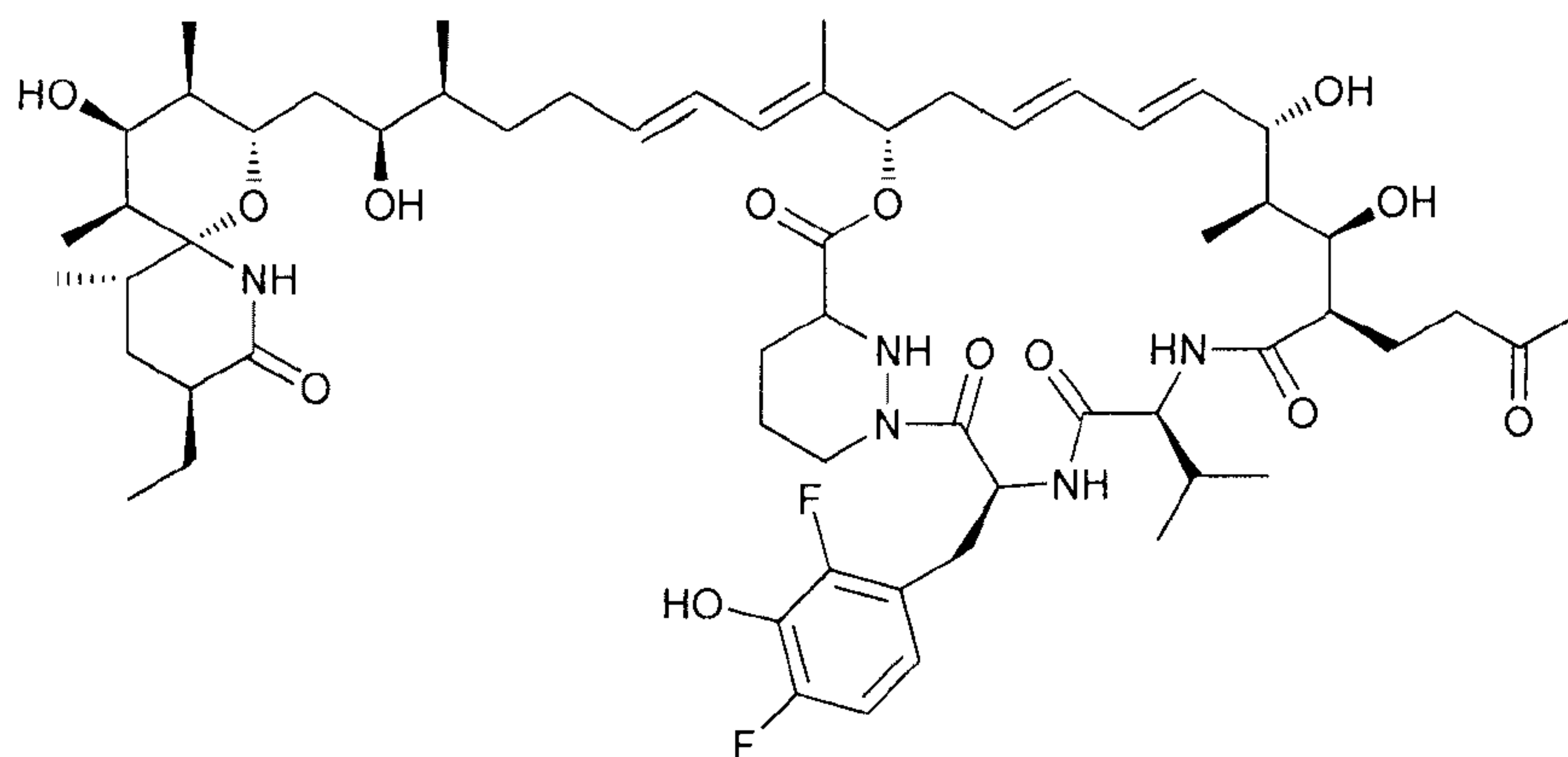
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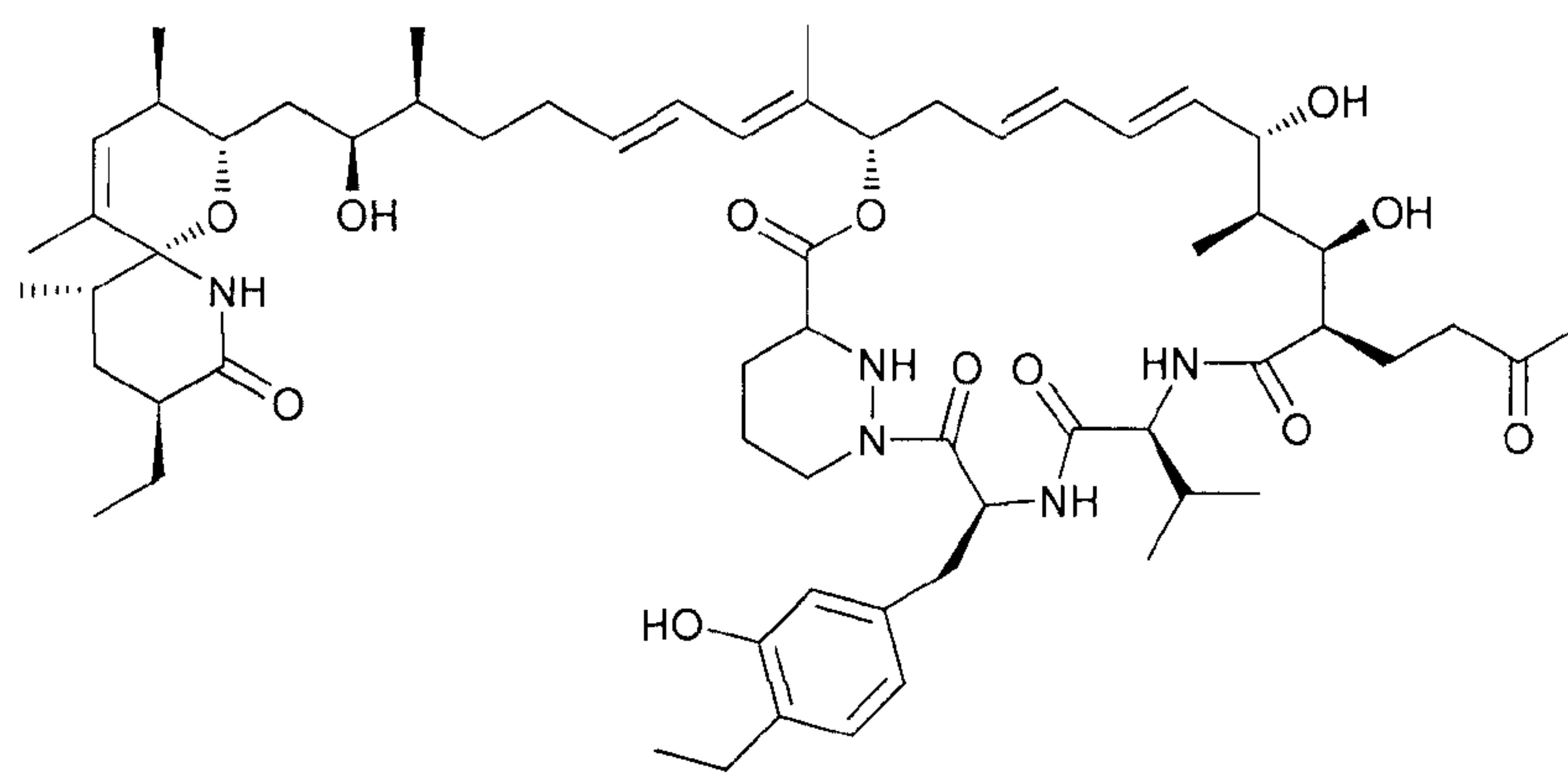
including any tautomer thereof; or an isomer thereof in which the C26, 27 C=C bond shown as *trans* is *cis*; and including a methanol adduct thereof in which a ketal is formed by the combination of the C-53 keto and the C-15 hydroxyl group and methanol;

or a pharmaceutically acceptable salt thereof.

2. A compound selected from the group consisting of:



and



;

including any tautomer thereof; or an isomer thereof in which the C26, 27 C=C bond shown as *trans* is *cis*; and including a methanol adduct thereof in which a ketal is formed by the combination of the C-53 keto and the C-15 hydroxyl group and methanol; or a pharmaceutically acceptable salt thereof.

3. The compound according claim 1 or 2 for use as a pharmaceutical for the treatment of a viral infection.
4. The compound according to claim 3, wherein the viral infection is a HCV or a HIV infection.
5. A pharmaceutical composition comprising a compound as defined in claim 1 or 2 together with a pharmaceutically acceptable diluent or carrier.
6. The pharmaceutical composition according to claim 5 further comprising a second or subsequent active ingredient.

7. Use of a therapeutically effective amount of a compound as defined in claim 1 or 2 for the treatment of a viral infection.
8. Use of a therapeutically effective amount of a compound as defined in claim 1 or 2 for the manufacture of a medicament for the treatment of a viral infection.
9. The use of claim 7 or 8, wherein the viral infection is a HCV or a HIV infection.
10. Use of a therapeutically effective amount of a compound as defined in claim 1 or 2 for the treatment of an inflammatory disorder.
11. Use of a therapeutically effective amount of a compound as defined in claim 1 or 2 for the manufacture of a medicament for the treatment of an inflammatory disorder.
12. The compound according to claim 1 or 2 for use as a pharmaceutical for the treatment of an inflammatory disorder.

Figure 1

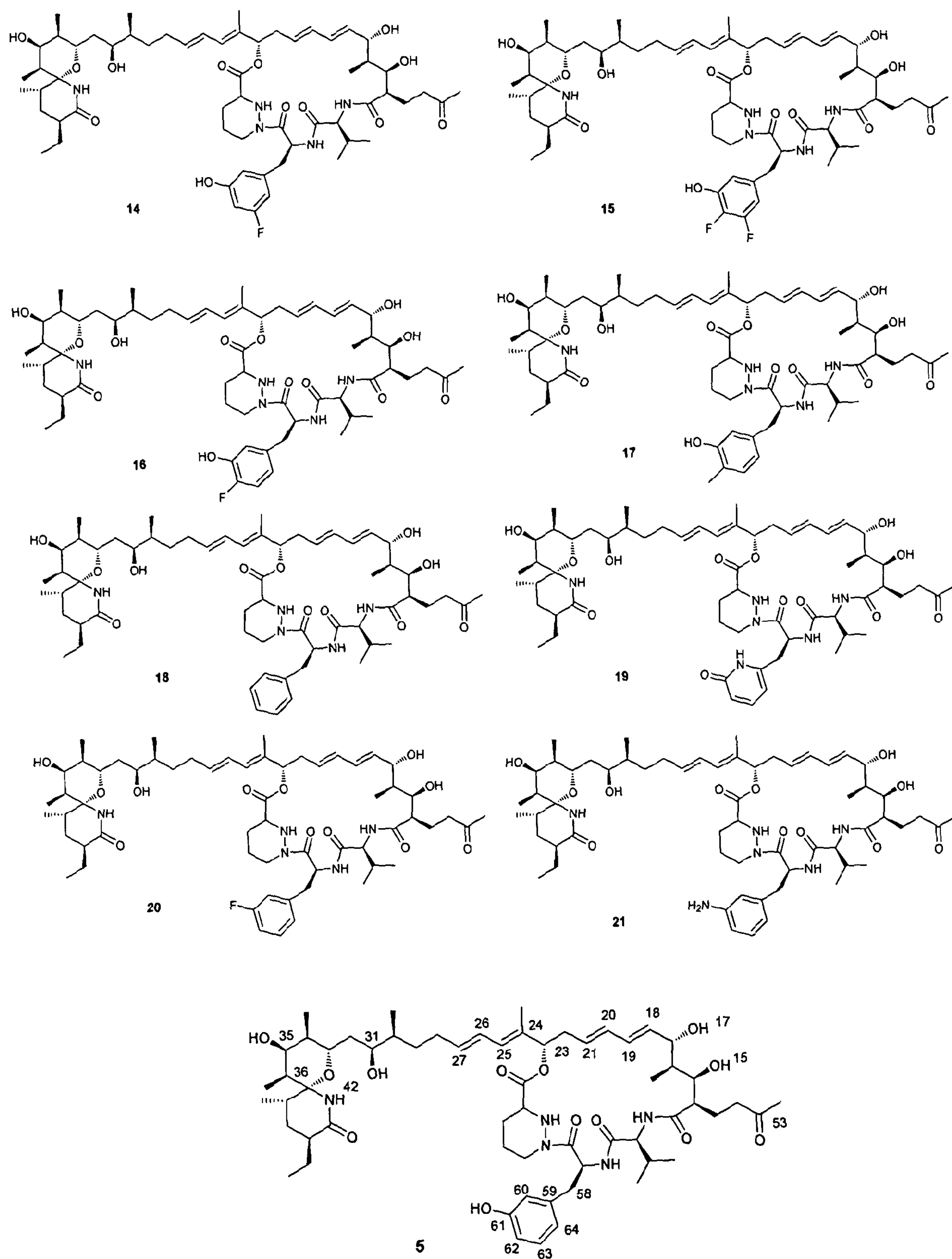


Figure 2

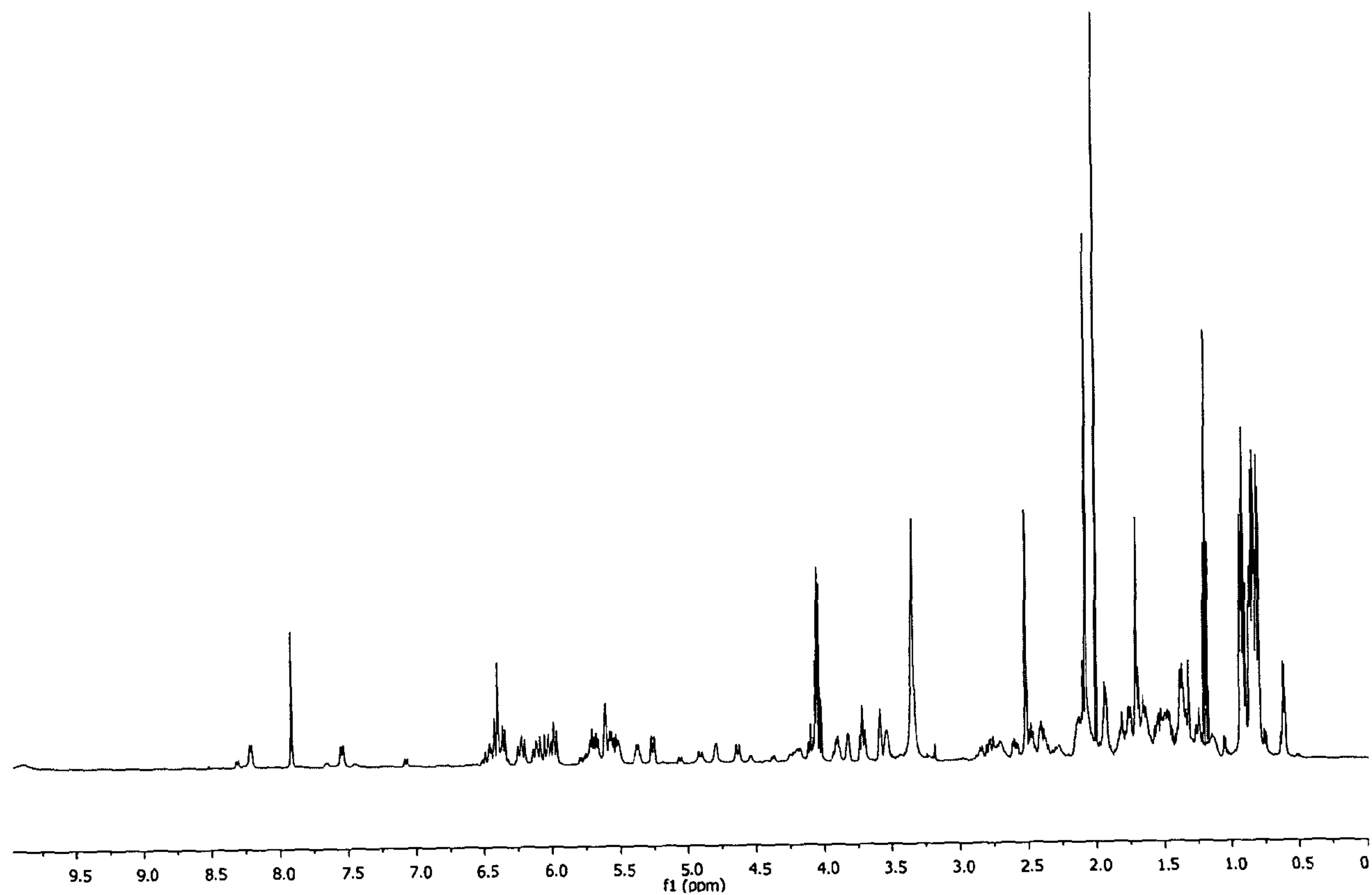


Figure 3

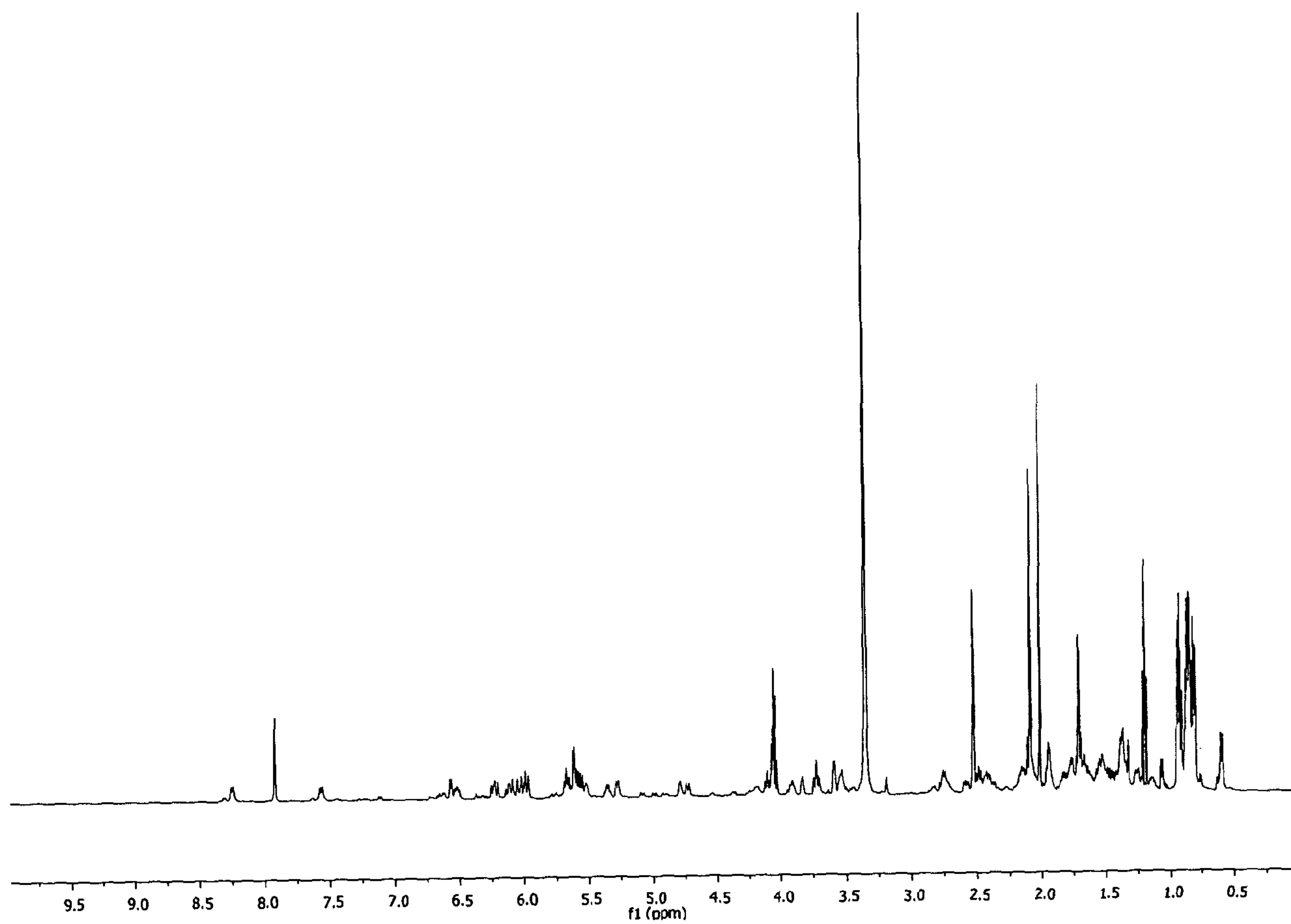


Figure 4

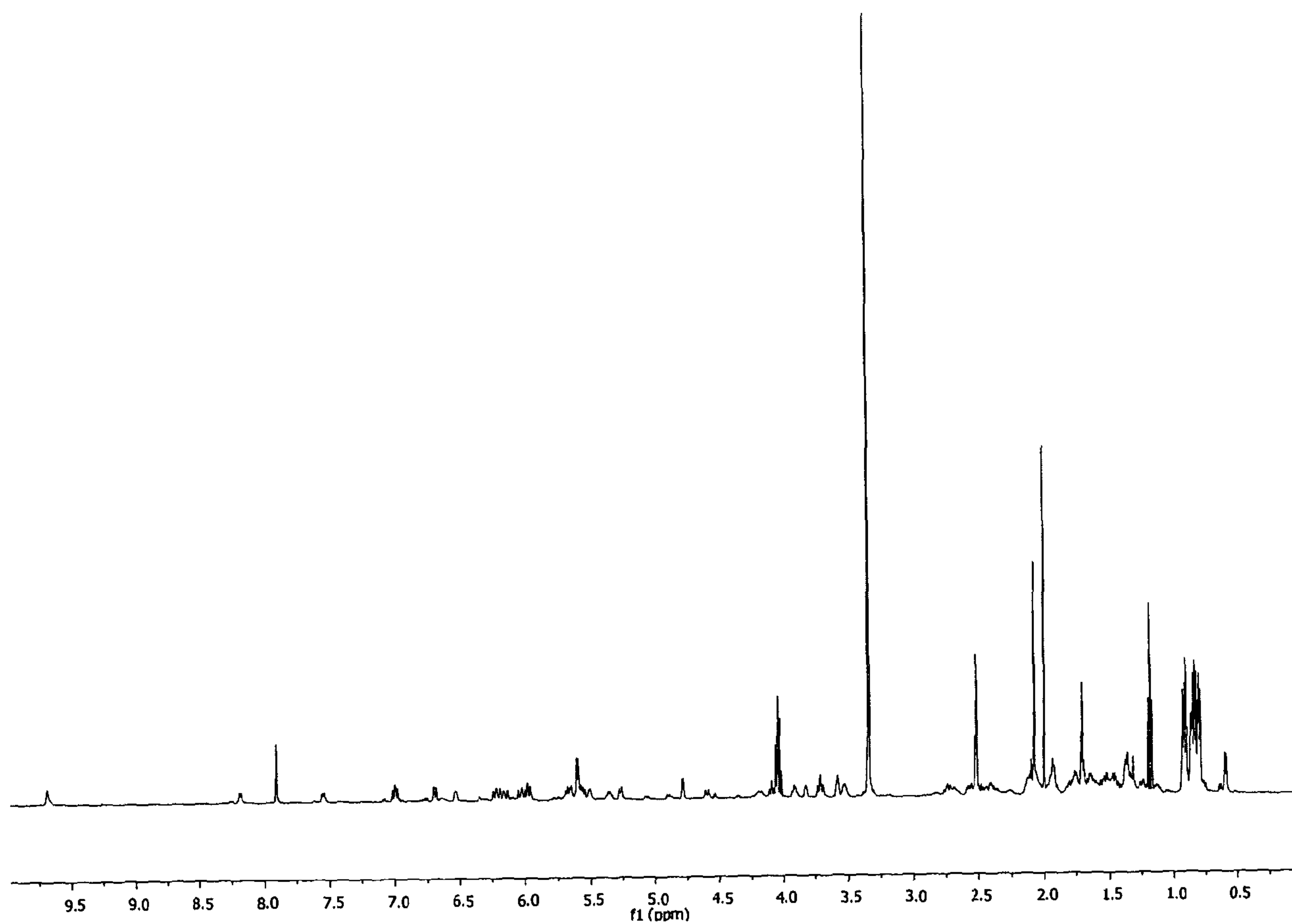


Figure 5

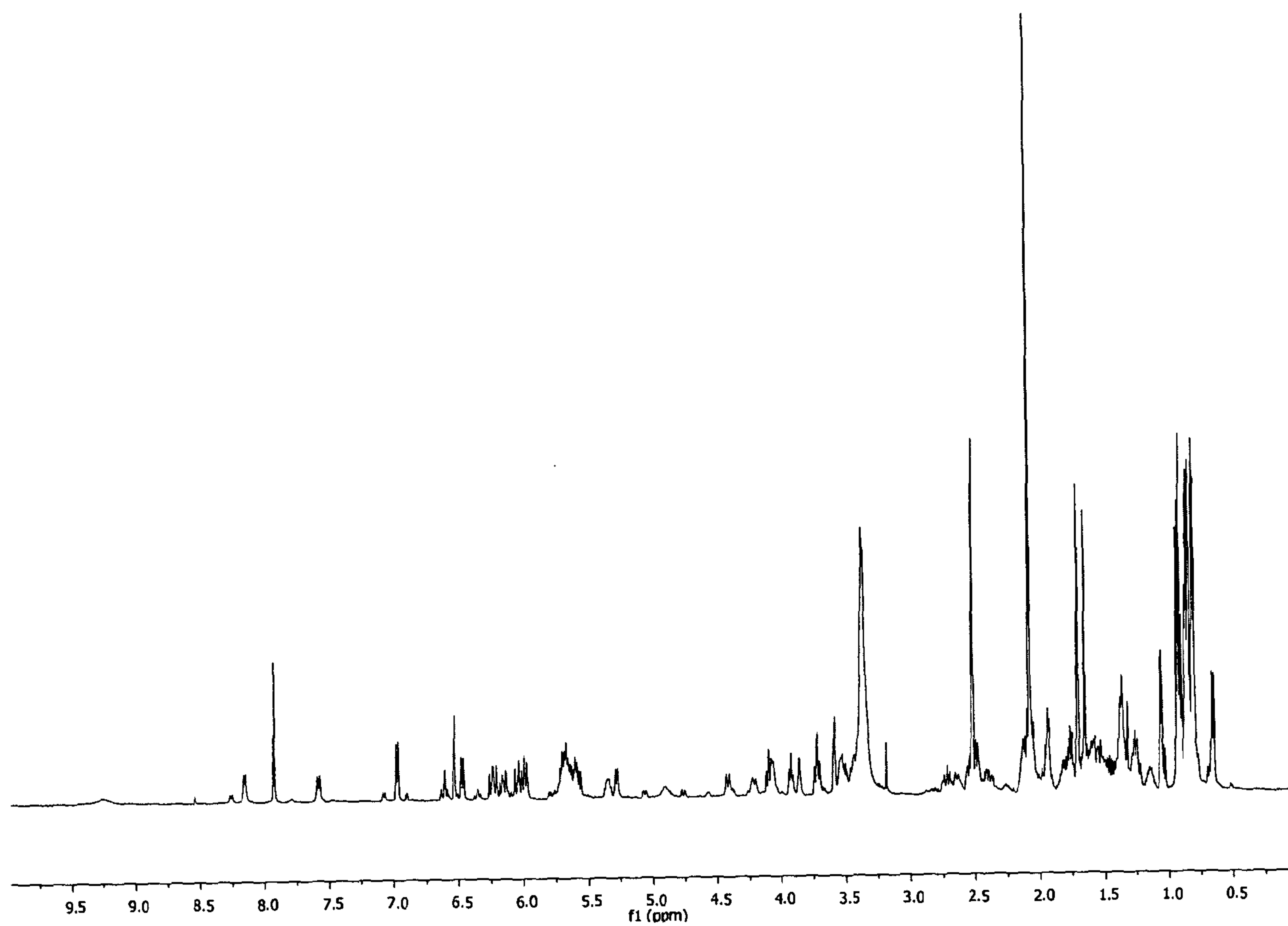


Figure 6

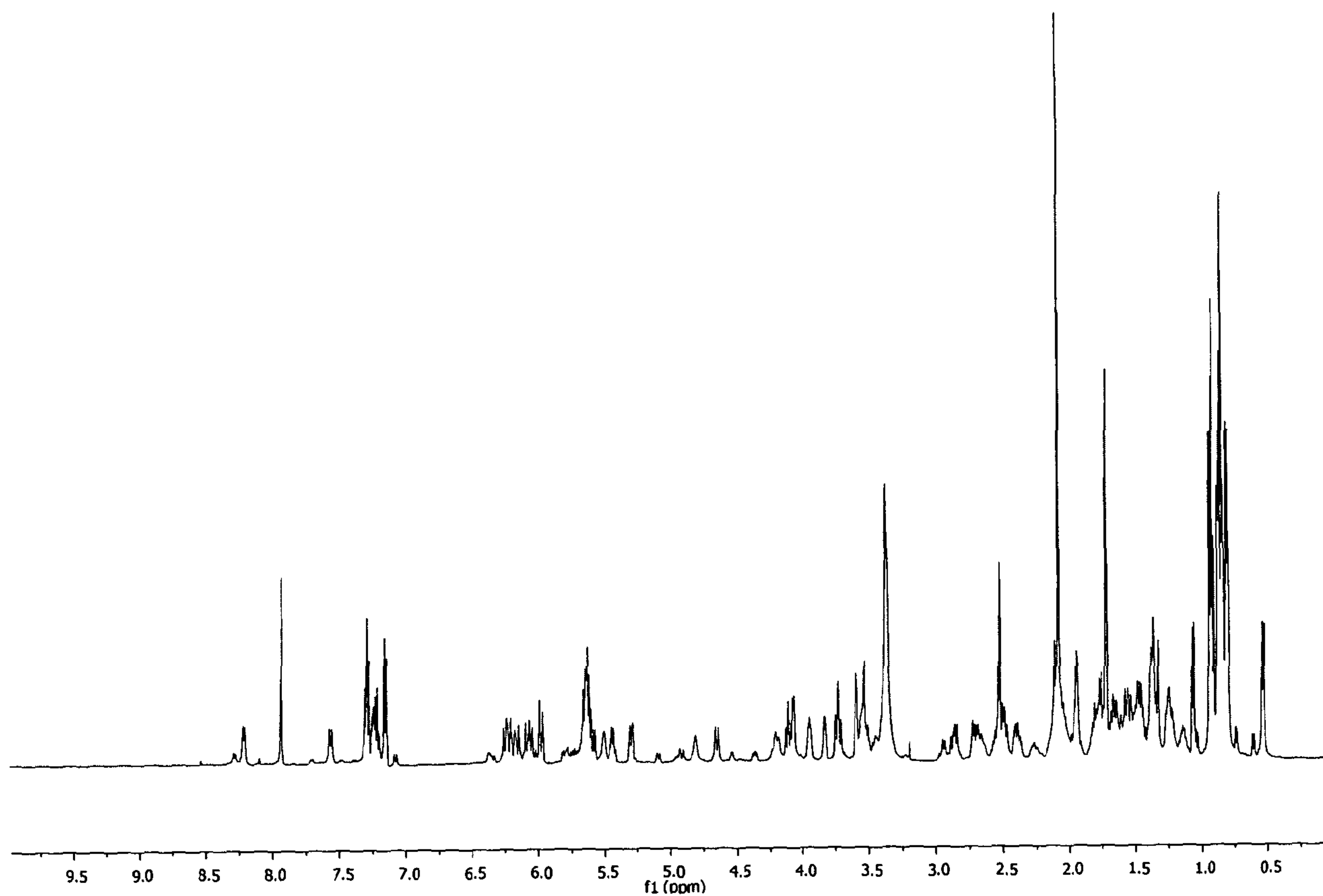


Figure 7

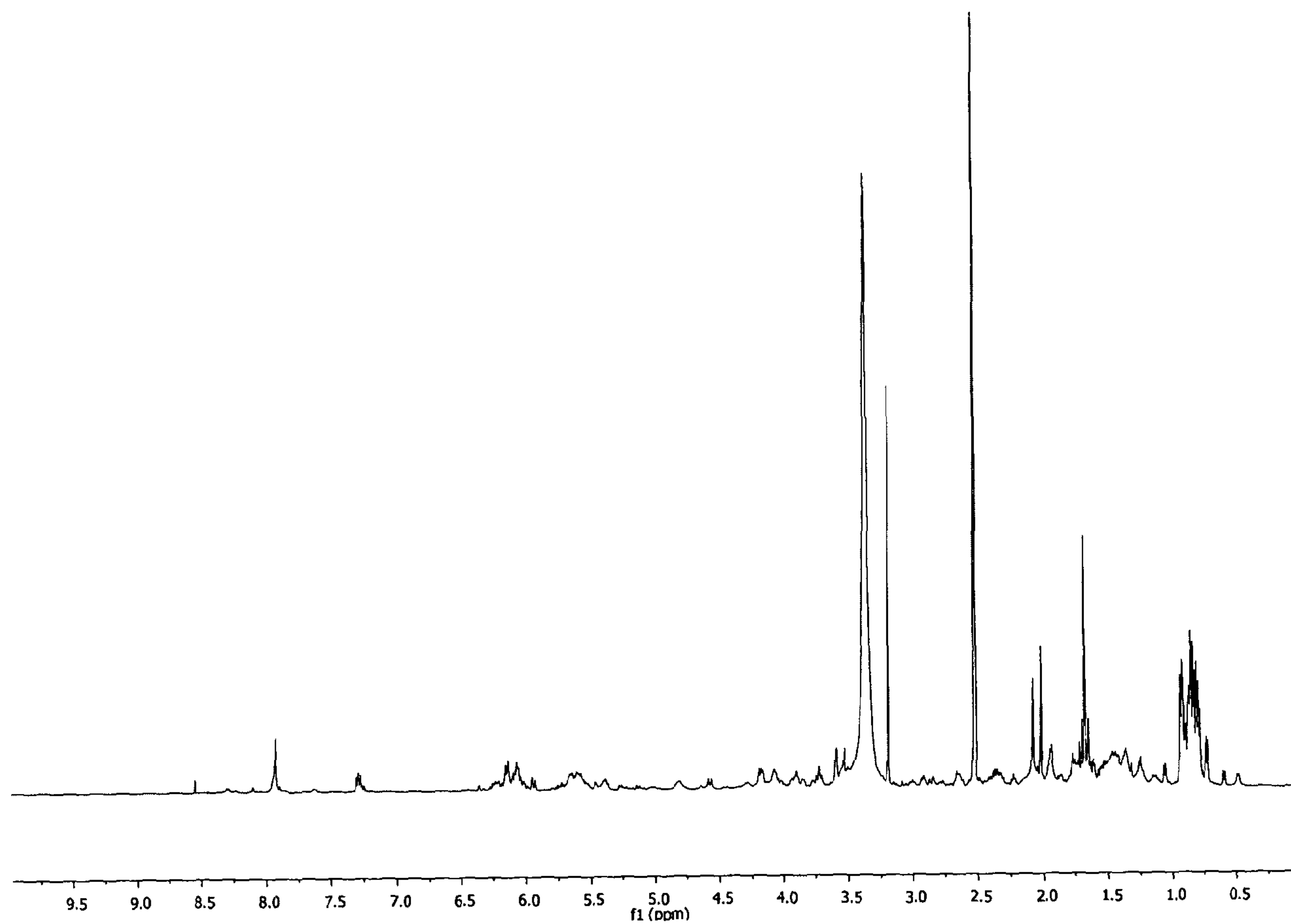
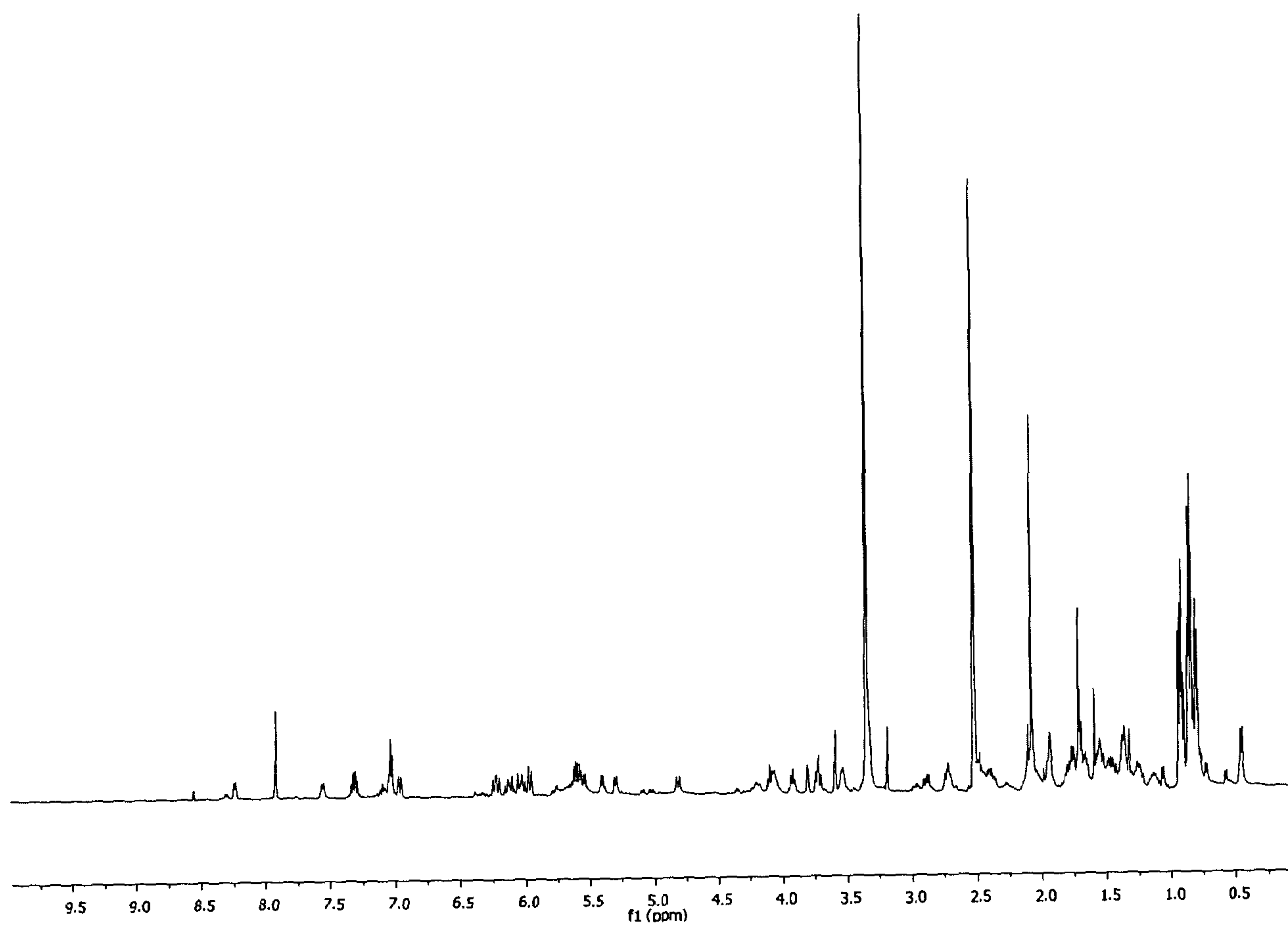
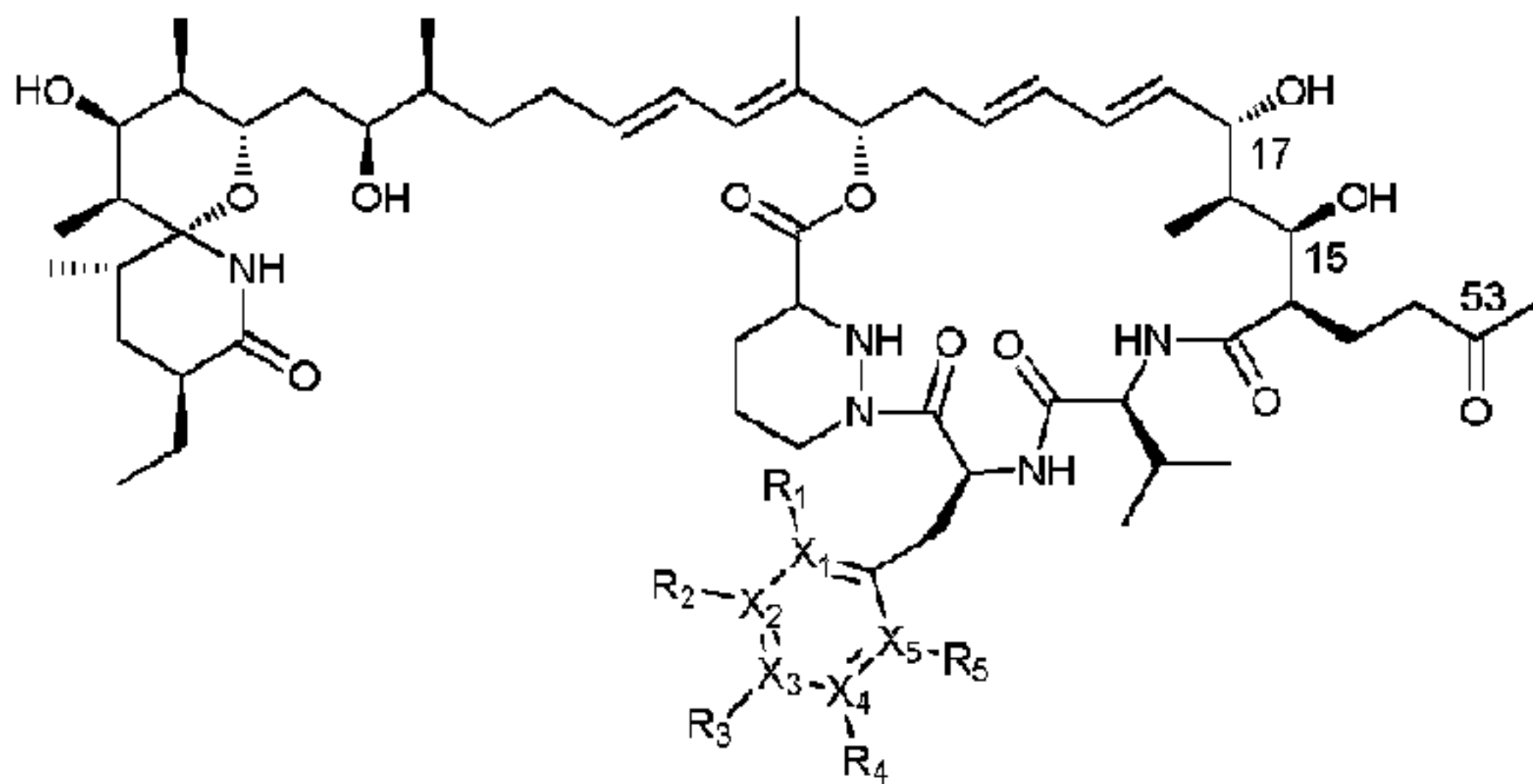
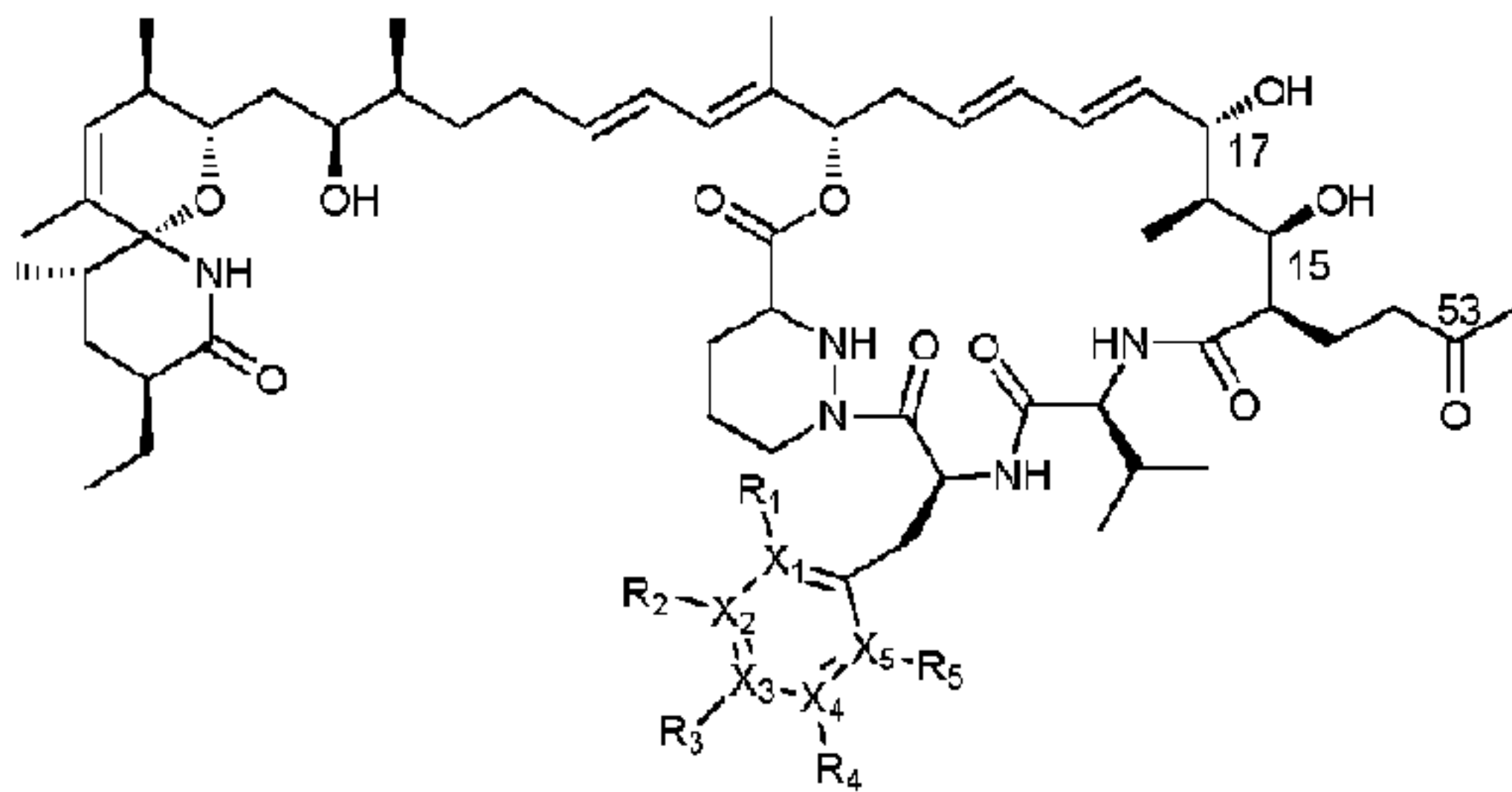


Figure 8





(I)



(II)