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Total synthesis of the *Mycobacterium tuberculosis* dideoxymycobactin-838 and stereoisomers: Diverse CD1a-restricted T cells display a common hierarchy of lipopeptide recognition

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Abstract: *Mycobacterium tuberculosis* produces dideoxymycobactin-838 (DDM-838), a lipopeptide that potently activates T cells upon binding to the MHC-like antigen-presenting molecule CD1a. *M. tuberculosis* produces DDM-838 in only trace amounts and a previous solid-phase synthesis provided sub-milligram quantities. We describe a high yielding solution-phase synthesis of DDM-838 that features a Mitsunobu substitution that avoids yield-limiting epimerization at lysine during esterification, and amidation conditions that prevent double-bond isomerization of the *Z*-C20:1 acyl chain, and provides material with equivalent antigenicity to natural DDM-838. Isomers of DDM-838 that varied in stereochemistry at the central lysine and the C20:1 acyl chain were compared for their ability to be recognised by CD1a-restricted T cell receptors (TCRs). These TCRs, derived from unrelated human donors, exhibited a similar spectrum of reactivity towards the panel of DDM-838 isomers, highlighting the exquisite sensitivity of lipopeptide-reactive T cells for the natural DDM stereochemistry.

Introduction

Humans express four antigen-presenting proteins (CD1a, CD1b, CD1c and CD1d) belonging to the CD1 family, which present lipid-based molecules to T cells via their T cell receptors (TCRs).[1] CD1a differs from the other CD1 molecules based on its constitutive, high-density expression on dendritic cells, termed Langerhans cells, in the skin and mucosa.[2] Moreover, distinct from the other human CD1 molecules, CD1a possesses a cytoplasmic tail that lacks lysosomal targeting motifs. Thus, CD1a localizes predominantly at the cell surface where it can promiscuously capture foreign or exogenous lipids. The only extensively-characterized type of foreign lipidic molecule that activates CD1a-restricted T cells is a family of lipopeptides isolated from *Mycobacterium tuberculosis*, termed dideoxymycobactins (DDMs).[3]

The initial discovery of the DDMs was achieved through bioassay-guided fractionation using a single T cell clone (CD8-2) obtained through stimulation and expansion with a chloroform/methanol *M. tuberculosis* extract.[4] A three-dimensional X-ray structure of CD1a with a silylated, DDM-like lipopeptide showed the fatty acyl chain buried in the A' pocket of CD1a with the peptide protruding to the surface of CD1a, where it presumably contacts the TCR.[5] The recent development of CD1a tetramers and dextramers that bind directly to polyclonal T cells in the *ex vivo* setting has allowed the detection and isolation of DDM-reactive T cell clones from small cohorts of tuberculosis patients.[6] This suggests that CD1a–DDM reactive T cells occur in unrelated individuals and that CD1a–DDM tetramers may be useful reagents for the *ex vivo* diagnosis of *M. tuberculosis* infection. Further, the existence of polyclonal T cells in small human cohorts raises the possibility that DDMs could be used to immunize or stimulate T cell responses in tuberculosis patients.[6] However, the key obstacle to these lines of new medical technology development is the absence of an abundant supply of DDM.

DDMs are extremely potent, but comprise less than 10 parts per million of the cell wall mass of *M. tuberculosis*, a

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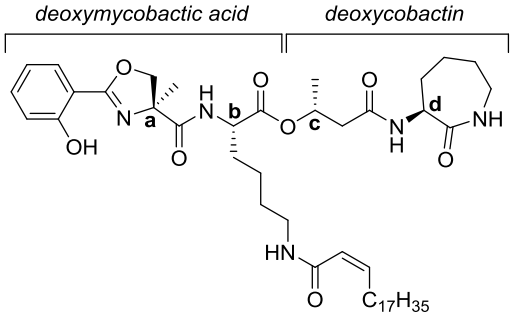
pathogen, whose laboratory cultivation is slow and cumbersome because it must be grown in biosafety level 3 conditions. Thus, isolation in high yield from natural sources remains impractical.[3] The key challenge for chemical synthesis is that naturally occurring DDMs possess a complex depsipeptide backbone bearing a range of saturated and unsaturated fatty acyl groups linked through an amide to the ε-amino group of a lysine residue, and an unusual α-methylserine-derived angular methyl group. The structure of the most reactive compound, DDM-838 (**1**, Figure 1) was proposed through mass spectrometric and NMR analysis,[3a] as well as the preparation of a range of possible stereoisomers and matching of their spectroscopic data and biological activity to the natural product,[3b] and is consistent with the stereochemistry of the biosynthetically-related mycobactins, iron-chelating hydroxamic acid siderophores.



	a	b	c	d	alkene
1 (DDM-838)	*S*	*S*	*R*	*S*	*Z*
2	*S*	*S*	*R*	*S*	*E*
3	*S*	*R*	*R*	*S*	*Z*
4	*S*	*R*	*R*	*S*	*E*

Figure 1. DDM-838 (**1**) and three stereoisomers.

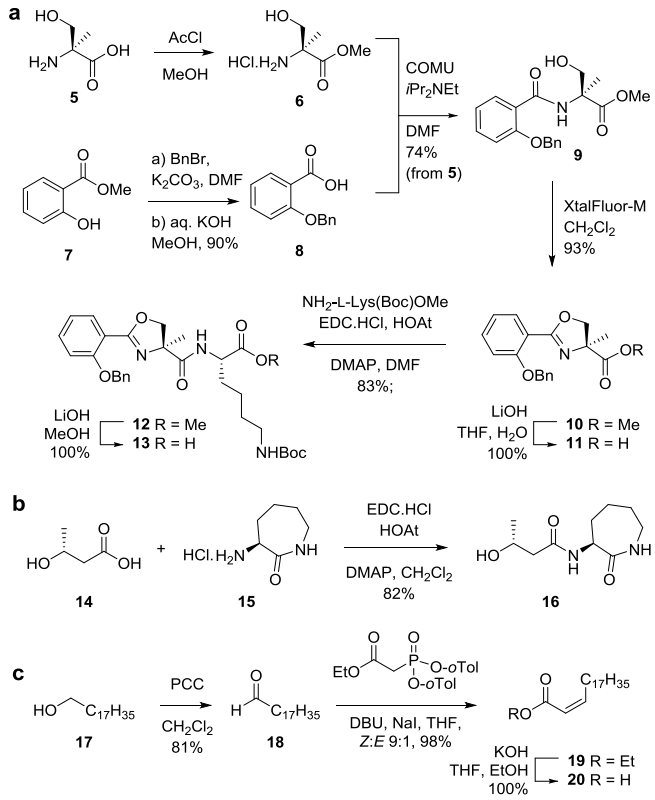
A previous synthetic approach[3b] to DDM-838 used solid-phase peptide chemistry to provide sufficient material to help clarify the structure of the natural product, yet the poor overall yield provided only sub-milligram quantities that are insufficient for *in vivo* studies. Prior work demonstrated that the correct stereochemistry at positions a and c (Figure 1), and the angular methyl group at position a are necessary for activating the DDM-reactive T cell clone CD8-2, and that natural DDMs lacking the unsaturation in the acyl chain are at least 10-fold less potent for T cell activation.[3] In the present work we investigate solution-based peptide-coupling approaches for the preparation of milligram quantities of DDM-838. Through careful isolation of by-products produced in this process, we identify difficulties with the establishment of the key ester linkage that occurs with partial epimerization at lysine, a problem that may have occurred in the previous approach of Young and coworkers (*unpublished*). This and other problems were overcome by the development of a new route that creates the challenging ester linkage through a Mitsunobu coupling and allows the preparation of milligram quantities of DDM-838 (**1**). Through the isolation of stereochemical isomers of DDM-838 (**2**-**4**) we have explored structure-activity relationships in antigen recognition by the prototypical DDM-reactive T cell clone CD8-2, as well as new DDM-reactive clones, and demonstrate the importance of peptide and lipid stereochemistry for T cell receptor recognition and T cell activation.

Results and Discussion

Total synthesis of DDM-838

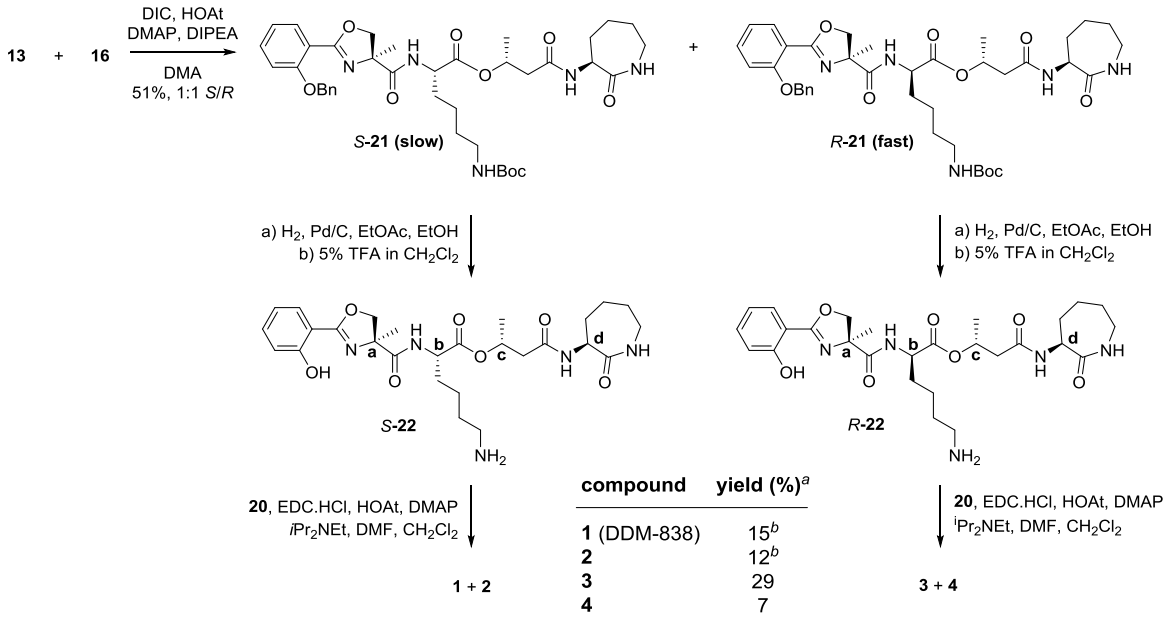
Young and co-workers reported the synthesis of DDM-838 and analogues, emphasizing solid-phase approaches.[3b] For assembly of DDM-838 itself, a linear depsipeptide bearing the fatty acyl chain was assembled on Fmoc-Lys Sasrin resin. Following release of the depsipeptide from the resin, the terminal lysine was cyclized to form the caprolactam moiety. After extensive HPLC purification, this approach yielded only trace quantities of DDM-838 (~95 μg or 2% overall yield), which we verified independently. As this synthesis was conducted on solid phase, it is difficult to identify the yield-limiting steps. In a solution-phase approach, used for the synthesis of analogues that lacked the distinguishing α-methyl group, an ε-amino-protected deoxymycobactic acid was coupled to deoxycobactin, and the depsipeptide was deprotected prior to attaching the fatty acyl chain. This resulted in low yields for both attachment of the fatty acyl chain and for formation of the ester linkage. Moreover, unexpected doubling of signals in NMR spectra of advanced intermediates was attributed to the presence of rotational isomers, but could alternatively reflect the formation of undefined stereoisomers. With the above issues in mind, we initially sought to develop a solution-based synthesis of DDM-838 broadly inspired by this earlier study,[3b] but with the intention to fully characterize all intermediates and identify yield-limiting steps in order to identify an improved synthetic approach.

The required building block fragments deoxymycobactic acid **13**, deoxycobactin **16**, and *Z*-C20:1 fatty acid **20** were prepared by optimization of the previously described literature approaches.[3b] Oxazoline acid **11** was prepared from α-methyl-L-serine **5**, by protection as the methyl ester **6**, which was condensed with 2-benzyloxysalicylic acid **8** using COMU to afford amide **9** (Scheme 1a).[7] Cyclization to the oxazoline **10** proceeded smoothly with XtalFluor-M in CH_2Cl_2.[8] Saponification of the ester of **10** with LiOH afforded the *O*-benzyl-protected acid **11**, which was coupled with NH_2-L-Lys(Boc)OMe using EDC.HCl/HOAt/DMAP. Saponification of **12** afforded deoxymycobactic acid **13**. Next, deoxycobactin **16** was prepared by coupling of (*R*)-3-hydroxybutyric acid **14** and L-α-amino-ε-caprolactam hydrochloride **15** in the presence of EDC.HCl/HOAt/DMAP (Scheme 1b).[3b] Finally, the *Z*-C20:1 fatty acid **20** was synthesized using Ando's reagent,[9] which enables a highly *Z*-selective Horner-Emmons-Wadsworth coupling. Treatment of octadecanol **17** with PCC in CH_2Cl_2 afforded stearylaldehyde **18**, which condensed with Ando's reagent[9-10] in the presence of DBU/NaI to afford a 9:1 *Z*:*E* ratio of alkenes **19** (use of the Still-Gennari reagent provided the alkenes in a 3:1 *Z*:*E* ratio) (Scheme 1c). These isomers could be separated by careful chromatography, prior to saponification to afford the acid **20**.



Scheme 1. Synthesis of deoxymycobactic acid **13**, deoxycobactin **16**, and *Z*-C20:1 fatty acid **20**.

Coupling of deoxymycobactic acid **13** with deoxycobactin **16** was explored under a range of esterification conditions using assorted peptide coupling reagents. In general, the esterification reactions were slow and low yielding, and occurred with varying amounts of epimerization at lysine. The best results were achieved using DIC/HOAt in the presence of 1.5 eq of DMAP, which afforded a 1:1 ratio of *S*-**21**/*R*-**21** (Scheme 2). Importantly, the *O*-benzyl protected epimeric intermediates **21** were separable by preparative t.l.c., but stereochemical assignment was not possible at this stage. Each isomer was individually carried through to the lipopeptide by deprotection of benzyl and Boc groups (to give *S*- and *R*-**22**), then coupled with the *Z*-C20:1 acid **20** using EDC.HCl/HOAt/DMAP in the presence of $^{i}Pr_2NEt$. Each product was formed as a mixture of *E* and *Z* isomers, which were separated by flash chromatography to afford four pure DDM isomers **1**-**4**, and in some cases small amounts of a contaminating trifluoroacetamide. The less polar isomer **21** afforded material whose spectral data matched the natural product DDM-838 (**1**). At this stage the stereochemistry of the central lysine was not known, but established later (*vide infra*).



compound	yield (%)[a]
1 (DDM-838)	15[b]
2	12[b]
3	29
4	7

Scheme 2. Preparation of DDM-838 (**1**) and stereoisomers **2**-**4**. Slow = higher polarity product; fast = lower polarity product. [a] yield over 3 steps from (*S*)- or (*R*)-**21.** [b] analysis of ^{1}H NMR of crude mixture revealed the formation of **1**:**2**:trifluoroacetamide byproduct at a ratio of 6:3:4, presumably derived from residual trifluoroacetate anion carried over from step iii.

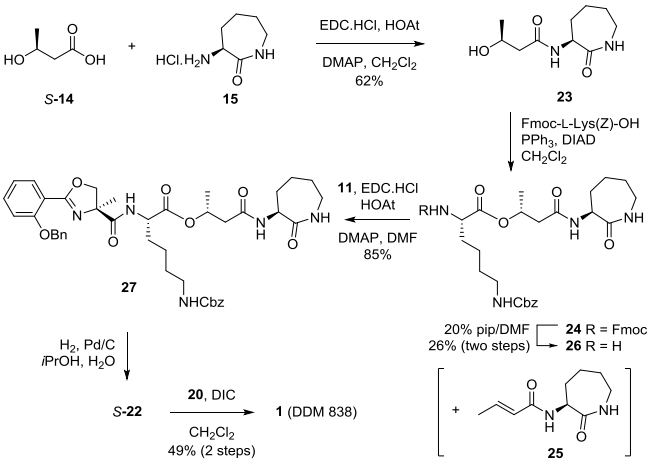
The above work, and an alternative approach utilizing a different order of assembly that gave improved yields yet also suffered from similar problems (see SI for details), highlighted two key problems that likely contributed to the low yield of the

prior solid- and solution-based syntheses of DDM-838.[3b] The first is the difficulty of effecting the esterification of 3-hydroxybutyramide, which occurred at best in only moderate yield and was complicated by epimerization at the central lysine. The difficulty of this coupling likely arises from the sterically-congested nature of the linkage, between an α-branched acid, and a secondary alcohol. The propensity for epimerization at the central lysine during esterification of the 3-hydroxybutyramide group means that the stereochemistry at this position had not been unambiguously secured, although the biosynthetic relationship of DDM-838 to the mycobactins allows assumption of the L-configuration.[11] The second problem was the unexpected isomerization of the unsaturated fatty acyl chain, and sometimes contamination with a trifluoroacetamide byproduct.

We initially focussed on solving the problem experienced with the unexpected formation of *E* and *Z* isomers upon acylation of *S*- and *R*-**22** with *Z*-C20:1 acid **20**, and the formation of the trifluoroacetamide by-product. By conversion of the trifluoroacetate salt of compound **22** to the free base, prior to acylation, we were able to avoid trifluoroacetamide formation. Treatment of a pure *Z*-C20:1-Lys model with $^{i}Pr_2NEt$ did not result in double bond isomerization, suggesting that isomerization was occurring to the activated acyl-intermediate. Attempts to acylate the model amine (Bz-L-Lys-OMe) using DIC/HOAt/DMAP, without iPr_2NEt, or with pyridine, still led to double bond isomerization, suggesting that base-catalyzed isomerization is faster than acylation of lysine. Hocking has shown that double bond isomerization of *cis*-crotonoyl chloride during reaction with ammonia does not occur when ammonia is passed over the surface of an ethereal solution of the acid chloride.[12] Inspired by this precedent, we examined pre-forming the activated ester by combining DIC and *Z*-C20:1 **20** in CH_2Cl_2, followed by addition of a CH_2Cl_2 solution of Bz-L-Lys-OMe, which afforded the *cis*-amide as the sole product.

To overcome the problem with epimerization at the central lysine, a Mitsunobu reaction was explored to form an ester bond and interchange the roles of the acid and alcohol to nucleophile and electrophile, respectively, thereby potentially alleviating the steric demands in forming this linkage. In related work, Miller and co-workers applied the Mitsunobu reaction in the synthesis of Mycobactins S and S2 through coupling of cobactin and mycobactic acid fragments in 49 and 50% yield, respectively.[13] In a recent article, Tsakos *et al.* reviewed a large number of challenging esterifications, and suggested that performing difficult esterification reactions on smaller fragments can often avoid yield-limiting side reactions by reducing the number of potentially interfering functional groups.[14]

Based on these considerations we devised a second linear approach to DDM-838. Coupling of (*S*)-3-hydroxybutyric acid ((*S*)-**14**) (rather than (*R*)-**14**) to L-α-amino-ε-caprolactam **15** using EDC.HCl/HOAt/DMAP and afforded the deoxycobactin **23** (Scheme 3).[3b] Mitsunobu coupling of **23** with Fmoc-L-Lys(Z)-OH in the presence of Ph_3P/DIAD afforded the ester **24** with the stereochemistry of 3-hydroxybutyrate inverted, and an elimination by-product **25** inseparable by flash chromatography. While the yield of this reaction was modest, no epimerization at lysine was observed. Removal of the Fmoc group with piperidine allowed separation of the alkene **25** by chromatography and afforded pure amine **26** in 26% yield over two steps. Condensation of amine **26** and oxazoline acid **11** afforded the depsipeptide **27**. Global deprotection of depsipeptide **27** with H_2 over Pd/C gave enantiomerically-pure amine *S*-**22,** which was isolated as a free base and then coupled with the *Z*-C20:1 acid **20**, this time using only DIC, and afforded DDM-838 **1** as a pure *Z* isomer in 49% yield over two steps. The spectral data were in agreement with the natural product. By this approach we unambiguously established the stereochemistry of DDM-838 (**1**) as *SSRS*, as well as the configuration of the three new stereoisomers (**2**–**4**) (Schemes 2 and SI).



Scheme 3. Mitsunobu route to DDM-838 (**1**).

Immunological investigation of CD1a-restricted T cell clones

We explored the ability of our synthetic compounds to mediate binding interactions between CD1a and TCRs using a series of immunological assays that measure either the biological potency of compounds in cellular assays (through the expression of activation marker CD69) or the mechanistic basis of TCR binding to CD1a-lipopeptide complexes (through tetramer staining and flow cytometry). We studied several CD1a-restricted TCR^+ clones including autoreactive clones that recognize CD1a alone, and those recognizing CD1a presenting foreign DDM and related antigens. Initially, we compared the biological activity of synthetic DDM-838 (**1**) prepared here, with natural DDM-838 isolated from *M. tuberculosis*, and material synthesized through the solid phase route of Young and coworkers.[3b] Titration of lipids into cultures of the native CD8-2 T cell clone with all three materials and quantification of IFNγ release by ELISA provided dose-response curves for the two synthetic compounds that were essentially identical (Figure 2). The slightly lower potency of natural DDM can be explained by the presence of small amounts of naturally occurring variants in acyl chain length and saturation, which are known to be less potent than DDM-838.[3] This result established the chemical identity of the synthetic compounds, and concordant activity with natural DDM-838.

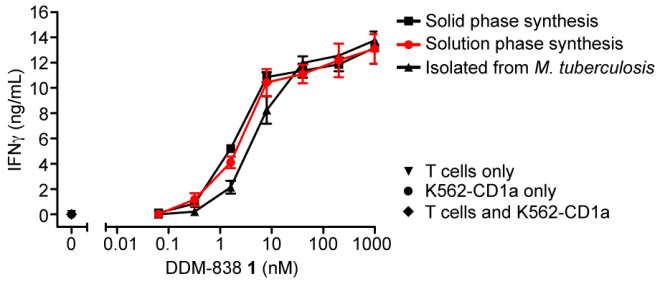


Figure 2. Synthetic and natural DDM-838 possess equivalent ability to activate the native CD8-2 T cell clone. Compound **1** prepared herein (solution phase); according to Young and co-workers (solid phase);[3b] and isolated from *M. tuberculosis* H37Ra, were incubated with the human T cell clone CD8-2 and CD1a-expressing K562 myeloid cells[15] over 24 h and IFNγ release measured by ELISA. DDM-838 isolated from *M. tuberculosis* contained 25% of the saturated analogue DDM-840,[3a] which is less stimulatory than DDM-838. Error bars indicate standard deviation among triplicates. Data representative of three independent experiments. Controls at 0 nM DDM-838 are for experiments including T cells alone, K562 cells alone, and both cell lines.

The BK6 clone[16] recognizes cellular CD1a when loaded with endogenous lipids and does not require DDM.[17] Jurkat cells expressing the BK6 TCR were stained brightly by CD1a loaded with endogenous lipids (endo, Figure 3) (these endogenous lipids are derived from the mammalian HEK293 GnT1$^-$ cells used for expression of CD1a monomers). By contrast, staining of Jurkat.BK6 cells was partially or completely inhibited by all four DDM analogs, suggesting that DDM lipids were loaded into CD1a and affected the TCR binding site. In contrast, Jurkat cells transfected with any of three TCRs derived from DDM-reactive T cell clones (CD8-2;[4] and Clone 8 and Clone 15, isolated from two different subjects with latent tuberculosis infection) stained more brightly with CD1a tetramers loaded with synthetic DDM-838, compared to CD1a-endo tetramers, indicating that synthetic lipopeptide enhances the interaction between CD1a and these TCRs. In comparing the heirarchy of CD8-2 staining, CD1a tetramers loaded with compound **2** (containing the *E*-C20:1 acyl lipid) stained less intensely than compound **1**. Compound **3** (bearing the opposite stereochemistry at the central lysine residue compared to DDM-838), and compound **4** (with unnatural stereochemistry at lysine and the acyl lipid) showed minimal, if any staining of the DDM-838-reactive T cell clones. A negative control cell line expressing the MR1-restricted TCR, Jurkat.M33,[18] provided only background staining levels with all of the CD1a tetramers, indicating that the interactions observed with CD1a-DDM tetramers were due to specific TCR interactions. These data indicate a role of peptide and stereochemistry in T cell response, and the highly similar patterns seen among 3 distinct TCRs suggests a conserved mechanism of DDM recognition.

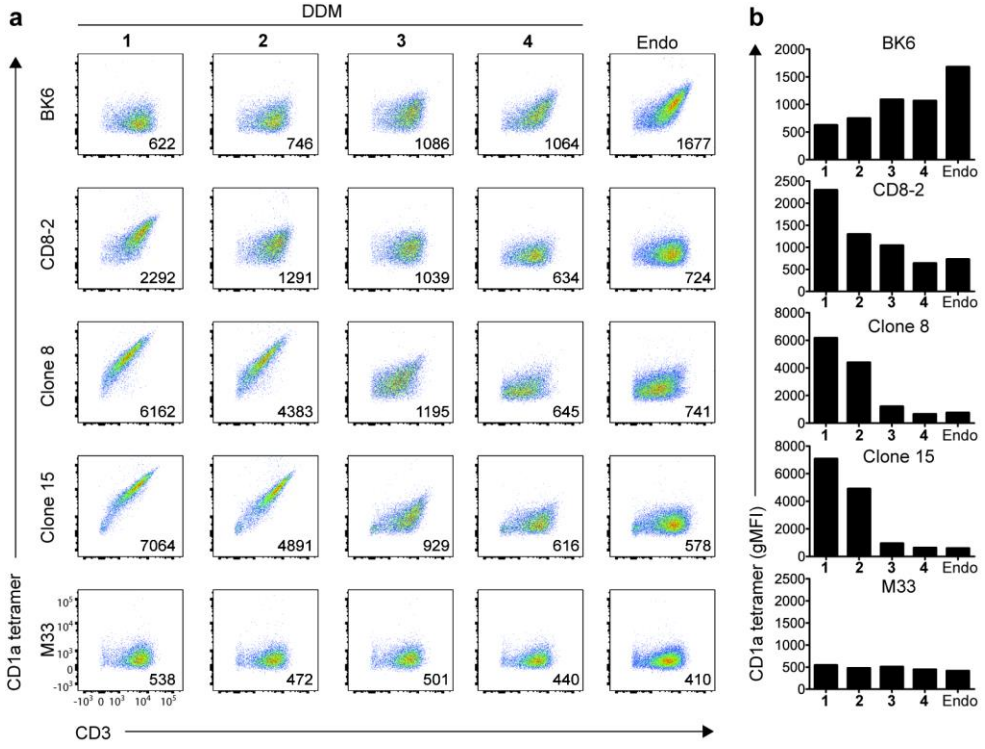


Figure 3. Reactivity of CD1a-restricted TCR$^+$ Jurkat cell clones to DDM isomers. (a) Flow cytometry (left) of BK6, CD8-2, Clone-8, Clone-15 and M33 TCR expressing cells labeled with CD1a-endo tetramers or CD1a tetramers loaded with DDM-838 (*cis-SSRS*) **1**, DDM *trans-SSRS* **2**, DDM (*cis-SRRS*) **3** and DDM (*trans-SRRS*) **4**, or endogenous lipid antigen (Endo). Numbers in the bottom right corner indicate geometric mean fluorescence intensity (gMFI) of staining with CD1a-DDM or CD1a-endo. (b) gMFI of each T cell line against endo and DDM isomers **1**-**4**. Data are representative of three independent experiments.

To determine if synthetic DDMs could also activate cells, we next investigated if compounds **1**–**4** to could cause TCR-transduced Jurkat cells to express an activation marker, CD69 (Figure 4). Titration of the DDM-reactive TCR$^+$ CD8-2 cell line with compounds **1**-**4** resulted in a concentration-dependent increase in activation with the same pattern of potency as was

observed in tetramer-based binding assays. CD1a-autoreactive BK6 cells showed no sign of activation by any of the compounds, and even showed a concentration-dependent reduction in activation. These data are consistent with lipopeptide loading into CD1a but reduced binding of CD1a-lipopeptide to the CD8-2 TCR for compounds **2**-**4**. Titration of the DDM-reactive TCR$^+$ clone 8 and clone 15 cell lines with **1**-**4** showed limited activation (data not shown).

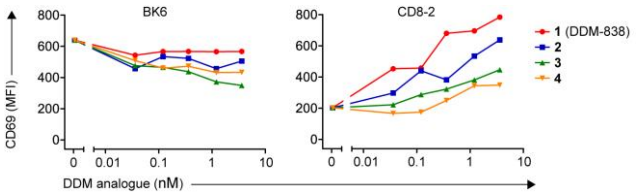


Figure 4. Activation of Jurkat cells expressing BK6 and CD8-2 TCRs by C1R cells transduced to express CD1a, measured by expression of the activation marker CD69. BK6 and CD8-2 expressing cells were co-cultured overnight with CD1a-expressing C1R cells in the presence of DDM isomers **1**-**4** at varying concentrations (0.036, 0.12, 0.36, 1.2, 3.6 nM). For BK6 and CD8-2 expressing cells with a high TCR to GFP ratio, the level of CD69 expression was measured as mean fluorescence intensity (MFI). Data is representative of three independent experiments.

Discussion

Although CD1a is broadly expressed on human Langerhans cells and myeloid dendritic cells, efforts to study foreign lipid-reactive CD1a-restricted T cells have been limited by the availability of the few known bacterial ligands. Although DDM-838 has been bound to CD1a and used in small-scale studies of tuberculosis patients,[6] this depsipeptidolipid has only been available in trace quantities from either natural sources or the reported synthetic route. The present work has investigated several synthetic approaches to DDM-838; demonstrated a new approach that markedly improves yield; and provides insight into a conserved mechanism of DDM-recognition by CD1a-restricted T cells. The new method utilizes the Mitsunobu reaction to construct the challenging ester linkage and provides access to milligram quantities of this important mycobacterial antigen. Notably, unlike direct esterification methods that caused epimerization at the central lysine, this approach generates the ester linkage of the depsipeptide in a substitution reaction without epimerization permitting an explicit assignment of the L-lysine stereochemistry of DDM-838.

The preparation of three diastereomers **2**-**4** of DDM-838 **1** permitted investigation of structure-activity relationships for recognition by DDM-838 reactive CD1a-restricted TCRs, as well as the CD1a-autoreactive BK6 TCR. The BK6 TCR$^+$ cell line is brightly stained by CD1a loaded with permissive self lipids,[17] but less brightly stained with CD1a tetramers treated with compounds **1**-**4**. Structural studies have revealed that the BK6 TCR binds to the A' roof of CD1a, whereas non-permissive ligands such as sphingomyelin and sulfatide protrude from the A' cleft and disrupt the BK6 TCR-CD1a contact zone.[17] An X-ray structure of a silylated mycobactin analogue revealed the alkyl chain inserted deep within the A' pocket of the antigen-binding groove, with the peptide folded over in the F' pocket and with both the caprolactam and salicyl moieties extending outward to the predicted plane of the TCR contact.[5] This binary structure, as well as molecular modelling studies of CD1a–DDM-838 complexes,[3] predict that the peptide moiety might block approach of the BK6 TCR to CD1a, based on the known docking mode of BK6 with CD1a.[17] This prediction is supported by our results, which also provides evidence that all four DDM analogs bind to CD1a. Furthermore, Jurkat cells expressing the CD8-2, Clone 8 and Clone 15 TCRs fail to bind to CD1a tetramers loaded with self lipids that are permissive to CD1a–BK6 TCR binding. This suggests an alternative TCR recognition mode compared to BK6 in which the peptide moiety likely protrudes from the groove to facilitate rather than block TCR binding.

Conclusions

Our data reveal a conserved spectrum of T cell recognition for the panel of DDM isomers for a set of three unrelated CD1a-DDM-reactive TCRs. The natural form, DDM-838 **1** is the most potent, while compound **2**, which varies in the lipid stereochemistry, maintained significant, albeit reduced, activity relative to **1**. This result is reminiscent of the effect seen upon saturation of the acyl chain of DDM-838 to generate DDM-840,[3a] demonstrating the importance of the *cis*-Δ^2-unsaturation of DDM-838 for its potent antigenicity. The stereochemistry and location of this unsaturation (at C2) is unusual and is generated by mycobactin synthase N (MbtN).[19] While the biochemical function of the unsaturation is unknown, this work confirms its importance for antigen recognition.

The inversion of stereochemistry at the lysine of the depsipeptide backbone compromises the activity of lipopeptide and this was reflected in the low reactivity of the DDM-reactive TCRs to CD1a-compound **3** tetramers, similar to prior studies of DDM-838 varying at either the oxazoline or butyrate moieties (positions a and c, Figure 1).[3b] The strong attenuation of TCR binding and T cell activation by compound **4**, which is both epimerized at the central lysine and contains the *trans* acyl chain, reinforces these structure-activity relationships. Collectively, these results reveal that the stereochemistry of the depsipeptide backbone is important for DDM-recognition by CD1a-restricted TCRs, and that variation in stereochemistry or saturation in the lipid chain leads to partial attenuation of reactivity. The recognition of DDM-838 **1** by three distinct T cell clones supports the biomedical development of these compounds to target the naturally occurring polyclonal T cells present in latent TB patients.[6] The ability to synthesize larger quantities of DDM-838 by the solution-phase scheme described here makes possible structural and functional studies of TCR recognition of CD1a–DDM-838 complexes, as well as *in vivo* studies that use DDM as a vaccine component or recall antigens for immunodiagnosis of TB.

Experimental Section

Expression and purification of CD1a

CD1a was expressed by recombinant methods in a mammalian expression system by cloning of constructs encoding CD1a and β_2-microglobulin in a pHLsec vector[20] as carboxyterminal fusions with the leucine zippers Jun (CD1a) and Fos (β_2-microglobulin) and a 'post-zipper' BirA-His_6 tag for CD1a. Fusion proteins were expressed by cotransfection of HEK293 GnT1$^-$ cells with the plasmids pHLsec-CD1a-Jun-BirA-His_6 and pHLsec-β_2m-Fos.[20] Purification of CD1a-β_2-microglobulin heterodimers was achieved by immobilized nickel affinity followed by size-exclusion chromatography.

Solubilization of DDM isomers

DDM SSRS *Z*-C20:1 **1**, DDM SSRS *E*-C20:1 **2**, DDM SRRS *Z*-C20:1 **3** and DDM SRRS *E*-C20:1 **4** were solubilized in a solution of 0.5% tyloxapol (Sigma) in Tris-buffered saline, pH 8.0 at 1 mg/mL. To ensure each lipopeptide was completely solubilized, 12 rounds of sonication (1 h, water bath temperature 25–45°C), vortex (10 sec) and freezing at –20°C (20 mins) were performed until the mixture was a homogenous suspension.

Production of CD1a-restricted Jurkat 76 cell lines

Parental Jurkat 76 cells lack endogenous TCR α-chains and β-chains,[22] and therefore TCR specificity is conferred by the transgene. CD1a-restricted BK6, CD8-2, Clone 8, Clone 15 and control MR1-restricted M33.64[23] Jurkat 76 cells were generated as described[24] using retroviral transduction whereby green fluorescent protein and an antibody to CD3ε (anti-CD3ε) (UCHT1; BD Biosciences) signified successful transduction. These have recently been tested and confirmed to be free of *Mycoplasma*.

Loading of CD1a, production of CD1a tetramers and staining of Jurkat 76 cells with CD1a tetramers

Soluble mammalian CD1a samples were enzymatically biotinylated with BirA biotin ligase as previously described.[21] Biotinylated CD1a was loaded overnight with each DDM isomer at a molar ratio of 1:6. CD1a tetramers were prepared by mixture of streptavidin-phycoerythrin (BD Biosciences) with DDM-loaded biotinylated CD1a at a molar ratio of 1:4. CD1a-DDM tetramers (35 μL of stock CD1a-DDM tetramers at 2.5 μg/mL) were incubated overnight with 3 x 10^4 CD1a-restricted BK6, CD8-2, Clone 8, Clone 15 and control MR1-restricted M33.64 Jurkat 76 cells, generated as described above. CD1a tetramer–positive cells were stained with antibody to CD3ε (anti-CD3ε) (UCHT1; BD Biosciences) and were analyzed with an LSR Fortessa (BD Biosciences). Data were processed with FlowJo software (TreeStar).

Assay of activation of the human BK6 and CD8-2 Jurkat 76 cell lines

CD1a-restricted BK6 and CD8-2 Jurkat 76 cells were generated as described above. CD1a-expressing C1R cells were generated as previously described.[17] 3.33 × 10^3 CD1a-expressing C1R cells were co-cultured overnight with 2 × 10^4 Jurkat cells (BK6 or CD8-2) and DDM isomers **1**-**4** at varying concentrations (0.036, 0.12, 0.36, 1.2, 3.6 nM). The cells were collected by centrifugation, the supernatant was removed and the cells were then stained with anti-CD3ε (BV421, UCHT1, BD Biosciences), anti-CD69 (PE, FN50, BD Biosciences), anti-CD19 (APC/Cy7, SJ25C1, BD Biosciences) and 7-AAD (Sigma Aldrich). Activation of $CD3^+$, GFP high (top 50%) BK6 and CD8-2 Jurkat 76 cells were assessed by upregulation of CD69. Cells were analyzed with an LSR Fortessa (BD Biosciences) and data were processed with FlowJo software (TreeStar).

Activation of human T cell clone CD8-2 by three sources of DDM-838 (1)

CD1a-expressing K562 myeloid cells have been described previously and are routinely tested by flow cytometry to confirm surface CD1a expression.[15] DDM-838 from solid-phase synthesis was provided by Dr David Young using the reported method.[3b] Natural DDM was purified from *M. tuberculosis* H37Ra as described previously[3b] and characterized by electrospray ionization mass spectrometry to be a mixture of DDM-838 (75%) and DDM-840 (25%). The concentrations of DDM-838 in natural DDM and of solution-phase DDM-838 were determined by UV spectroscopy absorbance at 306 nm, normalized to absorbance of 80 μM solid-phase DDM-838. 5 × 10^4 CD8-2 T cells were incubated with 2.5 × 10^4 CD1a-expressing K562 cells and serial dilutions of DDM-838 in 150 μl T cell media for up to 24 hrs at 37 °C. Secreted IFNγ in the undiluted cell culture media was quantified by standard sandwich ELISA using human IFNγ antibody clones 2G2 (Thermo-Fisher), biotin-conjugated B133.5 (Thermo-Fisher) and streptavidin-horse radish peroxidase (BD Pharmingen). Absorbance was measured at 405 nm using a VersaMax plate reader (Molecular Devices) and SoftMaxPro software. IFNγ concentrations were extrapolated from a standard curve in GraphPad Prism 6.

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Keywords: immunology • peptidolipid • antigens • natural product • T cell

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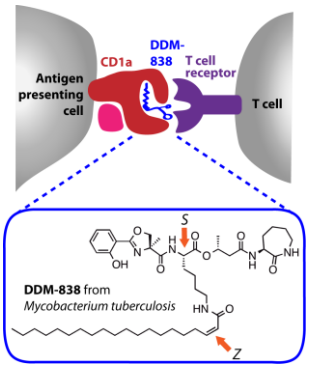
Entry for the Table of Contents

FULL PAPER

Sensing stereochemistry: *M. tuberculosis* dideoxymycobactin-838 (DDM-838) is an antigen involved in cell-mediated immunity. DDM-838 and stereoisomers epimeric in the depsipeptide backbone and isomeric in the C20:1 acyl-chain were synthesized. T cell receptors derived from unrelated human donors preferentially bind natural DDM-838 in complex with the antigen presenting molecule CD1a and display similar patterns of discrimination for DDM-838 stereoisomers.



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Total synthesis of the *Mycobacterium tuberculosis* dideoxymycobactin-838 and stereoisomers: Diverse CD1a-restricted T cells display a common hierarchy of lipopeptide recognition

SUPPLEMENTARY INFORMATION

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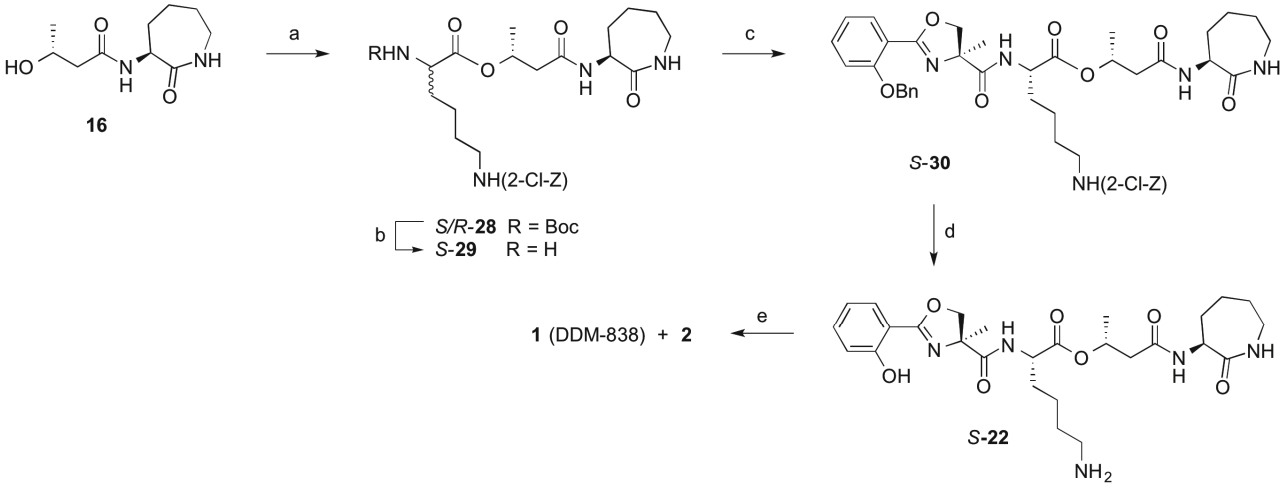
Additional Results and Discussion

An alternative order of assembly applied to the depsipeptide attempted in our laboratories provided improved yields yet still suffered from epimerization at lysine. The difficulty of this coupling likely arises from the sterically-congested nature of the linkage, between an α-branched acid, and a secondary alcohol. A bulky quaternary oxazoline acyl capping on lysine α-amino, as in **13**, may be the cause of the observed epimerisation. With this in mind, we first coupled deoxycobactin **16** with a Boc-protected L-lysine giving two inseparable forms of the products *S/R*-**28** (73:27 ratio of *S*/*R*, Scheme S1) that, after Boc-deprotection, were separable by rpHPLC. The major product *S*-**29** was progressed to give the natural antigen DDM-838 and its *E*-stereoisomer, confirmed as **1** and **2**, respectively. Similarly, D-lysine led to the opposite epimer (*R*-**29**) as the major product (21:79 ratio of *S*/*R*), which was progressed to *Z*- (24%) and *E*- (42%) stereoisomers with improved yields, confirmed to be **3** and **4**, respectively.



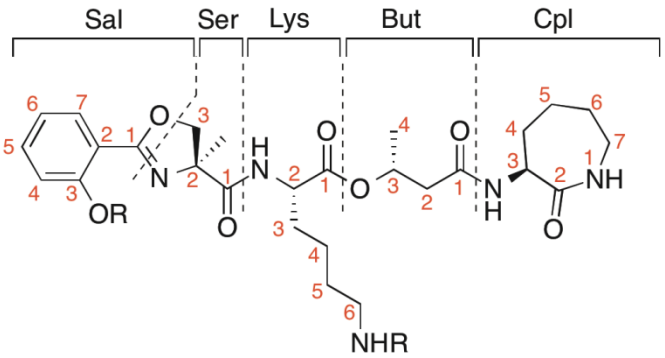
Scheme S1.

Reagents and conditions: (a) Boc-L-Lys(2-Cl-Z)OH, EDC.HCl, HOAt, DMAP, CH_2Cl_2, 72%, 73:27 ratio of *S*/*R*; (b) TFA, CH_2Cl_2, 80%; (c) **11**, EDC.HCl, HOAt, DMAP, CH_2Cl_2, 83%; (d) H_2 (1 atm), Pd/C, EtOH; (e) **20**, EDC.HCl, HOAt, DMAP, $^{i}Pr_2NEt$, DMF, CH_2Cl_2, 17% (**1**), 21% (**2**), yields over two steps.

Experimental

General

Proton nuclear magnetic resonance spectra (^{1}H NMR, 400, 500, 600 MHz) and proton decoupled carbon nuclear magnetic resonance spectra (^{13}C NMR, 100, 125, 150 MHz) were obtained in deuterochloroform, with residual protiated solvent as internal standard. Chemical shifts are followed by multiplicity, coupling constant(s) (*J*), integration and assignments where possible. Flash chromatography was carried out according to the procedure of Still *et al*.[1] using an automated system. Analytical thin layer chromatography (t.l.c.) was conducted on aluminium-backed 2 mm thick silica gel 60 GF_{254} and chromatograms were visualized with 10% H_2SO_4 in methanol. High resolution mass spectra (HRMS) were obtained by ionizing samples using electron spray ionization (ESI) and a time of flight mass analyzer. Dry THF and CH_2Cl_2 was obtained by the method of Pangborn *et al*.[2] Pet. spirits refers to petroleum ether, boiling range 40–60 °C. α-Methyl-L-serine was from Ontario Chemicals (Canada); all other reagents were from Sigma Aldrich (USA) and were used as received. Reaction progress was monitored by thin layer chromatography (TLC) and visualised under UV illumination (λ_{254nm}) or after staining with cerium ammonium molybdate (CAM) dip or ninhydrin dip (for compounds containing an amine functional group). Reversed phase high-performance liquid chromatography (rpHPLC) was performed on a Phenomenex Luna 5 µm, C18 250× 4.60 mm (analytical) or a Phenomenex Luna 15 µm C18 250 × 21.2 mm (preparative) column using specified gradient of solvent A (0.1% TFA in Milli-Q water) and solvent B (0.1% TFA in MeCN and Milli-Q water 9:1, v/v).



2-(Benzyloxy)benzoic acid (8)

(i) Methyl 2-(benzyloxy)benzoate

BnBr (3.10 mL, 26.3 mmol) was added to a mixture of methyl salicylate **7** (2.00 g, 13.1 mmol) and K_2CO_3 (5.40 g, 39.4 mmol) in DMF (65 mL). The mixture was stirred overnight at 60 °C. Water (150 mL) was added and the mixture was extracted with diethyl ether (100 mL) and the organic

layer was washed with water (100 mL × 2), sat. aq. $NaHCO_3$, brine, dried ($MgSO_4$) and concentrated to give methyl 2-(benzyloxy)benzoate (3.20 g) as a clear oil, which was used in the next step without purification.

(ii) 2-(Benzyloxy)benzoic acid (8)

A mixture of 40% aq. KOH (aq.) (45 mL) and crude methyl 2-(benzyloxy)benzoate (3.20 g) was refluxed for 1 h. The mixture was diluted with water and acidified with aq. 1 N HCl. The mixture was extracted with ethyl acetate (40 mL × 2), and the combined organic layers were washed with water, then brine, dried ($MgSO_4$) and concentrated *in vacuo*. Recrystallisation of the residue from EtOAc/pet. ether, 5/1, v/v) afforded compound **8** (2.71 g, 90% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (1 H, d, $J_{5,6}$ = 7.9 Hz, Ar), 7.57 (1 H, t, J = 8.0 Hz, Ar), 7.46–7.42 (5 H, m, Ar), 7.19-–7.13 (2 H, m, Ar), 5.31 (2 H, s, Sal-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.5 (Sal C=O), 154.3, 135.2, 134.4, 134.1, 129.4, 129.3, 128.1, 122.7, 118.3, 113.2 (Ar), 72.4 (Sal-CH_2); HRMS (ESI+) *m/z* calcd. for $[C_{14}H_{12}O_3+H]^+$: 229.0859, obsd.: 229.0859.

(*S*)-Methyl 2-(2-(benzyloxy)benzamido)-3-hydroxy-2-methylpropanoate (9)

(i) (*S*)-Methyl 2-amino-3-hydroxy-2-methylpropanoate hydrochloride (6)

α-Methyl-L-serine **5** (400 mg, 3.36 mmol) was added to a solution of acetyl chloride (1.2 mL) in dry methanol (8.0 mL) and the resulting solution was stirred under reflux for 20 h, after which the mixture was concentrated *in vacuo*, and co-evaporated with toluene (× 3). The resultant oil was used in the next step without purification. ^{1}H NMR (400 MHz, CD_3OD) δ 3.90 (1 H, d, J_{gem} = 11.8 Hz, Ser-H3), 3.80 (3 H, s, Ser-OMe), 3.64 (1 H, d, J_{gem} = 11.8 Hz, Ser-H3), 1.45 (3 H, s, Ser-Me).

(ii) (*S*)-Methyl 2-(2-(benzyloxy)benzamido)-3-hydroxy-2-methylpropanoate (9)

A mixture of acid **8** (843 mg, 3.36 mmol), hydrochloride **6** (3.36 mmol), COMU (2.16 g, 5.04 mmol) and DIPEA (1.73 mL, 10.1 mmol) in dry DMF (24.0 mL) was stirred for 20 h at rt. The mixture was diluted with Et_2O, washed with aq. 1 N HCl solution, sat. aq. $NaHCO_3$, water, brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (30-100% Et_2O/pet. ether) to give the amide **9** as a white solid (856 mg, 74% over two steps from α-methyl-L-serine). $[\alpha]_D^{27}$ = –9.7° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (1 H, s, NH), 8.16 (1 H, d, J = 7.8 Hz, Sal-H7), 7.47–7.37 (6 H, m, Ar, Sal-H5), 7.10–7.05 (2 H, m, Sal-H4,6), 5.19 (2 H, s, Sal-CH_2), 3.99–3.93, 3.76–3.70 (2 H, 2m, Ser-H3), 3.70 (3 H, s, Ser-OMe), 1.25 (3 H, s, Ser-Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.6 (Ser C=O), 165.4 (Sal C=O), 157.0, 135.5, 132.4, 133.4, 129.0, 128.5, 121.7, 121.3, 112.8 (Ar), 71.6 (Sal-CH_2), 67.1 (Ser-C3), 61.6 (Ser-C2), 52.7 (Ser-OMe), 20.7 (Ser-Me); HRMS (ESI+) *m/z* calcd. for $[C_{19}H_{21}NO_5+H]^+$: 344.1492, obsd.: 344.1497.

(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4-methoxycarbonyl-4,5-dihydrooxazole (10)

XtalFluor-M (414 mg, 1.70 mmol) was added to a solution of amide **9** (390 mg, 1.14 mmol) in 1,2-dichloroethane (11.0 mL) and the solution was stirred at rt for 2 h. The mixture was diluted with CH_2Cl_2 and washed sequentially with sat. aq. $NaHCO_3$, water, brine, dried ($MgSO_4$) and concentrated *in vacuo*. The crude residue was purified by flash chromatography (16-50% EtOAc/pet. ether) to afford oxazole **10** as a colorless oil (344 mg, 93%). $[\alpha]_D^{25}$ = +49.0° (c = 1.0, $CHCl_3$), lit.[10] $[\alpha]_D^{25}$ = +42.1° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (1 H, d, $J_{2',3'}$ = 7.7 Hz, Sal-H7), 7.52–7.27 (6 H, m, Ar), 7.01–6.97 (2 H, m, Sal-H4,6), 5.17 (2 H, s, Sal-CH_2), 4.81 (1 H, d, J_{gem} = 8.6 Hz, Ser-H3), 4.18 (1 H, d, J_{gem} = 8.6 Hz, Ser-H3), 3.77 (3 H, s, Ser-OMe), 1.64 (s, 3 H, Ser-Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0 (Ser C=O), 164.3 (C=N), 157.7, 137.1, 132.7, 131.7, 128.5, 127.7, 126.9, 120.9, 117.7, 113.8 (Ar), 75.8 (Ser-C3), 74.4 (Ser-C2), 70.7 (Sal-CH_2), 52.9 (Ser-OMe), 25.4 (Ser-Me); HRMS (ESI+) *m/z* calcd. for $[C_{19}H_{19}NO_4+H]^+$: 326.1387, obsd.: 326.1387.

(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4-hydroxycarbonyl-4,5-dihydrooxazole (11)

A mixture of $LiOH.H_2O$ (255 mg, 6.07 mmol) and oxazole **10** (395 mg, 1.21 mmol) in THF/H_2O (18 mL, 1/1, v/v) and stirred at rt for 15 h. H_2O (10 mL) was added to the reaction mixture and the THF was evaporated *in vacuo*. The aqueous solution was acidified to pH 4 with aq. 1 N citric acid solution and the solution was extracted with ethyl acetate (15 mL × 3). The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to afford the oxazoline acid **11** as a clear oil, which was not purified (365 mg, 96%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90–7.70 (1 H, br s, OH), 7.76 (1 H, d, *J* = 7.7 Hz, Sal-H7), 7.45–7.42 (3 H, m, Ar), 7.34 (2 H, *J* = 7.40 Hz, Ar), 7.28 (1 H, *J* = 7.4 Hz, Ar), 7.00–6.97 (2 H, m, Sal-H4,6), 5.19 (2 H, s, Sal-CH_2), 4.90 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 4.24 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 1.63 (s, 3 H, Ser-Me); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.3 (Ser C=O), 165.3 (C=N), 158.2, 136.4, 134.1, 131.7, 128.7, 128.1, 127.1, 120.9, 115.3, 113.7 (Ar), 76.6 (Ser-C3), 73.7 (Ser-C2), 70.9 (Sal-CH_2), 25.2 (Ser-Me); HRMS (ESI+) *m/z* calcd. for $[C_{18}H_{17}NO_4+H]^+$: 312.1230, obsd.: 312.1229.

N^{α}-[(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4,5-dihydrooxazol-4-ylcarbonyl]-N^{ε}-(*tert*-butyloxycarbonyl)-L-lysine methyl ester (12)

Acid **11** and H-Lys(Boc)-OMe hydrochloride (257 mg, 0.865 mmol) was co-evaporated with toluene (× 3) and dissolved in dry DMF (6.3 mL). HOAt (118 mg, 0.865 mmol), DMAP (106 mg,

0.865 mmol) and EDC.HCl (386 mg, 2.01 mmol) were added and the reaction mixture was stirred at rt for 48 h. The solution was diluted with water and extracted with EtOAc (60 mL × 3). The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (10-50% EtOAc/pet. ether) to give the amide **12** as a white solid (330 mg, 83% over 2 steps). $[\alpha]_D^{25}$ = –34.6° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (1 H, dd, *J* = 7.6, 1.8 Hz, Sal-H7), 7.52 (2 H, dd, *J* = 8.0, 1.0 Hz, Ar), 7.41 (1 H, ddd, *J* = 8.4, 7.4, 1.8 Hz, Sal-H5), 7.38–7.35 (2 H, m, Ar), 7.30–7.26 (1 H, m, Ar), 7.21 (1 H, d, $J_{2,NH}$ = 8.3 Hz, Lys-2-N**H**), 7.03–6.99 (2 H, m, Sal-H6,4), 5.25 (1 H, d, J_{gem} = 12.3 Hz, PhC**H**$_2$), 5.19 (1 H, d, J_{gem} = 12.3 Hz, PhC**H**$_2$), 4.64 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 4.48 (1 H, s, Lys-6-N**H**), 4.44 (1 H, td, $J_{2,NH}$ = 8.3, $J_{2,3}$ = 5.5 Hz, Lys-H2), 4.18 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 3.69 (3 H, s, OMe), 2.99–2.88 (2 H, m, Lys-H6), 1.70–1.63 (1 H, m, Lys-H3), 1.61 (3 H, s, Ser-Me), 1.40 (9 H, s, ^{t}Bu), 1.37–1.27 (3 H, m, Lys-H3,5), 1.21–1.13 (2 H, m, Lys-H4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.3 (Ser C=O), 172.3 (Lys C=O), 163.3 (C=N), 157.8 (Sal-C3), 156.0 (Boc C=O), 136.9 (Ar), 132.9 (Sal-C5), 131.3 (Sal-C7), 128.7, 127.9, 126.9 (Ar), 120.9 (Sal-C4/C6), 117.2 (Sal-C2), 113.9 (Sal-C4/C6), 79.1 (Boc **C**$(CH_3)_3$), 75.8 (Ser-C3), 75.0 (Ser-C2), 70.7 (Sal-CH_2), 52.3 (Lys-C2), 51.8 (Lys-OMe), 40.2 (Lys-C6), 31.6 (Lys-C3), 29.3 (Lys-C5), 28.5 (Boc-**C**H_3), 26.1 (Ser-Me), 22.7 (Lys-C4); HRMS (ESI+) *m/z* calcd. for $[C_{30}H_{39}N_3O_7+H]^+$: 554.2861, obsd.: 554.2867.

(*S*)-3-[(*R*)-3-Hydroxybutyramido)-hexahydro-2-azepinone (16)

(*R*)-3-Hydroxy-butyric acid **14** (25.0 mg, 0.240 mmol) and L-(–)-α-amino-ε-caprolactam hydrochloride **15** (47.0 mg, 0.288 mmol) were co-evaporated with toluene (3 mL × 3) and dissolved in dry CH_2Cl_2 (3.6 mL). HOAt (39.2 mg, 0.288 mmol), DMAP (35.2 mg, 0.288 mmol) and EDC.HCl (69.1 mg, 0.360 mmol) were added and the reaction mixture was stirred at rt for 24 h. The crude reaction mixture was applied directly to a silica gel column. Flash chromatography (100% EtOAc to 5% MeOH/EtOAc) afforded deoxycobactin **16** as a colourless oil (42.2 mg, 82%). $[\alpha]_D^{26}$ = +17.1° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 (1 H, d, $J_{3,NH}$ = 5.8 Hz, Cpl-3-N**H**), 6.39 (1 H, bs, Cpl-1-N**H**), 4.54 (1 H, dd, $J_{3,4}$ = 11.1, $J_{3,NH}$ = 5.8 Hz, Cpl-H3), 4.20–4.12 (1 H, m, But-H3), 4.03 (1 H, d, $J_{OH,3}$ = 3.0 Hz, OH), 3.33–3.20 (2 H, m, Cpl-H7), 2.40 (1 H, dd, $J_{2,2}$ = 15.3, $J_{2,3}$ = 3.1 Hz, But-H2), 2.30 (1 H, dd, $J_{2,2}$ = 15.3, $J_{2,3}$ = 8.8 Hz, But-H2), 2.08–1.97 (2 H, m, Cpl-H4,5), 1.87–1.75 (2 H, m, Cpl-H5,6), 1.53–1.30 (2 H, m, Cpl-H4,6), 1.20 (3 H, d, $J_{3,4}$ = 6.4 Hz, But-H4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 171.1 (But C=O, Cpl C=O), 64.9 (But-C3), 52.2 (Cpl-C3), 43.8 (But-C2), 42.3 (Cpl-C7), 31.6, 28.9, 28.1 (Cpl-C4,5,6), 22.7 (Ser-C2); HRMS (ESI+) *m/z* calcd. for $[C_{10}H_{18}N_2O_3+H]^+$: 215.1390, obsd.: 215.1381.

Ethyl *Z*-2-eicosenoate (19)

NaI (177 mg, 1.18 mmol) and DBU (161 μL, 1.08 mmol) was added to ethyl (di-*o*-tolyl-phosphono)acetate[3] (340 mg, 0.980 mmol) in dry THF (3 mL) at 0 °C and stirred for 10 min. The reaction mixture was cooled to -78 °C and stearyldehyde **18**[4] (290 mg, 1.08 mmol) in THF (2 mL) was added and the mixture was allowed to warm to 0 °C over 1.5 h. The solution was quenched with aq. NH_4Cl, and extracted with EtOAc (5 mL × 2). The combined organic layers were washed with sat. aq. $NaHCO_3$, water, brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (1-5% EtOAc/pet. ether) to afford the *cis* isomer **19** (123 mg, 37%) and mixture of *trans* and *cis* isomers (202 mg, 61%). Characterisation of *cis* isomer **19**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.21 (1 H, dt, $J_{\alpha,\beta}$ = 11.5, $J_{\beta,\gamma}$ = 7.4 Hz, C_{20}-Hβ), 5.74 (1 H, d, $J_{\alpha,\beta}$ = 11.5 Hz, C_{20}-Hα), 4.16 (2 H, q, $J_{CH2,CH3}$ = 7.2 Hz, OC**H**$_2$CH$_3$), 2.64 (2 H, q, $J_{\beta,\gamma} = J_{\gamma,\delta}$ = 7.4 Hz, C_{20}-Hγ), 1.46–1.39 (2 H, m, C_{20}-Hδ), 1.33–1.25 (31 H, m, OCH$_2$C**H**$_3$, C_{20}-CH_2), 0.87 (3 H, t, *J* = 6.9 Hz, C_{20}-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.7 (C=O), 150.8 (C_{20}-Cβ), 119.7 (C_{20}-Cα), 59.9 (O**C**H$_2$CH$_3$), 32.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.2, 22.8 (CH_2), 14.4, 14.2 (C_{20}-CH_3, OCH$_2$**C**H$_3$); HRMS (ESI+) *m/z* calcd. for $[C_{22}H_{42}O_2+H]^+$: 339.3258, obsd.: 339.3257.

***Z*-2-Eicosenoic acid (20)**

Aqueous 1 N KOH solution (1.4 mL, 0.474 mmol) and ester **19** (36.8 mg, 0.0947 mmol) were dissolved in THF/EtOH (1.4 mL, 1/1, v/v) and refluxed for 2 h. The reaction mixture was diluted with EtOAc and the organic layer washed with aq. 0.2 N HCl, water, brine, dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash chromatography (5% EtOAc/pet. ether) afforded *Z*-C20:1 acid **20** as an oil (29.5 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35 (1 H, dt, $J_{\alpha,\beta}$ = 11.4 Hz, $J_{\beta,\gamma}$ = 7.6 Hz, C_{20}-Hβ), 5.79 (1 H, d, $J_{\alpha,\beta}$ = 11.4 Hz, C_{20}-Hα), 2.66 (2 H, q, $J_{\beta,\gamma} = J_{\gamma,\delta}$ = 7.4 Hz, C_{20}-Hγ), 1.48–1.41 (2 H, m, C_{20}-Hδ), 1.35–1.22 (28 H, m, C_{20}-CH_2), 0.88 (3 H, t, *J* = 6.9 Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.7 (C=O), 153.8 (C_{20}-Cβ), 119.0 (C_{20}-Cα), 32.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.44, 29.38, 29.1, 22.9 (CH_2), 14.3 (C_{20}-CH_3); HRMS (ESI+) *m/z* calcd. for $[C_{20}H_{38}O_2+H]^+$: 311.2945, obsd.: 311.2944.

Coupling of 13 and 16: Synthesis of 21

A mixture of LiOH.H_2O (25.6 mg, 0.610 mmol) and methyl ester **12** (67.5 mg, 0.122 mmol) in THF/H_2O (1.8 mL, 1/1, v/v) and stirred at rt for 15 h. H_2O (1 mL) was added to the reaction mixture and the THF evaporated *in vacuo*. The aqueous solution was acidified to pH 4 with 1 N citric acid solution and the solution was extracted with ethyl acetate (10 mL × 3). The combined

organic layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to afford the acid **13** as a clear oil (53.6 mg, 0.0993 mmol, 81%), which was not purified. Acid **13** and deoxycobactin **16** (42.6 mg, 0.199 mmol) were co-evaporated with toluene (× 3) and dissolved dry DMA (1.2 mL). HOAt (16.2 mg, 0.119 mmol), DMAP (18.2 mg, 0.119 mmol) and DIC (31 μL, 0.199 mmol) were added and the reaction mixture was stirred at rt for 72 h. The solution was diluted with water and extracted with EtOAc (10 mL × 3). The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (50-100% EtOAc/pet. ether) to give ester **21** as a diastereomeric mixture of (1:1 ratio of *S/R* at the α position of lysine; 37.7 mg, 0.0513 mmol, 51%). Purification by preparative TLC (10% MeOH/EtOAc) afforded the *R* isomer **21 (fast)** and the *S* isomer **21 (slow)**.

Data for SRRS depsipeptide R-21 (fast)

$[\alpha]_D^{26}$ = +0.5° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79–7.75 (1 H, m, Ar), 7.51–7.15 (7 H, m, Ar, Lys-2-N**H**, Cpl-N**H**-3), 7.06–6.95 (3 H, m, Ar), 6.29 (1 H, bs, Lys-6-N**H**/Cpl-N**H**-1), 5.28–5.14 (3 H, m, Sal-CH_2, But-H3), 4.95 (1 H, bs, Lys-6-N**H**/Cpl-1-N**H**), 4.64 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 4.48–4.44 (2 H, m, Lys-H2, Cpl-H3), 4.18 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 3.27–3.16, 3.10–3.01 (4 H, 2 × m, Lys-H6, Cpl-H7), 2.46–2.33 (2 H, m, But-H2), 2.05–1.85 (2 H, m, Cpl-H5), 1.85–1.67 (3 H, m, Cpl-H6, Lys-H3), 1.61 (3 H, s, Ser-Me), 1.57–1.24 (16H, m, Boc-CH_3, Cpl-H4, Lys-H3,4,5), 1.22 (3 H, $J_{3,4}$ = 6.1 Hz, But-H4); ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.6, 175.4 (Ser C=O, Lys C=O), 171.1, 168.3 (2 C, But C=O, Cpl C=O), 164.1 (C=N), 157.8 (Sal-C3), 156.1 (Boc C=O), 117.5 (Sal-C2), 137.0, 132.9, 131.5, 128.6, 127.9, 127.0, 120.9, 114.0 (Ar), 79.0 (Boc **C**$(CH_3)_3$), 76.0 (Ser-C3), 74.8 (Ser-C2), 70.8 (Sal-CH_2), 69.2 (But-C3), 52.2, 51.9 (2 C, Lys-C2, Cpl-C3), 42.5 (But-C2), 42.2 (Cpl-C7), 40.2 (Lys-C6), 32.0 (Lys-C3), 31.6 (Cpl-C5), 29.1, 28.9, 28.0, 22.6 (4 C, Cpl-C4,6, Lys-C4,5), 28.6 (Boc-CH_3), 26.5 (Ser-Me), 19.7 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{39}H_{53}N_5O_9+H]^+$: 736.3916, obsd.: 736.3917.

Data for SSRS depsipeptide S-21 (slow)

$[\alpha]_D^{27}$ = +2.4° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (1 H, dd, J = 7.9, J = 1.7 Hz, Ar), 7.53–7.14 (8 H, m, Ar, Lys-2-N**H**, Cpl-3-N**H**), 7.04–7.01 (2 H, m, Ar), 6.30–6.29 (1 H, bs, Lys-6-N**H**/Cpl-1-N**H**), 5.31–5.20 (3 H, m, Sal-CH_2, But-H3), 4.91 (1 H, bs, Lys-6-N**H**/Cpl-1-N**H**), 4.66 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 4.52 (1 H, dd, J = 10.6, 5.9 Hz, Cpl-H3), 4.43 (1 H, dd, J = 13.7, 8.3 Hz, Lys-H2), 4.19 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 3.28–3.17 (2 H, m, Cpl-H7), 3.05–2.93 (2 H, m, Lys-H6), 2.52–2.51 (2 H, m, But-H2), 2.06–1.97 (2 H, m, Cpl-H5), 1.84–1.74 (3 H, m, Cpl-H6, Lys-H3), 1.62 (3 H, s, Ser-Me), 1.50–1.26 (16 H, m, Boc-CH_3, Cpl-H4, Lys-H3,4,5), 1.30 (3 H, $J_{3,4}$ = 6.4 Hz, But-H4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.5, 175.4 (Ser C=O, Lys C=O), 171.1, 168.4 (2 C, But C=O, Cpl C=O), 163.4 (C=N), 157.8 (Sal-C3), 156.0 (Boc C=O), 137.0,

132.9, 131.4, 128.8, 127.9, 126.9, 120.9, 114.0 (Ar), 117.4 (Sal-C2), 79.1 (Boc **C**$(CH_3)_3$), 75.9 (Ser-C3), 75.0 (Ser-C2), 70.8 (Sal-CH_2), 69.1 (But-C3), 52.3, 52.3 (2 C, Lys-C2, Cpl-C3), 42.7 (But-C2), 42.2 (Cpl-C7), 40.1 (Lys-C6), 31.7 (Lys-C3), 31.3 (Cpl-C5), 29.2, 29.0, 28.1, 22.7 (4 C, Cpl-C4,6, Lys-C4,5), 28.6 (Boc-CH_3), 26.3 (Ser-Me), 19.5 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{39}H_{53}N_5O_9+H]^+$: 736.3916, obsd.: 736.3926.

Synthesis of DDM-838 1 and isomer 2 from *S*-21 (slow)

A mixture of compound *S*-**21 (slow)** (18.7 mg, 0.0254 mmol), 5 wt. % Pd/C (5.3 mg) in EtOAc/EtOH (v/v, 3/1, 4 ml) was stirred under a H_2 atmosphere (1 atm) for 3 h. The mixture was filtered through Celite, and the celite plug was washed with 0.5% AcOH in EtOAc/EtOH (v/v, 3/1). The solvent was removed *in vacuo* and the residue co-evaporated with toluene (× 3) to afford the corresponding crude amine. A solution of 5% TFA/CH_2Cl_2 (1.5 mL) was added to the amine and stirred at 0 °C for 5 mins and rt for 1.5 h. Two additional portions of 50% TFA/CH_2Cl_2 (290 μL) solution were added at 0 °C and stirred continued at rt for 3 h. The solvent was removed *in vacuo* and the residue co-evaporated with dry toluene (× 3) to afford fully deprotected depsipeptide *S*-**22** as a pale pink oil, which was used without further purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.38 (1 H, d, J = 7.7 Hz, Lys-2-N**H**), 7.93 (1 H, d, J = 7.0 Hz, Cpl-3-N**H**), 7.77 (1 H, dd, J = 7.1, 5.1 Hz, Cpl-1-N**H**), 7.63 (1 H, dd, J = 7.8, 1.7 Hz, Sal-H7), 7.46 (1 H, ddd, J = 8.3, 7.3, 1.7 Hz, Sal-H5), 7.00 (1 H, dd, J = 8.3, 0.8 Hz, Sal-H4), 6.94 (1 H, ddd, J = 8.3, 7.3, 1.1 Hz, Sal-H6), 5.08–5.15 (1 H, m, But-H3), 4.67 (1 H, d, J_{gem} = 8.7 Hz, Ser-H3), 4.39 (1 H, ddd, J = 11.1, 6.7, 1.2 Hz, Cpl-H3), 4.28 (1 H, d, J_{gem} = 8.7 Hz, Ser-H3), 4.21–4.17 (1 H, m, Lys-H2), 3.15 (1 H, ddd, J = 14.6, 11.5, 5.0 Hz, Cpl-H7), 3.08–3.02 (1 H, m, Cpl-H7), 2.72–2.61 (2 H, m, Lys-H6), 2.51 (1 H, dd, J_{gem} = 14.3 Hz, $J_{2,3}$ = 6.7 Hz, But-H2), 2.43 (1 H, dd, J_{gem} = 14.5, $J_{2,3}$ = 6.8 Hz, But-H2), 1.89–1.83 (1 H, m, Lys-CH_2/Cpl-CH_2), 1.77–1.57 (5 H, m, Lys-CH_2, Cpl-CH_2), 1.54 (3 H, s, Ser-Me), 1.34–1.53 (3 H, m, Lys-CH_2, Cpl-CH_2), 1.18–1.34 (3 H, m, Lys-CH_2, Cpl-CH_2), 1.18 (3 H, d, $J_{3,4}$ = 6.4 Hz, But-H4); ^{13}C NMR (150 MHz, DMSO-d_6) δ 174.2 (Cpl C=O), 172.9 (Ser C=O), 170.9 (Lys C=O), 167.9 (But C=O), 164.0 (C=N), 159.0 (Sal-C3), 134.1 (Sal-C5), 128.1 (Sal-C7), 119.1 (Sal-C6), 116.7 (Sal-C4), 110.1 (Sal-C2), 74.6 (Ser-C3), 73.9 (Ser-C2), 68.8 (But-C3), 52.2 (Lys-C2), 51.4 (Cpl-C3), 41.4 (But-C2), 40.6 (Cpl-C7), 38.8 (Lys-C6), 31.0 (CH_2), 29.7 (CH_2), 28.9 (CH_2), 27.7 (CH_2), 27.2 (CH_2), 25.9 (Ser-Me), 22.5 (CH_2), 19.5 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{27}H_{39}N_5O_7+H]^+$: 546.2922, obsd.: 546.2926.

The crude depsipeptide *S*-**22** and *Z*-C20:1 acid **20** (9.5 mg, 0.0305 mmol) was co-evaporated with dry toluene (× 2) and dissolved in a 1:1 v/v mixture of dry CH_2Cl_2 and dry DMF (1.6 mL). HOAt

(4.2 mg, 0.0305 mmol), DMAP (3.7 mg, 0.0305 mmol) and DIC (5.1 μL, 0.0331 mmol) were added and the mixture was stirred at rt for 40 h. The mixture was diluted with EtOAc (20 mL) and washed with H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo*. Analysis of the crude mixture by ^{1}H NMR spectroscopy revealed a mixture of *cis*-C20:1/*trans*-C20:1/*N*-trifluoroacetamide (6:3:4). Purification by flash chromatography (10-100% EtOAc/pet. ether to 1% MeOH/EtOAc) afforded *SSRS*-DDM *cis*-C20:1 **1** (3.2 mg, 0.00382 mmol, 15% over 3 steps) and *SSRS*-DDM *trans*-C20:1 **2** (2.4 mg, 0.286 μmol, 12% over 3 steps).

Data for DDM-838 1 (cis-SSRS)

$[\alpha]_D^{26}$ = +10.1° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (1 H, dd, J = 7.9, J = 1.6 Hz, Sal-H7), 7.62 (1 H, d, J = 8.4 Hz, Lys-2-N**H**/Cpl-3-N**H**), 7.42 (1 H, dt, J = 7.3, J = 1.7 Hz, Sal-H5), 7.22–7.21 (1 H, m, Lys-2-N**H**/Cpl-3-N**H**), 7.02 (1 H, d, J = 8.5 Hz, Sal-H4), 6.92 (1 H, t, J = 7.5 Hz, Sal-H6), 6.52 (1 H, t, J = 6.3 Hz, Lys-6-N**H**/Cpl-1-N**H**), 5.92 (1 H, dt, $J_{\alpha,\beta}$ = 11.6, $J_{\beta,\gamma}$ = 7.4 Hz, C_{20}-Hβ), 5.74 (1 H, t, J = 5.4 Hz, Lys-6-N**H**/Cpl-1-N**H**), 5.61 (1 H, dt, $J_{\alpha,\beta}$ = 11.6, $J_{\alpha,NH}$ = 1.5 Hz, C_{20}-Hα), 5.32–5.26 (1 H, m, But-H3), 4.76 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 4.58–4.50 (1 H, m, Lys-H2, Cpl-H3), 4.22 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 3.30–3.16 (4 H, m, Lys-H6, Cpl-H7), 2.64–2.59 (3 H, m, But-H2, C_{20}-Hγ), 2.50 (1 H, dd, $J_{2,2}$ = 15.1, $J_{2,3}$ = 6.6 Hz, But-H2), 2.07–1.76 (7 H, m, Lys-CH_2, Cpl-CH_2), 1.56 (3 H, s, Ser-Me), 1.54–1.22 (35 H, m, C_{20}-CH_2, Lys-CH_2, Cpl-CH_2), 1.41–1.36 (2 H, m, C_{20}-Hδ), 1.31 (3 H, d, $J_{3,4}$ = 6.4 Hz, But-H4), 0.88 (3 H, t, J = 7.0 Hz, C_{20}-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6 (Cpl C=O), 174.0 (Ser C=O), 170.9 (Lys C=O), 168.5 (But C=O), 166.7 (C_{20} C=O), 165.6 (C=N), 159.5 (Sal-C3), 146.0 (C_{20}-Cβ), 134.2 (Sal-C5), 128.6 (Sal-C7), 122.2 (C_{20}-Cα), 119.4 (Sal-C6), 117.1 (Sal-C4), 110.5 (Sal-C2), 75.4 (Ser-C3), 74.4 (Ser-C2), 69.1 (But-C3), 52.7, 52.4 (2 C, Lys-C2, Cpl-C3), 42.7, 42.4 (2 C, But-C2, Cpl-C7), 38.7 (Lys-C6), 32.1, 31.6, 31.2, 29.82, 29.78, 29.69, 29.58, 29.56, 29.52, 28.91, 28.86, 28.1 (CH_2), 26.8 (Ser-Me), 23.05, 22.85 (CH_2), 19.5 (But-C4), 14.3 (C_{20}-CH_3); HRMS (ESI+) *m/z* calcd. for $[C_{47}H_{75}N_5O_8+H]^+$: 838.5688, obsd.: 838.5691.

Data for trans-SSRS 2

$[\alpha]_D^{24}$ = –16.5° (c = 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (1 H, dd, $J_{2',3'}$ = 7.9, $J_{2',4}$ = 1.7 Hz, Sal-H7), 7.60 (1 H, d, J = 8.3 Hz, Lys-2-N**H**/Cpl-3-N**H**), 7.42 (1 H, dt, J = 7.7, 1.7 Hz, Sal-H5), 7.22–7.21 (1 H, m, Lys-2-N**H**/Cpl-3-N**H**), 7.03 (1 H, d, J = 8.1 Hz, Sal-H4), 6.92 (1 H, t, J = 7.6 Hz, Sal-H6), 6.80 (1 H, dt, $J_{\alpha,\beta}$ = 15.3, $J_{\beta,\gamma}$ = 6.9 Hz, C_{20}-Hβ), 6.54 (1 H, t, J = 5.0 Hz, Lys-6-N**H**/Cpl-1-N**H**), 5.78 (1 H, t, J = 5.5 Hz, Lys-6-N**H**/Cpl-1-N**H**), 5.72 (1 H, d, $J_{\alpha,\beta}$ = 15.3 Hz, C_{20}-Hα), 5.31–5.28 (1 H, m, But-H3), 4.76 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 4.58–4.50 (1 H, m, Lys-H2, Cpl-H3), 4.22 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 3.30–3.21 (4 H, m, Lys-H6, Cpl-H7), 2.61 (1 H, dd,

$J_{2,2}$ = 15.3, $J_{2,3}$ = 4.1 Hz, But-H2), 2.51 (1 H, dd, $J_{2,2}$ = 15.3, $J_{2,3}$ = 6.8 Hz, But-H2), 2.16–1.77 (7 H, m, Lys-CH_2, Cpl-CH_2), 1.57 (3 H, s, Ser-Me), 1.54–1.25 (37 H, m, C_{20}-CH_2, Lys-CH_2, Cpl-CH_2), 1.32 (3 H, d, $J_{3,4}$ = 6.4 Hz, But-H4), 0.88 (3 H, t, J = 7.0 Hz, C_{20}-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6 (Cpl C=O), 174.0 (Ser C=O), 170.9 (Lys C=O), 168.5 (But C=O), 166.3 (C_{20} C=O), 165.6 (C=N), 159.5 (Sal-C3), 144.9 (C_{20}-Cβ), 134.2 (Sal-C5) 128.6 (Sal-C7), 123.7 (C_{20}-Cα), 119.4 (Sal-C6), 117.1 (Sal-C4), 110.5 (Sal-C2), 75.4 (Ser-C3), 74.4 (Ser-C2), 69.2 (But-C3), 52.7, 52.4 (2 C, Lys-C2, Cpl-C3), 42.6, 42.4 (2 C, But-C2, Cpl-C7), 39.0 (Lys-C6), 32.2, 32.1, 31.64, 31.59, 31.3, 30.5, 30.4, 29.83, 29.81, 29.78, 29.74, 29.68, 29.63, 29.56, 29.51, 29.41, 28.94, 28.91, 28.88, 28.5, 28.1 (CH_2), 26.8 (Ser-Me), 23.0, 22.9 (CH_2), 19.5 (But-C4), 14.3 (C_{20} CH_3); HRMS (ESI+) *m/z* calcd. for $[C_{47}H_{75}N_5O_8+H]^+$: 838.5688, obsd.: 838.5687.

Synthesis of DDM-838 isomers 3 and 4 from *R*-21 (fast)

A mixture of compound *R*-**21 (fast)** (27.0 mg, 0.0367 mmol), 5 wt. % Pd/C (7.9 mg) in EtOAc/EtOH (v/v, 3/1, 4 ml) was stirred under a H_2 atmosphere (1 atm) for 3 h. The mixture was filtered through Celite, and the Celite plug was washed with 0.5% AcOH in EtOAc/EtOH (v/v, 3/1). The solvent was removed *in vacuo* and the residue co-evaporated with toluene (× 3) to afford the corresponding crude amine. A solution of 23% TFA/CH_2Cl_2 (2.6 mL) was added to the amine and stirred at 0 °C for 1.5 h and rt for 1.5 h. The reaction mixture was evaporated and co-evaporated with dry toluene (× 3) to afford the fully deprotected depsipeptide *R*-**22** as a pale pink oil, which was used without further purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.27 (1 H, d, J = 8.0 Hz, Lys-2-N**H**), 7.87 (1 H, d, J = 6.9 Hz, Cpl-3-N**H**), 7.78 (1 H, dd, J = 6.8, 5.1 Hz, Cpl-1-N**H**), 7.65 (1 H, dd, J = 7.9, 1.7 Hz, Sal-H7), 7.47 (1 H, ddd, J = 8.3, 7.3, 1.7 Hz, Sal-H5), 7.01 (1 H, dd, J = 8.3, 0.7 Hz, Sal-H4), 6.95 (1 H, ddd, J = 7.9, 6.8, 1.0 Hz, Sal-H6), 5.06–5.12 (1 H, m, But-H3), 4.64 (1 H, d, J_{gem} = 8.7 Hz, Ser-H3), 4.35 (1 H, ddd, J = 11.2, 7.3, 1.2 Hz, Cpl-H3), 4.29 (1 H, d, J_{gem} = 8.7 Hz, Ser-H3), 4.22 (1 H, ddd, J = 13.0, 8.3, 5.0 Hz, Lys-H2), 3.14 (1 H, ddd, J = 14.5, 10.8, 5.2 Hz, Cpl-H7), 3.07–3.02 (1 H, m, Cpl-H7), 2.71–2.78 (2 H, m, Lys-H6), 2.48 (1 H, dd, J_{gem} = 14.5 Hz, $J_{2,3}$ = 7.7 Hz, But-H2), 2.37 (1 H, dd, J_{gem} = 14.5 Hz, $J_{2,3}$ = 5.8 Hz, But-H2), 1.83–1.89 (1 H, m, Lys-CH_2/Cpl-CH_2), 1.66–1.77 (4 H, m, Lys-CH_2, Cpl-CH_2), 1.47–1.64 (3 H, m, Lys-CH_2, Cpl-CH_2), 1.52 (3 H, s, Ser-CH_3), 1.27–1.42 (3 H, m, Lys-CH_2, Cpl-CH_2), 1.16–1.23 (1 H, m, Lys-CH_2/Cpl-CH_2), 1.14 (3 H, d, $J_{3,4}$ = 6.3 Hz, But-H4); ^{13}C NMR (150 MHz, DMSO-d_6) δ 174.1 (Cpl C=O), 172.9 (Ser C=O), 171.0 (Lys C=O), 167.9 (But C=O), 164.4 (Sal C=N), 159.1 (Sal-C3), 134.1 (Sal-C5), 128.1 (Sal-C7), 119.1 (Sal-C6), 116.7 (Sal-C4), 110.1 (Sal-C2), 74.9 (Ser-C3), 73.8 (Ser-C2), 68.9 (But-C3), 51.9 (Lys-C2), 51.4 (Cpl-C3), 41.3 (But-C2), 40.7 (Cpl-C7), 38.6 (Lys-

C6), 31.1 (CH_2), 29.8 (CH_2), 28.9 (CH_2), 27.7 (CH_2), 26.6 (CH_2), 25.9 (Ser-Me), 22.6 (CH_2), 19.5 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{27}H_{39}N_5O_7+H]^+$: 546.2922, obsd.: 546.2928.

The crude depsipeptide *R*-**22** and *Z*-C20:1 acid **20** (14.0 mg, 0.0451 mmol) was co-evaporated with dry toluene (× 2) and dissolved in a 1:1 v/v mixture of dry CH_2Cl_2 and dry DMF (2.0 mL). HOAt (5.5 mg, 0.0404 mmol), DMAP (4.9 mg, 0.0404 mmol) and DIC (7.4 μL, 0.0477 mmol) were added and the mixture was stirred at rt for 40 h. The mixture was diluted with EtOAc (20 mL) and washed with H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo*. Analysis of the crude mixture by ^{1}H NMR spectroscopy revealed a mixture of *cis*-C20:1/*trans*-C20:1 (6:2). Purification by flash chromatography (10-100% EtOAc/pet. ether to 1% MeOH/EtOAc) afforded *SRRS*-DDM *cis*-C20:1 **3** (9.0 mg, 0.0107 mmol, 29% over 3 steps) and *SRRS*-DDM trans C20:1 **4** (2.1 mg, 0.00251 mmol, 7% over 3 steps).

Data for cis-SRRS 3

$[\alpha]_D^{26}$ = +4.5° (c = 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (1 H, dd, *J* = 7.8, 1.6 Hz, Sal-H7), 7.41 (1 H, dt, *J* = 8.0, 1.6 Hz, Sal-H5), 7.23 (1 H, d, $J_{NH,2}$ = 7.9 Hz, Lys-2-N**H**), 7.02 (1 H, d, *J* = 8.5 Hz, Sal-H4), 6.97 (1 H, d, $J_{NH,3}$ = 6.3 Hz, Cpl-3-N**H**), 6.90 (1 H, t, *J* = 7.7 Hz, Sal-H6), 6.21–6.19 (2 H, m, Lys-6-N**H**, Cpl-1-N**H**), 5.97 (1 H, dt, $J_{\alpha,\beta}$ = 11.5, $J_{\beta,\gamma}$ = 7.4 Hz, C_{20}-Hβ), 5.73 (1 H, d, $J_{\alpha,\beta}$ = 11.5 Hz, C_{20}-Hα), 5.24 (1 H, sext, $J_{2,3}$ = $J_{3,4}$ = 6.2 Hz, But-H3), 4.67 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 4.49 (1 H, dt, $J_{NH,2}$ = 7.9, $J_{2,3}$ = 5.0 Hz, Lys-H2), 4.44 (1 H, dd, $J_{3,4}$ = 10.7, $J_{NH,3}$ = 6.3 Hz, Cpl-H3), 4.24 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 3.38–3.20 (4 H, m, Lys-H6, Cpl-H7), 2.71–2.62 (2 H, m, C_{20}-Hγ), 2.46 (2 H, d, $J_{2,3}$ = 6.0 Hz, But-H2), 1.96–1.47 (7 H, m, Lys-CH_2, Cpl-CH_2), 1.62 (3 H, s, Ser-Me), 1.44–1.37 (2 H, m, C_{20}-Hδ), 1.33–1.22 (35 H, m, C_{20}-CH_2, Lys-CH_2, Cpl-CH_2), 1.28 (3 H, d, $J_{3,4}$ = 6.3 Hz, But-H4), 0.87 (3 H, t, *J* = 6.9 Hz, C_{20}-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.0 (Cpl C=O), 173.7 (Ser C=O), 170.8 (Lys C=O), 168.2 (But C=O), 166.8 (C_{20} C=O), 166.4 (C=N), 159.7 (Sal-C3), 145.9 (C_{20}-Cβ), 134.2 (Sal-C5), 128.5 (Sal-C7), 122.1 (C_{20}-Cα), 119.1 (Sal-C6), 116.9 (Sal-C4), 110.2 (Sal-C2), 76.8 (Ser-C3), 75.6 (Ser-C2), 74.2 (Sal-C3), 69.3 (But-C3), 52.4, 52.3 (2 C, Lys-C2, Cpl-C3), 42.3, 42.1 (2 C, But-C2, Cpl-C7), 38.7 (Lys-C6), 31.9, 31.5, 29.67, 29.65, 29.63, 29.55, 29.46, 29.43, 29.35, 28.78, 28.73, 28.64, 27.8 (CH_2), 26.6 (Ser-Me), 22.7, 22.5 (CH_2), 19.5 (But-C4), 14.1 (C_{20} CH_3); HRMS (ESI+) *m/z* calcd. for $[C_{47}H_{75}N_5O_8+H]^+$: 838.5688, obsd.: 838.5695.

Data for trans-SRRS 4

$[\alpha]_D^{25}$ = –5.6° (c = 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 (1 H, dd, *J* = 7.8, 1.6 Hz, Sal-H7), 7.42 (1 H, d, *J* = 8.0, 1.6 Hz, Sal-H5), 7.24 (1 H, d, $J_{NH,2}$ = 7.9 Hz, Lys-2-N**H**), 7.03 (1 H, d, *J*

= 8.5 Hz, Sal-H4), 6.96 (1 H, d, $J_{NH,3}$ = 6.1 Hz, Cpl-3-N**H**), 6.91 (1 H, t, J = 7.8 Hz, Sal-H6), 6.84 (1 H, dt, $J_{\alpha,\beta}$ = 15.3, $J_{\beta,\gamma}$ = 7.0 Hz, C_{20}-Hβ), 6.18 (1 H, t, J = 5.4 Hz, Lys-6-N**H**/Cpl-1-N**H**), 6.13 (1 H, t, J = 5.4 Hz, Lys-6-N**H**/Cpl-1-N**H**), 5.82 (1 H, dt, $J_{\alpha,\beta}$ = 15.3, $J_{\beta,\gamma}$ = 1.4 Hz, C_{20}-Hα), 5.25 (1 H, sext, $J_{3,4}$ = $J_{2,3}$ = 6.2 Hz, But-H3), 4.68 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 4.50 (1 H, dt, $J_{NH,2}$ = 7.9, $J_{2,3}$ = 4.9 Hz, Lys-H2), 4.44 (1 H, dd, $J_{3,4}$ = 11.4, $J_{NH,3}$ = 6.1 Hz, Cpl-H3), 4.24 (1 H, d, J_{gem} = 9.0 Hz, Ser-H3), 3.40–3.20 (4 H, m, Lys-H6, Cpl-H7), 2.46 (2 H, d, $J_{2,3}$ = 6.0 Hz, But-H2), 2.18–2.13 (2 H, m, C_{20}-Hγ), 1.96–1.70 (7 H, m, Lys-CH_2, Cpl-CH_2), 1.62 (3 H, s, Ser-Me), 1.45–1.38 (2 H, m, C_{20}-Hδ), 1.31–1.22 (35 H, m, C_{20}-CH_2, Lys-CH_2, Cpl-CH_2), 1.28 (3 H, d, $J_{3,4}$ = 6.3 Hz, But-H4), 0.87 (3 H, t, J = 6.8 Hz, C_{20}-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.1 (Cpl C=O), 173.9 (Ser C=O), 171.0 (Lys C=O), 168.3 (But C=O), 166.51, 166.46 (2 C, C_{20} C=O,C=N), 159.9 (Sal-C3), 144.8 (C_{20}-Cβ**)**, 134.3 (Sal-C5), 128.7 (Sal-C7), 123.8 (C_{20}-Cα**)**, 119.3 (Sal-C6), 117.1 (Sal-C4), 110.4 (Sal-C2), 75.8 (Ser-C3), 74.3 (Ser-C2), 69.4 (But-C3), 52.4, 52.3 (2 C, Lys-C2, Cpl-C3), 42.4, 42.3 (2 C, But-C2, Cpl-C7), 39.1 (Lys-C6), 32.2, 32.1, 31.72, 31.68, 31.66, 29.86, 29.84, 29.81, 29.79, 29.78, 29.74, 29.72, 29.71, 29.70, 29.69, 29.68, 29.64, 29.52, 29.41, 28.90, 28.81, 28.5, 28.0, 26.8 (Ser-Me), 22.9, 22.6, 19.7 (But-C4), 14.3 (C_{20} CH_3); HRMS (ESI+) *m/z* calcd. for $[C_{47}H_{75}N_5O_8+H]^+$: 838.5688, obsd.: 838.5690.

Alternative order of assembly

Compound *S*-29

Deoxycobactin **16** (40.6 mg, 0.189 mmol) and Boc-L-Lys(2-Cl-Z)-OH (94.3 mg, 0.227 mmol) in dry CH_2Cl_2 (2.8 mL) was treated with HOAt (25.8 mg, 0.189 mmol), DMAP (27.8 mg, 0.227 mmol) and EDC.HCl (43.6 mg, 0.227 mmol) at rt for 2 d. The solution was diluted with CH_2Cl_2 (4.0 mL) and washed with sat. aq. $NaHCO_3$ (5 mL), and the CH_2Cl_2 phase was concentrated *in vacuo*. The residue was dissolved in MeCN–H_2O (1:1, 8 mL) and purified by preparative rpHPLC with isocratic 50%B (t_R 12.4–18.5 min) and lyophilized to give amide *S/R*-**28** as a white powder (83 mg, 72%). Analysis by ^{1}H NMR revealed a mixture of two epimers (73:27 ratio of *S*/*R*), which was used without further purification. Compound *S/R*-**28** (24.0 mg, 0.0393 mmol) was stirred in TFA (0.5 mL) and CH_2Cl_2 (2 mL) at rt for 2 h, and then concentrated *in vacuo*. Analytical rpHPLC indicated two separable products in a ratio of 73:27 (t_R = 13.1, 11.9 min, respectively, isocratic 30%B for 20 min). The residue was dissolved in MeCN–H_2O (1:1, 4 mL) and purified by preparative rpHPLC with isocratic 30%B followed by lyophilization to give the less polar product (white powder 2.5 mg, 10%) as a mixture of two isomers, and the more polar product (white powder, 19.5 mg, 80%) as *S*-**29** TFA salt. ^{1}H NMR (600 MHz, $CDCl_3$) two rotamers in a ratio of ~10 : 90, major isomer: δ 8.48 (3 H, br s, Lys-2-NH_3^+), 7.53 (1 H, d, J = 6.1 Hz, Cpl-3-N**H**), 7.32–

7.38 (2 H, m, Ar), 7.22–7.26 (3 H, m, Ar, Cpl-1-N**H**), 5.64 (1 H, br s, Lys-6-N**H**, two carbamate rotamers), 5.30–5.33 (1 H, m, But-H3), 5.15–5.18 (2 H, m, OC**H₂**Ar), 4.50–4.54 (1 H, m, Cpl-H3), 3.94–4.00 (1 H, m, Lys-H2), 3.12–3.22 (4 H, m, Lys-H6, Cpl-H7), 2.62 (1 H, dd, J_{gem} = 14.3, $J_{2,3}$ = 4.1 Hz, But-H2), 2.49 (1 H, dd, J_{gem} = 14.3, $J_{2,3}$ = 6.5 Hz, But-H2), 1.83–1.93 (4 H, m, Lys-CH_2, Cpl-CH_2), 1.66–1.77 (2 H, m, Lys-CH_2, Cpl-CH_2), 1.40–1.52 (5 H, m, Lys-CH_2, Cpl-CH_2), 1.28–1.32 (1 H, m, Lys-CH_2/Cpl-CH_2), 1.31 (3 H, d, $J_{3,4}$ = 6.3 Hz, But-H4); ^{13}C NMR (150 MHz, DMSO-d_6) δ 176.4 (Cpl-C2), 169.1 and 169.0 (Lys-C1, But-C1), 156.7 (C), 134.4 (C), 133.4 (C), 129.6 (CH), 129.5 (CH), 129.4 (CH), 127.0 (CH), 70.8 (But-C3), 64.0 (O**C**H**₂**Ar), 53.2 (Lys-C2), 52.3 (Cpl-C3), 42.0 (Cpl-C7 or Lys-C6), 41.9 (But-C2), 40.4 (Cpl-C7 or Lys-C6), 30.8 (CH_2), 29.8 (CH_2), 29.2 (CH_2), 28.5 (CH_2), 28.0 (CH_2), 21.9 (CH_2), 19.4 (CH_3, But-C4); Partial spectrum for minor rotamer: ^{1}H NMR δ 7.57 (1 H, br s), 6.27 (1 H, br s); ^{13}C NMR δ 157.4 (C), 134.1 (C), 64.5 (CH_2), 40.7 (CH_2), 29.6 (CH_2), 28.6 (CH_2), 21.6 (CH_2); HRMS (ESI+) *m/z* calcd. for $[C_{24}H_{35}ClN_4O_6+H]^+$: 511.2318, obsd.: 511.2314.

Compound *S*-30

Oxazoline acid **11** (24.6 mg, 0.0790 mmol) and amine *S*-**29** (40.0 mg, 0.0658 mmol) in dry CH_2Cl_2 (1.1 mL) were treated with HOAt (9.0 mg, 0.0658 mmol), DMAP (17.7 mg, 0.145 mmol) and EDC.HCl (13.9 mg, 0.0724 mmol) at rt for 1 d. The crude reaction mixture was purified using preparative t.l.c. (EtOAc) to give the depsipeptide *S*-**30** as a white powder (43.9 mg, 83%). ^{1}H NMR (600 MHz, $CDCl_3$) two rotamers in a ratio of 80:20. Major isomer δ 7.76 (1 H, dd, *J* = 7.9, 1.6 Hz, Sal-H7), 7.51 (2 H, d, *J* = 7.6 Hz, Ar), 7.35–7.42 (5 H, m, Ar), 7.21–7.30 (4 H, m, Ar, Lys-2-N**H**), 7.18 (1 H, d, *J* = 5.9 Hz, Cpl-3-N**H**), 6.99–7.02 (2 H, m, Ar), 6.19 (1 H, t, *J* = 5.6 Hz, Cpl-1-N**H**), 5.65 (1 H, t, *J* = 5.6 Hz, Lys-6-N**H**), 5.13–5.29 (5 H, m, But-H3, 2× OCH_2Ar), 4.65 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 4.49 (1 H, ddd, *J* = 10.7, 6.4 Hz, Lys-H2), 4.40–4.46 (1 H, m, Cpl-H3), 4.17 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 2.92–3.18 (4 H, m, Cpl-H7, Lys-H6), 2.54 (1 H, dd, J_{gem} = 14.8, $J_{2,3}$ = 4.9 Hz, But-H2), 2.47 (1 H, dd, J_{gem} = 14.8, $J_{2,3}$ = 6.0 Hz, But-H2), 1.60–2.05 (6 H, m, Lys-CH_2, Cpl-CH_2), 1.61 (3 H, s, Ser-CH_3), 1.20–1.50 (6 H, m, Lys-CH_2, Cpl-CH_2), 1.28 (d, *J* = 6.2 Hz, But-H4); ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.6, 175.4, 171.0, 168.4 (5 C, C=O), 163.4 (C, Sal-C1), 157.8 (C), 156.3 (C), 137.0 (C), 134.5 (C), 132.9 (CH), 131.4 (CH), 130.2 (CH), 129.7 (CH), 129.6 (CH), 128.8 (CH), 127.9 (CH), 127.1 (CH), 126.9 (CH), 120.9 (CH), 117.3 (C), 114.0 (CH), 75.9 (Ser-C3), 75.0 (Ser-C2), 70.8 (O**C**H_2Ar), 69.1 (But-C3), 64.0 (O**C**H_2Ar), 52.3 (2 × CH, Lys-C2, Cpl-C3), 42.7 (But-C2), 42.1 (Cpl-C7), 40.3 (CH_2, Lys-C6), 31.7 (CH_2), 31.1 (CH_2), 28.91 (CH_2), 28.87 (CH_2), 28.0 (CH_2), 26.3 (Ser-Me), 22.6 (CH_2), 19.3 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{42}H_{50}ClN_5O_9+H]^+$: 804.3370, obsd.: 804.3366.

Synthesis of DDM-838 isomers 1 and 2 from *S*-30

Global deprotection of *S*-**30** (26 mg, 0.0323 mmol) was completed in 2 h with hydrogen, 40 mg 10% Pd/C in ethanol (4 mL) at room temperature to give crude *S*-**22**, which showed only one set of signals in the ^{1}H and ^{13}C NMR spectra, providing evidence in support of the observation of rotamers in the ^{1}H and ^{13}C NMR spectra of compounds *S*-**29** and *S*-**30**. Following the above coupling procedure with Z-C20:1 fatty acid **20** the crude *S*-**22** gave *SSRS*-DDM *cis*-C20:1 **1** (4.6 mg, 17% over 2 steps) and *SSRS*-DDM *trans*-C20:1 **2** (5.7 mg, 21% over 2 steps).

Compound *R*-29

Following the above procedure for the synthesis of *S*-**29**, Boc-D-Lys(2-Cl-Z)-OH gave mainly R-epimer (21 : 79 ratio of *S/R*). After rpHPLC purification, pure *R*-**29** was obtained as TFA salt (white powder 23 mg, 70%). ^{1}H NMR (600 MHz, $CDCl_3$) two rotamers in a ratio of 30 : 70, major one: δ 8.65 (3 H, br s, Lys-2-NH_3^+), 7.59 (1 H, d, J = 6.8 Hz, Cpl-3-N**H**), 7.33–7.40 (2 H, m, Ar), 7.22–7.26 (2 H, m, Ar), 6.91 (1 H, br s, Cpl-1-N**H**), 5.76 (1 H, br s, Lys-6-N**H**), 5.25–5.31 (1 H, m, But-H3), 5.14–5.26 (2 H, m, OC**H$_2$**Ar), 4.53–4.57 (1 H, m, Cpl-H3), 3.96 (1 H, br s, Lys-H2), 3.05–3.20 (4 H, m, Lys-H6, Cpl-H7), 2.61–2.69 (1 H, m, But-H2), 2.42–2.46 (1 H, m, But-H2), 1.82–1.98 (4 H, m, Lys-CH_2, Cpl-CH_2), 1.62–1.82 (2 H, m, Lys-CH_2, Cpl-CH_2), 1.25–1.62 (6 H, m, Lys-CH_2, Cpl-CH_2), 1.32 (3 H, d, $J_{3,4}$ = 6.1 Hz, But-H4); ^{13}C NMR (150 MHz, DMSO-d_6) δ 176.7 (C, Cpl-C2), 169.1 (C) and 168.2 (C, Lys-C1, But-C1), 156.7 (C), 134.3 (C), 133.5 (C), 129.8 (CH), 129.6 (CH), 129.5 (CH), 127.1 (CH), 71.0 (CH, But-C3), 64.1 (CH_2, O**C**H$_2$Ar), 53.5 (CH, Lys-C2), 52.2 (CH, Cpl-C3), 42.0 (CH_2, Cpl-C7 or Lys-C6), 41.8 (CH_2, But-C2), 40.2 (CH_2, Cpl-C7/Lys-C6), 30.6 (CH_2), 29.9 (CH_2), 28.9 (CH_2), 28.4 (CH_2), 27.9 (CH_2), 21.4 (CH_2), 19.1 (CH_3, But-C4); Partial spectrum for minor rotamer: ^{1}H NMR δ 7.71 (1 H, d, J = 6.2 Hz, Cpl-3-NH), 7.64 (1 H, br s, Cpl-1-NH), 6.37 (1 H, br s, Lys-6-NH), 4.60–4.64 (1 H, m, Cpl-H3), 4.01 (1 H, br s, Lys-H2); ^{13}C NMR (150 MHz, DMSO-d_6) δ 177.2 (C, Cpl-C2), 167.9 (C), 157.4 (C), 134.1 (C), 133.4 (C), 129.4 (CH), 71.1 (CH, But-C3), 64.5 (CH_2, O**C**H$_2$Ar), 41.5 (CH_2), 40.0 (CH_2), 30.4 (CH_2), 29.4 (CH_2), 28.2 (CH_2), 20.4 (CH_2), 18.8 (CH_3, But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{24}H_{35}ClN_4O_6 + H]^+$: 511.2318, obsd.: 511.2320.

Compound *R*-30

Following the above procedure for the synthesis of *R*-**30**, amine *R*-**29** was coupled with oxazoline acid **11** to give *R*-**30** in 83% yield. ^{1}H NMR (600 MHz, $CDCl_3$) two rotamers in a ratio of 80:20. Major one δ 7.78 (1 H, d, J = 7.7 Hz, Sal-H7), 7.50 (2 H, d, J = 7.4 Hz, Ar), 7.33–7.42 (6 H, m, Ar,

Lys-2-N**H**/Cpl-3-N**H**), 7.17–7.30 (3 H, m, Ar), 6.99–7.02 (3 H, m, Ar, Lys-2-N**H**/Cpl-3-N**H**), 6.21 (1 H, t, J = 5.9 Hz, Cpl-1-N**H**), 5.67 (1 H, t, J = 5.2 Hz, Lys-6-N**H**), 5.18–5.25 (5 H, m, But-H3, 2× OCH_2Ar), 4.64 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 4.42–4.50 (2 H, m Lys-H2, Cpl-H3), 4.18 (1 H, d, J_{gem} = 8.8 Hz, Ser-H3), 3.02–3.22 (4 H, m, Cpl-H7, Lys-H6), 2.43 (1 H, dd, J_{gem} = 15.0 Hz, $J_{2,3}$ = 7.0 Hz, But-H2), 2.37 (1 H, dd, J_{gem} = 15.0 Hz, $J_{2,3}$ = 4.7 Hz, But-H2), 1.68–1.98 (5 H, m, Cpl-H7, Lys-H6), 1.59 (3 H, s, Ser-CH_3), 1.24–1.58 (7 H, m, Cpl-H7, Lys-H6), 1.25 (3 H, d, J = 7.3 Hz, But-H4); ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.4 (C), 175.2 (C), 171.2 (C), 168.3 (2× C), 164.1 (C), 157.8 (C), 156.4 (C), 137.0 (C), 134.6 (C), 132.9 (CH), 131.5 (CH), 129.9 (CH), 129.6 (CH), 129.4 (CH), 128.6 (CH), 127.9 (CH), 127.0(1) (CH), 126.9(6) (CH), 120.9 (CH), 117.4 (C), 113.9 (CH), 76.0 (CH_2, Ser-C3), 74.8 (C, Ser-C2), 70.8 (CH_2, OCH_2Ar), 69.2 (CH, But-C3), 63.8 (CH_2, OCH_2Ar), 52.2 (CH) and 51.9 (CH, Lys-C2, Cpl-C3), 42.4 (CH_2, But-C2), 42.1 (CH_2), 40.6 (CH_2, Cpl-C7, Lys-C6), 31.9 (CH_2), 31.6 (CH_2), 28.8 (2× CH_2), 27.9 (CH_2), 26.4 (CH_3, Ser-Me), 22.3 (CH_2), 19.7 (CH_2, But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{42}H_{50}ClN_5O_9 + H]^+$: 804.3370, obsd.: 804.3372.

Synthesis of DDM-838 isomers 3 and 4 from *R*-30

Following the above global deprotection procedure, *R*-**30** (53 mg, 0.0659 mmol) gave crude *R*-**22**, which again showed only one set of signals in ^{1}H and ^{13}C NMR spectra, further confirming the rotameric nature of intermediates *R*-**29** and *R*-**30**. Following the above coupling procedure with Z-C20:1 fatty acid **16**, the crude *R*-**22** gave *SRRS*-DDM *cis*-C20:1 **3** (4.6 mg, 24%) and *SRRS*-DDM *trans*-C20:1 **4** (8.0 mg, 42%),

Synthesis of DDM-838 1 via the Mitsunobu approach

(*S*)-3-[(*S*)-3-Hydroxybutyramido]-hexahydro-2-azepinone (23)

(*S*)-3-Hydroxy-butyric acid *S*-**14** (100 mg, 0.961 mmol) and L-(–)-α-amino-ε-caprolactam hydrochloride **15** (158 mg, 0.961 mmol) were co-evaporated with toluene (3 mL × 3) and then dissolved in dry CH_2Cl_2 (15 mL). HOAt (131 mg, 0.961 mmol), DMAP (117 mg, 0.961 mmol) and EDC.HCl (276 mg, 1.44 mmol) were added and the reaction mixture was stirred at rt for 24 h. The crude reaction mixture was applied directly to a silica gel column. Flash chromatography (100% EtOAc to 5% MeOH/EtOAc) afforded deoxycobactin **23** as a colourless oil (128 mg, 0.597 mmol, 62%). $[\alpha]_D^{26}$ = +76.3° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (1 H, d, $J_{NH,3}$ = 6.4 Hz, Cpl-3-N**H**), 6.73 (1 H, bs, Cpl-1-N**H**), 4.52 (1 H, dd, $J_{NH,3}$ = 6.4, $J_{3,4}$ = 11.0 Hz, Cpl-H3), 4.17–4.11 (1 H, m, But-H3), 4.03 (1 H, d, $J_{OH,3}$ = 3.0 Hz, OH), 3.30–3.19 (2 H, m, Cpl-H7), 2.40 (1 H, dd, $J_{2,2}$ = 14.8, $J_{2,3}$ = 3.0 Hz, But-H2), 2.30 (1 H, dd, $J_{2,2}$ = 14.8, $J_{2,3}$ = 9.1 Hz, But-H2), 2.04–1.97 (2 H, m,

Cpl-H4,5), 1.85–1.73 (2 H, m, Cpl-H5,6), 1.52–1.30 (2 H, m, Cpl-H4,6), 1.19 (3 H, d, $J_{3,4}$ = 6.3 Hz, But-H4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.8, 171.9 (2 C, But C=O, Cpl C=O), 65.0 (But-C3), 52.3 (Cpl-C3), 44.5 (But-C2), 42.1 (Cpl-C7), 31.3, 28.9, 28.0 (3 C, Cpl-C4,5,6), 22.9 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{10}H_{18}N_2O_3+H]^+$: 215.1390, obsd.: 215.1390.

Compound 26

Fmoc-L-Lys(Cbz)-OH (136 mg, 0.271 mmol) and deoxycobactin **23** (58.0 mg, 0.271 mmol) were co-evaporated with dry toluene (× 3) and dissolved in dry THF (2.40 mL). The reaction mixture was cooled to 0 °C and PPh_3 (92.3 mg, 0.352 mmol) was added. DIAD (69.1 μL, 0.352 mmol) was added over 5 min and the mixture was stirred at 0 °C for 30 min and then at rt overnight. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (30–100% EtOAc/pet. ether, then 0.5% MeOH/EtOAc) to afford a mixture of the desired ester **24** and the elimination by-product **25** (80.6 mg, 3:1 molar ratio), which could not be separated by flash chromatography. The mixture of ester **24** and alkene **25** was dissolved in a solution of 20% v/v piperidine in MeCN (1.3 mL) and stirred at rt for 30 min. The solvent was evaporated and the residue was purified by flash chromatography (1% NEt_3 in 20% MeOH/EtOAc) to afford amine **26** (33.4 mg, 26%) as a clear oil and alkene **25** (10.5 mg). Characterisation of amine **26**: $[\alpha]_D^{26}$ = +30.2° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) 7.35–7.29 (5 H, m, Ar), 7.16 (1 H, d, J = 5.4 Hz, Cpl-3-N**H**), 6.26 (1 H, bs, Cpl-1-N**H**), 5.56 (1 H, bs, Lys-6-N**H**), 5.29–5.23 (1 H, m, But-H3), 5.10 (1 H, d, J_{gem} = 12.5 Hz, Cbz-C**H**$_2$), 5.05 (1 H, d, J_{gem} = 12.5 Hz, Cbz-C**H**$_2$), 4.50–4.46 (1 H, m, Cpl-H3), 3.41–3.38 (1 H, m, Lys-H2), 3.29–3.21, 3.17–3.07, 2.93–2.88 (4 H, 3 × m, Cpl-H7/Lys-H6), 2.53 (1 H, dd, $J_{2,2}$ = 15.0, $J_{2,3}$ = 4.4 Hz, But-H2), 2.49 (1 H, dd, $J_{2,2}$ = 15.0, $J_{2,3}$ = 6.5 Hz, But-H2), 2.03–1.93 (2 H, m, Cpl-CH_2, Lys-CH_2), 1.80–1.73 (3 H, m, Cpl-CH_2, Lys-CH_2), 1.58–1.35 (6 H, m, Cpl-CH_2, Lys-CH_2), 1.30 (3 H, d, $J_{3,4}$ = 6.5 Hz, But-H4), 1.27–1.24 (1 H, m, Cpl-CH_2/Lys-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6, 175.4 (2 C, But C=O, Cpl C=O), 168.5 (Lys C=O), 156.5 (Cbz C=O), 136.7, 128.7, 128.4, 128.3 (Ar), 68.5 (But-C3), 66.7 (Cbz-CH_2), 54.6 (Lys-C2), 52.3 (Cpl-C3), 42.8 (But-C2), 42.0, 40.6 (2 C, Lys-C6, Cpl-C7), 34.3, 31.7, 29.5, 28.9, 28.0, 22.9 (6 C, Cpl-C4,5,6, Lys-C3,4,5), 19.5 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{24}H_{36}N_4O_6+H]^+$: 477.2708, obsd.: 477.2709.

Compound 27

Oxazoline acid **11** (35.4 mg, 0.1137 mmol) and amine **26** (33.4 mg, 0.0701 mmol) were co-evaporated with toluene (× 3) and dissolved in dry DMF (1 mL). HOAt (11.4 mg, 0.0841 mmol), DMAP (10.3 mg, 0.0841 mmol) and EDC.HCl (26.9 mg, 0.140 mmol) were added and the mixture

was stirred at rt for 18 h. The mixture was diluted with EtOAc (10 mL), washed with 0.37 N HCl solution, H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (0.5% MeOH/EtOAc) to afford the protected depsipeptide **27** as a clear oil (45.8 mg, 0.0595 mmol, 85%). $[\alpha]_D^{26}$ = +10.5° (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (1 H, dd, *J* = 7.9, 1.7 Hz, Ar), 7.52–7.19 (13 H, m, Ar, Lys-2-N**H**, Cpl-3-N**H**), 7.02–6.99 (2 H, m, Ar), 6.16 (1 H, bs, Cpl-N**H**-1), 5.75 (1 H, bs, Lys-6-N**H**), 5.28–5.17 (3 H, m, Sal-PhC**H**$_2$, But-H3), 5.08 (1 H, d, J_{gem} = 12.2 Hz, Cbz-C**H**$_2$), 5.02 (1 H, d, J_{gem} = 12.2 Hz, Cbz-C**H**$_2$), 4.65 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 4.47 (1 H, dd, $J_{2,3}$ = 10.7, 6.1 Hz, Lys-H2), 4.43–4.39 (1 H, m, Cpl-H3), 4.17 (1 H, d, J_{gem} = 8.9 Hz, Ser-H3), 3.18–3.12, 3.01–3.95 (2 H, 2m, Lys-H6), 3.10–3.03, 2.75–2.70 (2 H, 2m, Cpl-H7), 2.55 (1 H, dd, $J_{2,2}$ = 14.9, $J_{2,3}$ = 4.9 Hz, But-H2), 2.46 (1 H, dd, $J_{2,2}$ = 14.9, $J_{2,3}$ = 6.0 Hz, But-H2), 2.01–1.91 (2 H, m, Cpl-H5, Lys-H4), 1.79–1.66 (3 H, m, Cpl-H4,5,6), 1.62 (3 H, s, Ser-Me), 1.48–1.14 (7 H, m, Lys-H3,4,5, Cpl-H4,6), 1.28 (3 H, d, $J_{3,4}$ = 6.4 Hz, But-H4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6, 175.5 (2 C, Ser C=O, Lys C=O), 171.0 (Cpl C=O), 168.4 (But C=O), 163.4 (C=N), 157.9 (Sal-C3), 156.5 (Cbz C=O), 137.0, 136.7, 132.9, 131.4, 128.72, 128.80, 128.5, 128.4, 127.9, 126.9, 120.9, 114.0 (Ar), 117.3 (Sal-C2), 75.9 (Ser-C3), 75.0 (Ser-C2), 70.8 (Sal-CH_2), 69.0 (But-C3), 66.8 (Cbz-CH_2), 52.32 (Cpl-C3), 52.26 (Lys-C2), 42.7 (But-C2), 42.0 (Cpl-C7), 40.1 (Lys-C6), 31.7, 31.1, 28.94, 28.88, 28.0, 22.6 (6 C, Lys-C3,4,5, Cpl-C4,5,6), 26.3 (Ser-Me), 19.2 (But-C4); HRMS (ESI+) *m/z* calcd. for $[C_{42}H_{51}N_5O_9+H]^+$: 770.3760, obsd.: 770.3759.

DDM-838 (*cis*-SSRS) 1

Peptide **27** (34.8 mg, 0.0452 mmol) was dissolved in isopropanol (1 mL) and 2 drops of H_2O and 5 wt. % Pd/C (9.6 mg, 0.00452 mmol, 10 mol%) were added. The mixture was stirred under H_2 at 38 atm for 1 d. Analysis of the reaction mixture by mass spectrometry revealed incomplete hydrogenolysis of both benzyl and Cbz protecting groups. An additional 15 mol% of Pd/C was added, and the mixture treated with hydrogen for another 2 d. The mixture was filtered (Celite) and washed with 10% iPrOH in EtOAc to afford the fully deprotected peptide *S*-**22**. A solution of *Z*-C20:1 acid **20** (12.4 mg, 0.0398 mmol) and DIC (8.4 μL, 0.0398 mmol) in dry CH_2Cl_2 (0.4 mL) was stirred at rt for 15 mins. To this solution was added a solution of the crude peptide *S*-**22** in dry CH_2Cl_2 (0.8 mL), and the resultant solution was stirred at rt for 18 h. At this stage an additional portion of a mixture of *Z*-C20:1 acid **20** (34.9 mg, 0.112 mmol) and DIC (4.5 μL, 0.0434 mmol) in dry CH_2Cl_2 (0.2 mL), which had been preactivated by stirring at room temperature for 15 min, was added to the reaction mixture and the resultant mixture was stirred for further 18 h. The reaction mixture was diluted with EtOAc and washed with 0.2 N aq HCl, 5% aq $NaHCO_3$ solution, brine,

dried ($MgSO_4$) and concentrated *in vacuo*. The crude residue was purified by flash chromatography (10-100% EtOAc/pet. ether) to afford DDM-838 **1** as a clear oil (14.7 mg, 49% over two steps).

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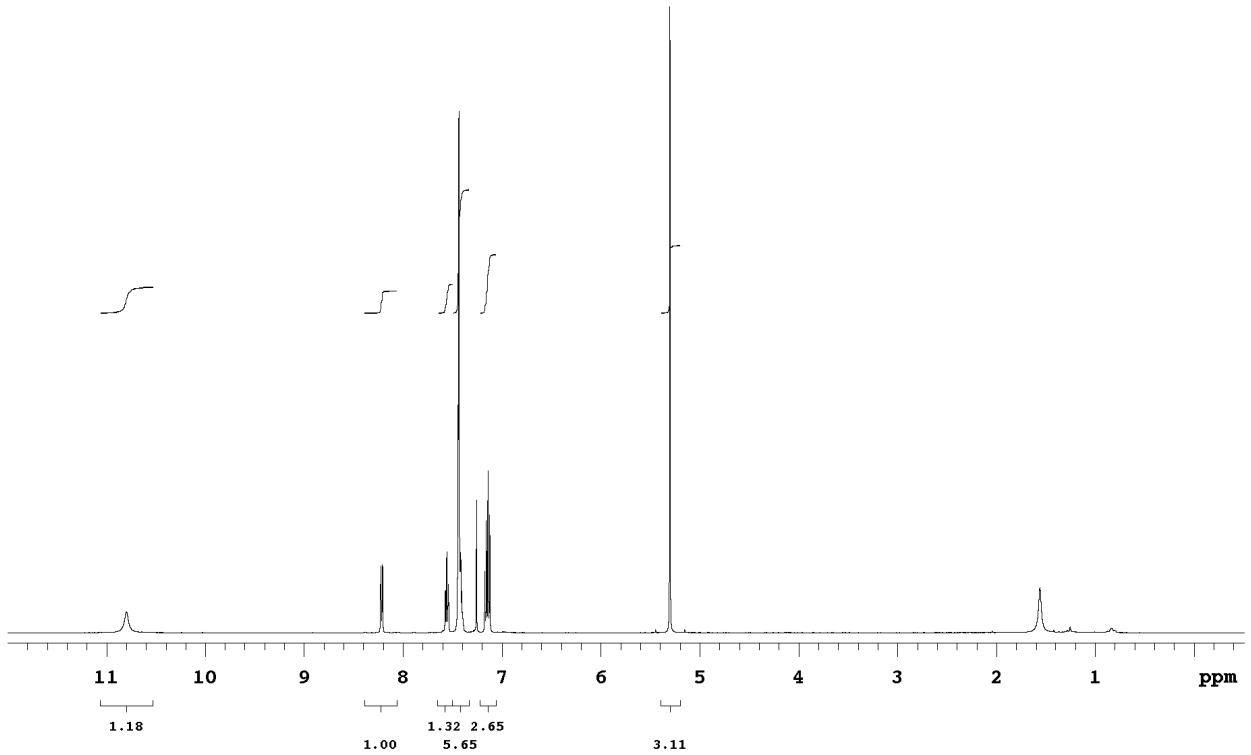
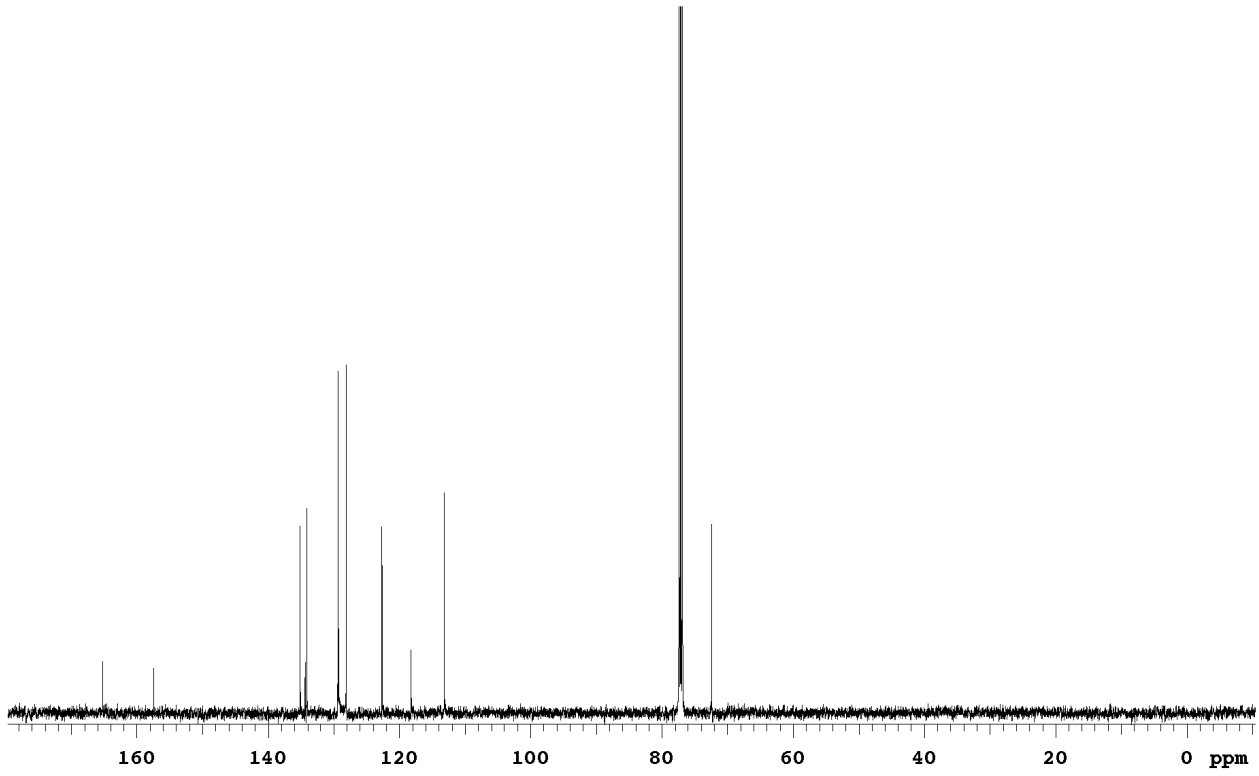
NMR Spectra
2-(Benzyloxy)benzoic acid (8)
^{1}H NMR

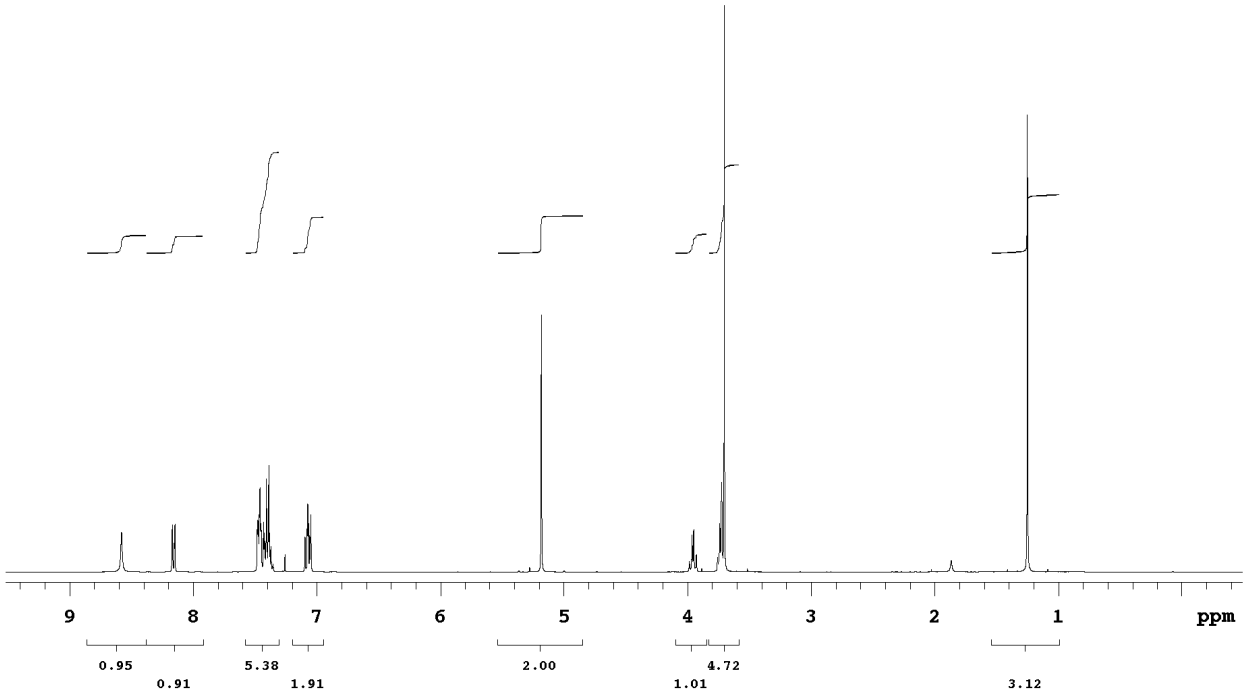


^{13}C NMR

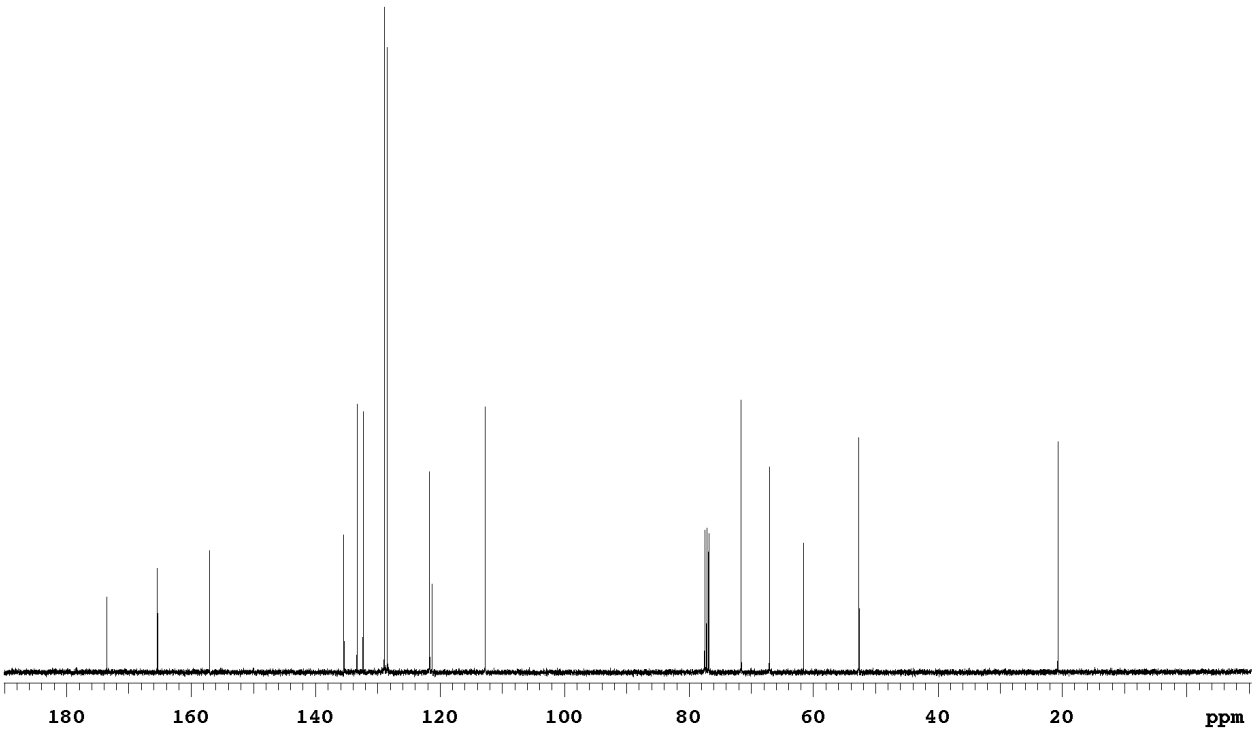


(*S*)-Methyl 2-(2-(benzyloxy)benzamido)-3-hydroxy-2-methylpropanoate (9)

^{1}H NMR

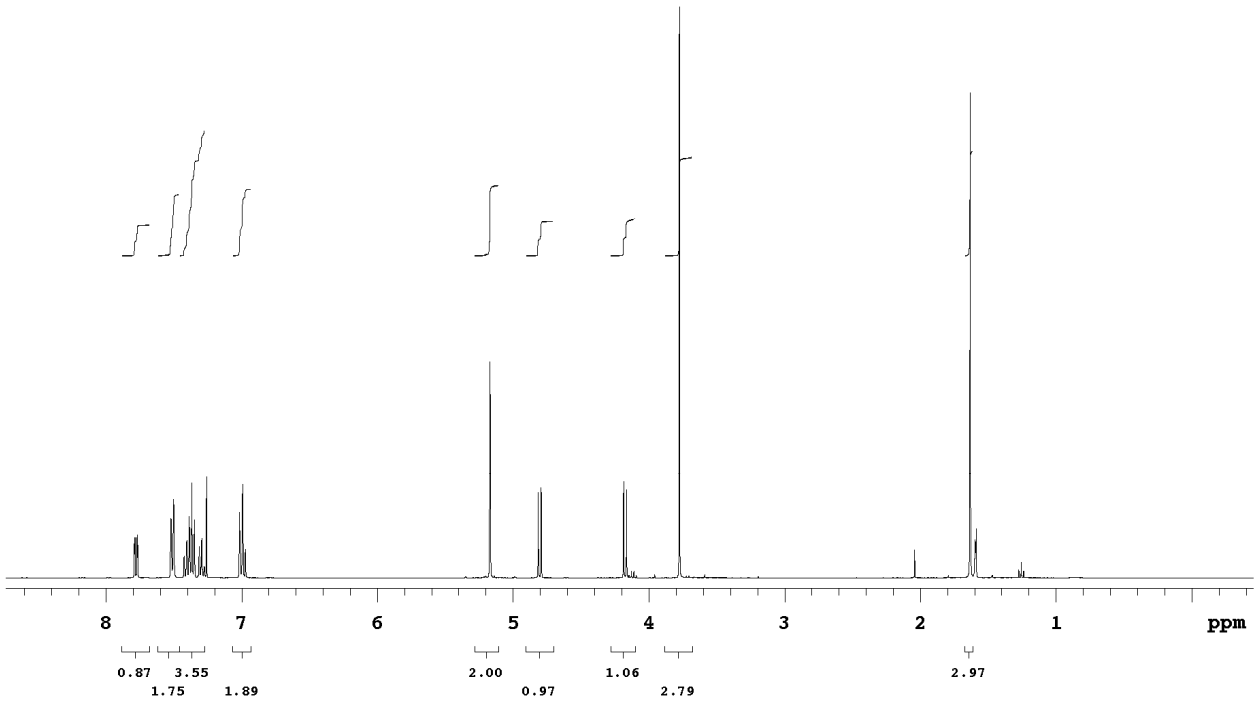


^{13}C NMR

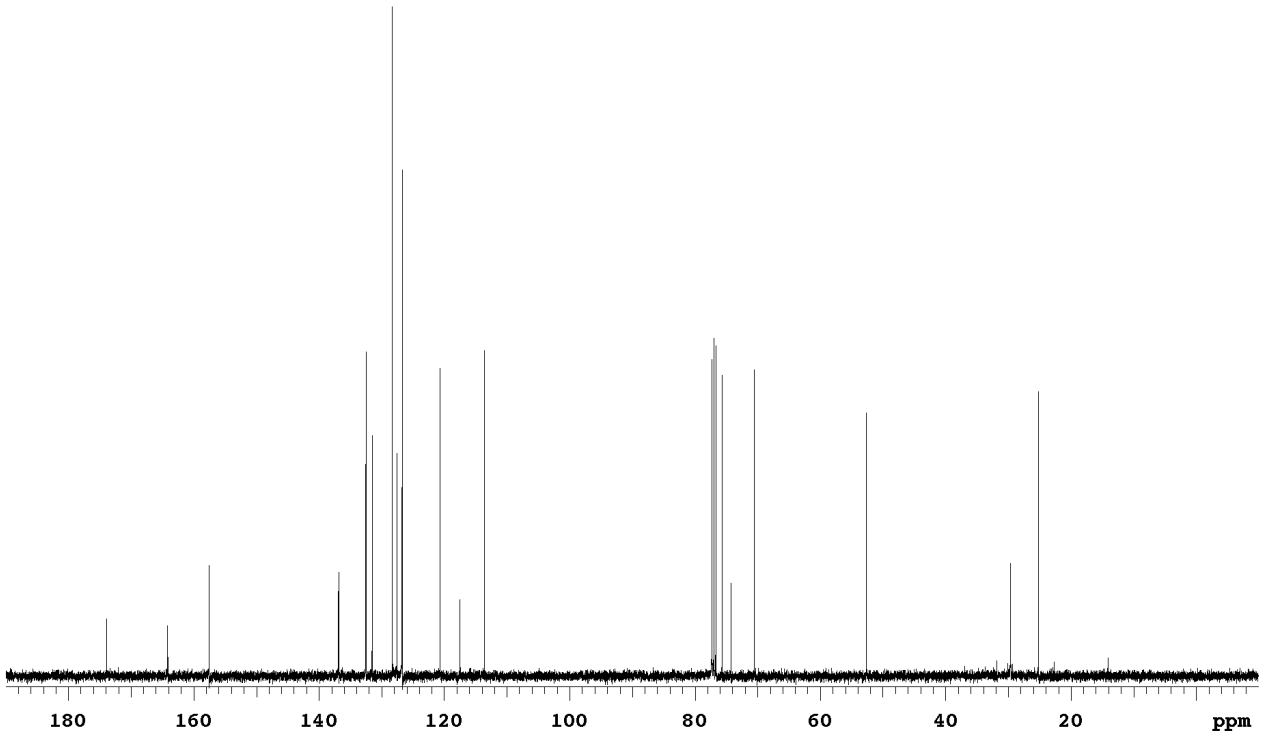


(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4-methoxycarbonyl-4,5-dihydrooxazole (10)

^{1}H NMR

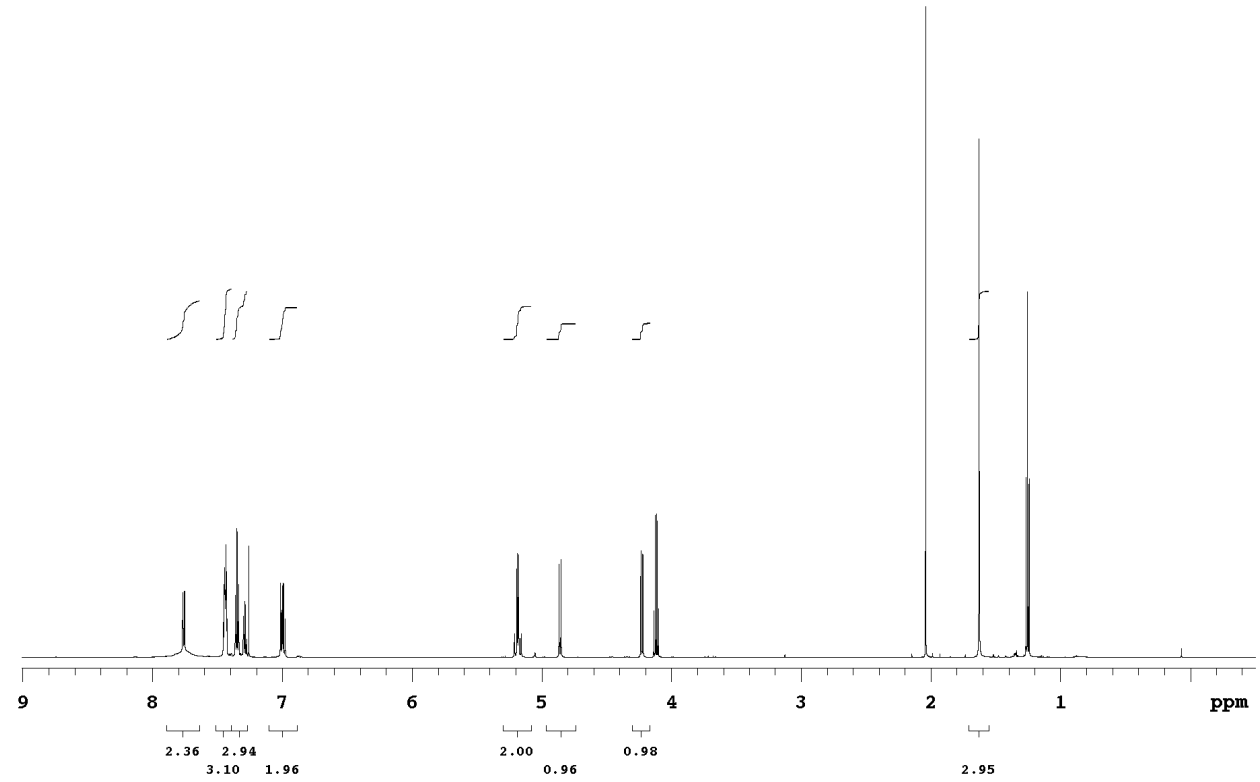


^{13}C NMR

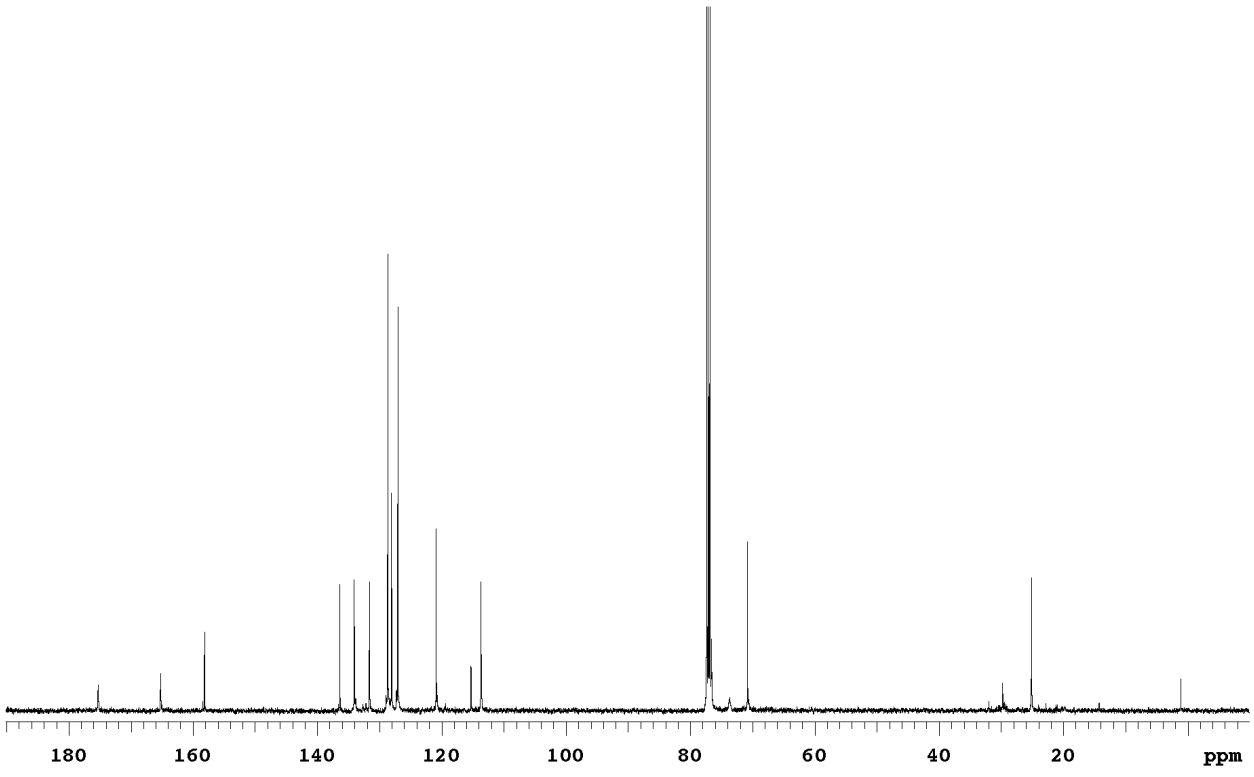


(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4-hydroxycarbonyl-4,5-dihydrooxazole (11)

^{1}H NMR

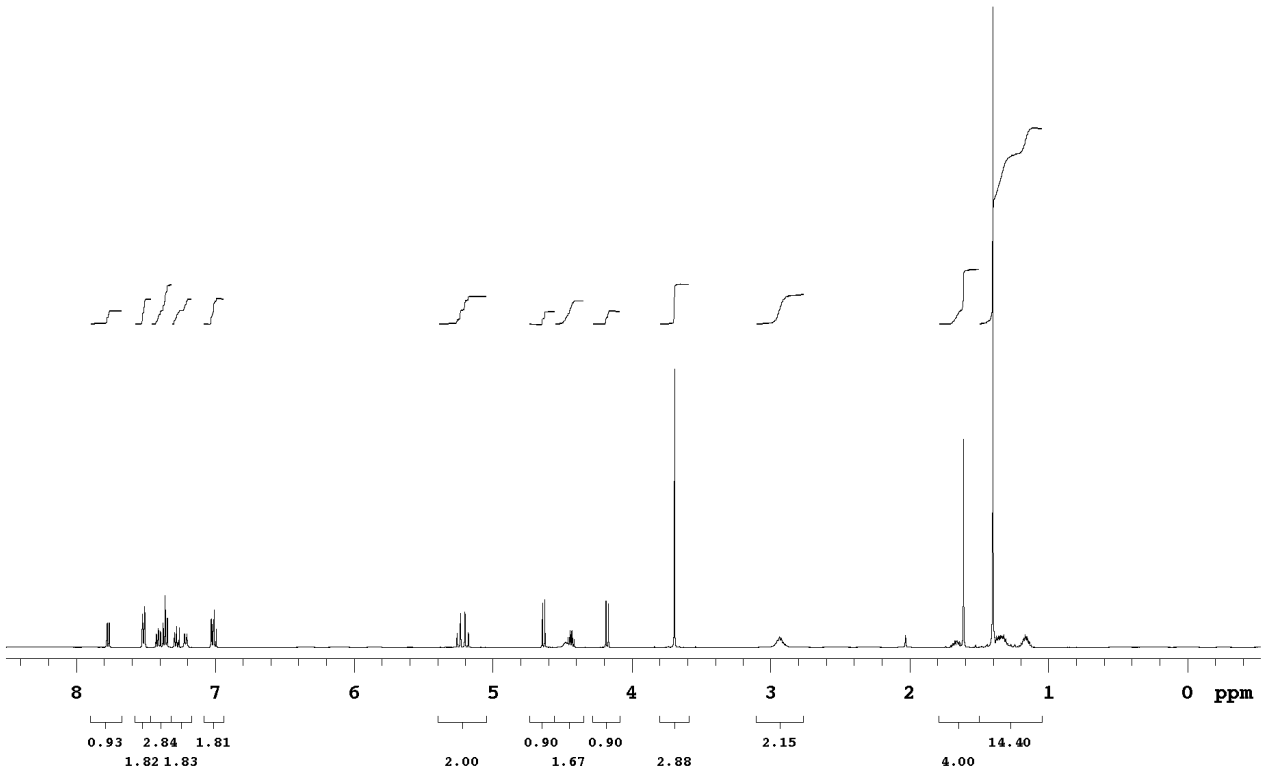


^{13}C NMR

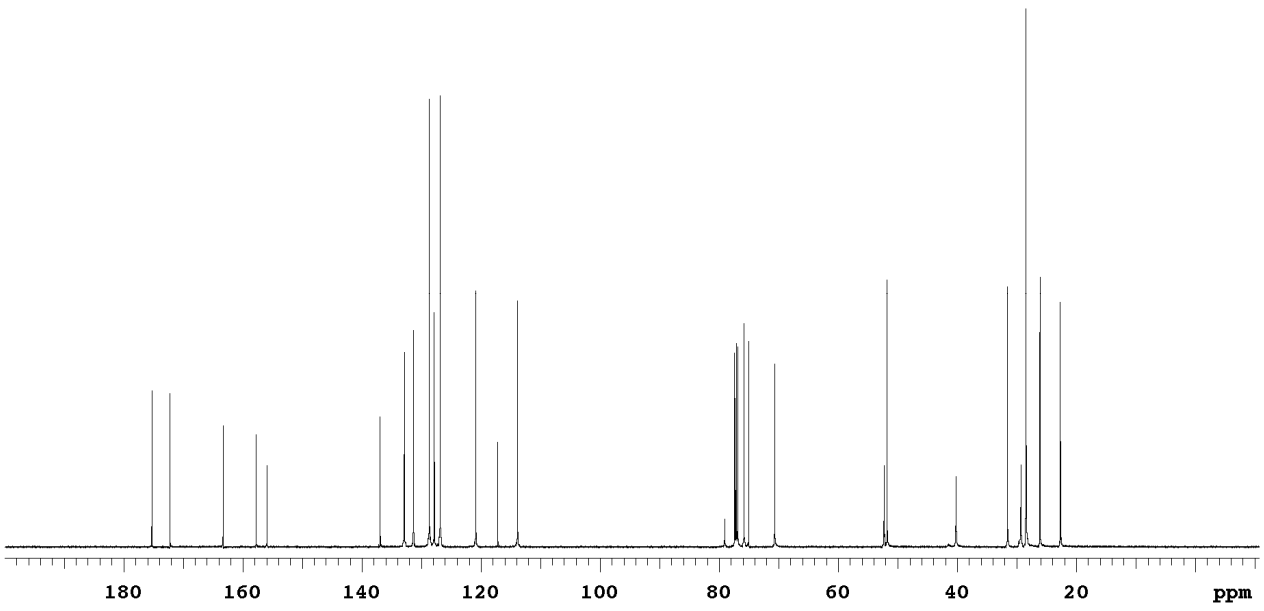


N^{α}-[(*S*)-2-(2-(Benzyloxy)phenyl)-4-methyl-4,5-dihydrooxazol-4-ylcarbonyl]-N^{ε}-(*tert*-butyloxycarbonyl)-L-lysine methyl ester (12)

^{1}H NMR

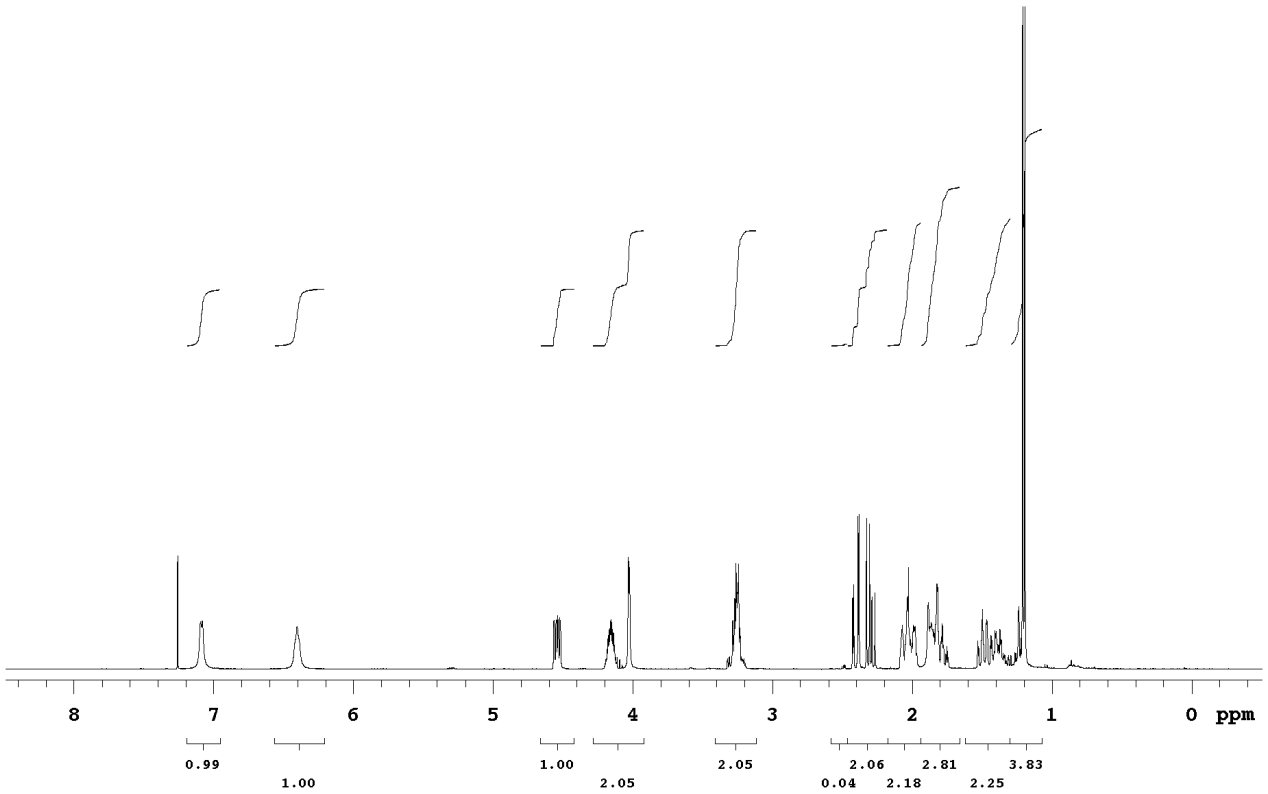


^{13}C NMR

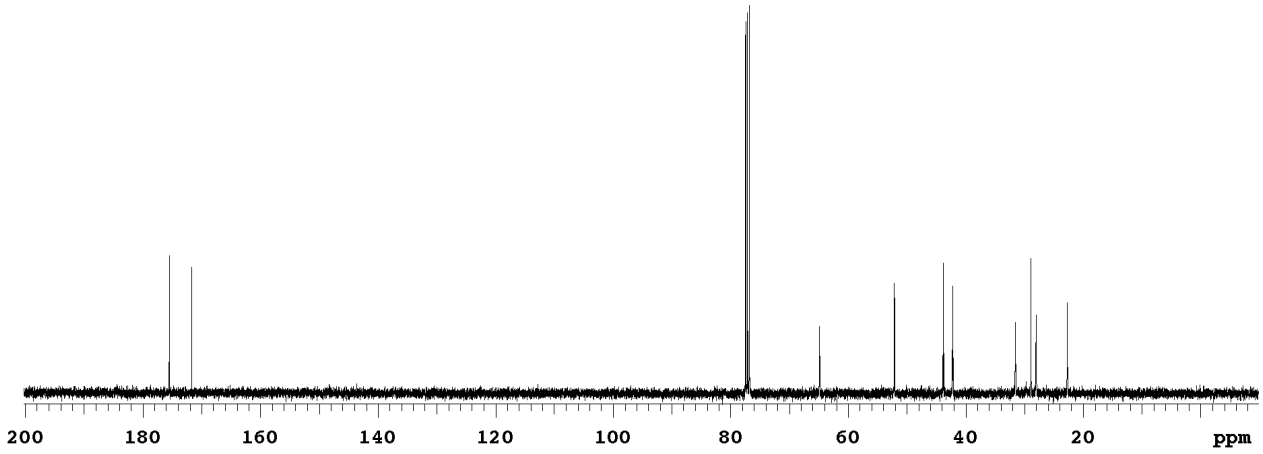


(*S*)-3-[(*R*)-3-Hydroxybutyramido)-hexahydro-2-azepinone (16)

^{1}H NMR

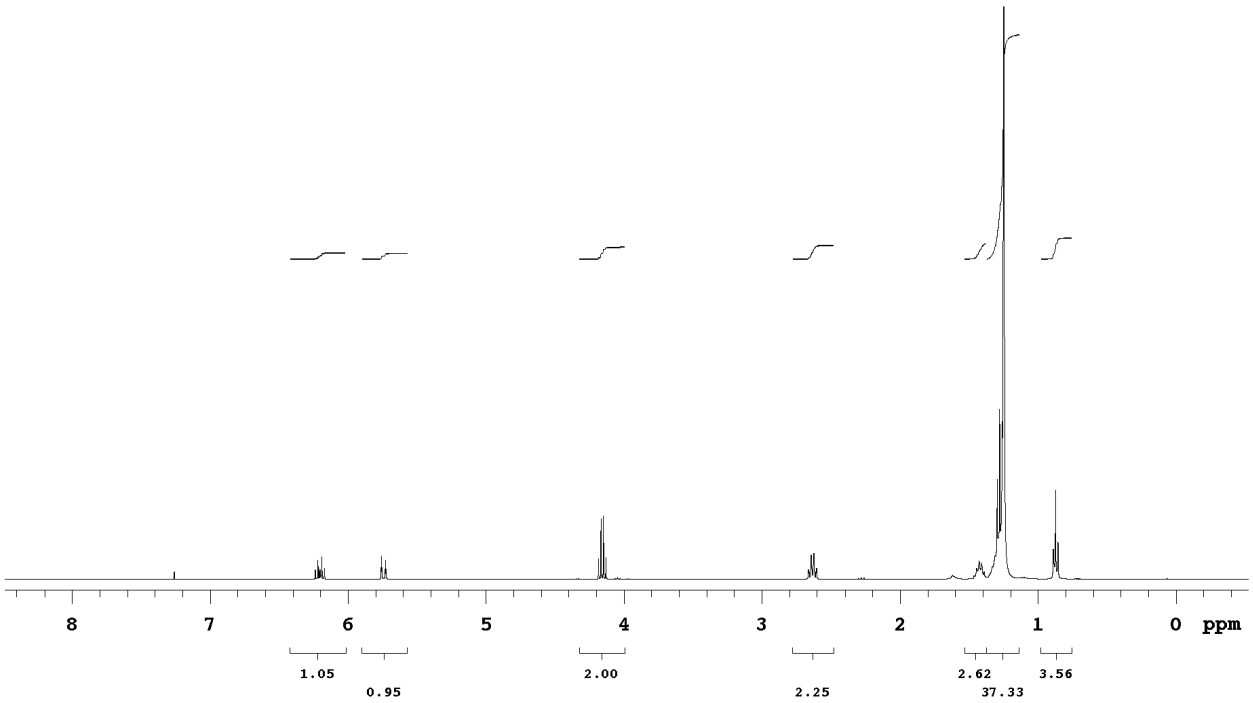


^{13}C NMR

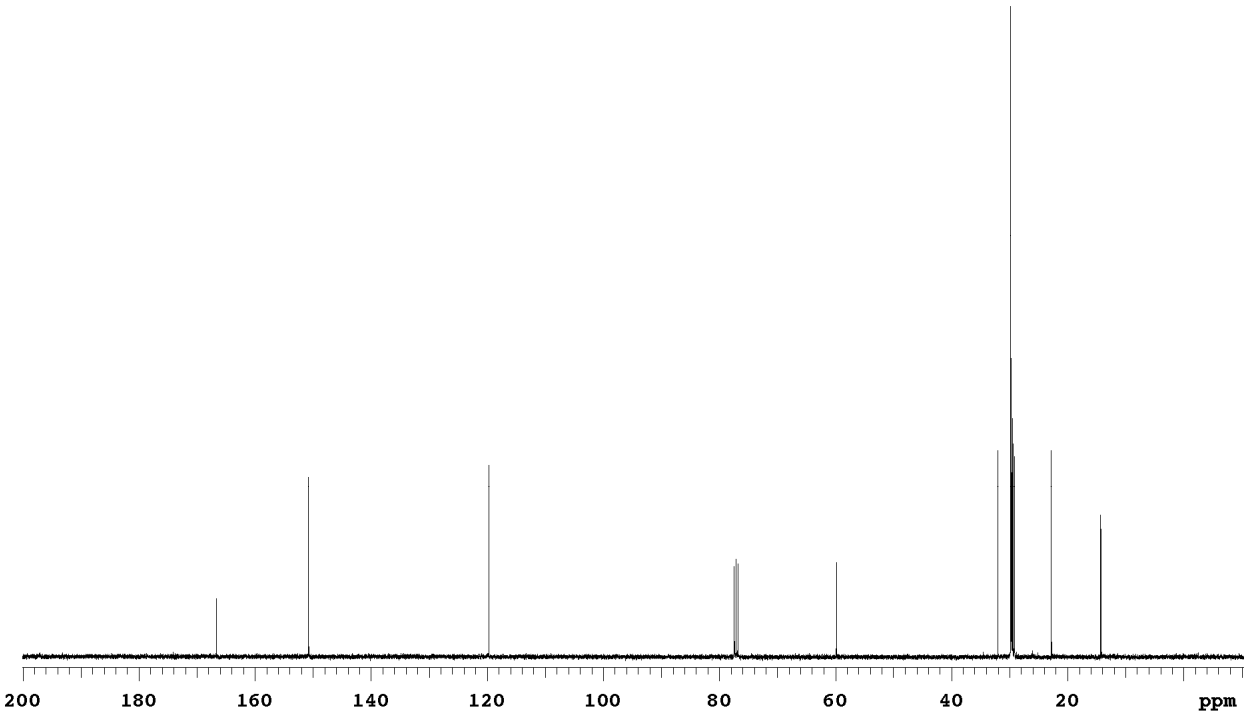


Ethyl *Z*-2-eicosenoate (19)

^{1}H NMR

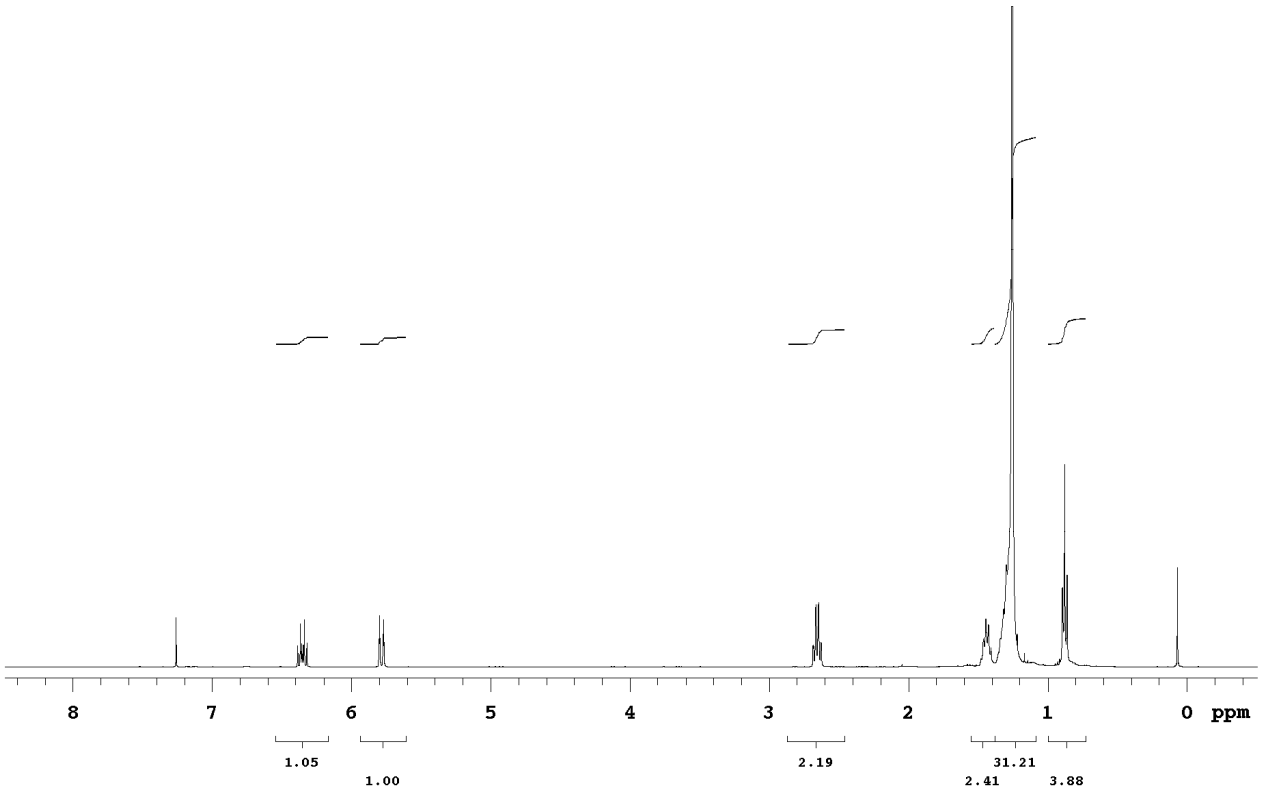


^{13}C NMR

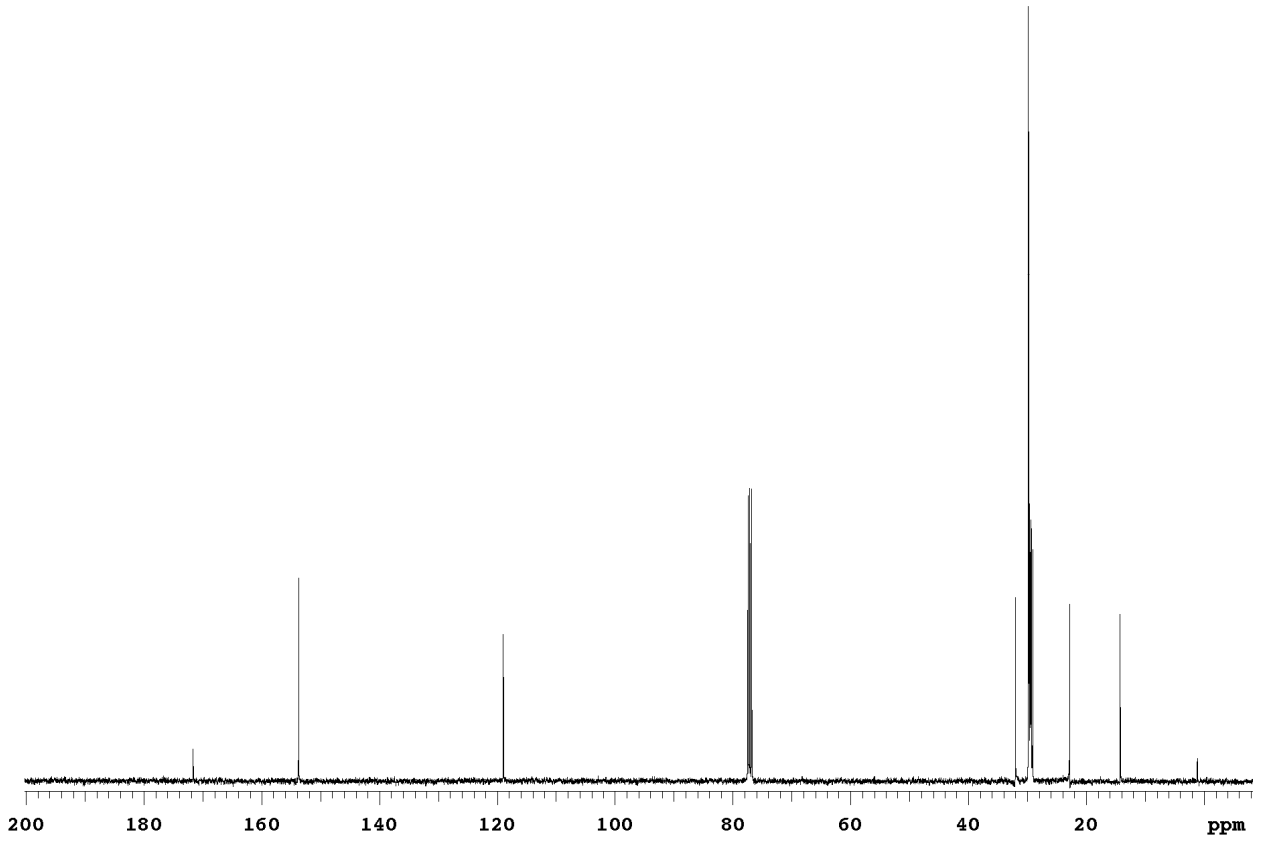


_Z_-2-Eicosenoic acid (20)

^{1}H NMR

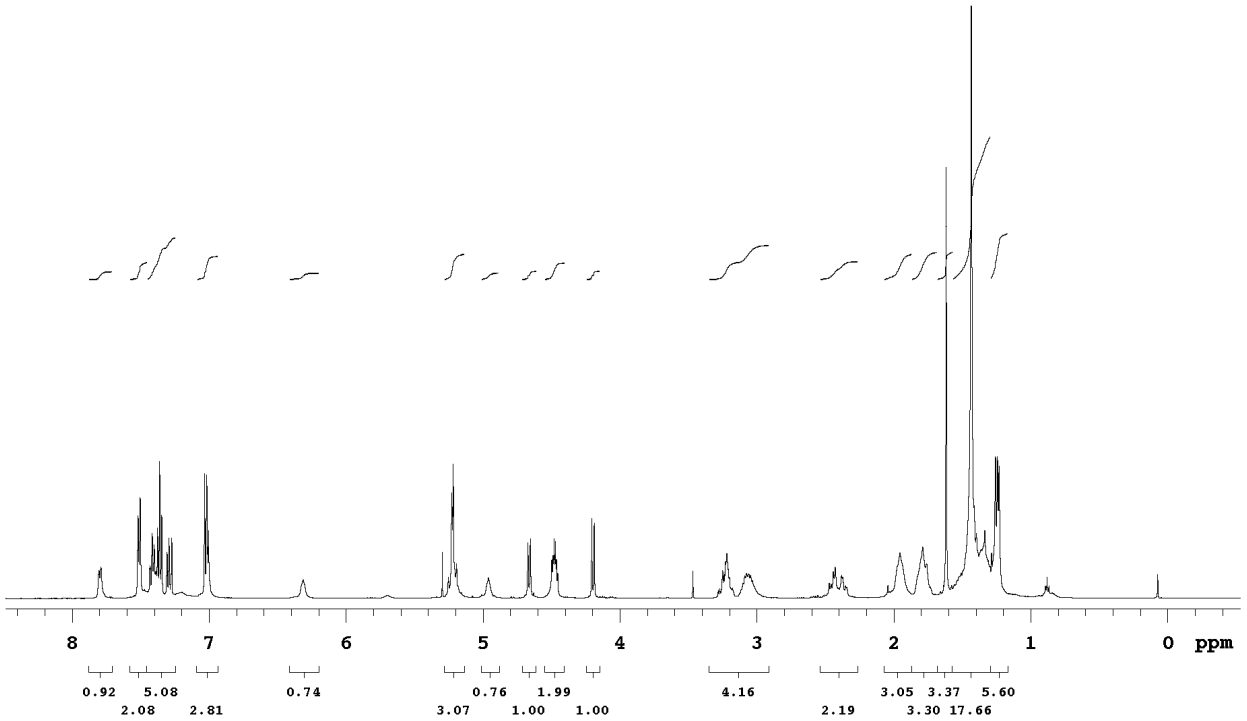


^{13}C NMR

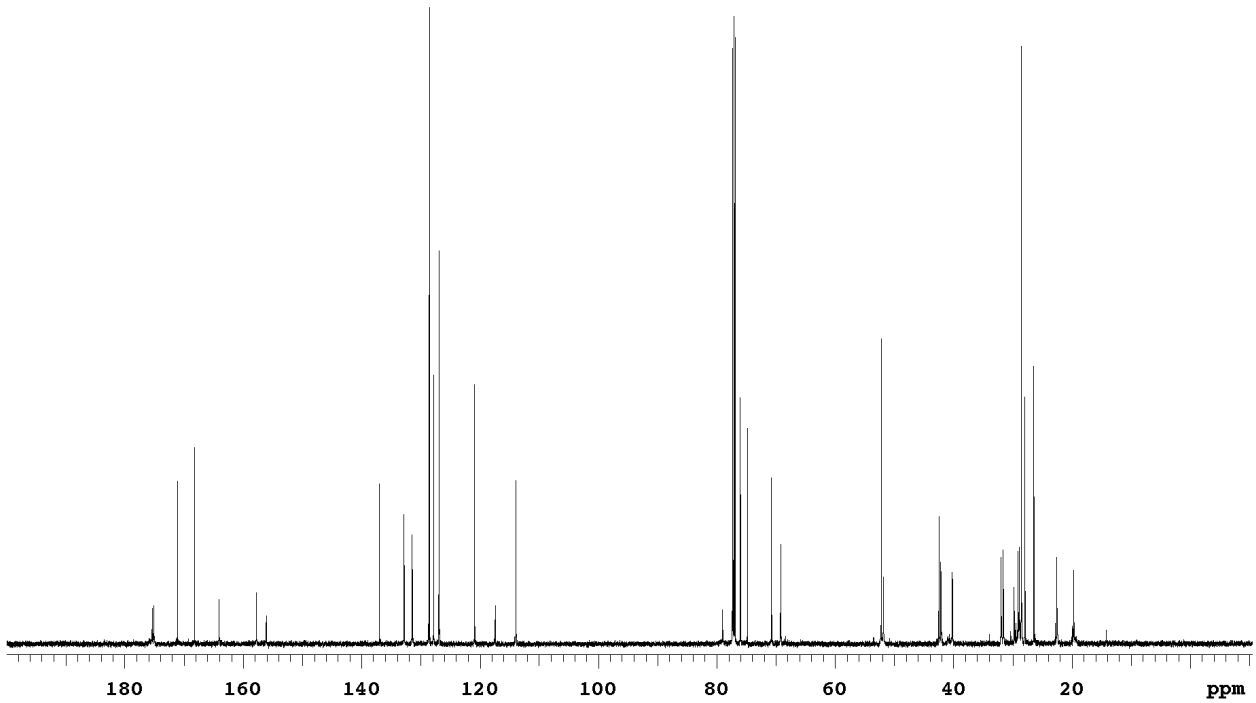


SRRS-depsipeptide _R_-21 (fast)

^{1}H NMR

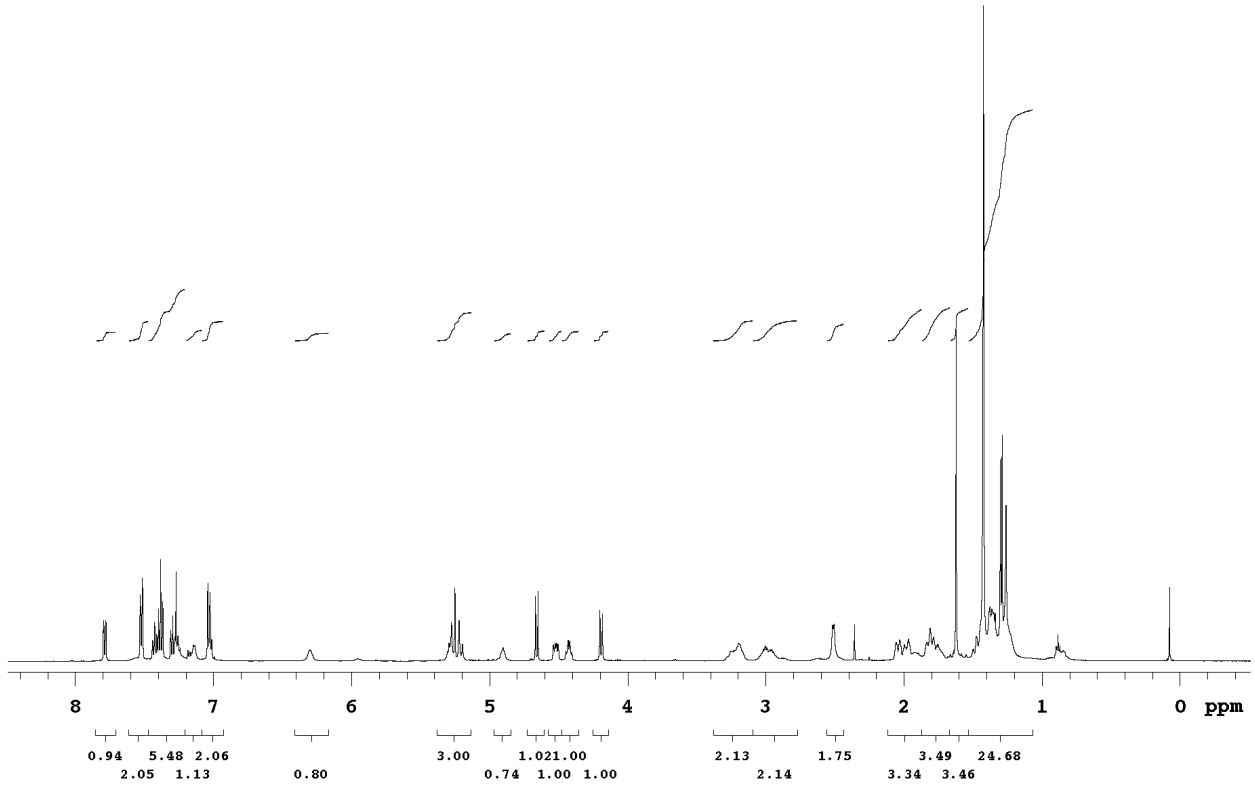


^{13}C NMR

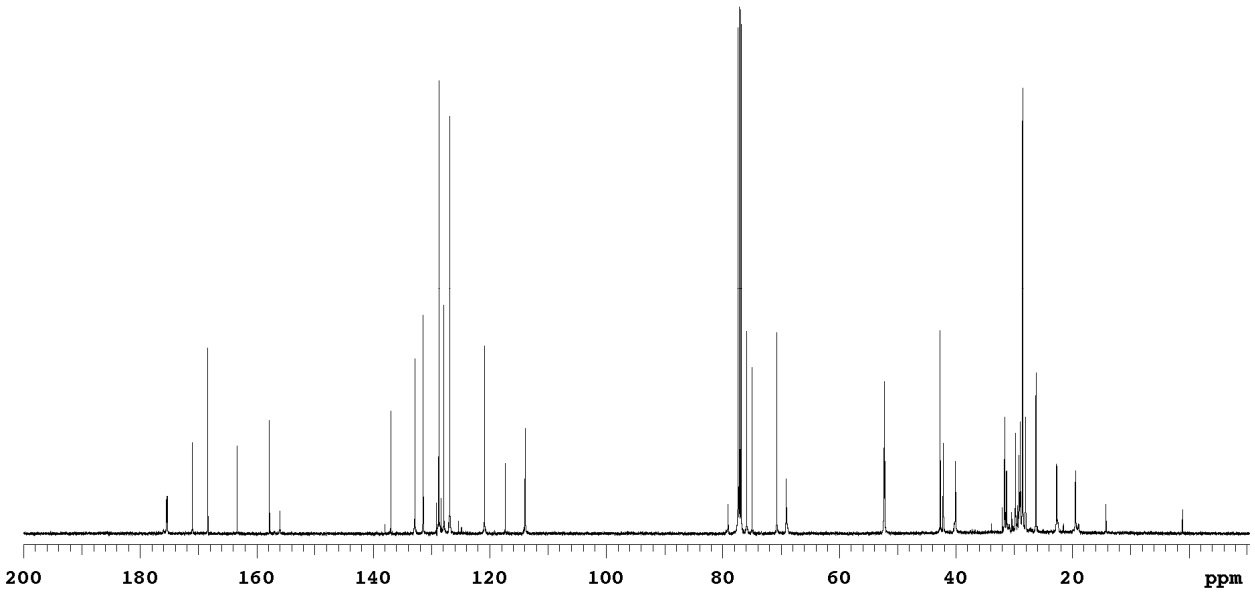


SSRS-depsipeptide _S_-21 (slow)

^{1}H NMR

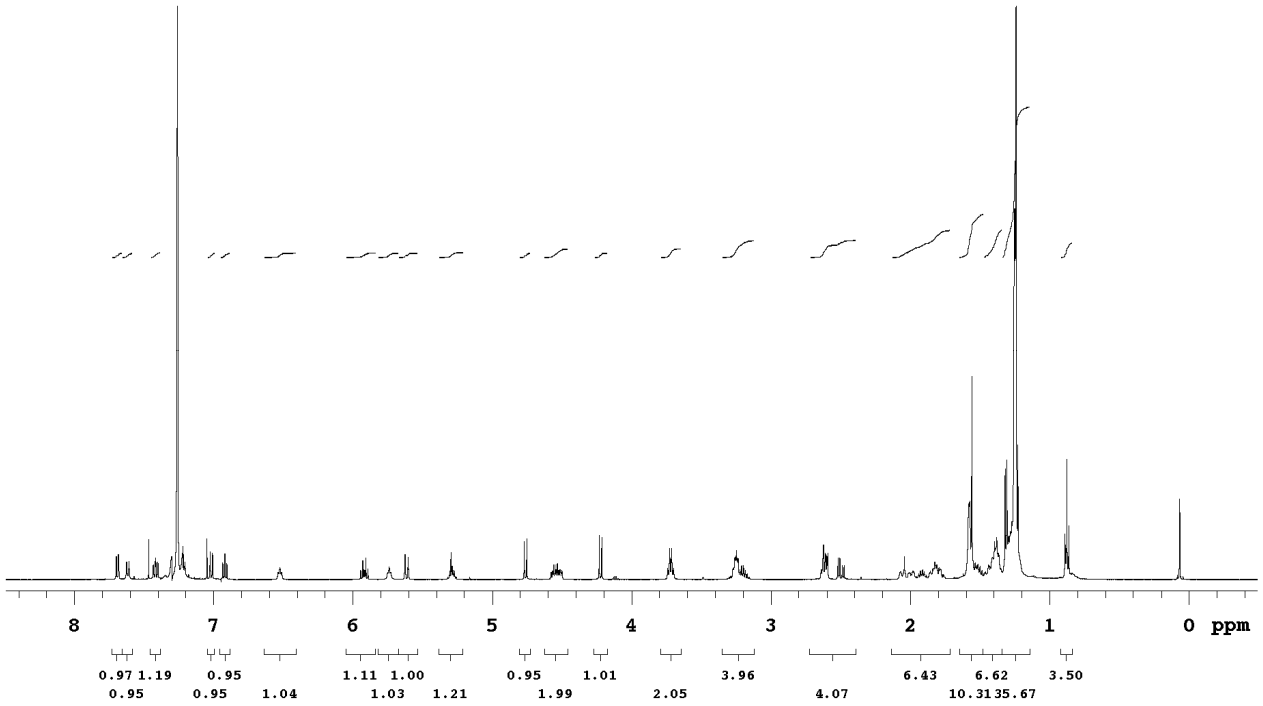


^{13}C NMR

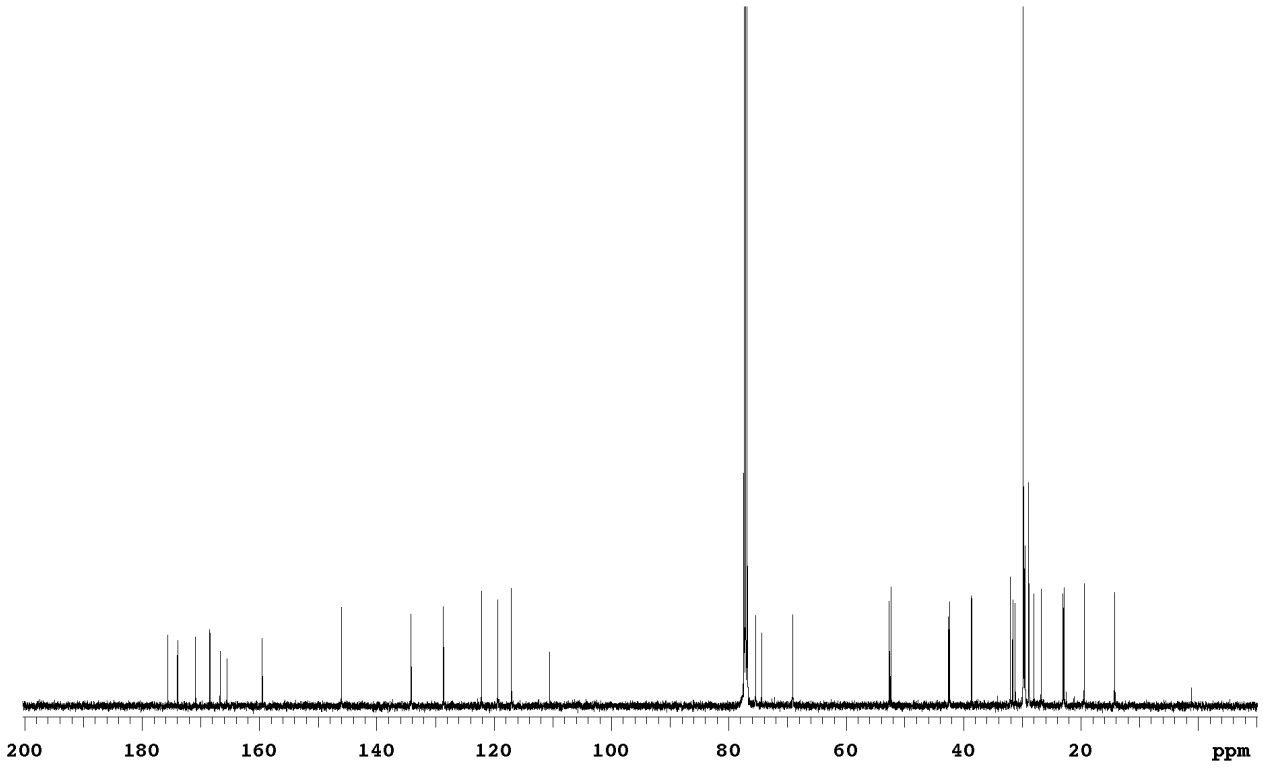


DDM-838 (1) (*cis-SSRS*)

^{1}H NMR

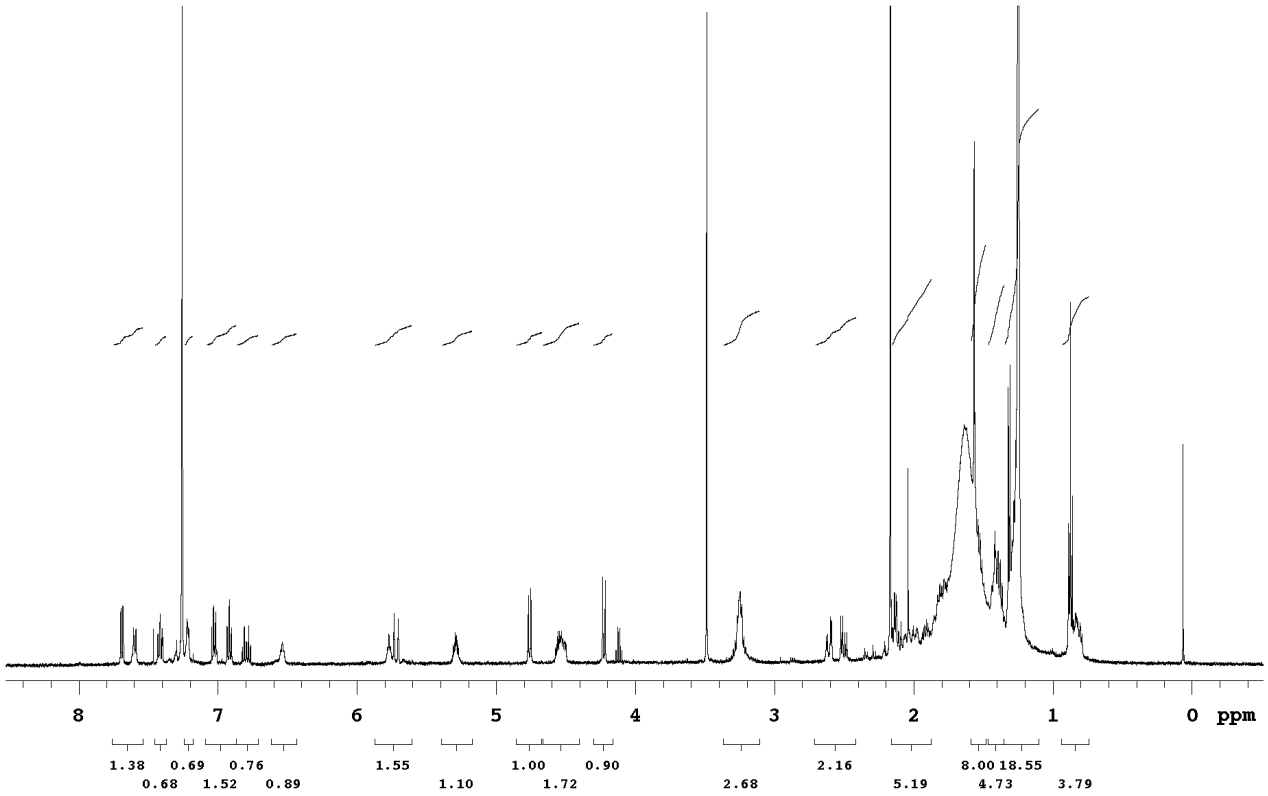


^{13}C NMR

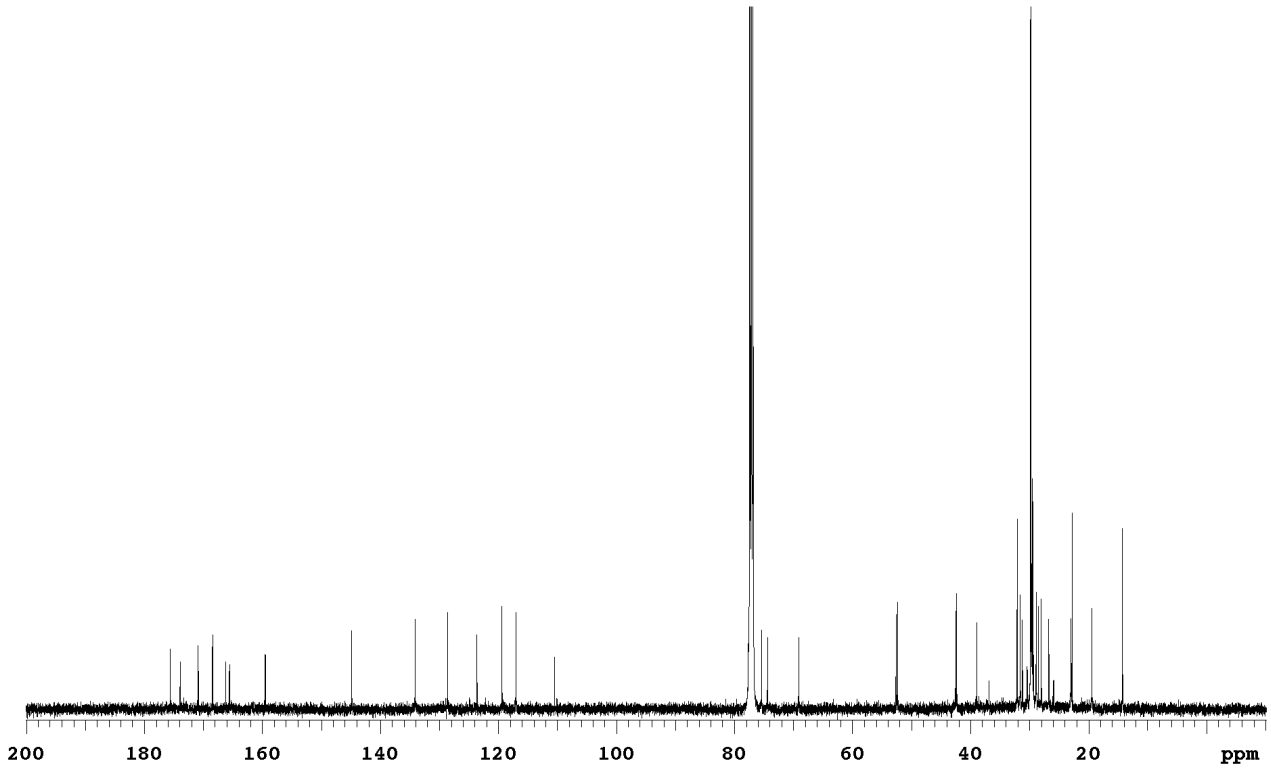


***trans-SSRS*-DDM (2)**

^{1}H NMR

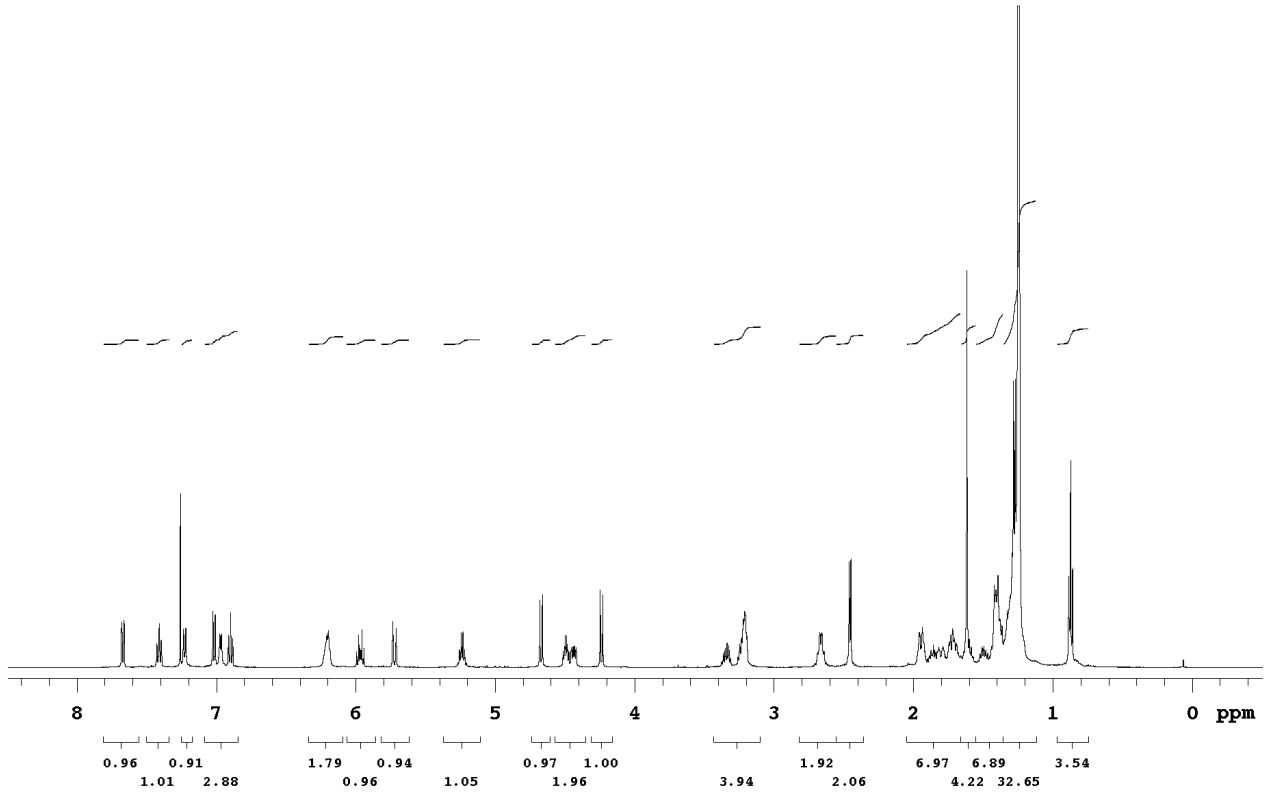


^{13}C NMR

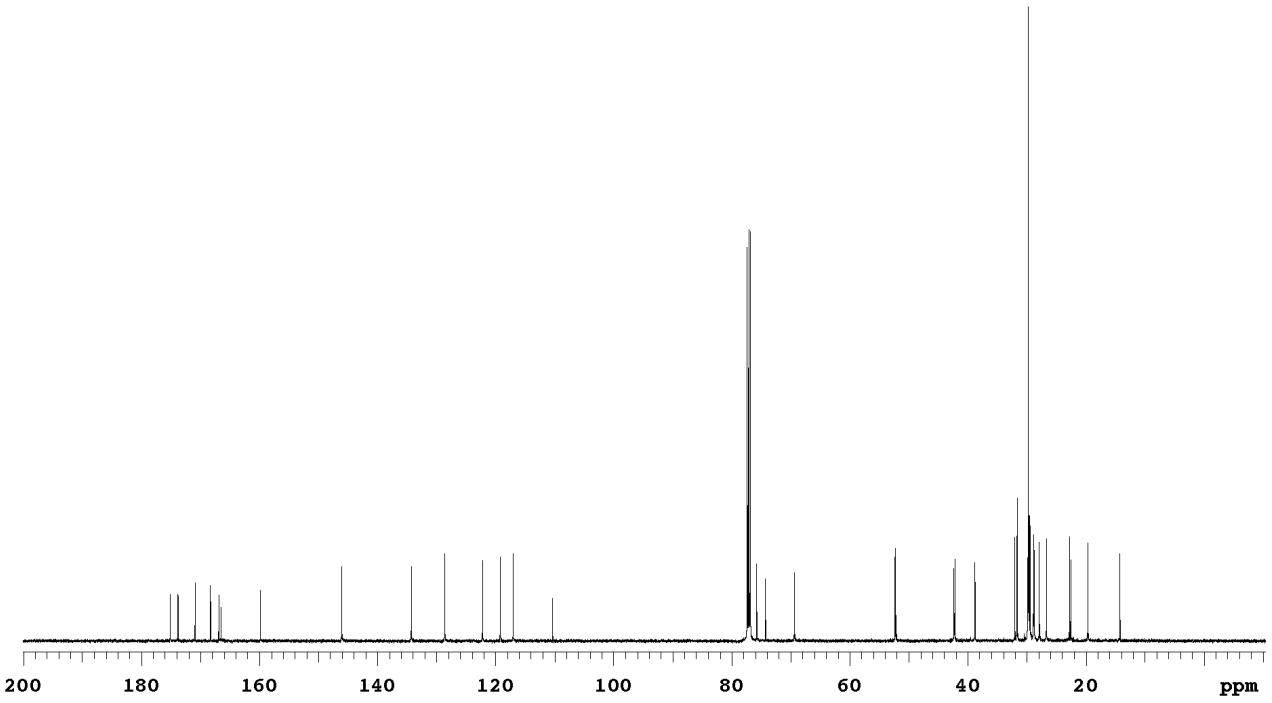


***cis-SRRS*-DDM (3)**

^{1}H NMR

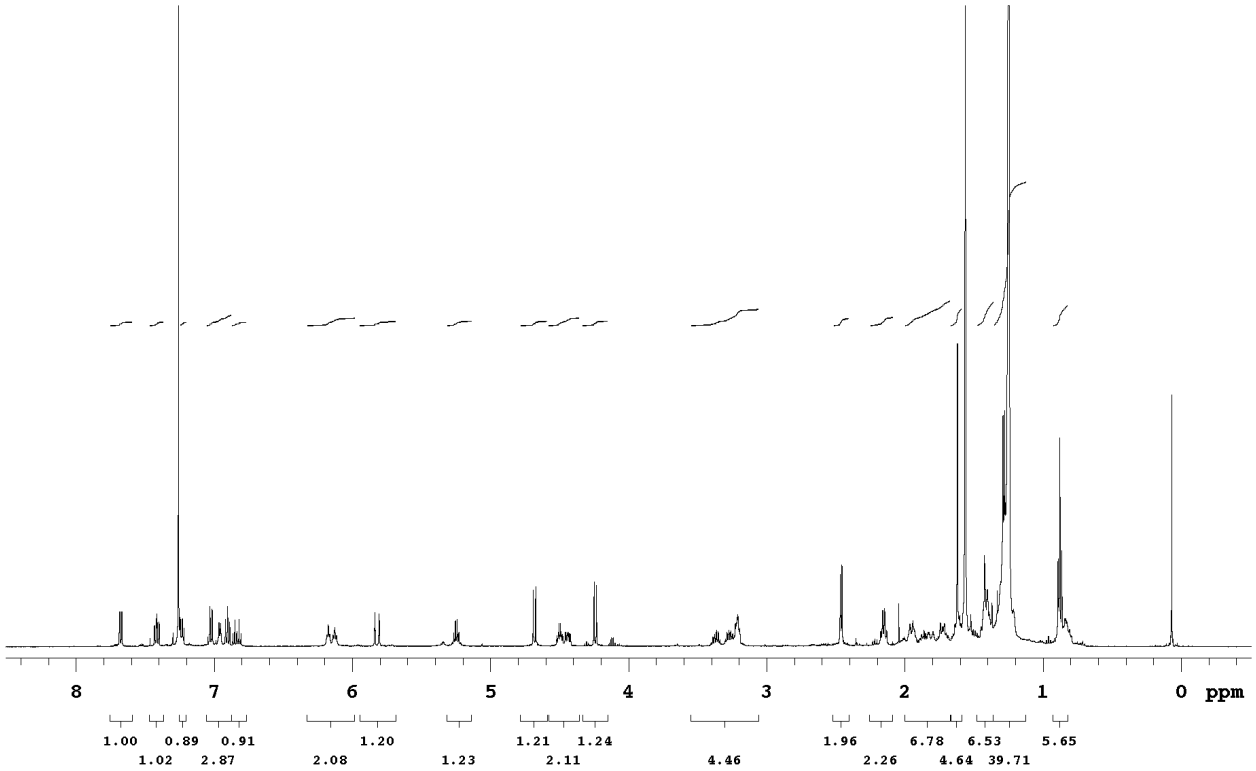


^{13}C NMR

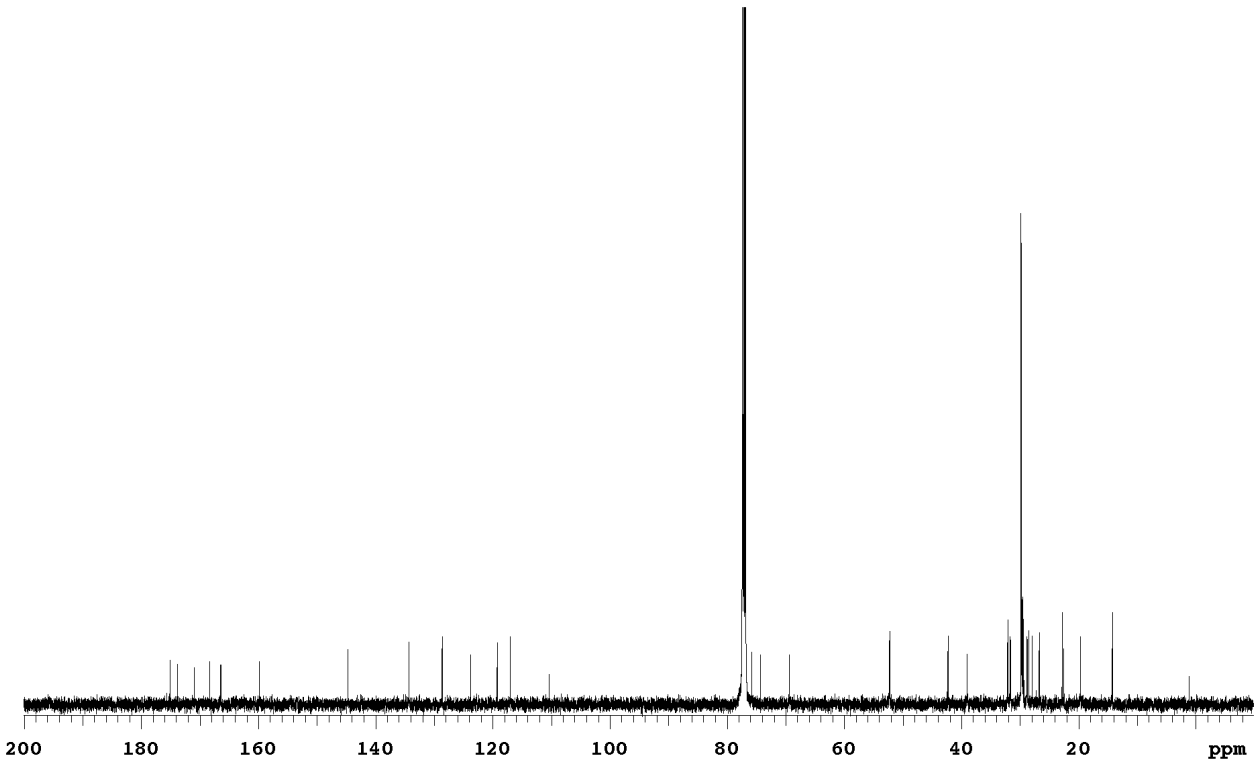


***trans-SRRS*-DDM (4)**

^{1}H NMR

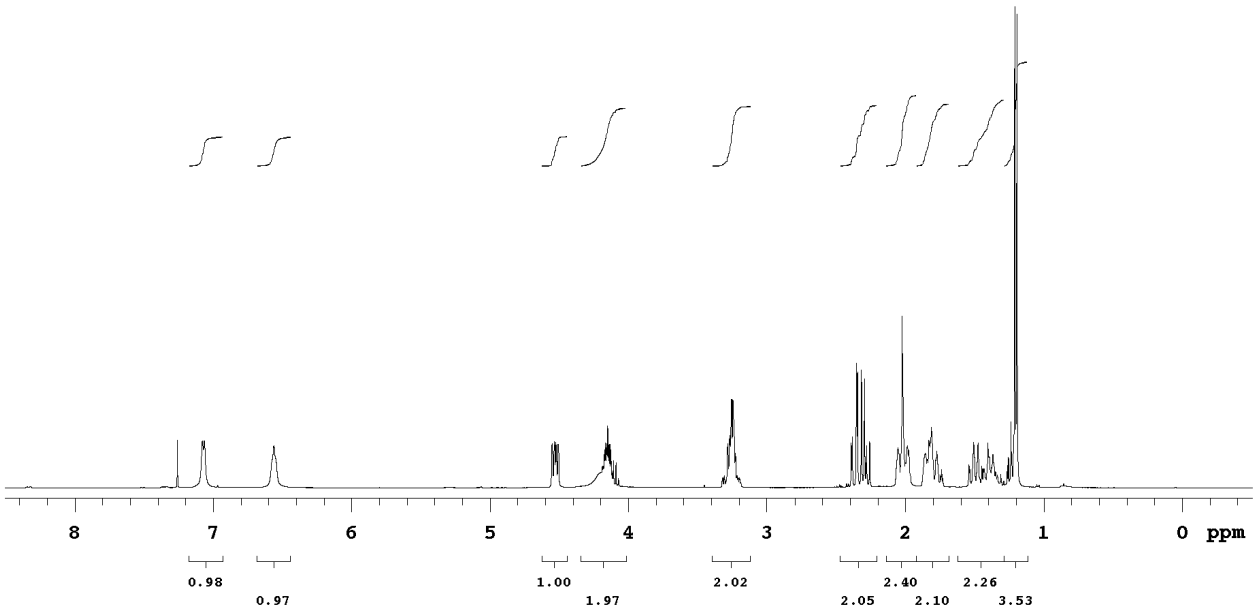


^{13}C NMR

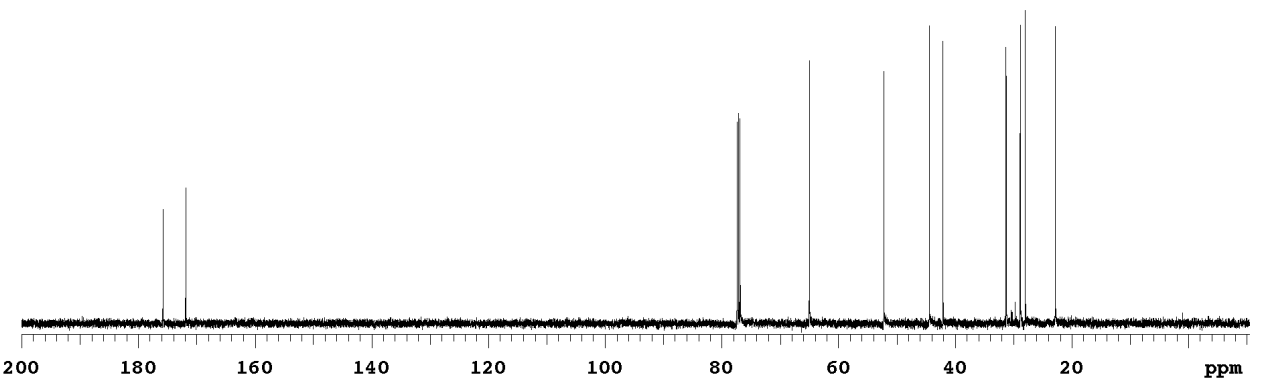


(*S*)-3-[(*S*)-3-Hydroxybutyramido]-hexahydro-2-azepinone (23)

^{1}H NMR

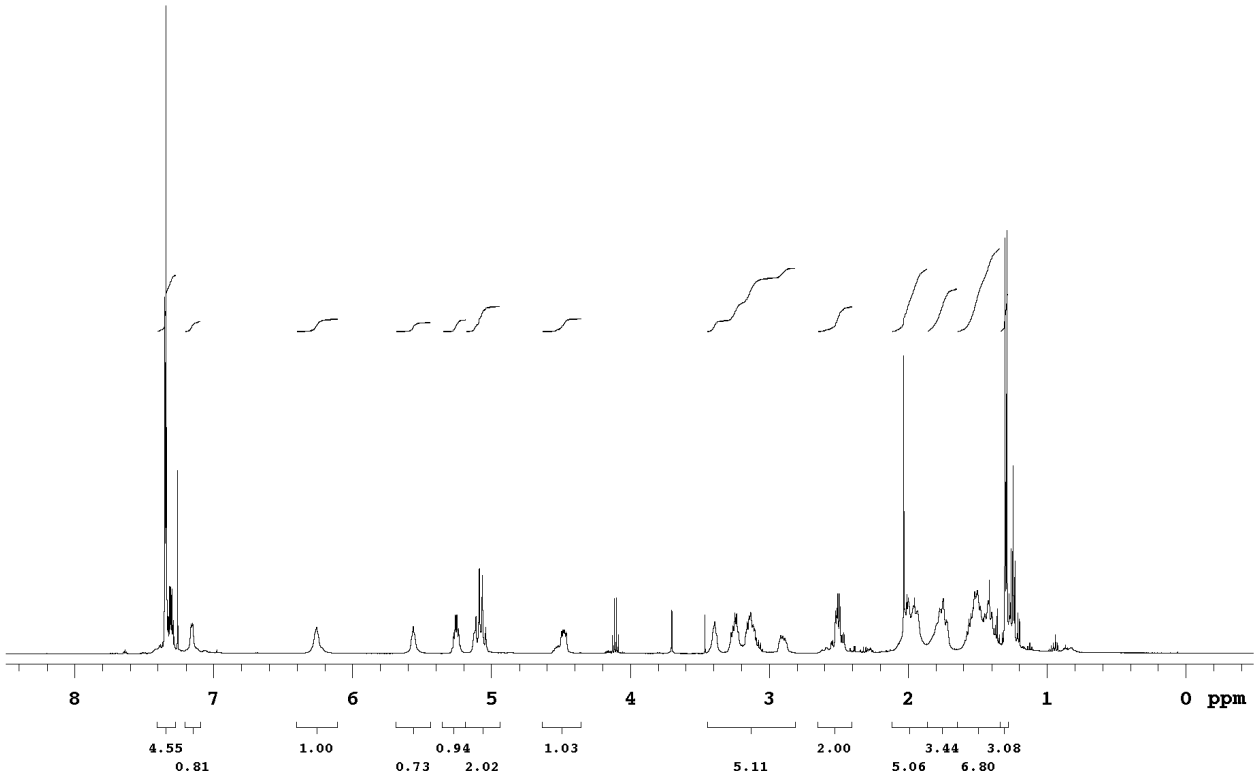


^{13}C NMR

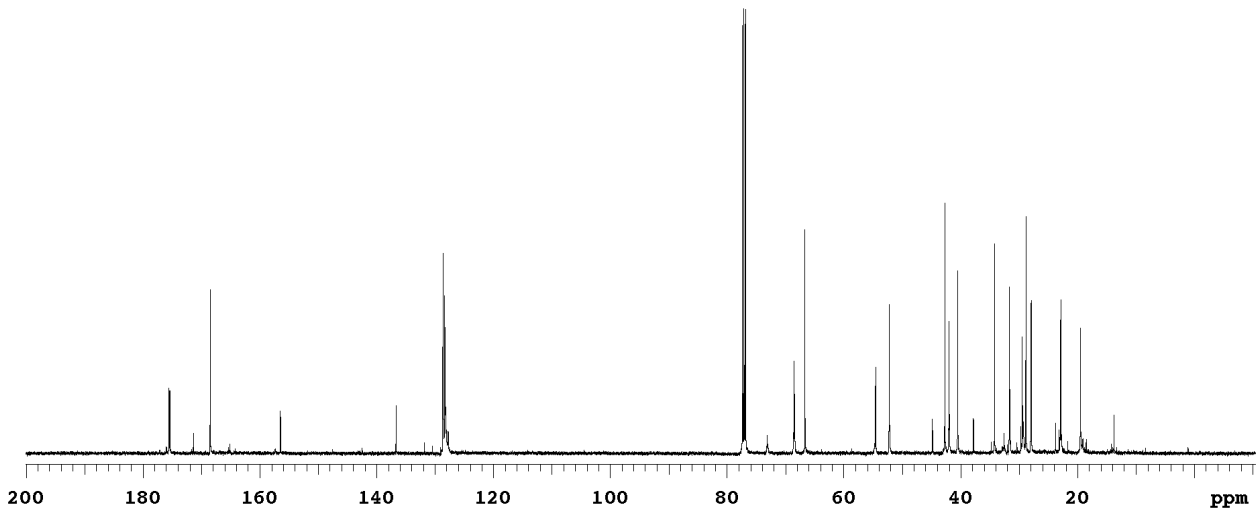


Compound 26

^{1}H NMR

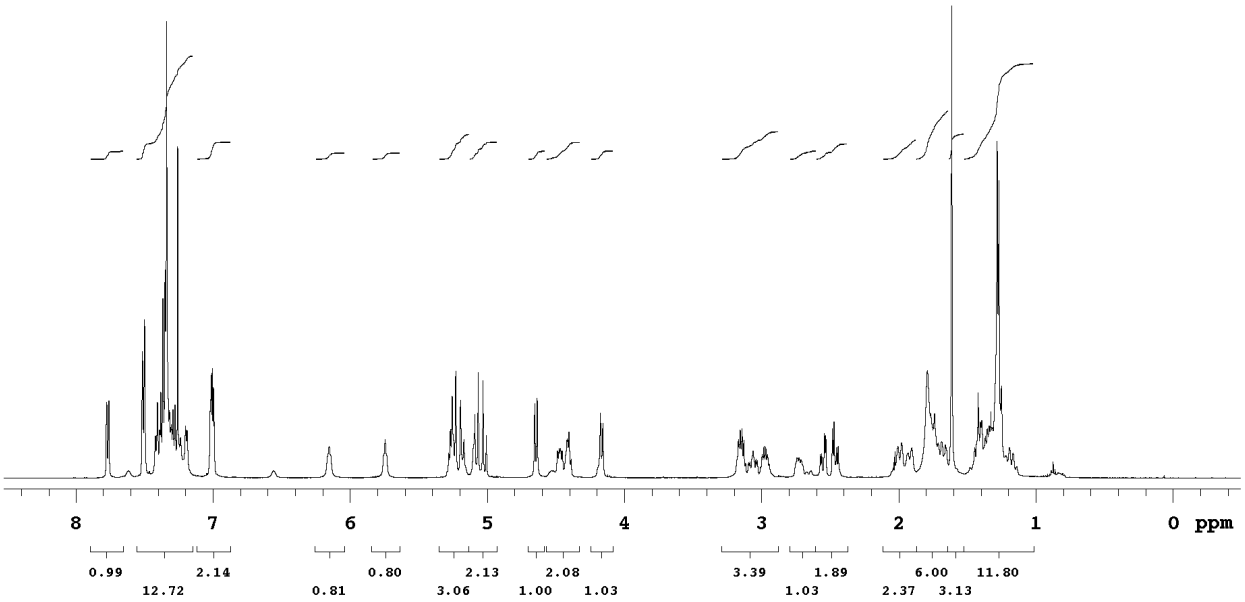


^{13}C NMR



Compound 27

^{1}H NMR



^{13}C NMR