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Total synthesis of a depsidomycin analogue by convergent solid-phase peptide synthesis and macrolactonization strategy for antitubercular activity

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Depsidomycin is a cyclic heptadepsi-peptide isolated from the cultured broth of *Streptomyces lavendofoliae* MI951-62F2. It exhibits significant antimicrobial and immunosuppressive activity. The total synthesis of a depsidomycin analogue in which 1,2-piperazine-3-carboxylic acid was substituted with proline is described. After several trials using different strategies, the desired depsidomycin analogue was obtained *via* stepwise synthesis starting by the amino acid 'head' and macrolactonization under Yamaguchi conditions. The cyclic depsipeptide was evaluated to have an minimum inhibitory concentration (MIC) of 4 μg/ml against H37RV and 16 μg/ml against MDR clinical strains of MTB (MDR-MTB), while the linear precursor 8 also had MICs of 4 and 16 μg/ml for the susceptible and resistant strains, respectively. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: antitubercular activity; cyclic depsipeptides; linear peptides; solid-phase peptide synthesis; macrolactonization reaction; HRMS characterization

Introduction

The rise in drug resistance is fuelling the ongoing search for new antibiotic agents [1]. In an attempt to discover new therapies, examination of the efficacies of naturally occurring compounds have led researchers to a range of peptidic molecules [2]. As the discovery of the didemnins, cyclic depsi-peptides continue to stimulate active research in synthetic and medicinal chemistry, as well as clinical oncology and cell biology [3]. Cyclic depsi-peptides by definition contain one or more amino acid(s) replaced by a hydroxy acid (seco-acid) resulting in up to four ester bonds in the core ring structure [4–7]. These are often secondary metabolites of fungi and plants or originate from the marine environment.

The cyclic heptadepsi-peptide antibiotic depsidomycin **1** has been isolated from the cultured broth of *Streptomyces lavendofoliae* MI951-62F2. It is primarily active against Gram-positive microorganisms and exhibits immunosuppressive activity [4]. These fascinating biological findings coupled with the fact that a synthesis of depsidomycin has not been reported make it a very attractive target for medicinal chemists. Total synthesis is an alternative to fermentation that will facilitate the availability of natural and unnatural analogues [8–9]. The 1,2-piperazine-3-carboxylic acid moieties make **1** a very expensive molecule to synthesize. An analogue **2** was designed, which differs in its structure from depsidomycin **1** by substitution of 1,2-piperazine-3-carboxylic acids with proline.

Depsidomycin has a 'head to side-chain' cyclic structure, whose synthesis can be achieved on solid phase. The most convenient approach to synthesise a cyclic peptide requires the presence of a trifunctional amino acid which could be used for synthesis

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Abbreviations used: *ACN, acetonitrile; BSA; CSPPS, convergent solid-phase peptide synthesis; DCM, dichloromethane; DIC, N,N′-diisopropylcarbodiimide; DIPEA; DMAP; DMF; DMSO; HATU; HBTU; HOBt; HRMS, high-resolution mass spectrometry; MeOH, methanol; MDR, multi-drug resistance; MTB, mycobacterium tuberculosis; OADC, oleic acid-albumin-dextrose-catalase; PyBOP; RP-HPLC; TFA; THF.*

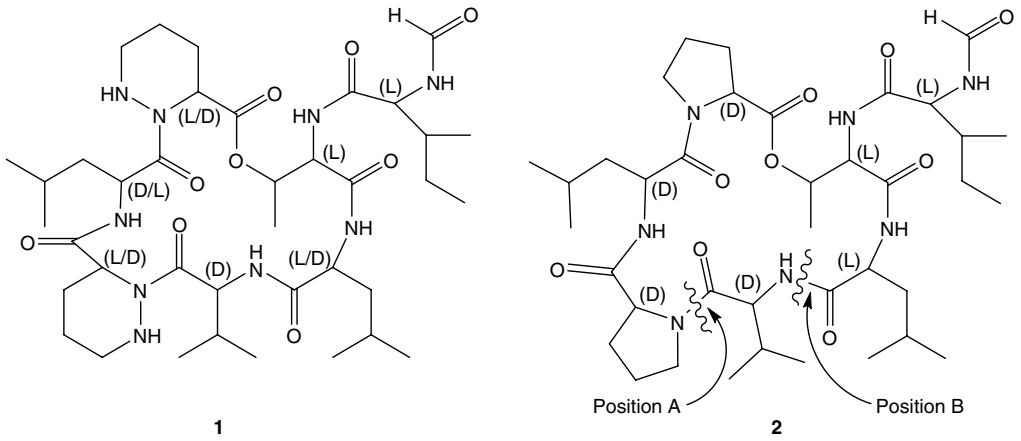
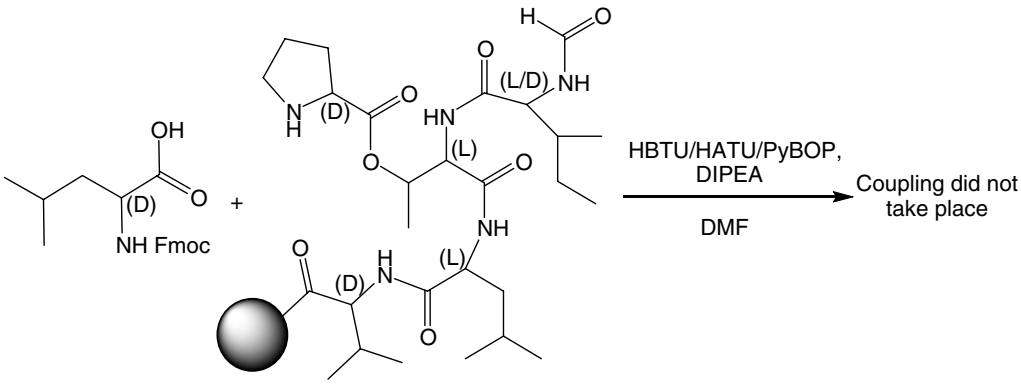


Figure 1. Depsidomycin **1** and a depsidomycin analogue **2**. Two possible positions for the final cyclization step are indicated.



Scheme 1. Synthesis of the linear depsipeptide and its final proposed cyclization at position **A** as illustrated in Figure 1.

followed by sequential amino acid addition and cyclization [10]. As the depsidomycin analogue **2** does not contain such a trifunctional amino acid, synthesis therefore has to start with an amino acid present in the middle of the cycle, followed by cleavage of the protected peptide from the resin and then cyclization in solution [11–13]. Even if both these strategies were possible, they show the drawback of requiring the synthesis of a branched peptide, whose synthesis could be problematic due to potential interactions of the side chain during the synthetic process. A third strategy involves the synthesis of a linear peptide starting by the 'head' amino acid, cleavage of the protected peptide from the resin and cyclization in solution. This strategy implies the cyclization through ester formation, which can be carried out by Yamaguchi [14] or Mitsunobu reactions [15]. Herein, a systematic study regarding the synthesis of the depsidomycin analogue **2** using different strategies is described.

Results and Discussions

The stereochemistry of the depsidomycin **1** was only partially elucidated [4] and to date no further reports are found on the synthesis of this compound. In the original communication, the stereochemistry of 1,2-piperizine-3-carboxylic acid was not elucidated. It was mentioned that one of the Leu's is of the L-form and the other one is D, but no further details were provided. It was therefore decided to attempt the synthesis of the linear sequences with alternating stereochemistry of the Leu residues, while the D isomer of Pro was employed in both positions and these configurations might not represent that of the natural depsidomycin. In an attempt to obtain **2** from cyclization at position **A** (Figure 1), in the first step Fmoc-D-Val-OH was loaded onto 2-chlorotrityl chloride resin and Fmoc-L/D-Leu-OH, Fmoc-L-Thr-OH and *N*-formyl-L-Ile-OH [16] were automatically coupled with microwave assistance. LCMS revealed that the Ile residue underwent complete racemization under these conditions.

The depsibond was manually prepared with Fmoc-D-Pro-OH and the reaction was only 90% complete after a 12-h reaction time. The Fmoc removal was only efficient with microwave assistance, which implies the presence intrachain interaction. Triple couplings (under microwave conditions) with Fmoc-D-Leu-OH were attempted with HBTU, HATU and PyBOP with the only observable products being the *N*-formyl-L/D-Ile-Thr(O-CO-D-Pro)-L-Leu-D-Val-OH (major) and *N*-formyl-L/D-Ile-L-Thr-L-Leu-D-Val-OH (minor) after elongated coupling periods (Scheme 1). So, this strategy was abandoned before cyclization.

Attempts to obtain **2** from cyclization at position **B** was met with difficulty when coupling Fmoc-D-Pro-OH to the second Leu unit. Coupling was attempted under microwave conditions with HBTU, HATU and PyBOP, but was unsuccessful (Scheme 2). This due to diketopiperazine formation as evident by the presence of the significant amounts of the *N*-formyl-L/D-Ile-L-Thr-L-Leu-OH fragment and intrachain interaction with the presence of *N*-formyl-L/D-Ile-L-Thr(O-CO-D-Pro-D-Leu-NH_2)-L-Leu-OH depsipeptides.

On the basis of the described attempts and observations, it was decided that a CSPPS strategy should be investigated employing

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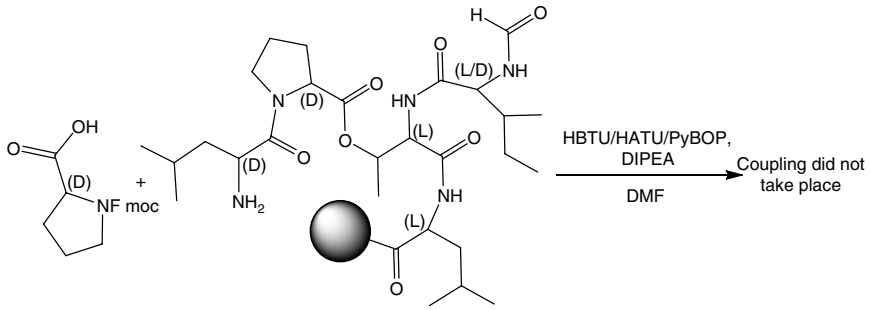
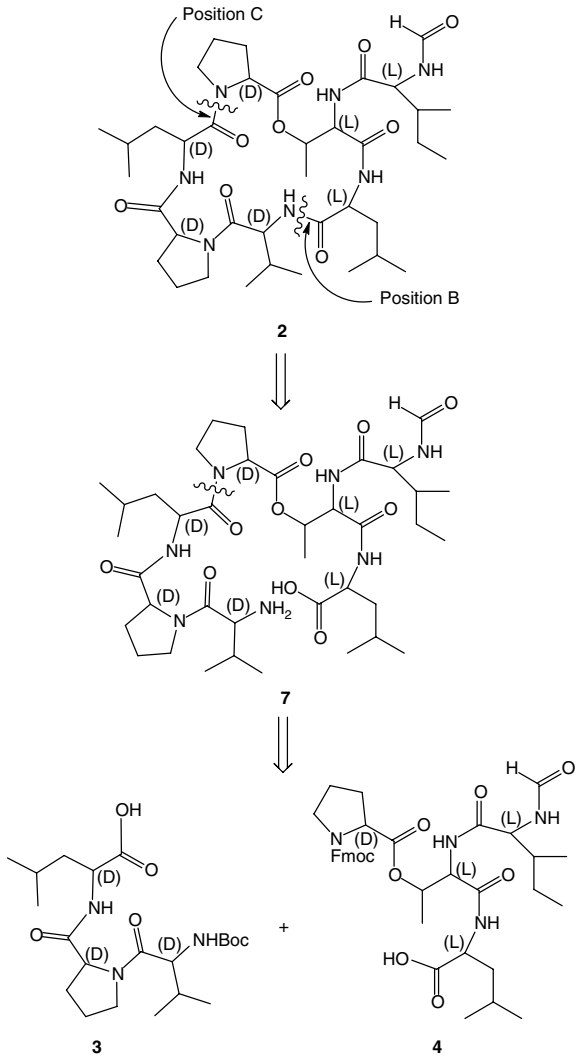


Scheme 2. Synthesis of the linear depsipeptide and its final proposed cyclization at position **B** as illustrated in Figure 1.



Scheme 3. Structure of depsidomycin analogue **2** and its retrosynthetic strategy according to CSPPS.

segments with the least intrachain interaction. We divided the target compound **2** into two peptide fragments, **3** and **4**, which can be combined to yield the linear peptide precursor **7** (Scheme 3).

Tripeptide **3** was prepared on 2-chlorotrityl chloride resin in an automated microwave assisted peptide synthesizer, cleaved from the resin under mild conditions to preserve the Boc protection and was of sufficient purity to be carried to the next step.

The linear depsi-tetrapeptide (*N*-formyl-L/D-Ile-L-Thr-(O-CO-D-Pro)-L-Leu-OH fragment **4**) was prepared on 2-chlorotrityl chloride resin. All couplings and deprotections were conducted in an automated microwave assisted peptide synthesizer. Preparation of the depsibond with Fmoc-D-Pro-OH on solid phase was achieved at room temperature in a manual peptide synthesis reactor. Following the successful couplings of fragments **3** and **4** (position **C** in Scheme 3), cleavage of the final linear depsipeptide from the resin yielded the heptadepsi-peptide **7**. Coupling of fragments **3** to **4** was most efficient with HATU and DIPEA for 12 h in DMF to yield **6** (Scheme 4). Cleavage of the linear heptadepsi-peptide **7** from the chlorotrityl chloride resin and simultaneous deprotection of the *N*-terminal Boc-protective group was achieved in 84% yield after purification. The optimized amide bond coupling for the cyclization was facilitated by PyBOP to give **2** in 42% yields after purification. Please note that the diastereomers as a result of racemization of the *N*-formyl-Leu were collected as a single product.

Although the convergent synthesis proved effective, we had simultaneously been investigating the more attractive sequential peptide synthesis approach to obtain **2**. The Yamaguchi esterification was employed in the preparation of large ring lactones from the corresponding open-chain hydroxy acids (seco-acids) [17]. As then, this esterification has found tremendous applications in the preparation of depsibonds on resin [18], macrolactonization of seco-acid peptides [14,18–20] and seco-acid non peptides [21–23].

In this strategy, we planned to cyclize the target peptide at position **D** (Scheme 5).

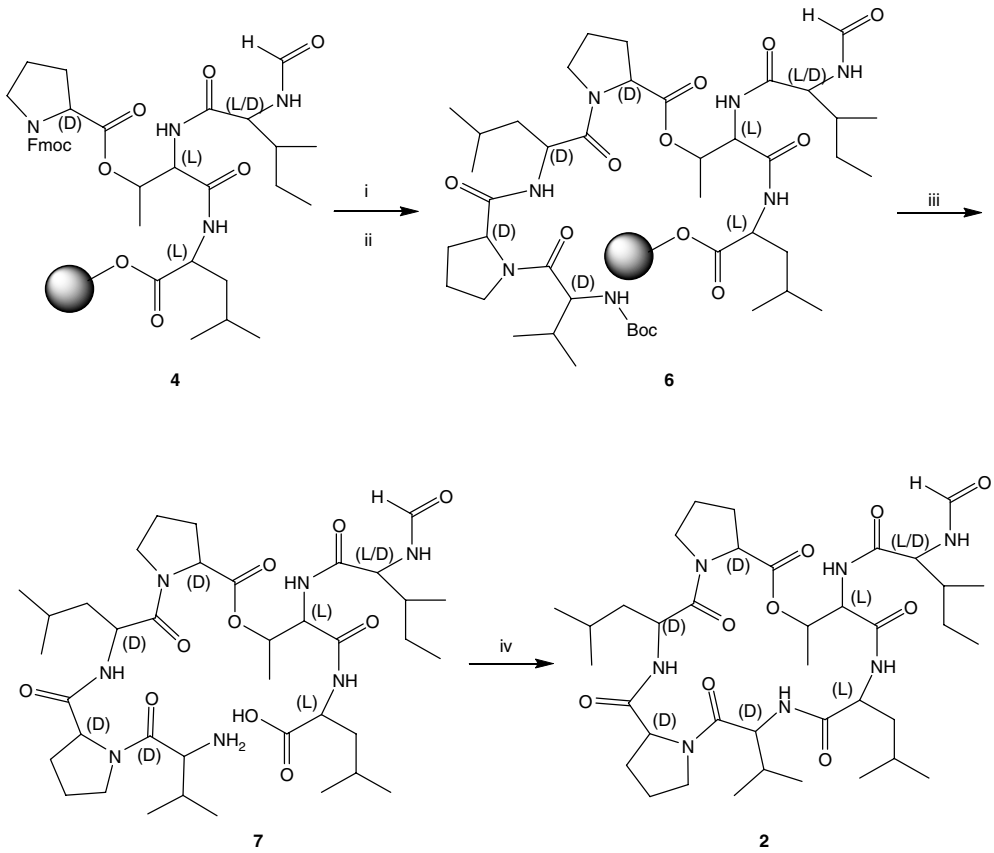
Synthesis of the hydroxy acid peptide (seco-acid) **8** was achieved on chlorotrityl chloride resin. The peptide chain was elongated by sequential coupling and Fmoc deprotection as depicted in Scheme 6. The peptide was cleaved from the resin upon treatment with 50% TFA in 67% yield after purification. The seco-acid **8** was subjected to a macrolactonization reaction [14] under Yamaguchi conditions to yield the title analogue **2** in 81% yield and the purity was 99% after purification on semi-preparative HPLC.

Depsipeptide **2** was found to inhibit H37RV and MDR strains of MTB with minimum inhibitory concentration (MICs) of 4 and 16 μg/ml, respectively. Its linear precursor **8** was also found to exhibit similar activity with MICs of 4 and 16 μg/ml for the susceptible and resistant strains, respectively.

Experimental Section

General Information

All reactions were conducted under an inert atmosphere (nitrogen). SPPS was performed on an automated CEM Liberty



Scheme 4. Synthesis of depsidomycin analogue **2** by CSPPS. Reagents and conditions: (i) Piperidine/DMF (2 : 8); (ii) Fragment **3** (2 equiv.), HATU (5 equiv.), DIPEA (10 equiv.), DMF, rt., 12 h; (iii) TFA/DCM (95 : 5), twice for 10 min; (iv) PyBOP (2 equiv.), DIPEA (4 equiv.), DMF, rt., 48 h.

Table 1. Microwave conditions for coupling and deprotection

Single coupling	Microwave power (Watts)	Temperature (°C)	Time (sec)
45 min coupling	0	25	1800
	35	73	900
Deprotection	35		180

microwave peptide synthesizer. The conditions for microwave couplings are provided in Table 1. The molar concentrations of amino acids, HBTU and DIPEA were 0.2, 0.5 and 1.0 mM, respectively. Analysis was performed on a Shimadzu LCMS 2020 mass spectrometer in the positive mode with a Waters Xbridge C18 column (150 mm × 4.6 mm × 5 µ) and UV/VIS detector (215 nm) at a flow rate of 1 ml/min. A two-buffer system was employed, utilizing formic acid as the ion-pairing agent. Buffer A consisted of 0.1% formic acid/H_2O (v/v) and buffer B consisted of 0.1% formic acid/ACN (v/v), with a liner gradient from 5 to 95% B in 7–16 min. Semi-preparative RP-HPLC was conducted on a Shimadzu 6AD instrument (Ace C18 column, 150 mm × 21.2 mm × 5 µ) with a UV/VIS detector (215 nm) at a flow rate of 15 ml/min. A two-buffer system was employed, utilizing formic acid as the ion-pairing agent. Buffer A consisted of 0.1% formic acid/H_2O (v/v) and buffer B consisted of 0.1% formic acid/MeOH (v/v), with a liner gradient from 5 to 95% B in 25 min. HRMS analysis was performed on a BrukerMicroTOF QII mass spectrometer in positive mode with internal calibration (see Supporting Information).

The Fmoc-L amino acids (Leu-OH, Ile-OH, Thr-OH), Fmoc-D amino acids (Leu-OH, Pro-OH, Val-OH), 2-chlorotrityl chloride resin, HATU, HBTU, HOBt, PyBOP and DIC were purchased from GL Biochem (Shanghai) Ltd. The Boc-D-Val-OH, DMAP, DIPEA, piperidine, 2,4,6-trichlorobenzoyl chloride, triethylamine and TFA were purchased from Sigma Aldrich. The solvents and reagents such as ACN, DCM, DMF, DMSO, formic acid, MeOH, THF and acetic anhydride were purchased from Merck KGaA. All the chemicals were used without further purification.

Details for the SPPS Approach

General Methods

The synthesis was performed on solid phase using standard Fmoc protocols [24] at 0.1 mmol (0.152 g) scales. The 2-chlorotrityl chloride resin was activated as per the reported procedure [25] and first amino acid was loaded onto chlorotrityl chloride resin (0.66 mmol/g calculated substitution) and the loading was evaluated with a Fmoc test [26]. Sequential couplings and Fmoc deprotections extended the peptide chain. All couplings (Schemes 1, 2 and 4–6) except for the depsibond (Schemes 1, 2 and 5) and CSPPS (Scheme 6) were performed in DMF on the microwave peptide synthesizer for 45 min. Deprotection was performed using 20% piperidine/DMF (v/v). An excess of each amino acid derivative (4 equiv.) was used. Activation took place *in situ* with standard HBTU/DIPEA reagents. DMF top and bottom washes were performed between deprotection and coupling steps.

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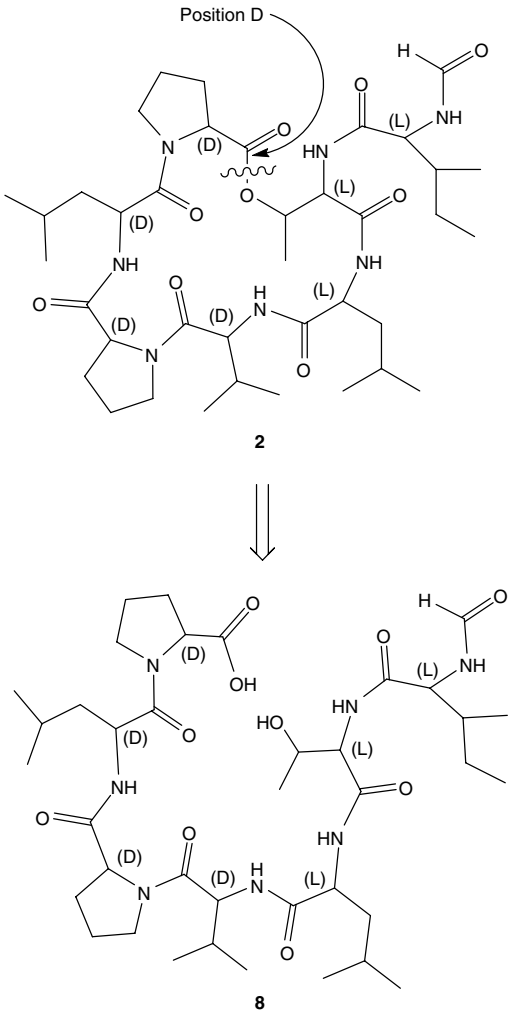


Scheme 5. Structure of depsidomycin analogue **2** and its retrosynthetic strategy according to macrolactonization reaction under Yamaguchi conditions.

*Synthesis of fragment **3** (Boc-D-Val-D-Pro-D-Leu-OH)*

2-Chlorotrityl chloride resin was activated according to the standard protocol and Fmoc-D-Leu-OH (4 equiv.) was loaded with DIPEA (6 equiv.) in dry DCM (5 ml) under nitrogen atmosphere for 8 h. Deprotection of Fmoc and coupling of Fmoc-D-Pro-OH and Boc-D-Val-OH was done in the microwave assisted peptide synthesizer. Boc-tripeptide **3** was cleaved from the resin using 4% TFA in DCM (10 ml) for 2 × 10 min and the solvent was removed *in vacuo*. The residue obtained was dissolved in ACN and DMSO (9 : 1) (8 ml), purified by RP-HPLC and lyophilized to yield the Boc-tripeptide **3** (41.41 mg, 97%) as a white amorphous solid in >99% according to analytical RP-HPLC; *R*t. 14.95 min. HRMS: calcd. for $C_{21}H_{37}N_3O_6$ $[M+H]^+$ 428.2755; found 428.2787.

*Synthesis of fragment **4** (N-formyl-L/D-Ile-L-Thr(O-CO-D-Pro)-L-Leu-OH)*

Fragment **5** on chlorotrityl chloride resin was treated with a solution of Fmoc-D-Pro-OH (2 equiv.), HOBt (1 equiv.), DIC (5 equiv.) and DMAP (catalytic) in dry DCM (5 ml) at room temperature and under nitrogen bubbling for 24 h. The excess reagents were washed with DMF (2 × 7 ml) and DCM (2 × 7 ml). A double coupling was employed to drive this reaction to completion. HRMS: calcd. for $C_{37}H_{48}N_4O_9$ $[M+H]^+$ 693.3494; found 693.3523.

*Synthesis of fragment **5** [N-formyl-L/D-Ile-L-Thr(OH)-L-Leu-OH]*

Activation of 2-chlorotrityl chloride resin was done according to the standard protocol and Fmoc-L-Leu-OH (4 equiv.) was loaded with DIPEA (6 equiv.) in dry DCM (5 ml) under nitrogen atmosphere. Deprotection of Fmoc and coupling of Fmoc-L-Thr-OH and *N*-formyl-L-Ile-OH was done on the peptide synthesizer. HRMS: calcd. for $C_{17}H_{31}N_3O_6$ $[M+H]^+$ 374.2286; found 374.2262.

*Synthesis of fragment **7** [N-formyl-L/D-Ile-L-Thr(O-CO-D-Pro-D-Leu-D-Pro-D-Val-NH$_2$)-L-Leu-OH]*

Fragment **3** (2 equiv.) was coupled with fragment **4** on chlorotrityl chloride resin in presence of HATU (5 equiv.) and DIPEA (10 equiv.) in DMF (10 ml) at room temperature with nitrogen bubbling for 12 h. Excess coupling reagents and fragment **3** were removed by washing with DMF (2 × 7 ml) and DCM (2 × 7 ml). Fragment **6** was removed from the resin with 95% TFA in DCM (10 ml) for 10 min (twice) and the solvent was removed *in vacuo*. The residue obtained was dissolved in ACN and DMSO (9 : 1) (8 ml), purified by RP-HPLC and lyophilized to yield the fragment **7** as a white amorphous solid (65.43 mg, 84%) in >99% purity according to analytical RP-HPLC; *R*t. 14.01 min. HRMS: calcd. for $C_{38}H_{65}N_7O_{10}$ $[M+H]^+$ 780.4866; found 780.4900.

*Cyclization of compound **7***

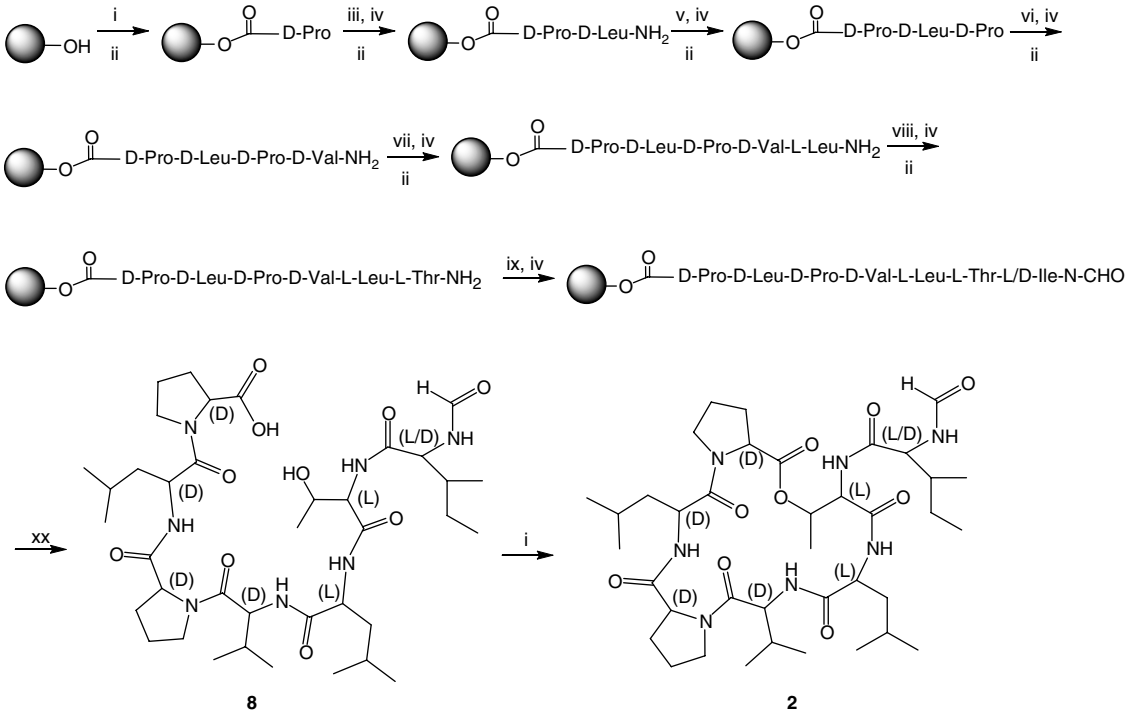
Compound **7** (1 equiv.) was treated with a solution of PyBOP (2 equiv.) and DIPEA (4 equiv.) in DMF (13 ml) (final concentration of compound **7** was 0.001 M) and the resulting mixture was stirred for 48 h at room temperature. The solvent was removed under reduced pressure. The residue obtained was dissolved in ACN and DMSO (9 : 1) (8 ml), purified by RP-HPLC and lyophilized to yield the title analogue **2** as a white amorphous solid (32.71 mg, 42%) in >99% according to analytical RP-HPLC; *R*t. 14.61 min. HRMS: calcd. for $C_{38}H_{63}N_7O_9$ $[M+H]^+$ 762.4760; found 762.4797.

*Synthesis of fragment **8** [N-formyl-L/D-Ile-L-Thr(OH)-L-Leu-D-Val-D-Pro-D-Leu-D-Pro-OH]*

The seco-acid peptide **8** was prepared by loading Fmoc-D-Pro-OH (4 equiv.) onto the resin with DIPEA (6 equiv.) in dry DCM (5 ml) under a nitrogen atmosphere for 8 h. Deprotection of the Fmoc and coupling of Fmoc-D-Leu-OH, Fmoc-D-Pro-OH, Fmoc-D-Val-OH, Fmoc-L-Leu-OH, Fmoc-L-Thr-OH and *N*-formyl-L-Ile-OH was done on the microwave assisted peptide synthesizer. The peptide was cleaved from the resin using 50% TFA in DCM (10 ml) for 2 × 10 min and the solvent was removed *in vacuo*. The residue obtained was dissolved in ACN and DMSO (9 : 1) (8 ml). Purification by semi-preparative RP-HPLC yielded the seco-acid peptide **8** as an amorphous solid (52.19 mg, 67%) in >99% according to analytical RP-HPLC; *R*t. 14.10 min. HRMS: calcd. for $C_{38}H_{65}N_7O_{10}$ $[M+H]^+$; 780.4866 found 780.4890.

*Macrolactonization of seco-acid **8***

Seco-acid **8** was dissolved in dry THF and added dropwise over 1 h to a solution of 2,4,6-trichlorobenzoyl chloride (5 equiv.), triethylamine (5 equiv.) and DMAP (6 equiv.) in dry THF (13 ml) (final concentration was 0.001 M) under inert conditions. After the



Scheme 6. Synthesis of depsidomycin analogue **2** by macrolactonization. Reagents and conditions: (i) Fmoc-D-Pro-OH (4 equiv.), DIPEA (6 equiv.), dry DCM, rt., 8 h, under N_2; (ii) Piperidine/DMF (2 : 8); (iii) Fmoc-D-Leu-OH; (iv) HBTU, DIPEA, DMF; (v) Fmoc-D-Pro-OH; (vi) Fmoc-D-Val-OH; (vii) Fmoc-L-Leu-OH; (viii) Fmoc-L-Thr-OH; (ix) *N*-Formyl-L-Ile-OH; (x) TFA/DCM (1 : 1), twice for 10 min; (xi) 2,4,6-Trichlorobenzoyl chloride (5 equiv.), triethylamine (5 equiv.), DMAP (6 equiv.), dry THF, rt., 72 h.

addition, the solution was stirred for 72 h. The solvent was then removed *in vacuo*, the crude product was dissolved in ACN and DMSO (9 : 1), purified by RP-HPLC and lyophilized to yield the title compound **2** as white amorphous solid (23.76 mg, 81%) in >99% purity according to analytical RP-HPLC; *R*t. 14.61 min. HRMS: calcd. for $C_{38}H_{63}N_7O_9$ $[M+H]^+$; 762.4760 found 762.4737.

Antitubercular Activity

The depsidomycin analogue **2** and linear precursor **8** were evaluated by an agar dilution method, performed in triplicate, against a MTB H37Rv strain (ATCC 25177, fully susceptible to antituberculosis drugs) and a well-characterized MDR–MTB strain. The test compound (5 mg) was first dissolved in 100% MeOH (1 ml) and sonicated at 40–45 °C for 3 min. Sterility of the stock solution was ensured by passing the compound through a 0.22 µ polycarbonate filter. Twofold serial dilutions were made of the test compounds to give final concentrations that ranged from 128 to 0.126 µg/ml, the solutions were aliquoted in cryovials and stored at −70 °C.

Determination of MIC by an Agar Dilution Method

MICs were performed according to standard laboratory methods [27] with slight modifications. All laboratory procedures were carried out in a Level II Biosafety cabinet, with full personal protective equipment, including a N95 MASK. MTB reference strain H37Rv (ATCC No. 25177) and a fully characterized clinical strain of MDR–MTB was cultured in Middlebrook 7H11 medium [28], enriched with OADC (0.005%, v/v, oleic acid; 0.5%, 171 w/v, BSA; 0.2%, w/v, glucose; 0.02%, v/v, catalase and 0.085%, w/v, NaCl) and incubated at 37 °C for 3 weeks. Thereafter, each of the two MTB cultures was added to sterile tubes containing 4.5 ml phosphate buffer, 0.05% tween 80 with glass beads (5-mm diameter) and vortexed. This solution was left for 45 min to allow bacterial clumps to settle. Sterile distilled water was then added to the supernatant to achieve a McFarland Number 1 which is equivalent to a bacterial concentration of 1×10^7 cfu/ml. This bacterial suspension was serially diluted tenfold in sterile water, and 100 µl of the 10^{-2} dilution was spotted onto Middelbrook 7H10 agar plates containing varying dilutions (128–0.126 µg/ml) of the test compound. The MIC of the compound was read 3 weeks after incubation at 37 °C. The MIC was defined as the minimum drug concentration that inhibited growth of the organism.

Conclusion

We have described two approaches for the first total synthesis of a depsidomycin analogue. The CSPPS and macrolactonization approaches gave the desired compounds in 42 and 81% yields (final cyclization step), respectively. The macrolactonization approach under Yamaguchi conditions was more efficient and required less manual intervention than the CSPPS approach thereby favouring it for a combinatorial approach for other analogues. The depsidomycin analogue was found to be active against MDR–MTB strains. Ongoing studies are aimed at the synthesis and evaluation of a library of depsidomycin analogues.

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