Solid-Phase Total Synthesis of Kahalalide A and Related Analogues

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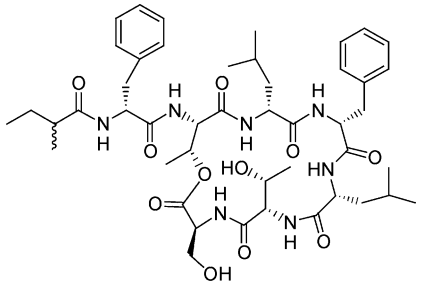
The marine natural product kahalalide A, a cyclic depsipeptide, was prepared by total synthesis on solid-phase. A backbone cyclization strategy was followed, using the Kenner sulfonamide safety-catch linker. By NMR comparison of synthetic and naturally isolated material, the stereochemistry of the methylbutyrate side chain was established as (*S*). Several analogues were synthesized and tested for antimycobacterial activity. The results indicate that the alcohol functional group in the serine and threonine residues is important, while the methylbutyrate side chain can be replaced by an achiral hexanoate with an increase in activity.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis,* is among the most important bacterial diseases.[1] It is estimated that eight million cases of active TB occur every year, predominantly in developing countries, while its prevalence is rising in the Western world particularly among immunocompromised patients (e.g. HIV active). Globally, TB is responsible for two to three million deaths annually. While a number of antimycobacterial medicines are available,[2] cost and patient compliance are major hurdles for successful treatment. Furthermore, resistance is an increasing problem. Some strains, resistant to as many as nine drugs, result in >50% fatality with current therapies. For these reasons, new antibiotics are desirable, particularly those acting by a novel mechanism of action.

Recently, 48 structurally diverse marine natural product and semisynthetic compounds were screened[3] for in vitro activity against *M. tuberculosis*. Within this set, kahalalide A (**1**) emerged as a promising lead, inhibiting 83% of the growth of *M. tuberculosis* at 12.5 μg/mL. Kahalalide A is one of a family of peptide natural products isolated[4] from the marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. Among these, the tridecapeptide kahalalide F has attracted the most attention[5] and is currently in phase I clinical trials as an anticancer and antipsoriatic agent, while kahalalide B has been the subject[6] of a total synthesis. Meanwhile, the structurally simpler kahalalide A is one of the few marine-derived cyclic peptides with antimycobacterial activity,[7] in addition to massetolides,[8] pitipeptolides,[9] cyclomarin and sulfactin.[10] Kahalalide A does not have significant homology to these other antimycobacterial cyclic peptides. Furthermore, it is devoid of obviously reactive functional groups, and it is not cytotoxic to various tumor cell lines, suggesting a selective antibacterial target. Here, we report the total synthesis of kahalalide A by a solid-phase route adaptable for analogue preparation. Besides the motivation of discovering biologically active compounds, there was a purely chemical impetus for the total synthesis. The stereochemistry of the methylbutyrate side chain was not established during the original isolation, and we believed this uncertainty could be resolved by synthesis.



kahalalide A (**1**)

There are two main approaches for the 'head-to-tail' solid-phase synthesis[11] of cyclic peptides and depsipeptides. In the first, an amino acid is immobilized by its side chain with orthogonal protection at the amine and carboxylic acid. After construction of the linear peptide, 'head-to-tail' intramolecular cyclization between the two ends followed by resin cleavage releases the cyclic peptide. In the second strategy, immobilization is through the carboxylic acid, but via a linker that is stable to the conditions of peptide synthesis. After assembly of the linear peptide, the site of immobilization is activated, permitting intramolecular cyclization. The first approach is fundamentally limited to amino acids that have suitable side-chain functionality for immobilization, while the second needs a linker that is robust during linear peptide synthesis and yet selectively activated upon demand. We chose the latter approach, taking advantage of recent developments with Kenner's sulfonamide 'safety-catch' linker. Originally designed[12] for linear peptide synthesis, Kenner's linker was later popularized[13] by Ellman for combinatorial chemistry applications. Subsequently, it was demonstrated[14] by Yang and Morriello at Merck that the linker is suitable for the 'head-to-tail' synthesis of cyclic

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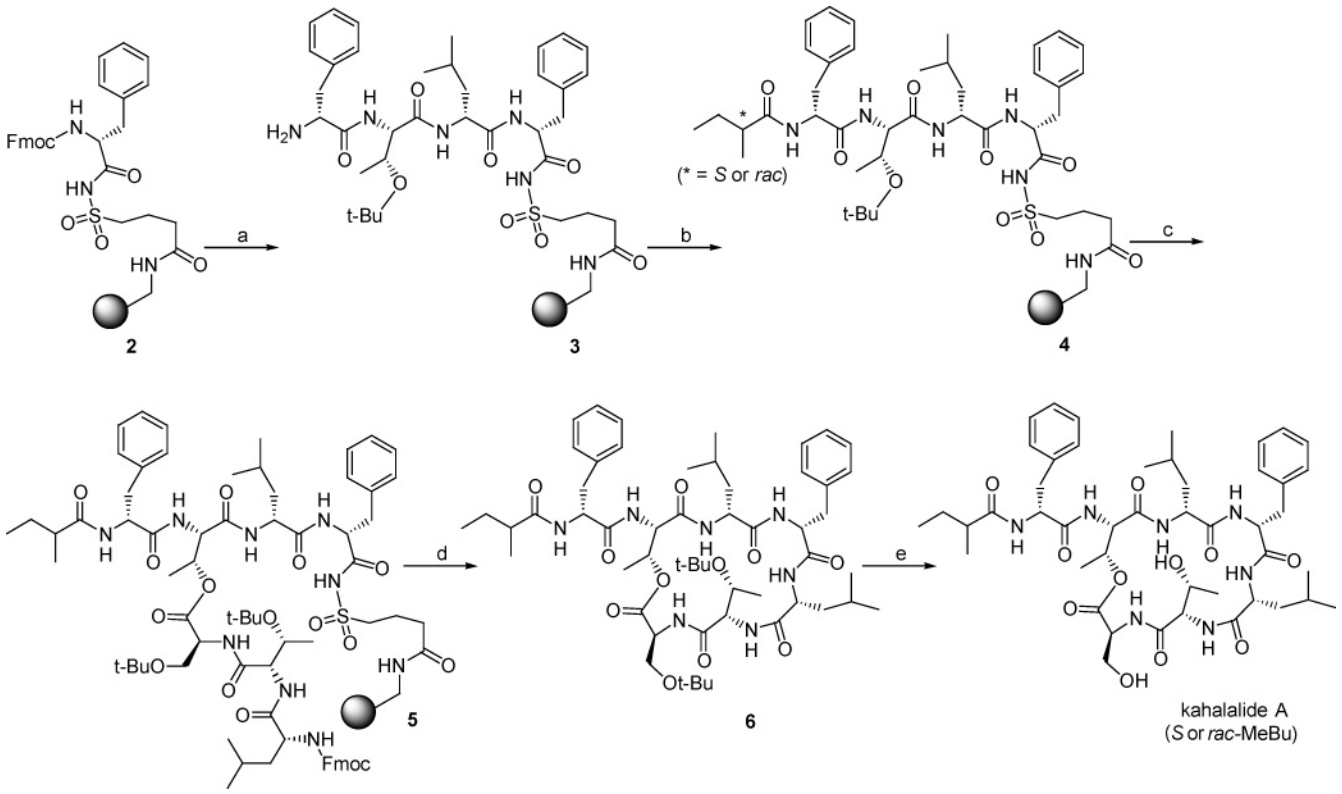
Scheme 1[a]



[a] Reagents: (a) (i) 20% piperidine/DMF; (ii) Fmoc-D-Leu-OH, DIC, HOBt (4 equiv each); (iii) 20% piperidine/DMF; (iv) Fmoc-L-Thr(*t*-Bu)-OH, DIC, HOBt (4 equiv each); (v) 20% piperidine/DMF; (vi) Fmoc-D-Phe-OH, DIC, HOBt (4 equiv each); (vii) 20% piperidine/DMF; (b) (*S*)-2-methylbutyric acid or (±)-2-methylbutyric acid, DIC, HOBt (4 equiv each); (c) (i) TFA/*i*-Pr_3SiH/H_2O: 95:2.5:2.5; (ii) Fmoc-L-Ser(*t*-Bu)-OH, DIC, (4 equiv each), 0.4 equiv of DMAP; (iii) 20% piperidine/DMF; (iv) Fmoc-L-Thr(*t*-Bu)-OH, DIC, HOBt (4 equiv each); (v) 20% piperidine/DMF; (vi) Fmoc-D-Leu-OH, DIC, HOBt (4 equiv each); (d) (i) 20% piperidine/DMF; (ii) 4 equiv of Trt-Cl, 8 equiv *i*-Pr_2NEt; (iii) 10 equiv of ICH_2CN, 12 equiv of *i*-Pr_2NEt; (iv) 5% TFA/CH_2Cl_2; 3 equiv of *i*-Pr_2NEt; (e) TFA/*i*-Pr_3SiH/H_2O.

peptides, and other groups[15] have recently employed sulfonamide linkers in this manner.

Results and Discussion

The total synthesis of kahalalide A began with the attachment of Fmoc-D-Phe to the commercially available sulfonamide resin, giving **2** (Scheme 1). This step was repeated to ensure high loading, after which peptide couplings (monitored throughout for completion using either 2,4,6-trinitrobenzenesulfonic acid (TNBS)[16] or ninhydrin[17] colorimetric reagents, and also by quantitative Fmoc removal analysis[18]) with Fmoc-D-Leu, Fmoc-L-Thr(*t*-Bu), Fmoc-D-Phe, and amine deprotection provided tetrapeptide **3**. The stage was now set for addition of the methylbutyrate (MeBu) side chain. Unfortunately, only the *S*-enantiomer of chiral 2-methylbutyric acid is commercially available. The synthesis was thus carried out twice, once with the *S*-enantiomer and once with racemic 2-methylbutyrate. The latter would ultimately result in a 50−50 mixture of kahalalide A and its MeBu diastereomer, while the first would produce a single diastereomer (possibly kahalalide A). Deprotection of the Thr side chain in **4** was followed by ester bond formation with Fmoc-L-Ser(*t*-Bu). A double coupling was employed to drive this reaction to completion. Further peptide extension then afforded the key linear heptapeptide **5** for safety-catch activation. According to Yang and Morriello, this is not compatible with the Fmoc group, and the nitrogen protection was first switched to trityl according to their procedure. Sulfonamide alkylation with iodoacetonitrile then activated the safety-catch linker, and trityl deprotection to the free amine resulted in macrocyclative cleavage of depsipeptide **6** into solution. Acidic cleavage of the *tert*-butyl ethers completed the synthesis of (*S*-MeBu)-kahalalide A and (±-MeBu)-kahalalide A. Although the overall yield was modest (15−20%, unoptimized), the crude material produced by the cyclative cleavage strategy[19] was unaccompanied by any significant peptide impurities. In this and later cyclizations, we did not observe the formation of dimers by intermolecular reaction. It is possible that the yields are dependent on which residue is chosen as the initial site of immobilization, although this was not investigated.

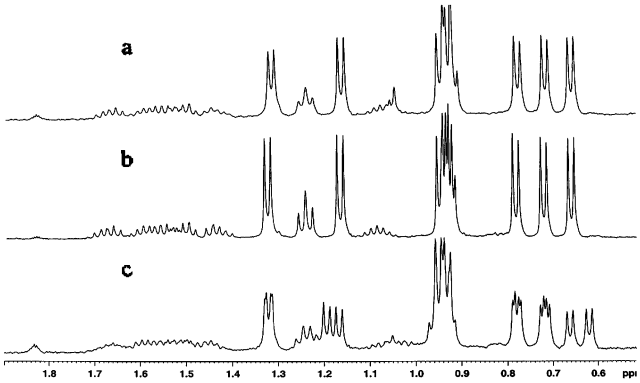


Figure 1. ^1H NMR spectrum in CD_3CN, showing the methyl region of (a) naturally isolated kahalalide A, (b) (*S*-MeBu)-kahalalide A and (c) (±-MeBu)-kahalalide A.

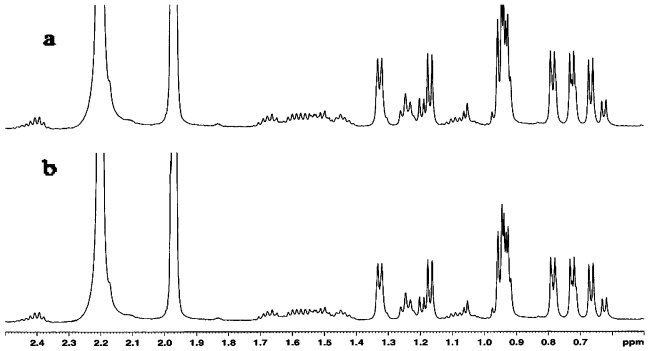


Figure 2. ^{1}H NMR spectrum of methyl region of a mixture of (a) naturally isolated kahalalide A and (±-MeBu)-kahalalide A, and (b) (*S*-MeBu)-kahalalide A and (±-MeBu)-kahalalide A.

HPLC separation of the two diastereomers of (±-MeBu)-kahalalide A with a variety of columns proved unsuccessful. Nevertheless, careful examination of ^{1}H NMR spectra allowed assignment of the MeBu stereochemistry. The ^{1}H NMR spectra of naturally isolated kahalalide A and synthetic (*S*-MeBu)-kahalalide A (Figure 1a and 1b, respectively) in CD_3CN are closely identical. These spectra differed significantly from the spectra of (±-MeBu)-kahalalide A (Figure 1c).

For comparison, ^{1}H NMR spectra of a mixture of natural kahalalide A and (±-MeBu)-kahalalide A (Figure 2a), and a mixture of synthetic (*S*-MeBu)-kahalalide A and (±-MeBu)-kahalalide A (Figure 2b) were also recorded. The 2-CH, 3-CH_2, 4-CH_3, and 5-CH_3 protons of the 2-methylbutyric acid moiety showed similar NMR patterns, but there were significant differences between the chemical shift sets for these signals in the two molecules. From these observations, it was possible to establish unambiguously the differences between (*S*-MeBu)-kahalalide A and (±-MeBu)-kahalalide A. For (±-MeBu)-kahalalide A, the 2-methyl group protons of 2-methylbutyric acid appeared as a multiplet at lower chemical shift than the corresponding signal for the *S*-isomer. On the other hand, the 2-methyl group protons of 2-methylbutyric acid for (*S*-MeBu)-kahalalide A appeared as a doublet and exactly matched with those for natural kahalalide A. From this study, it could be determined that the 2-methylbutyric acid of kahalalide A has the *S*-configuration. Our choice of *S*-methylbutyric acid for the synthesis, dictated by practical grounds, fortuitously turned out to provide the right diastereomer.

With the total synthesis successfully accomplished, we prepared two analogues aimed at probing the importance of the methylbutyrate side chain. In the first, the arm containing (MeBu)-Phe was deleted and replaced by a simple acetyl group. In the second, the methylbutyrate was replaced by the longer achiral hexanoate. In all these syntheses, depsipeptides with protected Ser and Thr residues are first produced by cyclative cleavage from the resin, and they were tested alongside with the deprotected material.

The full set of compounds (**7**−**14**, Figure 3) was tested for antimycobacterial activity. The results reveal a number of structure−activity relationships. First, all compounds containing protected Ser and Thr *tert*-butyl ethers were inactive, indicating the importance of the free alcohol at these positions. The truncated (Ac)-kahalalide **12**, in which a Phe residue and the methylbutyrate side chain are removed, was also inactive. Meanwhile, (*S*-MeBu)-kahalalide A and (±-MeBu)-kahalalide A were equally potent, implying that the stereochemistry of the methylbutyrate is not important for antimycobacterial activity. Indeed, replacement by

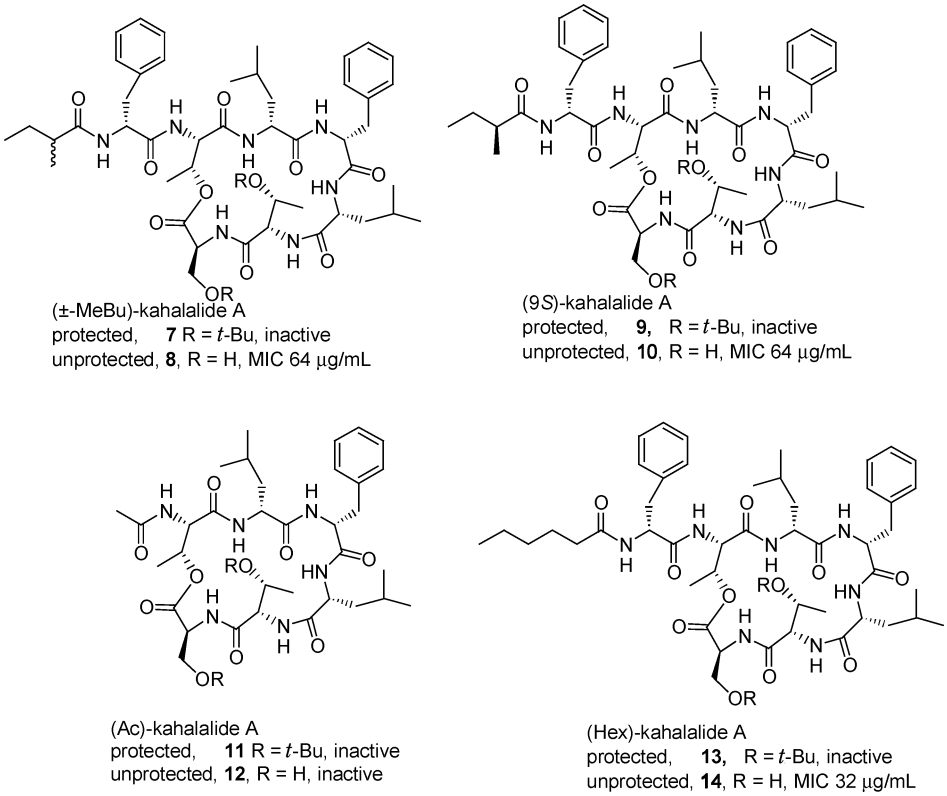
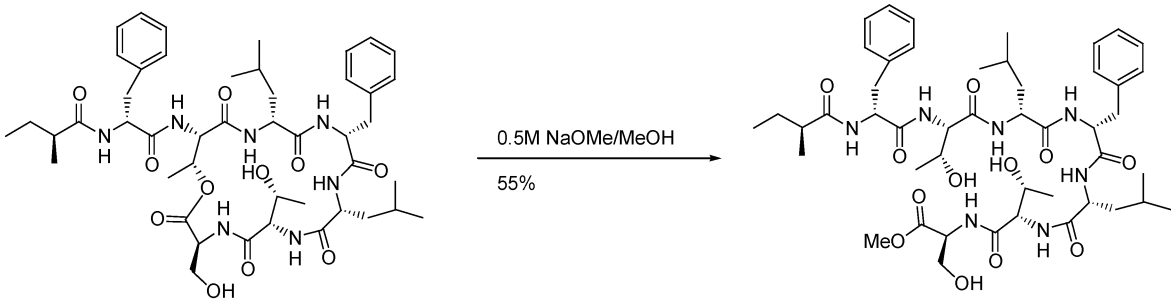


Figure 3. Compounds prepared, with antimycobacterial activity indicated.

Scheme 2



the achiral hexanoate (**14**) resulted in a 2-fold increase in potency over the natural product.

The increased conformational constraints placed by converting a linear peptide into a cyclic skeleton usually have a marked effect on its properties and biological activity. To test this in the present context, a small-scale methanolysis was carried out with synthetic kahalalide A to cleave the depsipeptide linkage (Scheme 2). The resulting 'linear' kahalalide A proved to be devoid of antimycobacterial activity, and this avenue was not pursued further.

To summarize, an efficient solid-phase total synthesis of kahalalide A was achieved. To the best of our knowledge, this is the first example where the Kenner safety-catch linker was used for the preparation of cyclic depsipeptides. By careful NMR analysis of synthetic kahalalide A containing either (*S*-MeBu) or (±-MeBu), we have established that the natural product contains a (*S*)-2-methylbutyrate subunit, thus resolving the only ambiguity in its structure. Our route is readily amenable to the preparation of libraries for antimycobacterial testing, by amino acid substitutions during the synthesis. The data from our first analogues highlight the importance of the free Ser and Thr side chains and the constrained depsipeptide framework for biological activity. The methylbutyrate side chain can be replaced by other hydrophobic groups, as evidenced by increased activity with the hexanoate. On the basis of these preliminary results, we plan the synthesis of additional analogues and mechanistic studies to identify the target of action.

Experimental Methods

Materials. All Fmoc amino acids, coupling reagents, and resin were purchased from Novabiochem, and other chemicals are from Aldrich. Dichloromethane and triethylamine were distilled over CaH_2 and THF distilled over Na in the presence of benzophenone. The inside surfaces of peptide synthesis reactors were pretreated by dichlorodimethylsilane, then rinsed with methanol and dichloromethane.

General Procedure for Depsipeptide Synthesis. Resin loading: To the 4-sulfamylbutyryl AM resin (Novabiochem, 500 mg, 0.56 mmol) preswollen in DMF was added Fmoc-D-Phe-OH (697 mg, 1.8 mmol), PyBOP (936 mg, 1.8 mmol) and Hünig's base (942.8 μL, 5.4 mmol) in 5 mL of DMF. The reaction mixture was left overnight, and the coupling repeated twice more. The efficiency of loading was determined by Fmoc removal analysis.

Peptide coupling: The Fmoc-peptidyl resin, swollen in DMF, was deprotected by 20% piperidine in DMF for 2 min, and the deprotection repeated for 20 min. The resin is washed (3 × DMF) and the presence of the amino group checked with TNBS. At the same time, the next Fmoc amino acid (2.24 mmol) is activated in 5 mL of DMF by DIC (2.24 mmol, 351 μL) and HOBt (2.24 mmol, 351 mg). The solution is filtrated in case of precipitation, before addition to the deprotected peptidyl resin. Reaction completion is checked by TNBS or ninhydrin. The coupling of acetic, 2-methylbutyric, or hexanoic acids was carried out under identical conditions.

Depsipeptide bond formation: The threonine *tert*-butyl ether is deprotected by a TFA/TIS/water (95/2.5/2.5) mixture for 2 min. The procedure was repeated for 30 min and the resin washed (CH_2Cl_2, THF). The alcohol resin was incubated with DMAP (27.3 mg, 0.224 mmol) and reacted in 4 mL of THF with Fmoc-L-Ser(*t*-Bu)-OH (859 mg, 2.24 mmol) and DIC (351 mg, 2.24 mmol). This coupling was first done for 2 h and repeated overnight to ensure complete esterification as quantified by Fmoc removal analysis. After resin washing (3 × THF, 3 × DMF), elongation of the linear peptide is continued, until the Fmoc group on the last residue is removed.

Safety-catch activation and cyclative cleavage of cyclic depsipeptides: The amine resin is treated with trityl chloride (624 mg, 2.24 mmol) and Hünig's base (782 μL, 4.48 mmol) in 2.5 mL of CH_2Cl_2 for 2 h or left overnight. After CH_2Cl_2 washings and TNBS test, the sulfonamide linker is activated by iodoacetonitrile (405 μL, 5.6 mmol) and Hünig's base (1.73 mL, 6.72 mmol) in 2.5 mL of *N*-methylpyrrolidinone for 12 h. This activation step was repeated under the same conditions. After resin washing (*N*-methylpyrrolidinone, CH_2Cl_2), the trityl group is removed by 5% TFA in CH_2Cl_2 treatment for 2 min. The deprotection was repeated for 2 h, followed by a TNBS test. The linear peptidyl resin is then washed (3 × CH_2Cl_2, 3 × THF, once by 1% Hünig's base in THF). Immediately afterward, the resin is swollen in THF and Hünig's base (293 μL, 1.68 mmol) added. The cyclization proceeds overnight, releasing the cyclic depsipeptide.

Peptide purification: The supernatant from cyclative cleavage is concentrated to the crude cyclic peptide, whose identity is checked by ES-MS and purity by HPLC and TLC. The compound is then purified by column chromatography (silica) to give peptides with protected Ser/Thr residues. Side-chain deprotection is performed by TFA/i-Pr_3SiH/water (95/2.5/2.5) for 30 min, followed by precipitation of the peptide in Et_2O dried over CaH_2. Deprotected peptides are further purified by preparative RP-HPLC (C-18 Nucleosil 300 mm × 25 mm, 3 mL/min, detection at 230 nm, eluent A: Water/TFA: 99.95/0.05, eluent B: acetonitrile/water/TFA 80/19.95/0.05, gradient B from 0 to 100% in 100 min).

(*R*/*S*)-Methyl-2-butyryl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr(*t*-Bu)-L-Ser(*t*-Bu)] (7). Overall yield: 19% (104 mg). ES-MS: 1028.5 $[M + Na]^+$; HPLC purity: 98% by ELSD, 90% at 220 nm; TLC (CH_2Cl_2/AcOEt/TEA: 6/4/0.05) R_f: 0.3.

(*R*/*S*)-Methyl-2-butyryl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr-L-Ser] ((±-MeBu)-kahalalide A, 8). Deprotection yield: 95%, overall yield: 18% (17 mg). ES-MS: 916.7 $[M + Na]^+$; HPLC purity: >99% by ELSD, 87% at 220 nm.

(*S*)-Methyl-2-butyryl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr(*t*-Bu)-L-Ser(*t*-Bu)] (9). Overall yield: 15% (85 mg). ES-MS: 1029.0 $[M + Na]^+$; HPLC purity: 98% by ELSD, >99% at 220 nm; TLC (CH_2Cl_2/AcOEt/NH_4OH aq: 7/3/0.05) R_f: 0.3.

(*S*)-Methyl-2-butyryl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr-L-Ser] (kahalalide A, 10). *tert*-Butyl ether deprotection yield: 75%, overall yield: 11% (12 mg). ^{1}H NMR δ 8.21 (Thr2-NH, d, J = 9.59 Hz), 7.63 (Phe1-NH, d, J = 9.45 Hz), 7.63 (Thr1-NH, d, J = 9.45 Hz), 7.45 (Leu2-NH, d, J = 9.37 Hz), 7.37 (Phe2-NH, d, J = 4.68 Hz), 7.25 (Phe2-H$_{5,5',6,6',7}$, Phe1-H$_{5,5',6,6',7}$, m), 7.23 (Leu1-NH, m), 6.87 (Ser-NH, d, J = 5.70 Hz), 5.44 (Thr2-H$_3$, dq, J = 6.16, 2.18 Hz), 5.04 (Phe2-H$_2$, dt, J = 7.98, 4.90 Hz), 4.77 (Phe1-H$_2$, m), 4.70 (Leu1-H$_2$, m), 4.48 (Thr1-H$_3$, dq, J = 6.49,1.89 Hz), 4.40 (Thr2-H$_2$, dd, J = 9.56, 2.16 Hz), 4.29 (Leu2-H$_2$, q, J = 9.27 Hz), 4.02 (Thr1-H$_2$, m), 4.02 (Ser-H$_2$, m), 3.52 (Ser-H$_3$, dd, J = 5.16, 1.63 Hz), 3.24 (Phe1-H$_3$, dd, J = 14.18, 5.13 Hz), 3.00 (Phe2-H$_3$, dd, J = 14.18, 5.13 Hz), 2.80 (Phe1-H$_{3'}$, dd, J = 14.27, 10.4 Hz), 2.4 (MeBu-H$_2$, m), 1.65 (Leu1-H$_4$, m), 1.55 (MeBu-H$_3$, m), 1.50 (Leu1-H$_3$, m), 1.29 (Thr1-H$_4$, d, J = 6.51 Hz), 1.20 (Leu2-H$_3$, t, J = 7.63 Hz), 1.13 (MeBu-H$_5$, d, J = 6.90 Hz), 1.02 (leu2-H$_4$, m), 0.91 (Leu1-H$_5$, d, J = 6.42 Hz), 0.90 (MeBu-H$_4$, t, J = 7.40 Hz), 0.89 (Leu1-H$_6$, d, J = 6.42 Hz), 0.74 (Leu2-H$_5$, d, J = 6.53 Hz), 0.68 (Leu2-H$_6$, d, J = 6.53 Hz), 0.61 (Thr2-H$_4$, d, J = 6.58 Hz); ^{13}C NMR δ 180.6 (MeBu-C$_1$), 175.3 (Leu1-C$_1$), 174.3 (Phe2-C$_1$), 172.1 (Leu2-C$_1$), 171.9 (Phe1-C$_1$), 170.9 (Thr1-C$_1$), 170.0 (Ser-C$_1$), 169.3 (Thr2-C$_1$), 138.0 (Phe1-C$_4$), 137.4 (Phe2-C$_4$), 130.2 (Phe2-C$_{5,5'}$), 129.7 (Phe1-C$_{5,5'}$), 129.3 (Phe2-C$_{6,6'}$), 129.3 (Phe1-C$_{6,6'}$), 127.8 (Phe2-C$_7$), 127.6 (Phe1-C$_7$), 69.9 (Thr2-C$_3$), 66.4 (Thr1-C$_3$), 61.7 (Ser-C$_3$), 60.8 (Thr1-C$_2$), 57.2 (Ser-C$_2$), 56.86 (Phe2-C$_2$), 56.86 (Thr2-C$_2$), 55.5 (Phe1-C$_2$), 54.2 (Leu2-C$_2$), 52.4 (Leu1-C$_2$), 43.4 (Leu1-C$_3$), 42.9 (MeBu-C$_2$), 42.3 (Leu2-C$_3$), 39.8 (Phe1-C$_3$), 37.7 (Phe2-C$_3$), 28.3 (MeBu-C$_3$), 25.5 (Leu2-C$_4$), 25.2 (Leu1-C$_4$), 23.0 (Leu2-C$_6$), 22.8 (Leu1-C$_6$), 22.6 (Leu1-C$_5$), 21.9 (Leu2-C$_5$), 20.8 (Thr1-C$_4$), 17.9 (MeBu-C$_5$), 16.0 (Thr2, C$_4$), 12.3 (MeBu-C$_4$); ES-MS: 916.17 [M + Na]$^+$; HPLC purity: 97% by ELSD, >99% at 220 nm.

Ac-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr(*t*-Bu)-L-Ser(*t*-Bu)] (11). Overall yield: 5% (26 mg). ES-MS: 855 [M + K]$^+$; HPLC purity: >99% by ELSD, 68% at 220 nm; TLC (CH_2Cl_2/AcOEt/TEA: 5/5/0.05) R_f: 0.3.

Ac-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr-L-Ser] (12). Deprotection yield: 59%, overall yield: 3% (7 mg). ^{1}H NMR δ 9.08 (Thr1-NH, d, J = 7.57 Hz), 8.12 (Phe1-NH, m), 8.05 (Leu1-NH, d, J = 6.94 Hz), 7.90 (Ser-NH, m), 7.78 (Thr2-NH, d, J = 9.18 Hz), 7.33 (Leu2-NH, m), 7.25 (Phe-H$_{5,5',6,6',7}$, m), 5.28 (Thr2-H$_3$, d, J = 5.76 Hz), 4.63 (Thr2-H$_2$, d, J = 8.93 Hz), 4.59 (Leu1-H$_2$, d, J = 6.95 Hz), 4.29 (Phe-H$_2$, m), 4.27 (Thr1-H$_3$, dd, J = 6.62, 2.45 Hz), 4.16 (Leu2-H$_2$, m), 4.02 (Thr1-H$_2$, m), 3.53 (Ser-H$_{2,3}$, m), 3.17 (Phe-H$_{3'}$, dd, J = 13.92, 5.00 Hz), 3.00 (Phe-H$_3$, dd, J = 13.83, 10.39 Hz), 1.56 (Leu1-H$_3$, Leu2-H$_3$, m), 1.35 (Leu2-H$_4$, m), 1.09 (Thr1-H$_4$, d, J = 6.44 Hz), 1.04 (Thr2-H$_4$, d, J = 6.72 Hz); 0.91 (Leu1-H$_5$, d, J = 6.42 Hz), 0.90 (Leu1-H$_4$, m), 0.79 (Leu1-H$_{6'}$, d, J = 6.02 Hz), 0.74 (Leu1-H$_6$, d, J = 5.94 Hz), 0.55 (Leu2-H$_{5'}$, d, J = 6.54 Hz), 0.42 (Leu2-H$_5$, d, J = 6.55 Hz); ^{13}C NMR δ 174.0, 172.4, 171.5, 171.2, 170.8, 170.6, 170.1 (all C$_1$), 138.0, 130.2, 129.8, 129.1, 128.9, 127.2 (Phe1-C$_4$, C$_{5,5'}$, C$_{6,6'}$ and C$_7$), 71.3 (Thr2-C$_3$), 66.4 (Thr1-C$_3$), 61.3 (Ser-C$_2$), 61.2 (Ser-C$_3$), 60.4 (Thr1-C$_2$), 56.5 (Phe1-C$_2$), 56.4 (Leu2-C$_2$), 55.4 (Thr2-C$_2$), 52.2 (Leu1-C$_2$), 42.9 (MeBu-C$_2$), 40.6 (Leu1-C$_3$), 40.4 (Leu2-C$_3$), 37.3 (Phe1-C$_3$), 25.1, 24.8, 23.8; 23.5, 23.4, 23.3, 23.1, 22.9, 22.7, 221.4, 21.1 (Leu2-C$_4$, Leu1-C$_4$, Leu2-C$_6$, Leu1-C$_6$, Leu1-C$_5$, Leu2-C$_5$, Thr1-C$_4$, Thr2-C$_4$, Ac-C$_2$); ES-MS: 727.7 [M + Na]$^+$; HPLC purity: 96% by ELSD, 77% at 220 nm.

Hexanoyl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr(*t*-Bu)-L-Ser(*t*-Bu)] (13). Overall yield: 15% (88 mg). ES-MS: 1043.0 [M + Na]$^+$; HPLC purity: 98% by ELSD, 74% at 220 nm; TLC (CH_2Cl_2/AcOEt/TEA: 7/3/0.05) R_f: 0.3.

Hexanoyl-D-Phe-cyclo[L-Thr-D-Leu-D-Phe-D-Leu-L-Thr-L-Ser] (14). Deprotection yield: 93%, overall yield: 14% (14 mg). ^{1}H NMR δ 8.20 (Thr2-NH, d, J = 9.49 Hz), 7.78 (Phe1-NH, d, J = 9.73 Hz), 7.62 (Thr1-NH, d, J = 7.21 Hz), 7.54 (Leu2-NH, d, J = 9.46 Hz), 7.45 (Phe2-NH, d, J = 4.44 Hz), 7.27 (Phe2-H$_{5,5',6,6',7}$ Phe1-H$_{5,5',6,6',7}$, m), 7.25 (Leu1-NH, m), 6.86 (Ser-NH, d, J = 6.02 Hz), 5.47 (Thr2-H$_3$, dq, J = 6.50, 2.20 Hz), 5.03 (Phe2-H$_2$, dt, J = 8.18, 4.56 Hz), 4.81 (Leu1-H$_2$, m), 4.73 (Phe1-H$_2$, m), 4.51 (Thr1-H$_3$, dq, J = 6.53, 1.92 Hz), 4.40 (Thr2-H$_2$, dd, J = 9.57, 2.17 Hz), 4.31 (Leu2-H$_2$, q, J = 9.4 Hz), 4.02 (Thr1-H$_2$, m), 3.99 (Ser-H2, dd, J = 6.93, 1.63 Hz), 3.54 (Ser-H$_3$, d, J = 4.89 Hz), 3.23 (Phe1-H$_3$, dd, J = 14.04, 4.91 Hz), 3.02 (Phe2-H$_3$, dd, J = 7.85, 1.79 Hz), 2.86 (Phe1-H$_4$, dd, J = 14.10, 10.25 Hz), 2.3 (Hex-H$_2$, t, J = 7.5 Hz), 1.66 (Hex-H$_3$, m), 1.59 (Leu1-H$_4$, m), 1.50 (Leu1-H$_3$, m), 1.37 (Hex-H$_{4,5}$, m), 1.32 (Thr1-H$_4$, d, J = 6.48 Hz), 1.22 (Leu2-H$_3$, m), 1.06 (Leu2-H$_4$, m), 0.96 (Leu1-H$_5$, d, J = 6.37 Hz), 0.92 (Hex-H$_6$, t, J = 7.1 Hz), 0.92 (Leu1-H$_6$, d, J = 6.49 Hz), 0.79 (Leu2-H$_5$, d, J = 6.53 Hz), 0.73 (Leu2-H$_6$, d, J = 6.51 Hz), 0.61 (Thr2-H$_4$, d, J = 6.56 Hz); ^{13}C NMR δ 177.0 (Hex-C$_1$), 174.8 (Leu1-C$_1$), 173.6 (Phe2-C$_1$), 171.6 (Leu2-C$_1$), 171.4 (Phe1-C$_1$), 170.4 (Thr1-C$_1$), 169.6 (Ser-C$_1$), 168.7 (Thr2, C$_1$), 137.6 (Phe1-C$_4$), 136.8 (Phe2-C$_1$), 129.8 (Phe2-C$_{5,5'}$), 129.4 (Phe1-C$_{5,5'}$), 128.9 (Phe2-C$_{6,6'}$), 128.9 (Phe1-C$_{6,6'}$), 127.4 (Phe2-C$_7$), 127.2 (Phe1-C$_7$), 69.5 (Thr2-C$_3$), 65.9 (Thr1-C$_3$), 61.3 (Ser-C$_3$), 60.5 (Ser-C$_2$), 56.7 (Thr1-C$_2$), 56.6 (Phe2-C$_2$), 56.3 (Thr2-C$_2$), 55.2 (Phe1-C$_2$), 53.8 (Leu2-C$_2$), 51.9 (Leu1-C$_2$), 42.6 (Leu1-C$_3$), 42.0 (Leu2-C$_3$), 39.2 (Phe1-C$_3$), 37.1 (Phe2-C$_3$), 36.2 (Hex-C$_2$), 31.4 (Hex-C$_4$), 26.5 (Hex-C$_3$), 25.0 (Leu1-C$_4$), 24.8 (Leu2-C$_4$), 22.8 (Leu1-C$_6$), 22.52 (Hex-C$_5$), 22.51 (Leu2-C$_6$), 22.3 (Leu1-C$_5$), 21.6 (Leu2-C$_5$), 20.2 (Thr1-C$_4$), 17.6 (Thr2-C$_4$), 15.5 (Hex-C$_6$); ES-MS: 930.6 [M + Na]$^+$, 946.7 [M + K]$^+$; HPLC purity: 96% by ELSD, 77% at 220 nm.

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Supporting Information Available: HPLC data and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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