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ORIGINAL PAPER

Application of the highly sensitive labeling reagent to the structural confirmation of readily isomerizable peptides

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Abstract

Thioamycolamide A (**1**) is a biosynthetically unique cytotoxic cyclic microbial lipopeptide that bears a D-configured thiazoline, a thioether bridge, a fatty acid side chain, and a reduced C-terminus. It has gained attention for its unique structure, and very recently we reported the total synthesis of **1** via a biomimetic route. The NMR spectra of synthetic **1** agreed with those of natural **1**. However, structural identity between peptidic natural and synthetic compounds is often difficult to confirm by comparison of NMR spectra because their NMR spectra vary depending on the conditions in the NMR tube, which often result in the structural misassignment of peptidic compounds. Especially, our total synthesis based on the putative biomimetic route potentially gives **1** as a diastereomixture at the final step. The problem is that the diastereomers of peptidic mid-sized molecules often exhibit similar properties (such as NMR spectra and bioactivities), and their separation procedures are often laborious. Herein we report the structural confirmation of synthetic **1** by the LC–MS-based chromatographic comparison with the use of our highly sensitive labeling reagent L-FDVDA; the highly sensitive-advanced Marfey's method (HS-advanced Marfey's method). This work demonstrated the utility of our highly sensitive labeling reagent for the structural determination of not only scarce natural products but also readily isomerizable synthetic compounds.

Keyword Natural products · Structural determination · Peptides · D-amino acid · Highly sensitive advanced marfey's method

Introduction

Peptide mid-size molecules, which have intermediate properties between those of antibodies and small molecule drugs, have gained much attention as ideal drug leads [1].The specific bioactivities of peptide drug leads are exerted by their unique structure, that often featured by the unusual amino acids including D-amino acids [2]. Therefore, the analyses of the absolute configurations are inevitable in the structural determination of newly identified peptide natural products.

Thioamycolamide A (**1**) is a biosynthetically unique cytotoxic cyclic microbial lipopeptide that bears a D-configured thiazoline, a thioether bridge, a fatty acid side chain, and a reduced C-terminus (Fig. 1) [3]. It has gained attention for its unique structure, and very recently we reported a concise total synthesis of **1** based on the putative biosynthetic pathway as summarized in Fig. 2 [4]. In general, peptides are chemically constructed from its C-terminus to N-terminus. Although the Fmoc-based solid-phase total synthesis of peptide that bears reduced C-terminus has been reported [5], peptide **1** possesses a thiazoline moiety, which was readily isomerized through the cleavage of Fmoc group under basic conditions [6–9]. Therefore, to avoid the isomerization of thiazoline, the linear peptide **7** was synthesized from Boc-L-cystine (**6**) under isomerization-suppressed manner in solution-phase, and then cyclized by one-pot reduction/thio-Michael addition under the neutral conditions to afford **1**.

Although the NMR spectra of synthetic **1** agreed with those of natural **1**, the spectroscopic data of peptides may

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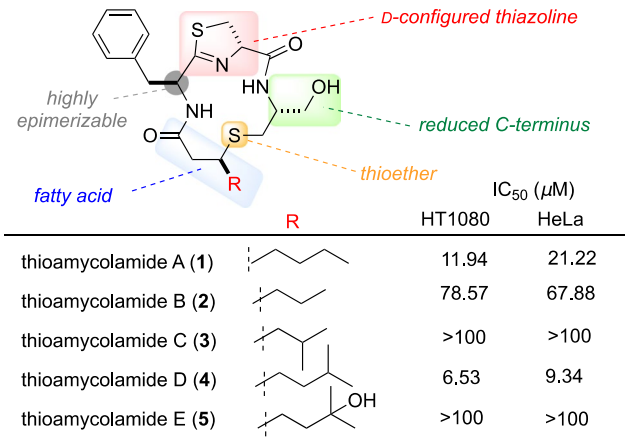

	R	IC_{50} (μM) HT1080	HeLa
thioamycolamide A (**1**)		11.94	21.22
thioamycolamide B (**2**)		78.57	67.88
thioamycolamide C (**3**)		>100	>100
thioamycolamide D (**4**)		6.53	9.34
thioamycolamide E (**5**)		>100	>100

Fig. 1 The structures and bioactivities of thioamycolamides A–E (**1**–**5**) [3]

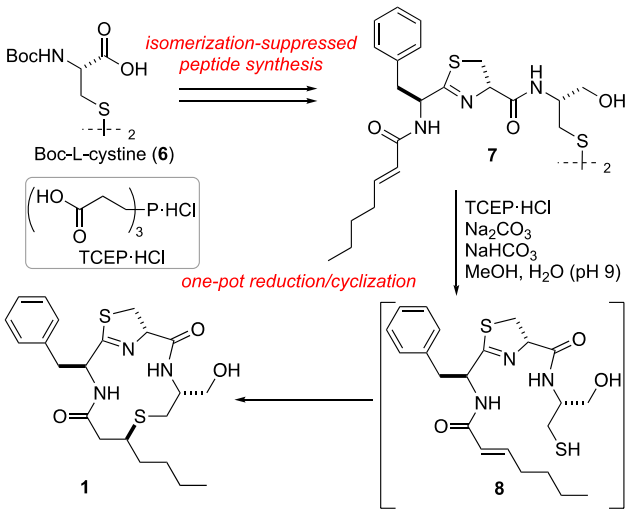

Fig. 2 The outline of the total synthesis of **1** [4]

vary depending on the solution conditions, and often result in structural misassignment of peptidic natural products. Structural identity between natural and synthetic compounds is often difficult to confirm only by comparison of NMR spectra because the NMR spectra of peptidic products varies depending on the conditions in the NMR tube (e.g., concentration, pH, and temperature) [10–15]. The essential task at this stage was the structural confirmation of the chemically constructed **1** because the thio-Michael addition used in the synthesis may give **1** as a diastereomeric mixture due to the absence of the chiral catalyst for thio-Michael addition. Additionally, the thiazoline moiety may be isomerized not only during the chemical conversions, but also during the purification steps [6–9] Especially, diastereomers of peptidic mid-sized molecules often exhibit similar properties (such as NMR spectra [10–15] and bioactivities [16]), and their separation procedures are often laborious. While the developed biomimetic route seemed to give **1** as a stereochemically pure form as with its biosynthesis, standard samples of the diastereomers for chromatographic comparison to confirm its purity could not be prepared by this synthetic route.

Results and discussions

To establish the efficiency of our bioinspired total synthesis, we had to determine the purity of synthetic **1**, which may be readily isomerized similar to natural **1**. Consequently, having developed a rapid synthetic entry to **1**, we then turned our attention to the structural validation of the chemically constructed **1**. Herein we report the structural confirmation of synthetic **1** by the combination of the chemical synthesis and LC–MS-based chromatographic comparison.

Among the strategies for the structural elucidation of peptides, Marfey's method [17] has been a powerful tool not only for the structural study on the new natural product but also the confirmation of the total synthesis [18]. Recently, we developed new modified Marfey's reagents such as 1-fluoro-2,4-dinitrophenyl-5-L-valine-*N*,*N*-dimethylethylenediamine-amide (L-FDVDA) for "scarce natural products" inspired by a scarce natural product yaku'amide B [19]. The highly sensitive-advanced Marfey's method (HS-advanced Marfey's method) by using new improved reagents enabled the detection of component amino acids resulting from mild chemical degradation of smaller amounts of peptide, which would be useful for the structural analysis of peptide **1** (Fig. 3).

In our preceding study (Fig. 4a) [4], first, natural **1** was carefully hydrolyzed (2 M HCl, 90 °C, 4 h) to determine the stereochemistry of C5 and C8 by Marfey's method [17].

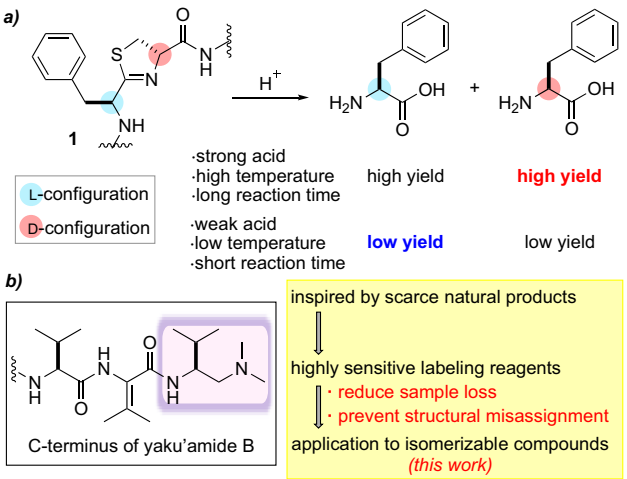

Fig. 3 **a** The problem in the amino acid analyses of **1**. **b** The outline of this study

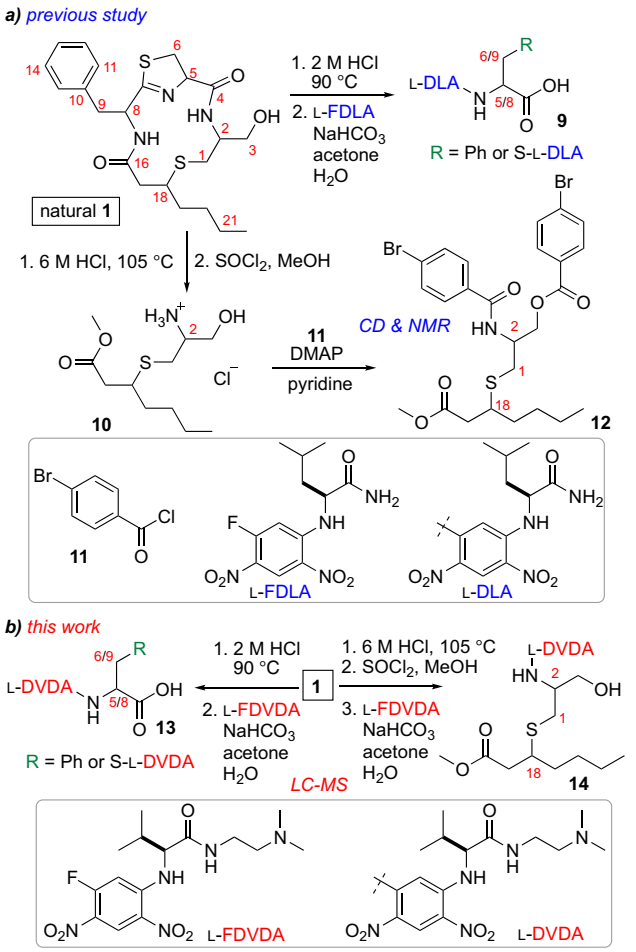

Fig. 4 **a** Structural studies on **1** from a previous work; **b** Structural studies on **1** in this work

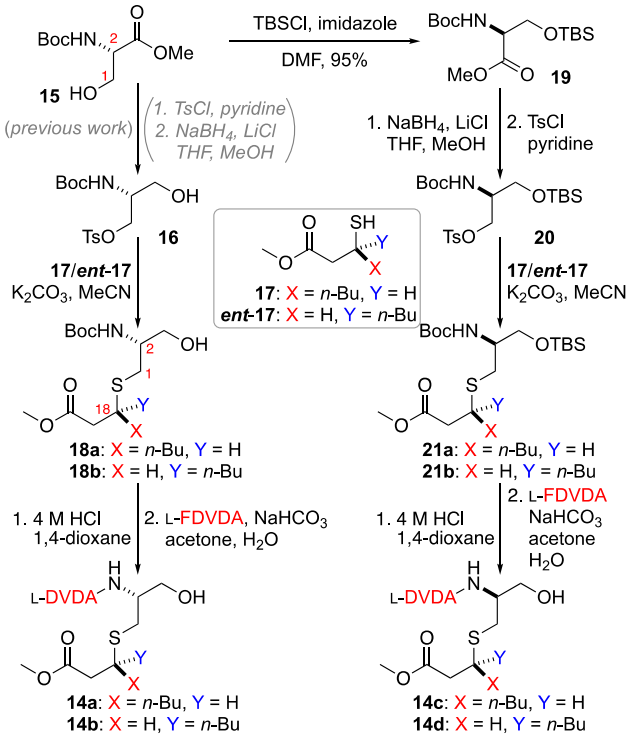

Fig. 5 Synthesis of four possible isomers of thioether **14**

Then the absolute configuration of C2 and C18 were evaluated by a combination of the spectroscopic analyses (CD [20] and NMR) and chemical synthesis. Although a single plausible structure of **1** could be determined by these experiments, a multi-milligram scale of **1**, which was totally consumed by the chemical degradation, was required for CD and NMR analyses. Conversely, in this study (Fig. 4b), acid hydrolysates of natural/synthetic **1** were derivatized with the new labeling reagent L-FDVDA and the corresponding derivatives were chromatographically compared with the chemically synthesized standard samples. A minute amount (< 0.1 mg) of **1** was enough for the LC–MS-based chromatographic comparison of the 2,4-dinitrophenyl-5-L-valine-*N*,*N*-dimethylethylenediamine-amide (DVDA) derivatives (See Fig. S1 in electronic supplementary materials for details including structural confirmation of C5/C8 and C6/C9).

The synthesis of all four possible isomers of **14** is summarized in Fig. 5. Treatment of the previously synthesized thioethers **18a/18b** [4] with HCl liberated the corresponding amines, which were labeled with L-FDVDA by a nucleophilic aromatic substitution (S_NAr) reaction leading to **14a/14b**. In the first step of the synthesis of **14c/14d**, the hydroxy function of **15** was protected with a TBS group, which can be cleaved simultaneously with a Boc group by HCl. Second, reduction of the methyl ester of **19**, followed by the tosylation of the generated primary alcohol furnished **20**. Then, **20** was converted to **14c/14d** in the same fashion as **14a/14b**.

Finally, the key labeled thioether **14** derived from natural/synthetic **1** was chromatographically compared with the standard thioethers **14a–d** by LC–MS experiments. The retention time of authentic **14** was matched with that of **14a**, confirming unambiguously the structure of natural/synthetic thioamycolamide A was that shown for **1** (Fig. 6). Moreover, no detectable peaks for **14b–14d** were observed

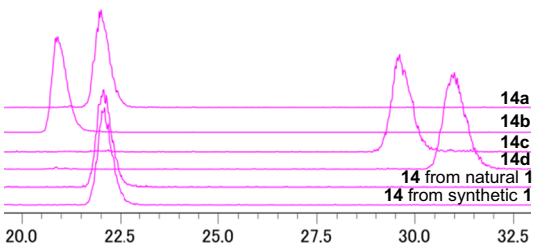

Fig. 6 The LC–MS charts of thioether **14**. See Supplementary Materials in the detail

in the LC–MS experiments of authentic samples even with the use of the highly sensitive labeling reagent, confirming the stereochemical purity of **1**. The thioether bridge was stereoselectively constructed in previous study, which strongly supports our proposed thio-Michael addition pathway for the thioether biosynthesis.

In summary, the structural identity of natural and synthetic **1** was confirmed by LC–MS experiments using our improved new labeling reagent L-FDVDA. In the LC–MS experiments, the stereochemical purity of chemically constructed **1** was verified, which corroborates the efficiency of our bioinspired synthesis and provides insight into the proposal that the thioether bridge can be stereoselectively formed by thio-Michael addition. The utility of the newly developed highly sensitive labeling reagent in the structural determination of unstable natural and synthetic products was also demonstrated. Further studies including the identification of the thiomycolomide A (**1**) biosynthetic gene cluster and detailed structure–activity relationship (SAR) studies are currently underway and will be reported in due course.

Materials and methods

General remarks ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) spectrometer. Chemical shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standard ($CDCl_3$, ^{1}H δ 7.25, ^{13}C δ 77.2). ESI–MS and LC–MS experiments were recorded on a Shimadzu LCMS-IT-TOF. Optical rotations were recorded on a JASCO P-2200 polarimeter. High performance liquid chromatography (HPLC) experiments were performed with a SHIMADZU HPLC system equipped with an LC-20AD intelligent pump. All reactions sensitive to air and/or moisture were conducted under nitrogen atmosphere using dry, freshly distilled solvents, unless otherwise noted. All reagents were used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F_{254} pre-coated plates. Silica gel column chromatography was performed using 40–50 μm Silica Gel 60 N (Kanto Chemical Co., Inc.).

Structural confirmation of 1

Natural **1** (0.1 mg) and synthetic **1** (0.1 mg) were hydrolyzed with 2 M HCl (100 μL) for 4 h at 90 °C under N_2 and then dried *in vacuo*, respectively. Each hydrolysate was dissolved in H_2O (100 μL), and then 1 M $NaHCO_3$ (20 μL) was added to the solution. To the resulting solution was added L-FDVDA (0.5% w/v in acetone, 20 μL), and then stirred at 50 °C for 2 h. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL), evaporated, dissolved in MeCN (100 μL), filtered through a membrane filter (SHIMADZU, TORAST™ DISC, PTFE 0.22 μm), and then analyzed by LC–MS [column: Cadenza CD-C18, 3.0 × 150 mm; eluent: MeCN/H_2O/formic acid = 50/50/0.1 to 100/0/0.1 (0–30 min), 0.2 mL/min; detection: ESI-positive].

Ester 19

To a solution of Boc-L-Ser-OMe (**15**) (500 mg, 2.28 mmol) in DMF (10 mL) were added imidazole (466 mg, 6.84 mmol) and TBSCl (516 mg, 3.42 mmol) at 0 °C. After being stirred at room temperature for 0.5 h, saturated aqueous NH_4Cl (20 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (30 mL × 2). The combined organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/hexane = 1:20) to afford **19** (719 mg, 95%) as a colorless oil: $[\alpha]^{20}_{D} = 7.0$ (*c* 2.22, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.33 (d, $J = 8.5$ Hz, 1H), 4.34 (dt, $J = 8.8$, 2.7 Hz, 1H), 4.03 (dd, $J = 10.0$, 2.6, 1H), 3.81 (dd, $J = 10.0$, 3.0, 1H), 3.73 (s, 3H), 1.45 (s, 9H), 0.85 (s, 9H), 0.02 (s, 3H), 0.01 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.4, 155.6, 80.2, 63.9, 55.7, 52.4, 28.5, 25.8, 18.3, −5.4, −5.5; HRMS (ESI) calcd for $C_{15}H_{31}NO_5SiNa^+$ $[M + Na]^+$ 356.1864, found 356.1845.

Tosylate 20

To a solution of **19** (700 mg, 2.10 mmol) in THF (2 mL) were added $NaBH_4$ (238 mg, 6.30 mmol), LiCl (267 mg, 6.30 mmol), and MeOH (6 mL) at 0 °C. After being stirred at 0 °C for 0.5 h and at room temperature for 1 h, aqueous citric acid solution (10%, w/w, 20 mL) was added to the reaction mixture. The resulting solution was extracted with CH_2Cl_2 (20 mL × 2). The combined organic layer was washed with saturated aqueous $NaHCO_3$ (40 mL) and brine (40 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford practically pure alcohol **S1** in supplementary material (664 mg, Figs. S5, S6, and S10) as a colorless oil, which was used in the next reaction without further purification.

To a stirred solution of the above alcohol **S1** (500 mg, ca 1.64 mmol) in pyridine (10 mL) was added TsCl (650 mg, 3.41 mmol) at 0 °C. After being stirred at room temperature overnight, saturated aqueous NH_4Cl (40 mL) was added to the reaction mixture. The resulting solution was extracted with EtOAc (40 mL × 2). The combined organic layer was washed with brine (80 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/hexane = 1:10) to afford **20** (581 mg) as a colorless oil: $[\alpha]^{20}_{D} = -2.2$ (*c* 0.05, MeOH); ^{1}H NMR (400 MHz,

$CDCl_3$) δ 7.73 (d, $J = 8.1$ Hz, 2H), 7.29 (d, $J = 8.1$ Hz, 2H), 4.75 (d, $J = 8.3$, 1H), 4.03 (m, 2H), 3.80 (s, 1H), 3.62 (dd, $J = 10.0$, 3.7 Hz, 1H), 3.48 (dd, $J = 10.0$, 6.2 Hz, 1H), 2.39 (s, 3H), 1.36 (s, 9H), 0.77 (s, 9H), −0.04 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 145.0, 132.6, 130.0, 128.0, 79.8, 67.9, 60.9, 50.4, 28.3, 25.8, 21.7, 18.1, 14.2, 5.6; HRMS (ESI) calcd for $C_{21}H_{37}NO_6SiSNa^+$ $[M + Na]^+$ 482.2003, found 482.1961.

Thioethers 14a–d

To a solution of **16** (1.5 mg, 4.4 μmol) and **17** (0.6 mg, 3.4 μmol) in MeCN (100 μL) was added K_2CO_3 (1.0 mg) at room temperature. After being stirred for 24 h, the mixture was filtered and concentrated under reduced pressure. To the residue was added 4 M HCl in dioxane (0.2 mL). After being stirred at room temperature for 1 h, the solvent was removed under reduced pressure. The obtained hydrolysate in H_2O (100 μL) were added L-FDVDA (5 mg/mL in acetone, 40 μL) and 1 M $NaHCO_3$ aqueous solution (20 μL). After being stirred at 50 °C for 2 h, the reaction was quenched with 2 M HCl (20 μL), evaporated, dissolved in MeCN (100 μL), filtered through a membrane filter (SHIMADZU, TORAST™ DISC, PTFE 0.22 μm) to give **14a** for the LC–MS analysis. Diastereomers **14b**–**14d** were prepared by the same procedure.

Natural **1** (ca 0.1 mg) and synthetic **1** (ca 0.1 mg) were hydrolyzed with 6 M HCl (200 μL) for 12 h at 105 °C, and then dried *in vacuo*. The obtained hydrolysate was dissolved in MeOH (100 μL), to which $SOCl_2$ (10 μL) was added at 0 °C. After 30 min the solution was dried *in vacuo*. The obtained mixture was dissolved in H_2O (50 μL), to which 1 M $NaHCO_3$ (20 μL) was added. The hydrolysate added L-FDVDA (0.5% w/v in acetone, 20 μL), and the mixtures were stirred for 2 h at 50 °C. The solution was cooled to room temperature, neutralized with 2 M HCl (20 μL), evaporated, dissolved in MeCN (100 μL), filtered through a membrane filter (SHIMADZU, TORAST™ DISC, PTFE 0.22 μm), and then analyzed by LC–MS with **14a**–**14d** [column: Cadenza CD-C18, 3.0 × 150 mm; eluent: MeCN/H_2O/formic acid = 60/40/0.1 (isocratic min), 0.2 mL/min; detection: ESI-positive].

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