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Thioamycolamides A–E, Sulfur-Containing Cyclicliopeptides Produced by the Rare Actinomycete *Amycolatopsis* sp.

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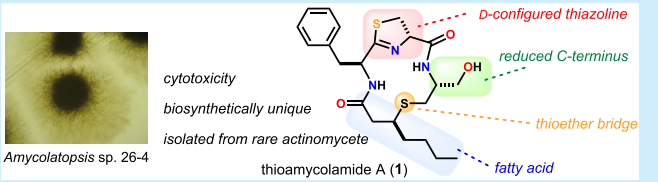
ABSTRACT: A series of novel sulfur-containing cyclicliopeptides named thioamycolamides A–E, with thiazoline, thioether rings, and fatty acid moieties, were identified from the culture broth of the rare actinomycete *Amycolatopsis* sp. 26–4. The planar structural elucidation was accomplished by HRMS and 1D/2D NMR spectroscopic data analyses. The absolute configurations were unambiguously determined by Marfey's method, CD spectroscopy, and synthesis of partial structures. Moreover, their growth inhibitory activities against human tumor cell lines were investigated.



Organosulfur compounds, such as thiols, sulfoxides, sulfones, thioesters, and thioamides, because of their reactive functions, have always been a research hotspot in organic chemistry.[1] As an essential element of organisms, sulfur also plays a critical role in primary metabolism, which has been discussed in various publications.[2] However, compared with other natural products, sulfur-containing characteristic secondary metabolites are relatively rare,[3] and we only have limited knowledge about their biological significance and biosynthetic mechanisms. Moreover, because of their novel structures and unique biological activities, the unusual enzyme involved in organosulfur natural products synthesis is becoming the focus of many researchers worldwide.[4]

As part of our research for structurally unique and biologically interesting natural products, we previously reported the isolation of several novel bioactive compounds from actinomycetes.[5] As a continuation of these studies, we investigated bioactive natural products from a library of rare actinomycetes derived from Iriomote Island near Okinawa, Japan. Based on our preliminary screenings using physicochemical properties including both mass spectrometry and UV spectroscopy, we isolated and identified a series of novel sulfur-containing heterocyclic lipopeptides named thioamycolamides A–E from the culture broth of *Amycolatopsis* sp. 26-4. Among these five metabolites with unprecedented carbon skeletons, thioamycolamide A (**1**) was produced as the major product by this Gram-positive bacterium (Figure 1). Herein, we describe the discovery, structure determination, cytotoxicity, and plausible biosynthetic route of thioamycolamides.

Thioamycolamide A (**1**) was isolated from the culture broth of *Amycolatopsis* sp. 26-4 (see Supporting Information), and then its structure was unveiled. The molecular formula was determined to be $C_{22}H_{31}N_3O_3S_2$ by the analysis of HRMS measurement (m/z 450.1853 $[M + H]^+$ calcd 450.1880). In order to elucidate the planar structure, detailed 1D and 2D

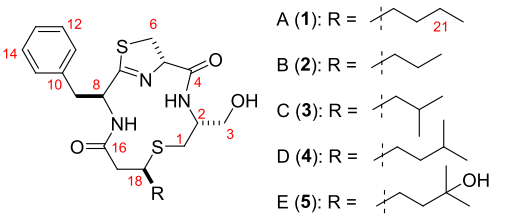


Figure 1. Structures of thioamycolamides A–E (**1**–**5**).

NMR experiments (COSY, HMBC, and HSQC) were carried out (Table S1, Figures S5–S9). The $^1H-^1H$ COSY cross peaks of H13/H12 and H12/H11 suggested the existence of a monosubstituted benzene ring. The HMBC correlations from methylene H9 to C10 and C11 confirmed a connection between C10 and C9. The COSY signals for H9/H8 and H8/8-NH, accompanied by the HMBC cross peaks from H9, H8, and 8-NH to C7, indicated a phenylalanine moiety. The carbonyl carbon at C16 was positioned beside 8-NH, which was inferred from the strong cross peak of H8 and 8-NH to C16 in the HMBC spectrum. The presence of an aliphatic chain moiety was established by the analysis of the $^1H-^1H$ COSY correlations; H17/H18, H18/H19, H19/H20, and H20/H21, and the HMBC data as shown in Figure 2. Moreover, from the HMBC signals, it was determined that C1 was connected with the aliphatic chain at C18 through a heteroatom. The $^1H-^1H$ COSY spectrum also revealed

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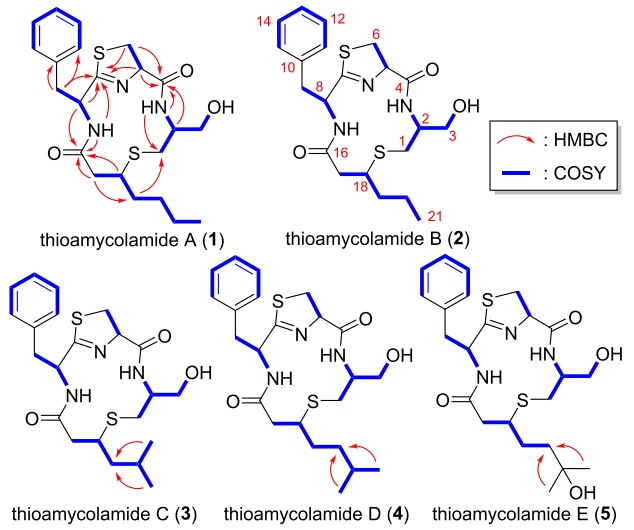


Figure 2. Planar structures, key HMBC correlations, and COSY correlations of thioamycolamides A–E (**1**–**5**).

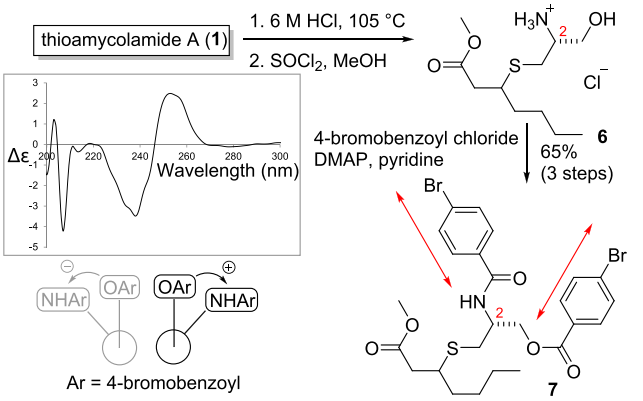
correlations between H1/H2, H2/H3, H3/3-OH, and H2/2-NH, and the HMBC correlations from H2 to C4 and 2-NH to C4 corroborated linkages between C1/C2, C2/C3, C3/3-OH, 2/2-NH, and 2-NH/C4. The C5–7 moiety was confirmed to be thiazoline from ^{1}H–^{1}H COSY correlations for H5/H6 and the HMBC correlations from H5 to C4/C7 and from H6 to C4/C5/C7, which would be generated by dehydration of cysteine. The heteroatom between C1 (δ 34.9 ppm) and C18 (δ 45.7 ppm) was assigned to be sulfur by the analysis of HRMS data and the chemical shifts of C1 and C18. Thus, the planar structure of thioamycolamide A was elucidated as shown in Figure 2 and included one phenylalanine, two cysteines, and a lipid moiety.

The absolute configurations of C5 and C8 were determined by the LC–MS-based Marfey's analysis.[6] At this stage, the peptide **1** should be carefully derivatized for the chromatographic analysis because the C_α isomerization of peptides would often be problematic, leading to the structural misassignments.[7] In particular, the reversible isomerizations of thiazoline have been exemplified under acidic or basic conditions.[8] To suppress the isomerization, the peptide was hydrolyzed by 2 M aqueous hydrogen chloride at 90 °C for 4 h. Then the hydrolysates were derivatized by L/D-FDLA[6] and analyzed by LC–MS. The comparison of the retention times between the standards and obtained derivertives shed light on the presence of L-Phe and D-Cys in **1** (Figure S2).

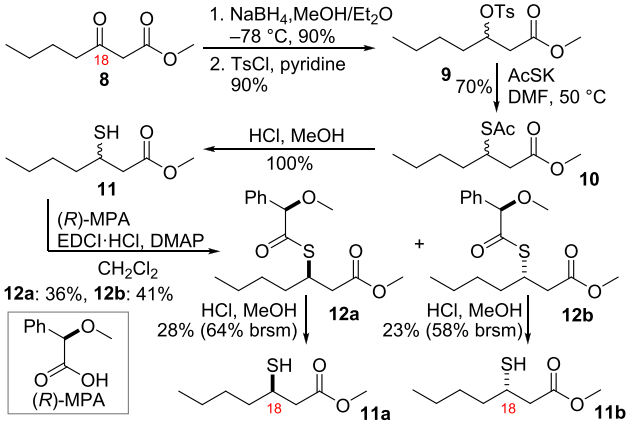
To determine the configuration of C2, 4-bromobenzoate **7** was synthesized (Scheme 1). Total hydrolysis of **1** followed by methyl esterification provided the key fragment **6**. Then **6** was condensed with 4-bromobenzoyl chloride to deliver **7** in 65% yield over three steps. Bromobenzoate **7** exhibited positive exciton split CD bands at 255 nm ($\Delta\varepsilon$ 2.4) and 238 nm ($\Delta\varepsilon$ −3.5), thereby indicating *R* configuration at C2 (Figure S1).[9]

The chirality of C18 was determined by the combination of the chemical synthesis and NMR analysis, which was the most challenging task in this structural elucidation because the thioether bridge is rare in nature. The required standard samples for the spectroscopic comparison, namely, the 2*R*18*R* and 2*R*18*S* isomers of **7**, were prepared by the chemical synthesis and optical resolution (Scheme 2). The selective reduction of β-ketoester **8** by $NaBH_4$ gave corresponding

Scheme 1. Synthesis of 7 from 1



Scheme 2. Synthesis and Optical Resolution of 11[a]



[a]brsm = based on recovered starting material.

racemic alcohol, and then the alcohol was converted to the tosylate **9**. The nucleophilic substitution reaction between **9** and AcSK (potassium thioacetate) delivered the thioester **10**. Hydrolysis of **10** furnished thiol **11**, and then racemic thiol **11** was treated with (*R*)-MPA (2-methoxy-2-phenylacetic acid), EDCI·HCl, and DMAP led to the diastereomixture of **12a**/**12b**, which could be separated by reversed-phase HPLC. Hydrolysis of thioesters **12a**/**12b** led to optically pure thiols **11a**/**11b**, respectively.

The absolute configuration of the thiols **11a** and **11b** were determined by applying Riguera's method in chiral thiols **11a**/**11b**.[10] Thioester **12b** is the enantiomer of **11a**-(*S*)-MPA, which could be considered as the same compound in ^{1}H NMR. The difference of the ^{1}H NMR chemical shifts ($\Delta\delta$) between **12a** and **12b** validated the absolute configurations of C18 as depicted in **11a**/**11b**, respectively (Figure 3).

With optically pure thiols **11a** and **11b** in hand, the standard diastereomers **7a** and **7b** for the structural determination were chemically constructed (Scheme 3). At the outset, Boc-L-Ser-OMe (**13**) was transformed into the corresponding tosylate,

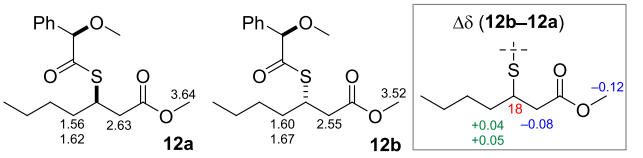


Figure 3. Stereochemical assignment of **11a** and **11b**.

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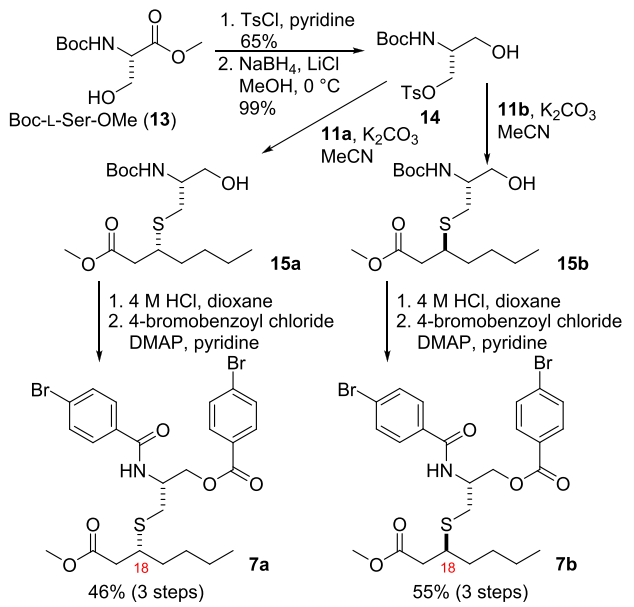
Scheme 3. Synthesis of 7a and 7b



and then its methyl ester was reduced by in situ generated $LiBH_4$, leading to **14**. Subsequently, tosylate **14** was separately coupled with enantiomeric thiols **11a** and **11b** to furnish thioethers **15a** and **15b**, respectively. Treatment of **15a**/**15b** with 4 M HCl liberated the corresponding amino alcohols, which were then condensed with 4-bromobenzoyl chloride to give **7a**/**7b**.

The ^{1}H NMR spectra of synthesized two diastereomers **7a** and **7b** were then compared with that of authentic **7** derived from natural **1**. As charted in Figure 4, the NMR data of **7b**

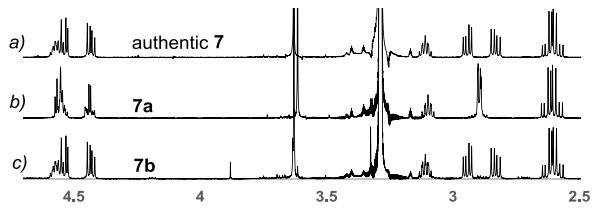


Figure 4. ^{1}H NMR spectra of authentic **7** and synthetic **7a**/**7b**. (a) Authentic **7**. (b) Synthetic **7a**. (c) Synthetic **7b**.

with C18(*S*)-configuration was identical to that of authentic **7**. Unifying the all data of our structural studies, the complete structure of **1** was elucidated as drawn in Figure 1.

The alkyl chain at C18 of each metabolite varied in terms of the number of carbon atoms and the branching pattern of the methyl groups. The carbon numbers found in the side chains of **1**−**5** were three, four, and five, and the terminal structure of the side chains was normal- (without branched methyl) or iso-type. These structural moieties were confirmed by HRMS and NMR analyses (Figure S3). The congeners were named in the same way as thioamycolamides B, C, D, and E, respectively. The peptides **1**−**5** were isolated from the same strain and exhibited almost identical NMR data in the main skeleton (Table S1), which suggests that they may originate from a similar biosynthetic pathway; the optical rotation of **1** ($[\alpha]_D^{20}$ −75.0) is close to those of **2** ($[\alpha]_D^{20}$ −91.9), **3** ($[\alpha]_D^{20}$ −54.1°), **4** ($[\alpha]_D^{20}$ −94.1), and **5** ($[\alpha]_D^{20}$ −114.1). Additionally, the ECD spectrum of **1** showed one positive Cotton effect at 222 nm and one negative Cotton effect at 252 nm, which were in good agreement with the other experimental curves, indicating the same absolute configurations at the chiral center of **2**−**5** (Figure S4).

The cytotoxicities of all isolated compounds were evaluated with two human cancer cell lines, fibrosarcoma HT1080 and cervix adenocarcinoma HeLa S3 (Table 1).[5d] Adriamycin was used as a positive control, with IC_{50} values of 0.77 and 0.43 μM, respectively. Peptide **1** and **4** with a four carbon chain length (*n*-butyl- or 2-methylbutyl) at C18 exhibited moderate cytotoxicity with IC_{50} values of 6.53−21.22 μM, whereas **5** with a 2-dimethylbutanol side chain had an IC_{50} value of greater than 100 μM, indicating the importance of the aliphatic property for their biological activities. Moreover, **2** and **3**, both of which have a three carbon chain length at C18, showed weak (IC_{50}, 78.57, 67.88 μM, respectively) or no cytotoxicity even at 100 μM, respectively. These structure−activity relationships (SAR) suggest that both hydrophobicity and the length of the side chain at C18 are crucial for exhibiting the cytotoxicity presumably based on the difference of membrane permeability.

Table 1. Cytotoxicties of 1−5 against Human Cancer Cells (IC_{50}, μM).[a]

sample	HT 1080	Hela S3
1	11.94 ± 1.43	21.22 ± 2.57
2	78.57 ± 12.86	67.88 ± 15.31
3	>100	>100
4	6.53 ± 0.36	9.34 ± 0.21
5	>100	>100
adriamycin	0.77 ± 0.06	0.43 ± 0.05

[a] IC_{50} values are shown as mean ± SD (n = 4).

Although the biosynthetic gene cluster of these peptides **1**−**5** has not been identified and the possibility of RiPPs pathway[11] cannot be ruled out, based on their structural features, the biosynthesis of thioamycolamides is reasonably proposed to be assembled by nonribosomal peptide synthetase (NRPS) using α,β-unsaturated fatty acids as the starter unit (Scheme S1).[12] The putative cyclization (Cy) domain catalyzes the generation of the thiazoline ring between L-Phe and the adjacent D-Cys residues via three chemical steps (condensation, cyclization, and dehydration).[4a,13] Then the second cysteine would be incorporated into the peptide chain, which would form the thioether linkage by Michael-type addition. In the canonical cases, a thioesterase (TE) domain is involved in the peptide release from the carrier protein. However, the C-terminus alcohol at C3 implies that these cyclic peptides would be released through reductase (R) domain-mediated four-electron reduction.[14] Identification of the biosynthetic gene cluster of thioamycolamides is currently underway in our laboratory.

In summary, the sulfur-containing cytotoxic cyclicpeptidyl metabolites, thioamycolamides A−E, were isolated from the culture broth of the rare actinomycete *Amycolatopsis* sp. 26−4. Their unprecedented structures were precisely elucidated by the combination of extensive spectroscopic analyses and chemical synthesis. These compounds exhibited varying cytotoxicities toward two human cancer cells. The differences in the activities seemed to be related to the length and methylation/hydroxylation patterns of the C18 side chain, which suggests that an expanded SAR study would be helpful

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for possible anticancer development of this class of compounds. The biosynthetic mechanisms and physiological functions of the thioamycolamides in the producing organism are also of considerable interest because of their highly modified skeletons.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c00776.

General experimental procedures, extraction and isolation of thioamycolamides A–E (**1**–**5**), cytotoxicity assays, putative biosynthetic pathway of **1**–**5**, synthesis of bromobenzoates (**7**, **7a**, **7b**), acid hydrolysis and Marfey's method of **1**, 1D NMR and HRMS spectra for compounds **1**–**5**, **7**, **9**–**12**, **14**; COSY, HSQC, HMBC, and CD spectra for compounds **1**–**5** (PDF)

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Notes

The authors declare no competing financial interest.

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