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Isolation, Structure Elucidation, and Total Synthesis of Tryptopeptins A and B, New TGF-β Signaling Modulators from *Streptomyces* sp.

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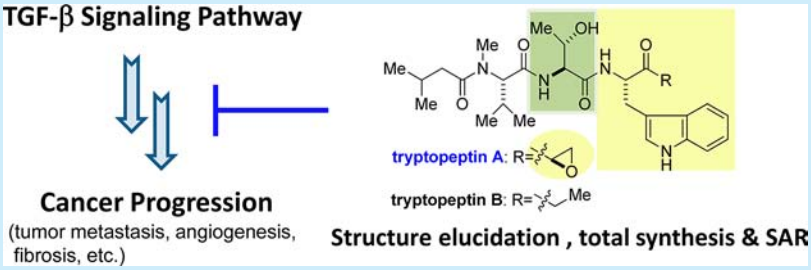
Supporting Information



ABSTRACT: Two new microbial metabolites, tryptopeptins A (**1**) and B (**2**), were isolated from the cultured broth of *Streptomyces* sp. KUSC-G11, as modulators of the transforming growth factor-β (TGF-β) signaling pathway. Their chemical structures consisting of isovalerate, *N*-Me-L-Val, L-*allo*-Thr, and a tryptophan-related residue were elucidated on the basis of spectroscopic analyses, while they were unambiguously determined by total syntheses. A structure−activity relationship (SAR) study using natural and synthesized tryptopeptins revealed the importance of the α,β-epoxyketone function located at the *C* terminus. These new TGF-β signaling modulators would be highly useful for development of new drug leads targeting TGF-β-related diseases such as fibrosis and cancer.

Transforming growth factor-β (TGF-β) is a key molecule for cancer progression, since it facilitates cancer metastasis, organ fibrosis, angiogenesis and immunosuppression.[1] For example, epithelial−mesenchymal transition (EMT) induced by TGF-β is a crucial event in tumor metastasis; polarized and immotile epithelial cells differentiate into motile mesenchymal cells to be transferred to another organ via blood or lymph vessels. To combat cancer, small molecules inhibiting the TGF-β signaling pathway have been developed, some of which are under clinical trial.[2] However, most chemicals target TGF-β receptors, and a variety of chemicals with unique modes of action are requisite for exploring the therapeutic potential of modulation of TGF-β signaling. During the course of our screening for novel TGF-β modulators, we have found two novel microbial metabolites from *Streptomyces* sp. KUSC-G11, designated tryptopeptins A (**1**) and B (**2**). We herein describe the isolation, structure elucidation and total synthesis of tryptopeptins. The structure−activity relationship (SAR) is also discussed using natural and synthetic derivatives.

We conducted a cell-based screening against thousands of microbe culture extracts to find that the culture extract of *Streptomyces* sp. KUSC-G11 exhibited potent inhibitory activity against TGF-β signaling. Bioassay-guided fractionation by silica gel chromatography and repetitive reversed phase HPLC afforded novel metabolites **1** and **2** (Figure 1).

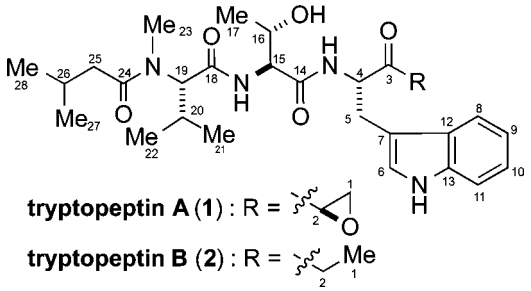


Figure 1. Structures of tryptopeptins A (**1**) and B (**2**).

Tryptopeptin A (**1**) was isolated as an optically active colorless oil ($[\alpha]_D^{27}$ = −94.3, *c* 0.010, MeOH). The molecular formula was determined to be $C_{28}H_{40}N_4O_6$ with 11 degrees of unsaturation, based on HR-FABMS (*m*/*z* 529.3028 $[M + H]^+$, Δ = +0.2 mmu, calcd. for $C_{28}H_{41}N_4O_6$, 529.3026) and 1D NMR spectra. Diagnostic signals in the NMR spectra indicated the peptidic nature of **1**; two amide protons (δ_H 7.12 and 6.78 ppm) and one *N*-methyl signal (δ_H 2.87 ppm) appeared in the ^{1}H NMR spectrum, while the ^{13}C NMR spectrum exhibited three amide carbonyl signals (δ_C 174.8, 171.6, and 171.1 ppm).

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2D NMR data including DQF-COSY, TOCSY, HSQC and HMBC spectra revealed the presence of one residue each of isovalerate, *N*-methylvaline (*N*-Me-Val) and threonine (Thr). The remaining portion was $C_{13}H_{13}N_2O_2$. Five aromatic proton signals (δ_H 7.62−7.08 ppm), one exchangeable NH signal (δ_H 9.17 ppm) and eight aromatic carbon signals (δ_C 137.5−110.2) indicated the presence of an indole ring, while a spin-system between an amide proton (4-NH), a methine proton (H-4) and methylene protons (H-5ab) was identified in the DQF-COSY spectrum, suggesting that **1** includes a tryptophan-related residue. The DQF-COSY and TOCSY spectra included another spin system consisting of one methine (H-2) and one methylene (H-1ab). The NMR signal that remains to be assigned was one carbonyl (C-3). HMBC correlations were observed from H-1, H-2, H-4 and H-5 to this carbon, indicating that the two spin systems H-1 to H-2 and 4-NH to H-5ab were connected through C-3 carbonyl. Finally, the unsaturation degree indicated the presence of an epoxide group, which was supported by characteristic chemical shift values for C-1 and C-2 (δ_C 47.7 and 53.0 ppm, respectively), deducing the structure of an *α,β*-epoxyketone functionality.

The sequence of amino acid residues was determined by interpretation of the HMBC data; the HMBC correlations from H-4, 4-NH and H-15 to C-14, from 15-NH and H-19 to C-18, and from H-19, H-23, H-25a and H-25b to C-24 allowed us to determine the sequence as follows: from *N*-terminus, isovalerate, *N*-Me-Val, Thr and *C*-terminally modified tryptophan-related residue possessing *α,β*-epoxyketone (Trp-EK) (Figure 2).

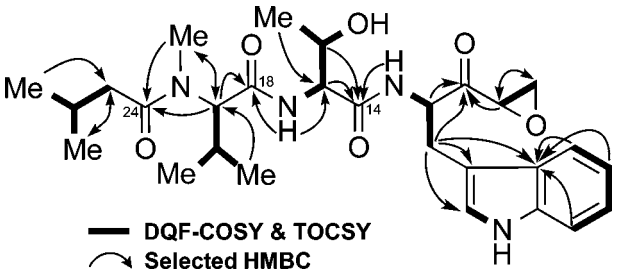


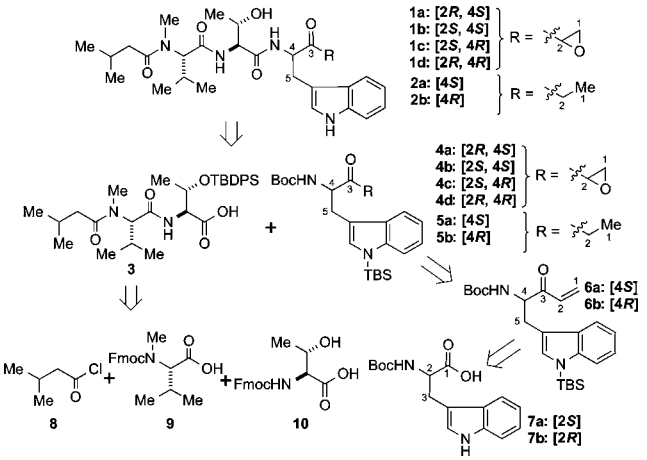
Figure 2. 2D NMR correlations in tryptopeptin A (**1**).

Tryptopeptin B (**2**) was obtained as a colorless oil ($[\alpha]_D^{25}$ = −96.0, *c* 0.010, MeOH). The molecular formula of $C_{28}H_{42}N_4O_5$, which was deduced by HR-ESIMS (*m/z* 537.3066 [M + Na]$^+$, Δ = +1.3 mmu, calcd. for $C_{28}H_{42}N_4NaO_5$, 537.3053), indicated loss of one oxygen atom and decrease of one degree of unsaturation when compared to the metabolite **1**. The ^{1}H and ^{13}C NMR spectra strongly suggested lack of an epoxide group. Instead, signals corresponding to an ethyl group (δ_H 0.89 and 2.45 ppm, and δ_C 7.6 and 33.7 ppm) were observed, indicating that the epoxyketone function of **1** was substituted by an ethyl ketone group. Detailed analysis of the 2D NMR data confirmed the presence of the ethyl ketone group, and we could determine the planar structure of **2** as depicted in Figure 1.

We next tried to determine the stereochemistry of tryptopeptins. The advanced Marfey's method[3] was applied to determine the absolute configuration of amino acids. The acid hydrolysate of **2** was condensed with Marfey's reagents L- or D-FDLA, and the products were analyzed by LC−MS. L-FDLA derivative of *N*-Me-valine was eluted faster than the D-FDLA derivative on reversed phase HPLC, which indicated the presence of *N*-Me-L-Val[3] in **2**. The configuration of Thr was determined to be L-*allo*-Thr by comparing the retention times with those of FDLA-derivatized authentic Thr and *allo*-Thr (Figure S15, Supporting Information). Unfortunately, however, we could not determine the configuration of C-4 since FDLA derivatives of the tryptophan-related residue were not detected. By contrast, the configuration of the Trp-EK in metabolite **1** was only speculatively deduced by the NMR analysis (Figure S14). To determine the absolute configuration of these tryptopeptins, we conducted the total synthesis of tryptopeptins.

Tryptopeptin A (**1**) seems to have the same configuration to that of **2**; these two molecules shared the same amino acid backbone, suggesting that they are biosynthesized by a common nonribosomal peptide synthetase (NRPS) machinery. In addition, they exhibited very similar ^{1}H NMR signals especially for the backbone protons. Retrosynthetic analysis of tryptopeptins **1** and **2** is shown in Scheme 1. We planned to

Scheme 1. Retrosynthetic Analysis of Four and Two Possible Diastereomers of Tryptopeptins A (1a−1d) and B (2a and 2b), Respectively



synthesize them by conjugating the corresponding *N*-terminal and *C*-terminal fragments; **1** could be synthesized with **3** and **4**, and **2** from **3** and **5**, respectively. The common segment **3** consisting of isovalerate, *N*-Me-L-Val and L-*allo*-Thr could be obtained by a conventional peptide synthesis methodology from **8**−**10**. In contrast, we had to synthesize a set of stereoisomers of the *C*-teminal fragment for determining the configuration of the natural products. We expected that four diastereomers **4a**−**4d** could be obtained by epoxidation of an *α,β*-enone **6a** or **6b** (**6a** and **6b** with 4*S* and 4*R* configuration, respectively), while another set of two diastereomers **5a** and **5b** could be synthesized by reduction of **6a** and **6b**, respectively. The *α,β*-enones **6a** and **6b** could be synthesized from commercially available *N*-Boc-L-tryptophan **7a** and its enantiomer **7b**, respectively.

We began the synthetic work by preparing chiral tryptophan-related residues (Scheme 2A). We first synthesized **6a**, a common intermediate for **4a**, **4b** and **5a**. *N*-Boc-L-Trp **7a** was converted to Weinreb's amide,[4] followed by protection of the indole nitrogen with a TBS group to afford **11**. Grignard reaction of **11** with vinylmagnesium chloride gave the corresponding enone **6a**. We next tried to synthesize epoxyketone **4a** and **4b** from **6a**. We first attempted Sharpless asymmetric epoxidation or dihydroxylation to an allylic alchol

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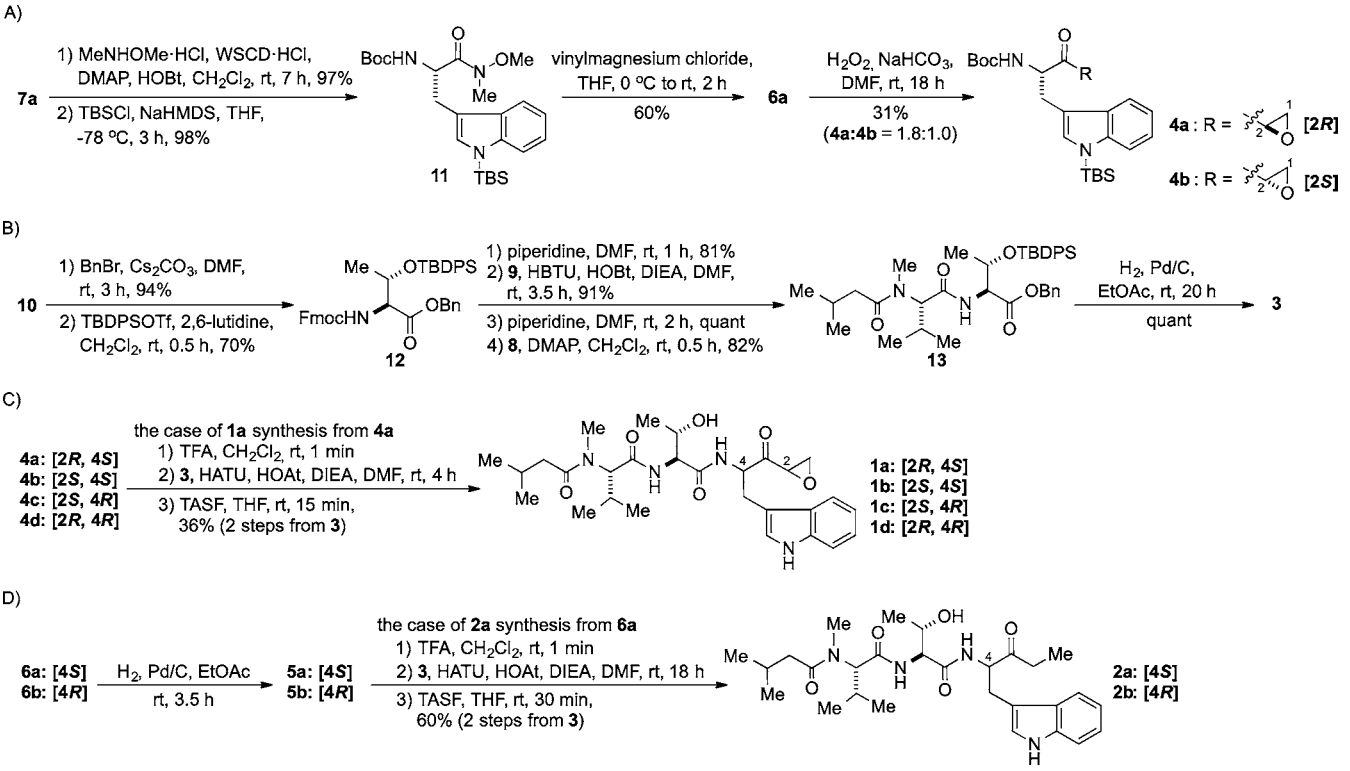
Scheme 2. Syntheses of Tryptopeptin A (1a−1d) and B (2a and 2b) Diastereomers[a]



[a](A) Syntheses of tryptopeptin A *C*-terminal fragments **4a** and **4b**. (B) Common *N*-terminal fragment synthesis. (C) Syntheses of tryptopeptin A diastereomers (**1a−1d**). (D) Syntheses of tryptopeptin B diastereomers (**2a** and **2b**).

derivative that was prepared from **6a** with $NaBH_4$; however, these strategies were not satisfactory due to the low reaction yields. We then tested a nonstereoselective route, which can conveniently furnish diastereomers. After testing several epoxidation conditions, we found that treatment of enone **6a** with H_2O_2/$NaHCO_3$ afforded diastereomers of α,β-epoxyketones **4a** and **4b** (31%, **4a**:**4b** = 1.8:1.0). We purely separated two products by reversed phase HPLC. The absolute configuration of the epoxide (C2-position) was determined by derivatization and spectroscopic analysis (Scheme S2, Figures S16−S17). Another set of diastereomers **4c** and **4d** were prepared from **7b** in a similar fashion (Scheme S3). On the other hand, hydrogenation of **6a** successfully furnished the ethyl ketone residue **5a**, in addition, the enantiomer **5b** was prepared from **6b** (Scheme 2D).

The *N*-terminal fragment was constructed by applying conventional Fmoc chemistry (Scheme 2B). Fmoc-protected L-*allo*-Thr (**10**) was converted to the benzyl ester and its secondary hydroxyl group was protected by the TBDPS group to give **12**. After removal of the Fmoc group, *allo*-Thr was coupled with **9**, followed by Fmoc deprotection and acylation with **8**, which afforded the acylated dipeptide **13**. Subsequent removal of the benzyl ester by hydrogenation yielded the *N*-terminal fragment **3**.

Synthesis of tryptopeptins was accomplished by conjugation of the corresponding *C*-terminal and *N*-terminal fragments followed by deprotection. To obtain four possible diastereomers of **1**, the *N*-terminal fragment **3** and the *C*-termical fragments derived from **4** were condensed; *C*-terminal fragments (**4a**, **4b**, **4c** or **4d**) were deprotected with TFA[5] and immediately coupled with the *N*-terminal fragment **3** using HATU. Finally, we carefully deprotected the secondary alcohol with TASF to afford the four diastereomers **1a−1d**, respectively[6] (Scheme 2C, Scheme S4). With four possible diastereomers of natural tryptopeptin A (**1**) in hand, we compared their physicochemical properties including NMR spectra to that of the natural one. We found that only synthetic **1a** exhibited similar properties to those of natural **1** (Table S3, Figures S19−S22, S55); for example, optical rotation values of natural **1** and synthetic **1a** were close (**1**: $[\alpha]_D^{27}$ = −94.3, *c* 0.010, MeOH, **1a**: $[\alpha]_D^{27}$ = −96.9, *c* 0.064, **1b**: $[\alpha]_D^{26}$ = −157.2, *c* 0.25, MeOH, **1c**: $[\alpha]_D^{26}$ = −127.1, *c* 0.25, MeOH, **1d**: $[\alpha]_D^{25}$ = −50.3, *c* 0.25, MeOH). Thus, we concluded that the natural product **1** consists of isovalerate, *N*-Me-L-Val, L-*allo*-Thr and a tryptophan-related residue (Trp-EK) with 2*R*,4*S* configurations.

Two possible diastereomers of tryptopeptin B (**2**) were synthesized from the *C*-terminal fragment **5a** or **5b** and the *N*-terminal **3** in a similar way as described above; *N*-Boc deprotection, peptide condensation and silyl-deprotection afforded **2a** and **2b**, respectively (Scheme 2D). We compared their physicochemical properties and found that **2a**, not **2b**, exhibited similar properties to those of natural **2** (Table S4, Figures S23−S27 and S56), concluding the configuration of 4*S*. As predicted above, tryptopeptins **1** and **2** shared common absolute configurations.

With two natural and six synthesized tryptopeptins in hand, SAR study was conducted. Among compounds tested, tryptopeptin A (**1**) showed the most potent inhibitory activity with the IC_{50} of 1.0 μM against TGF-β signaling without cytotoxicity (Figure 3 and S57). It is noteworthy that a loss of an epoxide moiety of tryptopeptins caused a significant decrease in the TGF-β inhibitory activities; the IC_{50} values for **2** and **2b** are 55 and 70 μM, respectively. We also found that

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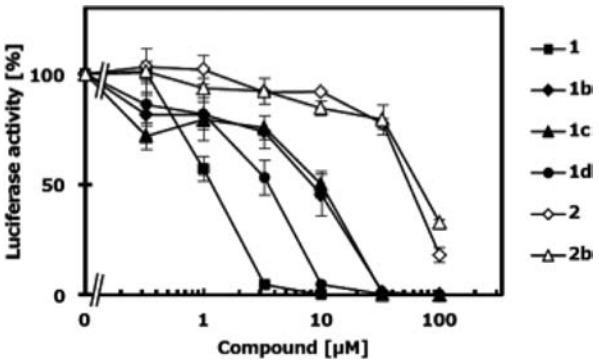

Figure 3. TGF-β signaling inhibitory activities of tryptopeptin A (**1**), tryptopeptin B (**2**) and their synthetic diastereomers (**1b**–**1d** and **2b**).

the configuration of the epoxide group affected the inhibitory activity; the IC_{50} values of **1**, **1b**, **1c**, and **1d** were 1.0, 8.0, 10.0, and 3.2 μM, respectively, indicating that the diastereomers with 2*S* configuration exhibited weaker inhibition than the compounds with 2*R* configuration (Figure 3). Similar tendency was observed in the growth inhibitory activities against HeLa cells (Figure S58). Importantly, natural tryptopeptins (**1** and **2**) and synthetic tryptopeptins with the same configuration (**1a** and **2a**) exhibited the same biological activities (Figures S55 and S56), which also confirmed the absolute configuration of the natural tryptopeptins. Taken together, tryptopeptin A (**1**) with 2*R*,4*S* configurations exhibited the most potent biological activities, suggesting that target molecule(s) in a cell might recognize the configurations of the epoxyketone.

So far, several compounds possessing a peptidyl α,β-epoxyketone function have been reported from microorganisms, e.g., histone deacetylase inhibitor trapoxins,[7] and 20*S* proteasome inihibitors epoxomicin[8a] and eponemycins.[8b] The α,β-epoxyketone group has been revealed to play a crucial role in the enzyme inhibition, by forming a covalent bond with the catalytic residue in the enzyme.[9] In this study, we showed the presence and the absolute configuration of epoxide in tryptopeptins affected the potency of TGF-β signaling inhibition. It is likely that we can fish the cellular targets taking advantage of the reactivity of the epoxyketone group. Synthesis and evaluation of tryptopeptin probes are under way in our laboratory.

Tryptopeptins consist of four blocks; among them, L-*allo*-Thr is a rare residue in nature, while there is no report on the *C*-terminally modified tryptophan-related residue. The peptide backbones might be constructed by NRPS and *C*-terminus of L-tryptophan is likely elongated by polyketide synthase (PKS), followed by some modifications to form α,β-epoxyketone group in compound **1**. Recently, the biosynthetic gene clusters of epoxomicin and eponemycin were reported, whereas the mechanism of the *C*-terminus modification is unknown.[10] Genomic sequencing of the tryptopeptin producer *Streptomyces* sp. KUSC-G11 would unveil the biosynthetic mechanism of tryptopeptins including generation of the Trp-EK.

In summary, we isolated two novel natural products, tryptopeptins A (**1**) and B (**2**), from the cultured broth of *Streptomyces* sp. as TGF-β signaling inhibitors. Chemical structures were unambiguously determined by spectroscopic analyses and total syntheses. Since TGF-β signaling is deeply involved in cancer progression, understanding modes of action of tryptopeptins would open a way for cancer chemotherapy.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compound data, tables of NMR data, 1D and 2D NMR spectra, and results of biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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