The Journal of Antibiotics (2017) 70, 718–720

www.nature.com/ja

NOTE

Precursor-directed *in situ* synthesis of Saccharothriolides G and H by the Actinomycete *Saccharothrix* sp. A1506

Shan Lu[1], Shinichi Nishimura[1], Masashi Ito[2], Taira Kato[2] and Hideaki Kakeya[1]

The Journal of Antibiotics (2017) **70,** 718–720; doi:10.1038/ja.2016.153; published online 21 December 2016

Rare actinomycetes are attractive resources of secondary metabolites that show biological activities with unprecedented chemical structures.[1,2] Genus *Saccharothrix* is a rare actinomycete that has furnished an increasing number of bioactive metabolites.[3–7] Our recent studies of *Saccharothrix* sp. A1506 have afforded saccharothriolides A–F (**3–8**) (Supplementary Figure S1).[8,9] Saccharothriolides possess unique phenyl-substituted 10-membered macrolide structures, and some exhibited moderate cytotoxicity to human fibrosarcoma HT1080 cells. Since they contain a variety of substituents at C-7, we expected the presence of 'precursor A', with an α,β-unsaturated ketone that can function as a Michael acceptor.[8] However, to date, our attempts to isolate precursor A from culture broth have not been successful, probably because of its high reactivity. Instead, we have investigated the presence of precursor A by modifying the culture conditions. When we removed tryptophan from the culture media, precursor A was still not observed in the LC-MS analysis, and moreover, no production of saccharothriolides A (**3**) and B (**4**) was observed (Figure 1b). As expected, saccharothriolides A (**3**) and B (**4**) were generated when aryl amines, predicted metabolites of tryptophan, were added as nucleophiles to the culture media (Figure 1). Additionally, new congeners saccharothriolides G (**1**) and H (**2**) were obtained when we used 2-methoxyaniline (*o*-anisidine) as a nucleophile. Herein, we report the precursor-directed *in situ* synthesis (PDSS) of saccharothriolides including new congeners, and their biological activities.

Saccharothriolides A (**3**) and B (**4**) are expected to be Michael addition products of precursor A and the amino aryl groups, anthranilic acid and 2-aminophenol, respectively. The biosynthetic origin of anthranilic acid and 2-aminophenol have been proposed to be the tryptophan which is abundant in the culture medium.[10–12] To avoid the effect of tryptophan on the production of main product **3**, we supplemented tryptophan-free media with the nucleophilic reagents anthranilic acid (10 g l^{-1}) or 2-aminophenol (10 g l^{-1}). There were obvious changes in the LC-MS profile, suggesting the production of **3** or **4** (Figure 1). These results supported the presence of precursor A as a Michael acceptor, and indicated that we can explore *in situ* synthesis to obtain further saccharothriolide analogs by simply adding nucleophilic substituents.

Previous structure–activity relationship (SAR) studies using saccharothriolides A–F (**3–8**, Supplementary Figure S1) revealed the importance of the substituent at C-2″ on their cytotoxicity;[8,9] metabolites possessing an alcohol group showed activity, while those possessing a carboxylic acid were less potent. To investigate the necessity of the free hydroxy group, we planned to obtain 2-methoxyaniline-substituted saccharothriolide analogs by PDSS. *Saccharothrix* sp. A1506 was cultivated in 3 l of tryptophan-free medium for 4 days, followed by the addition of 2-methoxyaniline (10 g l^{-1}) and acetone (1:1 *v/v*), then shaken at 4 °C for 1 day. The reaction mixture was extracted, and LC-MS-guided fractionation was carried out to yield two new analogs, saccharothriolide G (**1**) and its C-2 epimer saccharothriolide H (**2**) (Figure 2a).

Saccharothriolide G (**1**) was obtained as a light-yellow oil with $[\alpha]_D^{20}$ −81.6 ($c = 0.31$, MeOH). The molecular formula was determined to be $C_{26}H_{33}NO_7$ by HR-ESI-MS (*m/z* 472.2321 $[M+H]^+$, calcd 472.2335), revealing that its molecular size was 14 Da larger than saccharothriolide B (**4**). ^1H and ^{13}C NMR data resembled those of **4** (Supplementary Table S1, Supplementary Figure S2), except for the presence of a methoxy group (δ_H 3.92, δ_C 56.5) and chemical shift differences in the amino aryl signals. The downfield shift of C-2″ (δ_C 148.8 in **1**, δ_C 146.0 in **4**) and upfield shift of C-3″ (δ_C 111.4 in **1**, δ_C 115.2 in **4**) suggested that **1** possessed a methoxy group instead of a phenolic hydroxy group at C-2″. Installation of 2-methoxyaniline (o-anisidine) was unambiguously determined by HMBC correlations from H-6″ to C-2″, H-3″ to C-1″, and from the methoxy proton 2″-OCH_3 to C-2″, along with the ^1H-^1H COSY correlations from H-3″

[1]Division of Bioinformatics and Chemical Genomics, Department of System Chemotherapy and Molecular Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan and [2]Bioresource Laboratories, MicroBioPharm Japan Co Ltd (MBJ), Shizuoka, Japan
Correspondence: Professor H Kakeya, Division of Bioinformatics and Chemical Genomics, Department of System Chemotherapy and Molecular Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail: scseigyo-hisyo@pharm.kyoto-u.ac.jp
We celebrate the 2015 Novel Prize in physiology or Medicine of Professor Satoshi Ōmura and his pioneering work and long-lasting contributions to the splendid study on numerous microbial metabolites.
Received 28 October 2016; revised 16 November 2016; accepted 20 November 2016; published online 21 December 2016

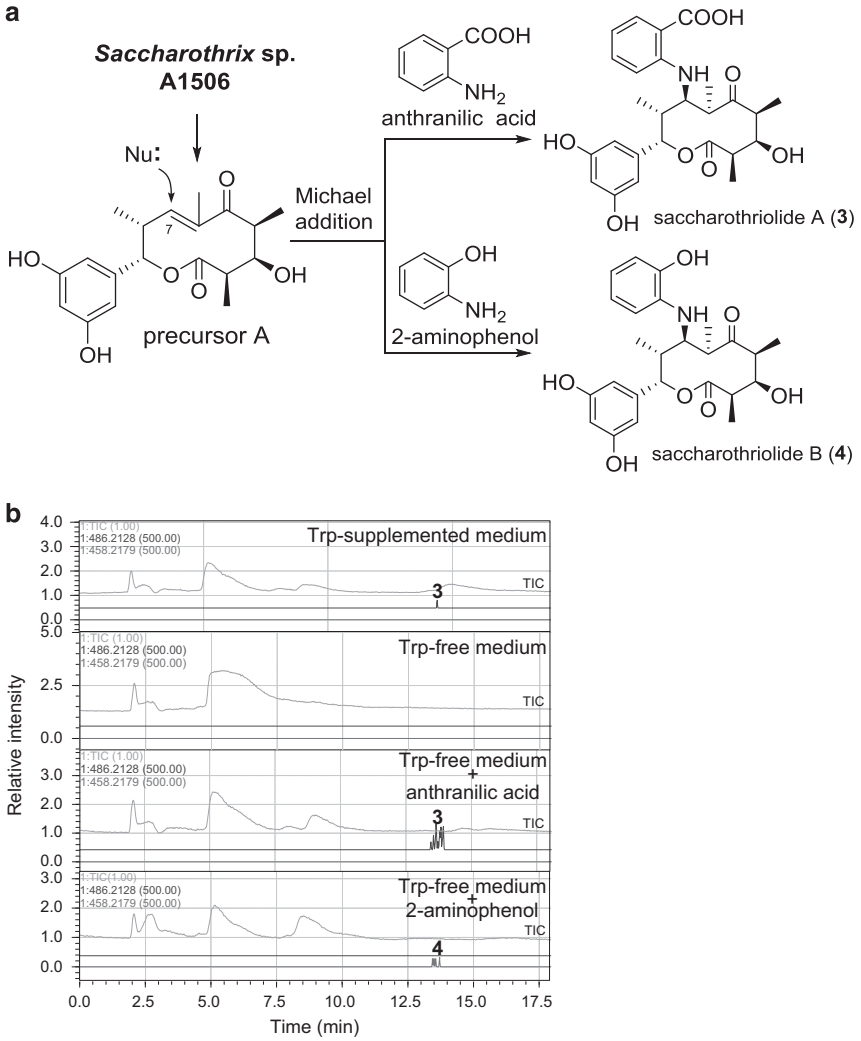

Figure 1 Structures of saccharothriolides A (**3**) and B (**4**) and their biosynthesis. (**a**) Plausible biosynthetic pathway of saccharothriolides. Precursor A is a Michael acceptor, to which anthranilic acid or 2-aminophenol attacks to yield saccharothriolide A (**3**) and B (**4**), respectively. (**b**) Production of saccharothriolides A (**3**) and B (**4**) in various culture conditions. LC-MS analyses were examined for crude extracts (PEGASIL ODS SP100, ϕ 3×250 mm, 0.2 ml min^{-1}, 40% aq. MeCN with 0.1% formic acid). Total ion chromatogram (TIC) and ion chromatograms for 486.2128 (up) and 458.2179 (down) (corresponding to $[M+H]^+$ for **3** and **4**, respectively) are shown. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

to H-6″. The planar structure of the molecule was deduced by the COSY, HMQC and HMBC data (Figure 2b). The ^{1}H-^{1}H COSY data revealed the presence of three spin systems: CH_3-10/H-2/H-3/H-4/CH_3-11, CH_3-12/H-6/H-7/H-8/CH_3-13, and H-3″/H-4″/H-5″/H-6″ (Figure 2b). HMBC correlations from CH_3-11/CH_3-12 to C-5 connected C-4 and C-6 through a ketone group. HMBC correlations from CH_3-10 to carbonyl C-1, and from H-9 to CH_3-13/C-1, combined with the down-field shifted chemical shift of H-9 (δ_H 5.49), connected C-2 and C-9 through an ester bond, leading to the formation of the 10-membered lactone ring (Figure 2b). The meta-disubstituted benzene ring was connected to C-9 based on the HMBC correlations from H-9 to the aromatic carbons C-2′/6′ and C-1′.

The relative stereochemistry of **1** was determined by the NOESY and $^3J_{H\text{-}H}$ data (Figure 2b). NOESY cross peaks between H-2 and H-4, and between H-3 and H-2/CH_3-10/H-4/CH_3-11, indicated that H-2, -3, and -4 are on the same α face. NOESY correlations between H-6 and CH_3-13/CH_3-11 indicated that H-6 and H-8 have α and β configurations, respectively. The NOESY correlations between H-7 and H-6/H-8/CH_3-12/CH_3-13 indicated that H-7 also has an α configuration, while the β-orientation of H-9 was revealed by the correlations between the aromatic proton H-6′ and H-8/CH_3-13. This result was also supported by the similar $^3J_{H\text{-}H}$ values between **1** and **4**. Thus, the relative configurations were deduced to be 2*R**, 3*R**, 4*S**, 6*R**, 7*R**, 8*R**, and 9*S**. The absolute stereochemistry of **1** was determined by comparing the ECD spectra of **1** and **4**. The ECD spectrum of **1** showed characteristic Cotton effects at 212 ($\Delta\varepsilon$, −29.3), 252 ($\Delta\varepsilon$, +15.9), and 296 ($\Delta\varepsilon$, −6.5) nm, which overlapped well with the spectrum of **4** (Figure 2c). Thus, the absolute configuration of **1** was established to be 2*R*, 3*R*, 4*S*, 6*R*, 7*R*, 8*R*, and 9*S*.

Saccharothriolide H (**2**) was obtained as a light yellow oil with $[\alpha]_D^{20}$ −81.2 (c = 0.05, MeOH). The molecular formula was determined to be $C_{26}H_{33}NO_7$ by HR-ESI-MS (*m/z* 472.2325 $[M+H]^+$, calcd 472.2335), being the same as that of saccharothriolide G (**1**). ^{1}H and ^{13}C NMR data of **2** resembled those of **1**, while differences were observed for the chemical shifts of H-2, H-3, CH_3-10, and CH_3-11 in the right half of the lactone ring (Supplementary Table S1). Notably, the chemical shifts of the lactone ring were identical to those of saccharothriolide E (**7**), a C-2 epimer of **4**. From these results, **2** was deduced to be an epimer of **1** at C-2. The absolute stereochemistry of

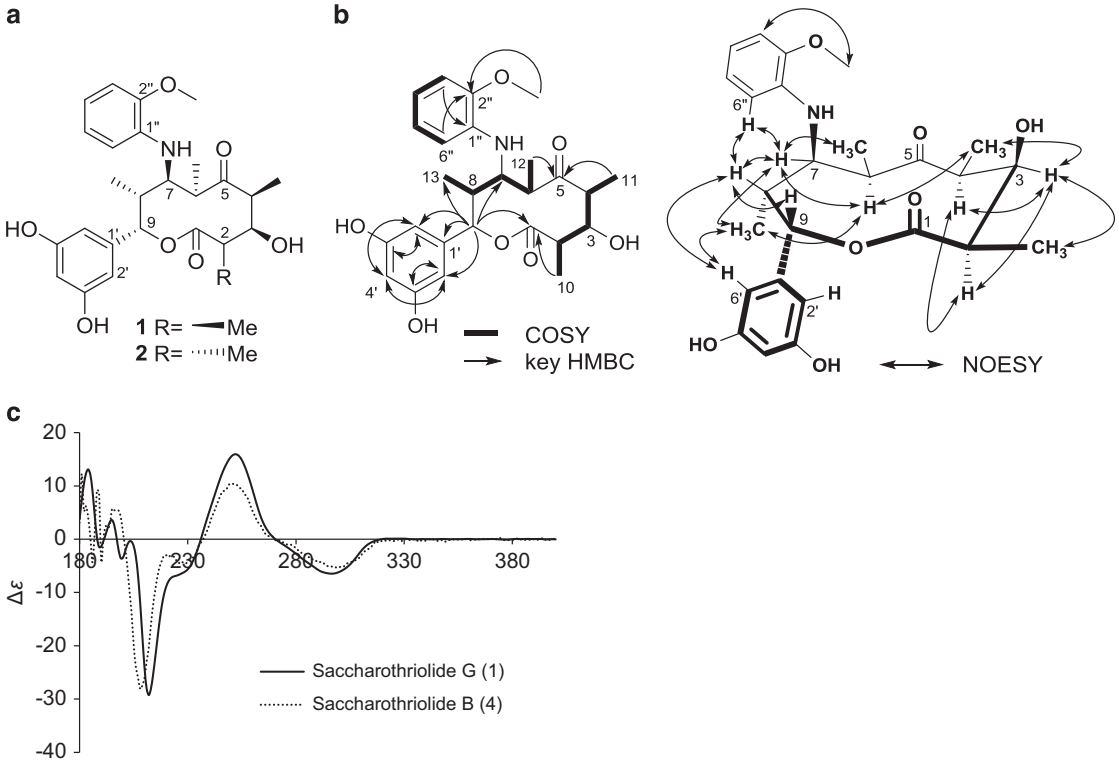

Figure 2 Structure elucidation of saccharothriolide G (**1**). (**a**) Chemical structures of saccharothriolides G (**1**) and H (**2**). (**b**) ^{1}H-^{1}H COSY (left, bold) correlations, and selected HMBC (left, arrow) and NOESY (right) correlations for saccharothriolide G (**1**). (**c**) Experimental ECD spectra of saccharothriolide G (**1**) (solid line) and saccharothriolide B (**4**) (dotted line).

2 was proven to be 2*S*, 3*R*, 4*S*, 6*R*, 7*R*, 8*R* and 9*S*, the same as that of **7** on the basis of the coupling constants and optical rotation values.[9]

We previously investigated the SAR of saccharothriolides A–F (**3**–**8**) which revealed that substitution at C-7 affects the cytotoxicity against human fibrosarcoma HT1080 cells.[8,9] Saccharothriolides B (**4**) and E (**7**), both of which have a 2-aminophenol group at C-7, exhibited moderate cytotoxicity (IC_{50} values, 13.9 and 29.2 μM, respectively). When the C-7 substituent was anthranilic acid or a hydroxyl group, that is, saccharothriolides A (**3**), D (**6**) or C (**5**), compounds were inactive even at 100 μM. To further analyze the structure-activity relationship of these 10-membered macrolides, we examined the cytotoxicity of saccharothriolides G (**1**) and H (**2**) against HT1080 cells. Saccharothriolide G (**1**) exhibited weak activity (IC_{50} value, 53.5 μM), making it less potent than its C-2 epimer saccharothriolide H (**2**) (IC_{50} value, 24.8 μM). These results not only confirmed the importance of the phenolic hydroxyl group at C-2″ in the observed cytotoxicity, but also suggested that the cytotoxicity was sensitive to the stereochemistry of C-2.

In summary, two new Michael addition products, saccharothriolides G (**1**) and H (**2**), were synthesized *in situ* and isolated. Their chemical structures were spectroscopically determined and their cytotoxicities were evaluated to determine the importance of the free hydroxyl group at C-2″ and the stereochemistry of C-2. Further precursor-directed *in situ* synthesis (PDSS) and isolation of precursor A are ongoing in our laboratory.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported in part by research grants from the Japan Society for the Promotion of Science (JSPS), the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and the Japan Agency for Medical Research and Development (AMED).

1 Bérdy, J. Bioactive microbial metabolites. *J. Antibiot.* **58**, 1–26 (2005).
2 Kakeya, H. Natural products-prompted chemical biology: Phenotypic screening and a new platform for target identification. *Nat. Prod. Rep.* **33**, 648–654 (2016).
3 Murakami, R. *et al.* Ammocidins B, C and D, new cytotoxic 20-membered macrolides from *Saccharothrix* sp. AJ9571. *J. Antibiot.* **62**, 123–127 (2009).
4 Merrouche, R. *et al.* Dithiolopyrrolone antibiotic formation induced by adding valeric acid to the culture broth of Saccharothrix algeriensis. *J. Nat. Prod.* **73**, 1164–1166 (2010).
5 Boubetra, D. *et al.* Taxonomy and chemical characterization of new antibiotics produced by *Saccharothrix* SA198 isolated from a Saharan soil. *Microbiol. Res.* **168**, 223–230 (2013).
6 Wang, X. L., Tabudravu, J., Jaspars, M. & Deng, H. Tianchimycins A–B, 16-membered macrolides from the rare actinomycete *Saccharothrix xinjiangensis*. *Tetrahedron* **69**, 6060–6064 (2013).
7 Gan, M. *et al.* Saccharothrixones A-D, tetracenomycin-type polyketides from the marine-derived Actinomycete *Saccharothrix* sp. 10-10. *J. Nat. Prod.* **78**, 2260–2265 (2015).
8 Lu, S. *et al.* Saccharothriolides A-C, novel phenyl-substituted 10-membered macrolides from a rare actinomycete *Saccharothrix* sp. *Chem. Commun.* **51**, 8074–8077 (2015).
9 Lu, S., Nishimura, S., Ito, M., Tsuchida, T. & Kakeya, H. Isolation and structure elucidation of cytotoxic saccharothriolides D to F from a rare actinomycete *Saccharothrix* sp. and their structure-activity relationship. *J. Nat. Prod.* **79**, 1891–1895 (2016).
10 Dalgliesh, C. E., Knox, W. E. & Neuberger, E. Intermediary metabolism of tryptophan. *Nature* **168**, 20–22 (1951).
11 Pan, E. *et al.* Precursor-directed generation of amidine containing ammosamide analogs: ammosamides E-P. *Chem. Sci.* **4**, 482–488 (2013).
12 Husted, R. R. Biosynthesis and reactions of cyclic hydroxamates in maize. *Retrospective Theses and Dissertations, Iowa State University. Paper 3477* (1968).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)