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The absolute stereochemistry of anachelins, siderophores from the cyanobacterium *Anabaena cylindrica*

Yusai Ito, Keishi Ishida, Shigeru Okada* and Masahiro Murakami

Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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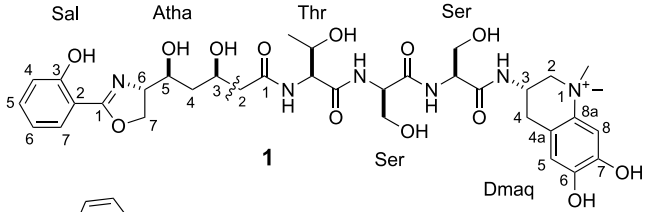
Abstract—The absolute stereochemistry of anachelins (**1** and **2**), siderophores isolated from the freshwater cyanobacterium *Anabaena cylindrica*, was determined via the application of Boc-phenylglycine and Mosher's method. Consequently, it was revealed that a 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-6,7-dihydroxyquinolinium unit (Dmaq) has 3*S* (eq.) configuration, and a 6-amino-3,5,7-trihydroxy-heptanoic acid unit (Atha) has 3*R*, 5*S*, 6*S* configuration. The 6*S* configuration of Atha suggested that L-Ser was a biosynthetic precursor of Atha.

1. Introduction

Siderophores are low molecular weight and highly specific Fe(III) chelating agents synthesized by microorganisms to sequester iron from the iron-limited environment.[1] The structures of siderophores are diverse and several hundred structures have been reported from various microorganisms. Recently, the study of siderophore biosynthesis has been eagerly pursued, because the ability of siderophores from pathogenic microorganisms to efficiently assimilate Fe(III) from host cells has proven to be an important virulence factor. Therefore, proteins involved in the biosynthesis of siderophores have potential to be targets of therapeutic drugs.[2] In the view of application for the designed biosynthesis of natural-product-like molecules, siderophores are quite attractive molecules because many of them are peptide-like molecules[3] that are nonetheless biosynthesized non-ribosomally by large, multidomain enzymes termed non-ribosomal peptide synthetases (NRPS).[2,4] Furthermore, it has been recently elucidated that some siderophores, including yersiniabactin from *Yersinia pestis*,[5] are biosynthesized through the combination of NRPS and its biosynthetic cousin, polyketide synthase (PKS).[6]



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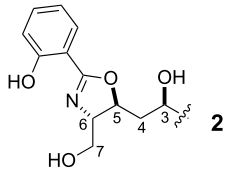

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Anachelin and anachelin-2 (**1** and **2**) were isolated as the first genuine cyanobacterial siderophores from the freshwater cyanobacterium *Anabaena cylindrica*.[7,8] The structures of **1** and **2** have the sequence of three hydrophilic amino acids (L-Thr-D-Ser-L-Ser) in the middle of molecule and it has been assumed that D-Ser plays a role to resist hydrolysis by endogenous proteases. Two unique units responsible for binding iron are attached through amide bonds to each terminus of the sequence. One unit is a 6-amino-3,5,7-trihydroxyheptanoic acid unit (Atha). A 2-(2-hydroxyphenyl)-2-oxazoline ring of **1** and **2** functions as an iron-binding group formed from cyclization of 6-NH_2 and 7- or 5-OH of Atha, respectively, with salicylic acid (Sal). Although the ring system has been found in other siderophores such as the mycobactins of Mycobacteria,[9] it has usually been derived from cyclization of Ser or Thr.[10] In fact, the long-chain polyhydroxy unit Atha, which enhances the hydrophilic nature of the molecule, is quite unique in siderophores that usually consist of small endogenous

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* Corresponding author. Tel.: +81-3-5841-5302; fax: +81-3-5841-8166; e-mail: aokada@mail.ecc.u-tokyo.ac.jp

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organic moieties such as amino acids and diamines. Furthermore, the structure of Atha strongly suggests a hybrid of non-ribosomal peptide and polyketide biosynthetic origin. The other unit is a 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-6,7-dihydroxyquinolinium unit (Dmaq), of which a catechol group works as another iron-binding group. The structure of Dmaq is similar to the chromophore of the pyoverdines, the main siderophores of fluorescent pseudomonads,[11] but the dimethylated quaternary nitrogen seen in Dmaq has never been reported in any siderophores, and is reminiscent of quinoline alkaloids from plants.

Thus, Atha and Dmaq units of anachelins (**1** and **2**) have unique structural features and their biosynthetic pathway is also interesting, but the stereochemistry of **1** and **2** remained to be determined. Herein, we describe the absolute stereochemistry of Atha and Dmaq of **1** and **2**.

2. Results

Anachelins (**1** and **2**) were prepared from the iron-deficient culture supernatant of *A. cylindrica* NIES-19 as described previously.[8] Previous study reported that the oxazoline ring of **1** and **2** was sensitive to hydrolysis and easily opened to afford a 5- or 7-salicyl ester of Atha in acidic conditions, respectively.[7,8] Treatment with dilute alkali finally led the salicyl esters to a salicyl amide of Atha by the O→N acyl shift.[8,12] Since both salicyl amide derivatives prepared from **1** and **2** were identical in spectral analyses, including NMR (data not shown), the configuration of all chiral centers of **1** and **2** were determined to be identical. Therefore, we used a suitable sample of **1** or **2** for the determination of the stereochemistry of each chiral center.

2.1. Dmaq

The relative configuration at C-3 of Dmaq was elucidated by the combination of *J* values and NOESY correlations. Large vicinal coupling constants of H-2a/H-3 and H-3/H-4a (11.7 and 11.1 Hz, respectively) were observed in ^{1}H NMR spectrum of **1** and NOESY experiment of **1** showed the cross peak between H-2a and H-4a, indicating that the secondary amine group was equatorially connected to C-3 of Dmaq (Fig. 1). The absolute configuration at C-3 was determined by a Boc-phenylglycine (BPG) method,[13] because the BPG amides give rise to much higher $\Delta\delta$ values than those given by 2-methoxy-2-trifluoromethyl-2-phenylacetyl (MTPA) ester of Mosher's method. After treatment of **2** with excess trimethylsilyldiazomethane ($TMSCHN_2$) in dry MeOH to protect the catechol group,[14] **2** was hydrolysed in acid condition. HPLC purification of the hydrolysate furnished the 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-6,7-dimethoxyquinolinium unit (MeDmaq, **3**), which was subsequently converted to (*R*)- and (*S*)-BPG amides (**4** and **5**, respectively). ^{1}H NMR spectra of **4** and **5** were recorded for calculation of anisotropic chemical shift differences (the $\Delta\delta=\delta_R-\delta_S$) for each proton. The $\Delta\delta$ values for two *N*Me-1 and H-2 were positive, while negative $\Delta\delta$ values were observed for H-4, H-5, and *O*Me-6, which indicated that C-3 possessed *S* configuration (Fig. 2). Thus, the absolute configuration of Dmaq was assigned as 3*S* (eq).

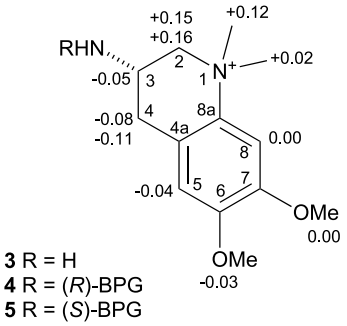


Figure 2. $\Delta\delta$ values [$\Delta\delta$ (in ppm)$=\delta_R-\delta_S$] obtained for (*R*)- and (*S*)-BPG amides of **3** (**4** and **5**, respectively).

2.2. Atha

To elucidate the relative configuration of C-3/C-5 of Atha, **1** was converted to a diacetonide derivative (**6**) with 2,2-dimethoxypropane in DMF. NOESY spectrum of **6** showed the cross peaks of H-3/H-5, H-3/H-4b, H-5/H-4b, H-3/Me (δ 1.42), and H-5/Me (δ 1.42) in Atha, indicating that the 1,3-dioxane ring in Atha existed in chair configuration to show the relative configuration of C-3/C-5 of Atha to be *syn* (Fig. 3). The oxazoline ring of **2** seemed to be convenient to determine the relative configuration of C-5/C-6 of Atha. Unfortunately, it was impossible to establish the configuration from the ^{1}H NMR spectrum of **2** recorded in DMSO-d_6, since the signals due to H-7 of Atha and H-3 of two Ser residues overlapped. After the trial of several solvents, however, the spectrum recorded in C_5D_5N gave the

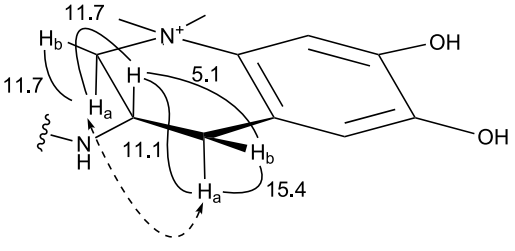


Figure 1. The relative stereochemistry of Dmaq of **1**. Coupling constants (Hz) and NOESY correlations are shown by plain lines and a dashed arrow, respectively.

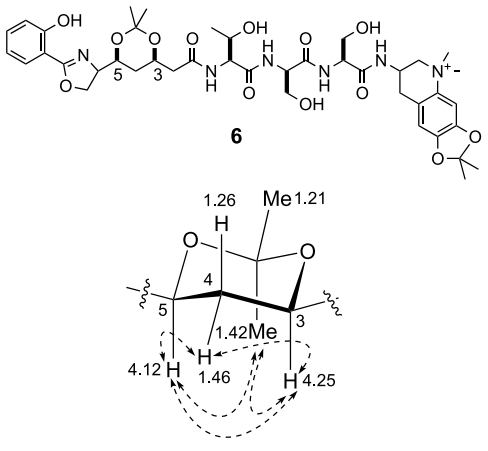


Figure 3. The relative stereochemistry of C3/C5 of Atha of a diacetonide derivative of **1** (**6**). NOESY correlations are shown by dashed arrows.

favorable separation between the signal of H-7 of Atha and other signals. The irradiation of the signal of H-7 of Atha changed that of H-6 to be a distinct doublet ($^3J_{H\text{-}5/H\text{-}6}=6.4$ Hz, Fig. 4). Previous study described that in a phenyl-oxazoline ring the large coupling constant ($^3J_{HH}=10.0$–11.0 Hz) corresponded to *cis* form and the small one ($^3J_{HH}=6.0$–7.0 Hz) to *trans* form.[15] Therefore, the conformation of the oxazoline ring of **2** was assigned to be *trans*, indicating the relative stereochemistry of C-5/C-6 of Atha to be *syn*.

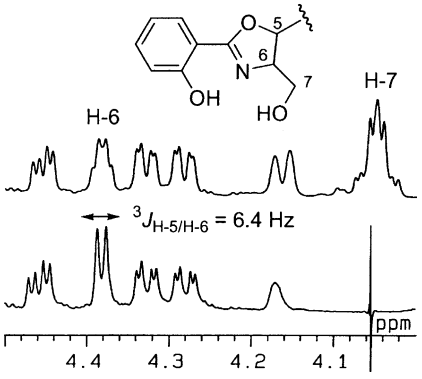


Figure 4. ^{1}H NMR spectra of **2** in C_5D_5N. Top: non-irradiation, bottom: irradiation of H-7 signal of Atha at δ_H 4.00.

The absolute stereochemistry of Atha was elucidated by Mosher's method.[16] It was found that acidic methanolysis of **1** gave a 7-*O*-salicyl-Atha methyl ester as a major degradation product (**7**, Scheme 1). Then, the methanolysate including **7** was treated in diluted NaOH solution to yield a *N*-salicyl Atha amide (**8**) by the O→N acyl shift.[12] Under acidic conditions, **8** was spontaneously cyclized to a delta lactone and therefore the application of Mosher's method for the secondary hydroxyl group at C-3 of the delta lactone was undertaken. However, the expected MTPA diester was not obtained because of the rapid dehydration between C-2 and C-3 of the delta lactone. Therefore, to prevent from the spontaneous cyclization, **8** was converted to a methyl ester (**9**) with $TMSCHN_2$ again, which also methylated the phenol group (Scheme 1). Then the methylester (**9**) was treated with 2 equiv of each (*R*)- and (*S*)-MTPACl in the presence of DMAP in dry C_5H_5N, followed by HPLC purification to furnish the 3,7-bis[(*S*)- and (*R*)-MTPA] esters of **9** (**10** and **11**, respectively, Scheme 1). The $\Delta\delta$ values ($\delta_S-\delta_R$) obtained from ^{1}H NMR data of **10** and **11** consistently fell into regions that lay to the left and right of the secondary MTPA ester group at C-3 except for that of Sal (Fig. 5), which was interpreted to be caused by turn of a branched Sal amide toward C-3. Thus the absolute stereochemistry at C-3 of Atha was confirmed to be *R* configuration. Consequently, the absolute stereochemistry at C-3, C-5, and C-6 of Atha unit were assigned as *R*, *S*, and *S*.

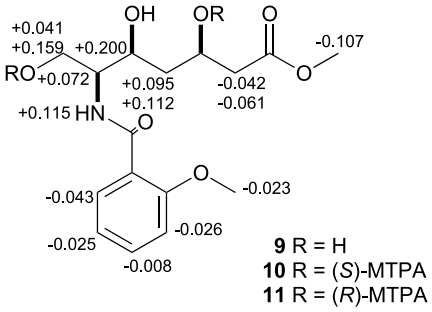
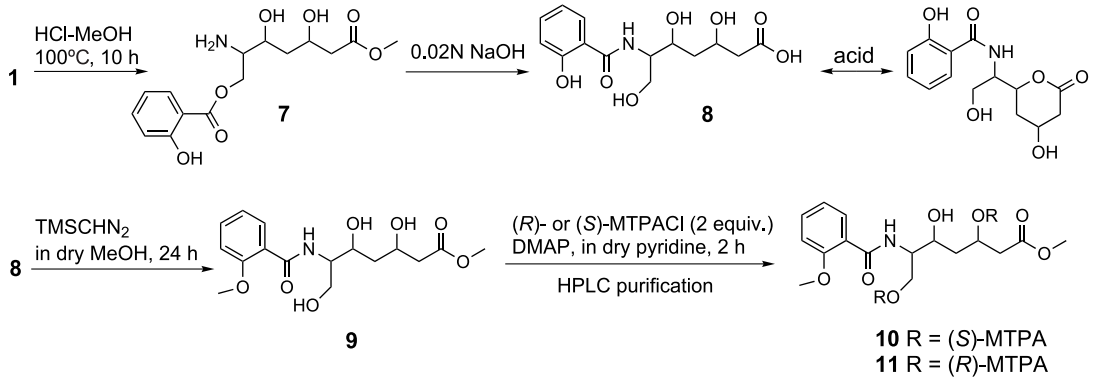


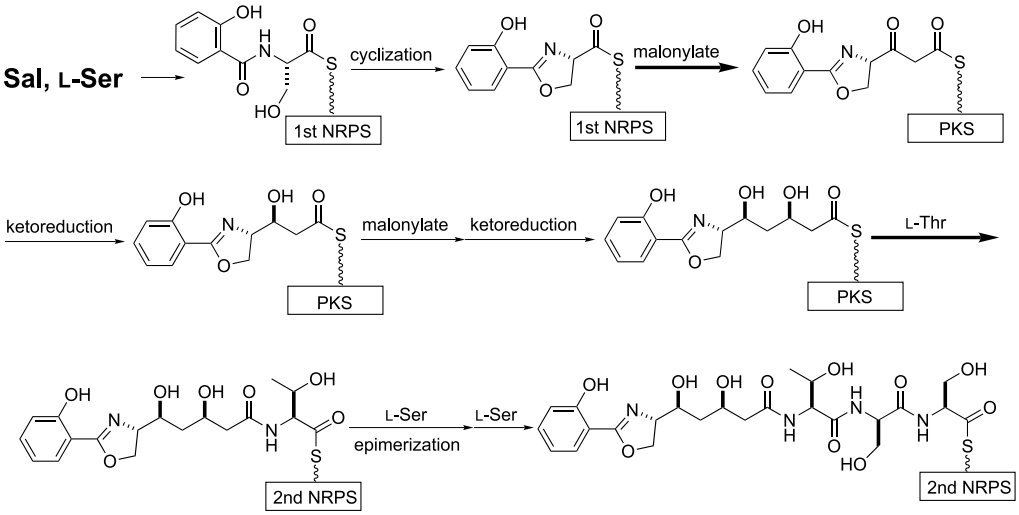
Figure 5. $\Delta\delta$ values [$\Delta\delta$ (in ppm)$=\delta_S-\delta_R$] obtained for 3,7-bis[(*S*)- and (*R*)-MTPA] esters of **9** (**10** and **11**, respectively).

3. Discussion

The stereochemistry of anachelins (**1** and **2**) was completely determined in this study. The heterocyclic oxazoline ring is a class of iron binding group in siderophores and it is usually derived from cyclization of L-Ser or L-Thr.[10] The *S* configuration at C-6 of Atha suggested that L-Ser was incorporated as a precursor in the biosynthesis of Atha. Furthermore, the structure of Atha also suggested that the carbon chain from C-1 to C-4 of Atha was incorporated by condensation of malonyl moieties by PKS. Recently, Walsh and co-workers revealed yersiniabactin, a siderophore containing three heterocycles (two thiazolines and a thiazolidine) from the plague bacterium *Yersinia pestis*,[5] to be produced by NRPS and PKS, which comprises Sal, three L-Cys, three methyl units from *S*-adenosyl-methionine, and a malonyl moiety.[6] Yersiniabactin synthetase was the first example of a NRPS/PKS hybrid assemble and involves two switching points from NRPS to PKS module. On the basis of suggested biosynthetic scheme of yersiniabactin, we propose the pathway to **1** that has two switching points from NRPS to PKS modules as follows (Scheme 2). At the beginning, L-Ser condenses to Sal on the first NRPS followed by cyclization to the oxazoline ring. The two-ring intermediate is elongated by condensation of



Scheme 1.



Scheme 2. Propose biosynthetic scheme for anachelin (**1**).

two malonyl groups on an intervening PKS module. Following ketone reduction, the molecule is transferred to the second NRPS module to attach the peptide sequence (L-Thr-D-Ser-L-Ser). However, one question arises from the scheme proposed above; when is the oxazoline of anachelin-2 (**2**) cyclized? If the carbon chain of Atha is elongated by the condensation of malonyl moieties on PKS, the oxazoline of **2** should be cyclized after the condensation of the first malonyl unit to Sal-Ser and the following reduction of its ketone to the hydroxyl group corresponding to 5-OH of Atha. However, some studies showed that cyclization to a phenyl-oxazoline or a phenyl-thiazoline occurred soon after the condensation of Sal and amino acids (L-Ser or L-Cys, respectively) on NRPS module.[2,6] Therefore, it is reasonable to surmise that **2** is an artificial compound formed from **1** during culture or purification. In our previous study, however, the conversion between **1** and **2** was not observed in the acidic solution used during HPLC purification and the culture supernatant in the fourth day showed the presence of both **1** and **2** in the ratio of 1:1.[8] Moreover, **1** dissolved in culture medium (pH 8.5) was incubated under a fluorescent light for 1 week, but the conversion from **1** to **2** was not observed (data not shown). Therefore, it is assumed that **2** is also a product of biosynthesis of *A. cylindrica* NIES-19, but we cannot exclude the possibility of the conversion between **1** and **2** in cell and other biosynthetic schemes. The structure of Dmaq is similar to the chromophore of pyoverdines, the main siderophore of fluorescent pseudomonads.[11] Feeding experiments and genetic studies revealed that Tyr was a precursor of the chromophore.[17] Therefore, it is possible that Tyr is also incorporated in Dmaq of **1** and **2**, and if so, its form could be L on the basis of the stereochemistry of the resulting siderophores.

4. Experimental

4.1. Instrumentation

NMR spectra were recorded on a JEOL JNM-A600 spectrometer at 27 °C. ^{1}H and ^{13}C NMR chemical shifts were referenced to residual solvent peaks of DMSO-d_6 at δ_H 2.49 and δ_C 39.5. FAB-MS spectra were measured using glycerol as matrix on a JEOL JMS SX-102 mass spectrometer.

4.2. Material

Anachelins (**1** and **2**) were prepared from the iron-deficient culture supernatant of *A. cylindrica* NIES-19 as described previously.[8] The obtained **1** and **2** were lyophilized completely and stored as powder at −20 °C. Boc-phenylglycine (BPG) was prepared from a commercial phenylglycine.

4.2.1. MeDmaq (3). To a solution of **2** (11.2 mg) in dry MeOH (100 μL) and dry benzene (500 μL), 8 equiv of $TMSCHN_2$ solution (68.8 μL) was added and stirred for 12 h under argon. The reacting mixture was dried in vacuo, followed by hydrolysis in 6 N HCl at 110 °C for 10 h. The solvent was removed in a stream of dry N_2, and the residue was applied to a YMC-ODS column (50×150 mm) and eluted with 0, 25, 50, and 100% MeOH. The concentrated H_2O fraction was purified by HPLC (Cosmosil C_{18} MS column, 10.0×250 mm; 0–20% MeCN containing 0.05% TFA in 20 min; flow rate 2 mL/min, UV detection at 210 nm) to yield MeDmaq (**3**, 2.4 mg). Retention time (min): **3** (25.6). HRFAB-MS *m/z* 237.1612 (M^+) calculated for $C_{13}H_{21}N_2O_2$ (Δ −0.8 mmu). ^{1}H NMR (DMSO-d_6), δ_H 3.56 (s, *N*Me-1a), 3.65 (s, *N*Me-1b), 3.66 (m, H-2a), 3.90 (m, H-2b), 4.45 (m, H-3), 2.75 (dd, J=16.2, 11.1 Hz, H-4a), 2.98 (s, J=16.2, 5.0 Hz, H-4b), 6.91 (s, H-5), 3.75 (s, *O*Me-6), 3.80 (s, *O*Me-7), 7.40 (s, H-8).

4.2.2. (*R*)-BPG amide of 3 (4). To solution of **3** (1.0 mg) in dehydrated DMF (1 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.0 mg), 1-hydroxybenzotriazole (HOBt, 2.0 mg) and (*R*)-BPG (2.0 mg) were added at 0 °C and stirred at room temperature for 10 h. The reacting mixture was dried in vacuo and redissolved in MeOH, and subjected to HPLC (Cosmosil C_{18} MS column, 10.0×250 mm; 32–37% MeCN containing 0.05% TFA in 10 min; flow rate 2 mL/min, UV detection at 210 nm) to afford (*R*)-BPG amide of **3** (**4**, 1.2 mg). Retention time (min): **4** (12.0). HRFAB-MS *m/z* 470.2647 (M^+) calculated for

$C_{26}H_{36}N_3O_5$ (Δ −0.8 mmu). ^{1}H NMR (DMSO-d_6), MeDmaq, δ_H 3.56 (s, *N*Me-1a), 3.65 (s, *N*Me-1b), 3.66 (m, H-2a), 3.90 (brd, J=12.0 Hz, H-2b), 4.45 (m, H-3), 2.78 (dd, J=16.2, 11.1 Hz, H-4a), 2.95 (dd, J=16.2, 5.0 Hz, H-4b), 6.91 (s, H-5), 3.75 (s, *O*Me-6), 3.80 (s, *O*Me-7), 7.40 (s, H-8), 7.40 (m, NH), BPG, 5.21 (d, J=4.2 Hz, H-2), 7.42 (d, J=7.5 Hz, H-4), 7.36 (t, J=7.5 Hz, H-5), 7.32 (t, J=7.5 Hz, H-6), 8.61 (d, J=4.2 Hz, NH), 1.40 (s, Me).

4.2.3. (*S*)-BPG amide of 3 (5). (*S*)-BPG amide of **3** (**5**, 1.0 mg) was prepared by the same manner as described above. Retention time (min): **5** (13.6). HRFAB-MS *m/z* 470.2652 (M^+) calculated for $C_{26}H_{36}N_3O_5$ (Δ −0.3 mmu). ^{1}H NMR (DMSO-d_6), MeDmaq, δ_H 3.54 (s, *N*Me-1a), 3.53 (s, *N*Me-1b), 3.51 (m, H-2a), 3.74 (brd, J=12.0 Hz, H-2b), 4.50 (m, H-3), 2.86 (dd, J=16.2, 11.1 Hz, H-4a), 3.06 (dd, J=16.2, 5.1 Hz, H-4b), 6.95 (s, H-5), 3.78 (s, *O*Me-6), 3.80 (s, *O*Me-7), 7.40 (s, H-8). 7.40 (m, NH), BPG, 5.21 (d, J=4.2 Hz, H-2), 7.41 (d, J=7.5 Hz, H-4), 7.36 (t, J=7.5 Hz, H-5), 7.32 (t, J=7.5 Hz, H-6), 8.61 (d, J=4.2 Hz, NH), 1.40 (s, Me).

4.2.4. Diacetonide derivative of 1 (6). To solution of **1** (10.4 mg) in dehydrated DMF (1.0 mL), *p*-TsOH (11.8 mg) and excess amount of 2,2-dimethoxypropane (500 μL) were added and stirred at room temperature for 24 h. The reacting mixture was dried in vacuo and dissolved in H_2O, and subjected to ODS open column chromatography, and eluted with 0, 20, 50 and 100% MeOH. The 50% MeOH fraction was lyophilized to obtain a diacetonide derivative (**6**, 7.5 mg). HRFAB-MS *m/z* 841.4024 (M^+) calculated for $C_{41}H_{57}N_6O_{13}$ (Δ +4.0 mmu). ^{1}H NMR (DMSO-d_6), Sal, δ_H 6.96 (d, J=7.7 Hz, H-4), 7.42 (t, J=7.7 Hz, H-5), 6.91 (t, J=7.7 Hz, H-6), 7.58 (d, J=7.7 Hz, H-7), Atha, 2.06 (m, H-2a), 2.35 (m, H-2b), 4.25 (m, H-3), 1.26 (m, H-4a), 1.46 (m, H-4b), 4.12 (m, H-5), 4.33 (m, H-6), 4.33 (m, H-7a), 4.46 (m, H-7b), 1.21 (s, Me), 1.42 (s, Me), Thr, 4.33 (m, H-2), 3.97 (m, H-3), 1.27 (m, H-4), 7.44 (m, NH), Ser (1), 4.16 (m, H-2), 3.65 (m, H-3a), 3.73 (m, H-3b), 8.50 (d, J=5.7 Hz, NH), Ser (2), 4.22 (m, H-2), 3.58 (m, H-3a), 3.64 (m, H-3b), 8.96 (d, J=5.6 Hz, NH), Dmaq, 3.51 (s, *N*Me-1a), 3.60 (s, *N*Me-1b), 2.97 (m, H-2a), 3.82 (m, H-2b), 4.50 (br, H-3), 2.85 (dd, J=15.4, 11.1 Hz, H-4a), 2.95 (dd, J=15.3, 5.1 Hz, H-4b), 6.56 (s, H-5), 7.27 (s, H-8), 7.83 (d, J=7.7 Hz, NH), 1.13 (s, Me), 1.29 (s, Me).

4.2.5. *N*-Salicyl-Atha amide (8). 10% HCl–MeOH solution (3.0 mL) containing **1** (10 mg) was heated to 100 °C in a sealed tube for 10 h and then cooled. The reaction mixture was evaporated and lyophilized to remove traces of HCl. The HPLC and following spectral analyses of a part of the resultant products showed that a 7-*O*-salicyl Atha methyl ester (**7**) was a major degradation compound **7**; FABMS *m/z* 328 $(M+H)^+$, ^{1}H NMR (DMSO-d_6). Sal, δ_H 6.88 (m, H-4), 7.35 (t, J=7.7 Hz, H-5), 6.88 (m, H-6), 7.91 (d, J=7.7 Hz, H-7), Atha, 2.16 (dd, J=15.0, 8.1 Hz, H-2a), 2.30 (dd, J=15.0, 3.9 Hz, H-2b), 4.00 (m, H-3), 1.48 (m, H-4a), 1.53 (m, H-4b), 3.98 (m, H-5), 3.62 (m, H-6), 4.44 (m, H-7a), 4.52 (m, H-7b). Then, the resultant compound was dissolved in 0.02 N NaOH solution and incubated at room temperature for 1 h, followed by resolution by HPLC (Cosmosil C_{18} MS column, 10.0×250 mm; 20–30% MeCN containing 0.05% TFA in 10 min; flow rate 2 mL/min, UV detection at 210 nm) to yield **8** (1.5 mg). Retention time (min): **8** (18.4). HRFAB-MS *m/z* 314.1263 $(M+H)^+$ calculated for $C_{14}H_{20}NO_7$ (Δ +2.3 mmu). ^{1}H NMR (DMSO-d_6), Sal, δ_H 6.88 (m, H-4), 7.35 (t, J=7.7 Hz, H-5), 6.88 (m, H-6), 7.91 (d, J=7.7 Hz, H-7), Atha, 2.16 (dd, J=15.0, 8.1 Hz, H-2a), 2.32 (dd, J=15.0, 3.9 Hz, H-2b), 4.00 (m, H-3), 1.48 (m, H-4a), 1.53 (m, H-4b), 3.98 (m, H-5), 3.99 (m, H-6), 3.44 (m, H-7a), 3.52 (m, H-7b), 8.34 (d, J=8.5 Hz, NH).

4.2.6. Methylester of 8 (9). To solution of **8** (1.5 mg) in dehydrated MeOH (1.0 mL), excess $TMSCHN_2$ (100 μL, 2.0 M solution) was added and stirred at room temperature for 24 h. The reacting mixture was directly subjected to HPLC (Cosmosil C_{18} MS column, 10.0×250 mm; 30–60% MeCN containing 0.05% TFA in 15 min; flow rate 2 mL/min, UV detection at 210 nm) to afford a methylester (**9**, 1.4 mg). Retention time (min): **9** (18.4). HRFAB-MS *m/z* 342.1570 $(M+H)^+$ calculated for $C_{16}H_{24}NO_7$ (Δ +1.8 mmu). ^{1}H NMR (CD_3OD), Sal, δ_H 3.98 (s, *O*Me), 7.15 (d, J=7.7 Hz, H-4), 7.50 (t, J=7.7 Hz, H-5), 7.06 (t, J=7.7 Hz, H-6), 7.98 (d, J=7.7 Hz, H-7), Atha, 3.61 (s, *O*Me), 2.43 (dd, J=15.2, 4.7 Hz, H-2a), 2.52 (dd, J=15.2, 8.1 Hz, H-2b), 4.24 (m, H-3), 1.66 (dt, J=14.1, 8.1 Hz, H-4a), 1.75 (dt, J=14.1, 5.1 Hz, H-4b), 4.21 (m, H-5), 4.12 (dt, J=1.7, 6.4 Hz, H-6), 3.70 (d, J=6.4 Hz, H-7, 2H).

4.2.7. 3,7-Bis[(*S*)-MTPA] ester of 9 (10). The methylester **9** (400 μg: 1.17 μmol) was dried in a reaction tube which was made by cutting off a NMR tube (5 mm). And the solution of DMAP in dehydrated pyridine (100 μL, 3.15 mg/mL, 2.58 μmol) was added to the reaction tube. (*R*)-MTPACl solution (0.48 mL, 2.58 μmol) was carefully added and placed for 2 h at room temperature. The reaction mixture was lyophilized and dissolved in MeOH and subjected to HPLC (Cosmosil C_{18} MS column, 10.0×250 mm; 76–100% MeCN containing 0.05% TFA in 12 min; flow rate 2 mL/min, UV detection at 210 nm) to afford a 3,7-bis[(*S*)-MTPA] ester derivative (**10**, 500 μg). Retention time (min): **10** (18.4), 5,7-bis[(*S*)-MTPA] ester (18.8), and 3,5,7-tris[(*S*)-MTPA] ester (22.4). HRFAB-MS *m/z* 774.2365 $(M+H)^+$ calculated for $C_{36}H_{38}NO_{11}$ (Δ +1.6 mmu). ^{1}H NMR (CD_3OD), Sal, δ_H 3.900 (s, *O*Me), 7.130 (d, J=7.7 Hz, H-4), 7.517 (t, J=7.7 Hz, H-5), 7.052 (t, J=7.7 Hz, H-6), 7.851 (d, J=7.7 Hz, H-7), Atha, 3.516 (s, *O*Me), 2.626 (dd, J=16.2, 9.0 Hz, H-2a), 2.715 (dd, J=16.2, 3.8 Hz, H-2b), 5.635 (m, H-3), 1.807 (m, H-4a), 1.995 (m, H-4b), 3.885 (m, H-5), 4.460 (m, H-6), 4.382 (dd, J=11.1, 5.6 Hz, H-7a), 4.614 (dd, J=11.1, 7.7 Hz, H-7b), 8.540 (br, NH).

4.2.8. 3,7-Bis[(*R*)-MTPA] ester of 9 (11). A 3,7-bis[(*R*)-MTPA] ester derivative (**11**, 500 μg) was prepared using (*S*)-MTPACl by the same procedure as described above. HRFAB-MS *m/z* 774.2360 $(M+H)^+$ calculated for $C_{36}H_{38}NO_{11}$ (Δ +1.1 mmu). ^{1}H NMR (CD_3OD), Sal, δ_H 3.923 (s, *O*Me), 7.156 (d, J=7.7 Hz, H-4), 7.525 (t, J=7.7 Hz, H-5), 7.077 (t, J=7.7 Hz, H-6), 7.894 (d, J=7.7 Hz, H-7), Atha, 3.516 (s, *O*Me), 2.687 (dd, J=16.2, 9.0 Hz, H-2a), 2.757 (dd, J=16.2, 3.8 Hz, H-2b), 5.623 (m, H-3), 1.695 (m, H-4a), 1.900 (m, H-4b), 3.685 (m, H-5), 4.388 (m, H-6), 4.223 (dd, J=11.1, 5.6 Hz, H-7a), 4.573 (dd, J=11.1, 7.7 Hz, H-7b), 8.425 (br, NH).

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