Conversion of (2*S*)-Arginine to (2*S*,3*R*)-Capreomycidine by VioC and VioD from the Viomycin Biosynthetic Pathway of *Streptomyces* sp. Strain ATCC11861

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Dedicated to Professor Christopher T. Walsh in celebration of his 60th birthday

The antibiotics of the tuberactinomycin family, which includes viomycin (**1a**), the tuberactinomycins (**1b**–**e**), and the capreomycins (**2a**–**d**) (Scheme 1), are important components of the drug regimen for the treatment of mycobacterial infections. Their medical relevance is reflected by **2a**–**d** being included on the World Health Organization's List of Essential Medicines for the treatment of multidrug-resistant tuberculosis infections[1] and by **1d** commonly being used in Asia for the treatment of *Mycobacterium tuberculosis*[2] and *M. avium* complex[3] infections. The tuberactinomycins target the ribosome,[4] and structural analysis of the moieties needed for their biological activity identified the cyclic portion of amino acid 5 of the peptide core as being essential for antimicrobial activity.[5,6] This amino

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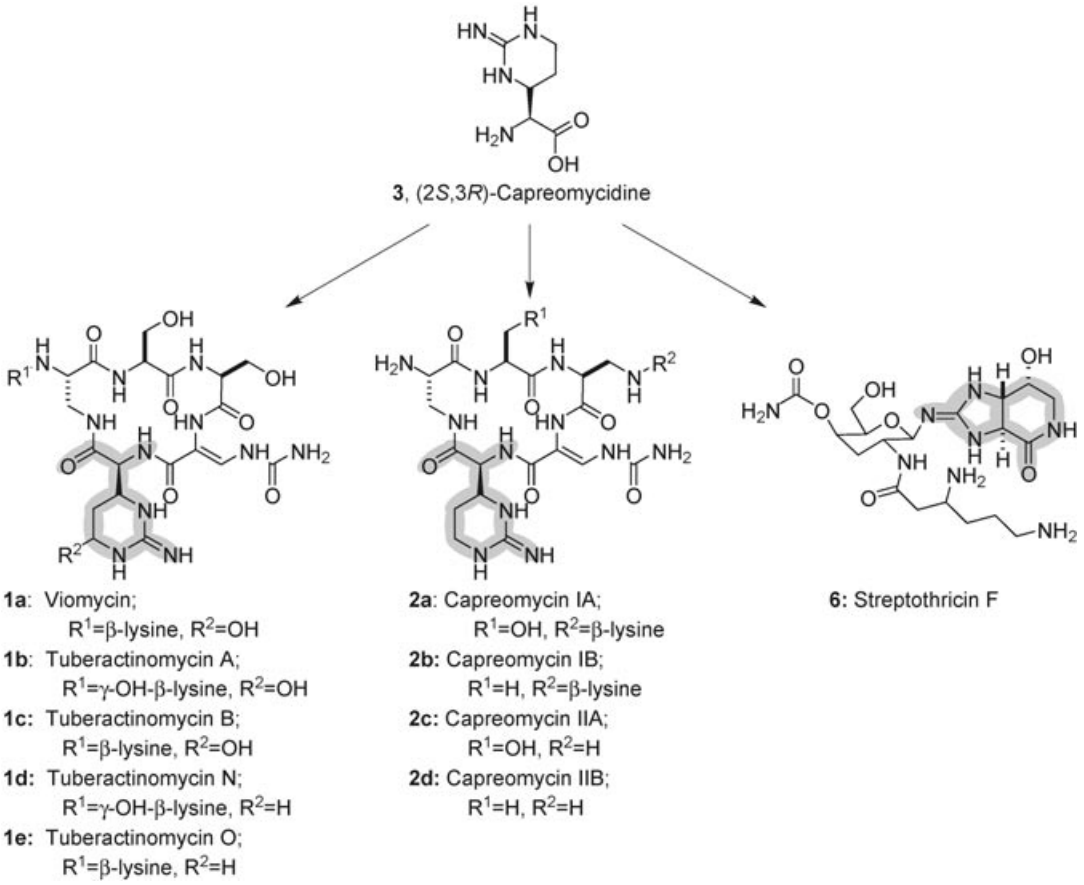
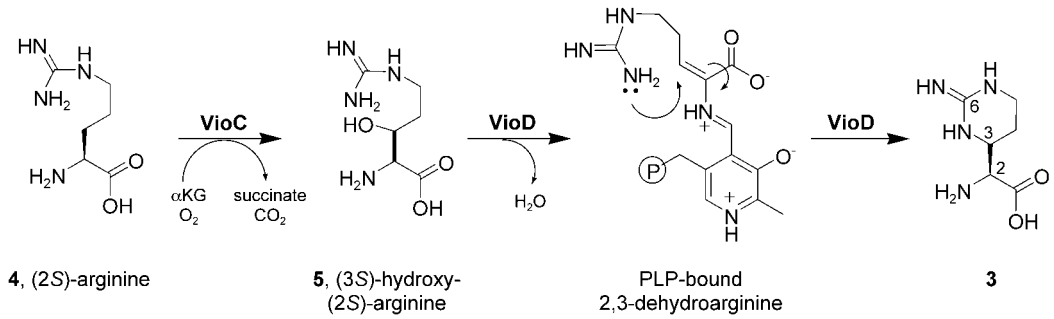


Scheme 1. *Chemical structures of antibiotics that incorporate (2S,3R)-capreomycidine. The (2S,3R)-capreomycidine moiety or moieties derived from (2S,3R)-capreomycidine are highlighted in gray. For compounds* ***1a–e*** *and* ***2a–d****, the shaded amino acids are residue 5 of the pentapeptide cores of the antibiotics. The shaded portion of* ***6*** *identifies the streptolidine lactam moiety.*

acid is either (2*S*,3*R*)-capreomycidine (**3**) or its hydroxylated derivative (2*S*,3*R*)-tuberactidine (Scheme 1). Based on the results of precursor-labeling studies,[7,8] these nonproteinogenic amino acids are derived from the novel intramolecular cyclization of the side chain of (2*S*)-arginine (**4**), which generates the 2-iminohexahydro-2-pyrimidyl ring. It is reasonable to presume that (2*S*,3*R*)-tuberactidine is formed by the subsequent hydroxylation of **3**.[9] While the structures of these unusual amino acids have been known for over 35 years,[10,11] the exact mechanism of conversion of **4** to **3** has remained an open question. Here we present the in vitro characterization of two enzymes, VioC and VioD, from the **1a** biosynthetic pathway from *Streptomyces* sp. strain ATCC11861 (previously *S. vinaceus*). We show that they catalyze the stepwise conversion of **4** to (3*S*)-hydroxy-(2*S*)-arginine (**5**) to **3** (Scheme 2), as predicted by our previous bioinformatic analysis, with the exception that the stereochemistry of the intermediate was hypothesized to be (3*R*)-hydroxy-(2*S*)-arginine.[9] This work establishes the steps in the formation of **3** and sets the groundwork for the use of these enzymes for combinatorial biosynthesis of natural products or for production of enantiomerically pure **3** for semisynthetic purposes.

In addition to being directly incorporated into the tuberactinomycins, **3** is also proposed to be an intermediate in the production of the streptolidine lactam moiety of the streptothricin



Scheme 2. *Schematic representation of the proposed (2S,3R)-capreomycidine biosynthetic pathway. The PLP-linked 2,3-dehydroarginine represents the anticipated VioD-bound intermediate. The C2, C3, and C6 positions of* ***3*** *are noted to aid in the interpretation of Table 1.*

broad-spectrum antibiotics, for example, streptothricin F (**6**) (Scheme 1). Precursor-labeling studies on **6** have also identified **4** as the precursor for **3** formation.[12,13] Based on the precursor-labeling studies of **1a**, **2a**–**d**, and **6**, along with the recent identification of the biosynthetic gene clusters for **1a**[9,14] and **6**,[15] three proposals have been made for the mechanism of conversion of **4** to **3**. Based on feeding experiments with [2,3,3,5,5-$^2H_5$]-(2*S*)-arginine, Gould and Minott first proposed that this conversion proceeds via a 2,3-dehydroargininyl intermediate.[8] Gould and Minott hypothesized that, to stabilize this proposed intermediate, the desaturation and cyclization of the side chain of **4** occurs after peptide synthesis. More recently, a second proposal was made for the initial steps in the production of **3**, whereby a nonribosomal peptide synthetase (NRPS), SttM, from the biosynthetic pathway to **6**, activates and tethers **4** to a peptidyl carrier protein, followed by C3 hydroxylation by an α-ketoglutarate (αKG)-dependent non-heme iron dioxygenase, SttL, to form a peptidyl carrier protein-linked **5.**[16] This NRPS-dependent mechanism of **4** hydroxylation was extended to the biosynthesis of **1a** based on the finding of an SttL homologue in *Streptomyces* sp. strain ATCC11861,[14] the **1a**-producing bacterium.[17] In neither of these two latter studies was a mechanism for conversion of the NRPS-linked **5** to **3** discussed.

We recently sequenced what we have proposed to be the complete **1a** biosynthetic gene cluster from *Streptomyces* sp. strain ATCC11861.[9] Based on our analysis of this gene cluster, we have made a third proposal, that is, an NRPS-independent two-enzyme pathway for the biosynthesis of **3** from **4**, involving the enzymes VioC and VioD (Scheme 2).[9] Our hypothesis was based on the homology of VioC to clavaminic acid synthases (CASs), which are αKG-dependent non-heme iron dioxygenases involved in clavulanic acid biosynthesis,[18] and the homology of VioD to pyridoxal phosphate (PLP) dependent enzymes that catalyze β-replacement reactions.[19] We have proposed that this pathway functions not only for **1a** biosynthesis, but that analogous pathways perform the same function for the biosynthesis of **1b**–**e**, **2a**–**d**, and **6.**[9] To test our hypothesis, we heterologously overproduced and purified VioC and VioD from the **1a** biosynthetic pathway and identified the substrate and product for each reaction. These data are consistent with our proposal shown in Scheme 2.

VioC was overproduced in *Escherichia coli* with an N-terminal hexahistidine affinity tag and purified to near homogeneity by using nickel-chelate chromatography (Figure 1 A). To assay for VioC turnover, we developed an assay that utilized *O*-phthalaldehyde (OPA) derivatization of primary amines in the reaction mixture, followed by separation of derivatized products by high-performance liquid chromatography (HPLC). Using this assay, we detected a new product with an elution time distinct from **4** (Figure 2). The appearance of this product peak correlated with the loss of the peak associated with **4**, and formation of this product required VioC, **4**, $FeSO_4$, and αKG (data not shown). Furthermore, repeating the reactions with [1-^{14}C]-αKG and trapping $^{14}CO_2$ released during VioC turnover, by using a protocol established for the analysis of CASs,[20] determined that $^{14}CO_2$ was released from the reaction in a VioC-, **4**-, and $FeSO_4$-dependent manner (data not shown). These data are all consistent with the hypothesis that VioC is an αKG-dependent non-heme iron dioxygenase. Collection of the eluted product peak from the HPLC and subsequent analysis of the product by positive electrospray ionization mass spectrometry (ESIMS) gave results consistent with the product being the OPA-derivatized and hydroxylated **4** ($[M+H]^+$: observed, 307.6; calculated, 307.1 for $C_{14}H_{18}N_4O_4$).

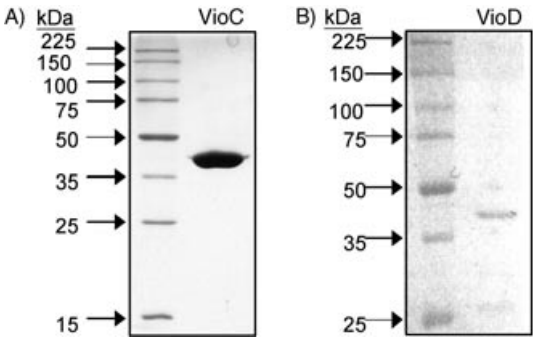


Figure 1. *A) Purified VioC and B) purified VioD analyzed by a 12% SDS-PAGE gel stained with Coomassie blue.*

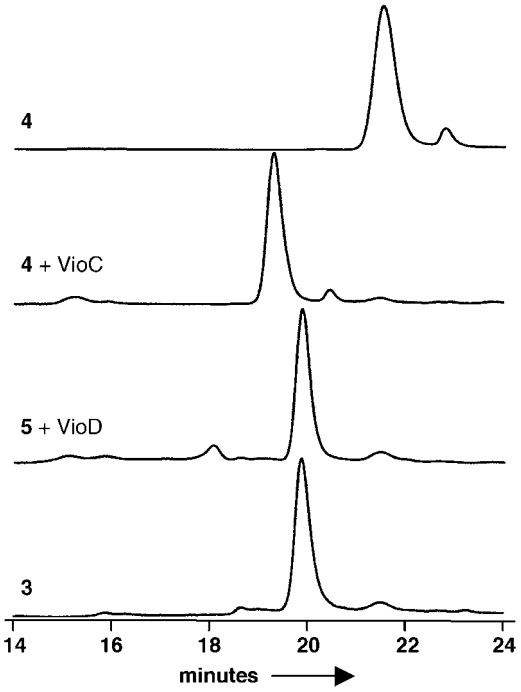


Figure 2. *HPLC elution profiles (from top to bottom) of OPA-derivatized **4**, OPA-derivatized VioC reaction, OPA-derivatized VioD reaction, and OPA-derivatized **3**. Elution of products was monitored at A_{340}. For each sample, OPA and the sample were combined in a 1:1 ratio (v/v) 5 minutes prior to injection.*

To identify the **4**-derived product of the VioC reaction, a scaled-up reaction was used to produce enough product for MS and NMR analysis. Cation-exchange chromatography followed by ethanol precipitation yielded the reaction product **5**. The positive ESIMS of **5** exhibited pseudomolecular ions at $m/z = 191.0$ for $[M+H]^+$ and $m/z = 213.0$ for $[M+Na]^+$; these are consistent with the molecular formula $C_6H_{14}O_3N_4$ for **5**. ^{1}H

and ^{13}C NMR spectra of the purified product [^1H NMR (D_2O, 500 MHz, 25 °C): $\delta = 4.22$ (dt, $J = 8.0$, 3.5 Hz, 1H; H3), 3.90 (d, $J = 3.5$ Hz, 1H; H2), 3.39 (m, 2H; H5), 1.83 (m, 2H; H4); ^{13}C NMR (D_2O, 125 MHz, 25 °C): $\delta = 172.7$ (C1), 158.1 (C6), 68.1 (C3), 60.6 (C2), 39.2 (C5), 31.3 (C4)] were in good agreement with previously reported ^1H and ^{13}C NMR spectra for chemically synthesized **5**.[21] While the site of hydroxylation was as expected, the surprising finding was the stereochemistry of the C3 hydroxylation. VioC is a homologue of CASs, enzymes that catalyze the hydroxylation of the comparable C3 position of their substrates. However, the hydroxylation by CASs results in (3*R*)-hydroxylation[20] rather than the (3*S*)-hydroxylation catalyzed by VioC. Further studies are ongoing to analyze the mechanistic and structural basis for this altered stereochemical hydroxylation. We note that OPA-derivatized **5** eluted from the HPLC with the same retention time as the previously discussed VioC reaction product peak (data not shown).

VioD is a homologue of PLP-dependent enzymes that catalyze β-replacement reactions. We have proposed that VioD catalyzes the replacement of the C3 hydroxyl of **5** with its own guanido group, thus catalyzing a novel intramolecular cyclization of the side chain of **5** (Scheme 2).[9] This proposed mechanism proceeds via a PLP-linked 2,3-dehydroarginine intermediate, consistent with prior precursor-labeling studies.[8] Furthermore, the PLP would stabilize this intermediate, thereby eliminating the need for peptide synthesis to occur prior to desaturation and cyclization of the side chain of **4** as previously proposed.[8]

VioD was overproduced in *E. coli* with an N-terminal hexahistidine affinity tag and purified to near homogeneity by using nickel-chelate chromatography (Figure 1B). Incubation of purified VioD with **5**, followed by OPA derivatization and HPLC separation, allowed the identification of a new product that eluted 0.5 min later than **5** (Figure 2). The formation of this product peak required VioD and **5**, and its formation correlated to the loss of the peak associated with **5** (data not shown). No change in the elution of **4** was observed if **4** and VioD were incubated together prior to OPA derivatization and HPLC separation (data not shown).

To identify the product of the VioD reaction, a scaled-up reaction was used to generate a sufficient quantity of product for MS and NMR analysis. The reaction product **3** was purified as the monoacetic acid salt (see Experimental Section). The positive ESIMS of **3** showed pseudomolecular ions at $m/z =$ 173.0 $[M+H]^+$, 195.0 $[M+Na]^+$, and 345.2 $[2M+H]^+$; these are consistent with the molecular formula $C_6H_{12}O_2N_4$. The ^1H NMR spectrum (500 MHz, D_2O) displayed six proton signals, and the ^{13}C NMR spectrum (125 MHz, D_2O) showed all six carbon atoms. A range of 2D (^1H,^1H gCOSY, HMQC, and gHMBC) NMR experiments was carried out that allowed complete assignments of protons attached to their respective carbons (Table 1). The ^1H,^1H gCOSY correlations shown in Table 1 revealed the connectivities from H2 to H5. HMBC correlations from H5a and H5b to C6, and from H2 to C1 established the planar structure of **3**. The optical rotation of **3** we obtained as the mono-acetic acid salt ($[\alpha]_D^{20} = +5.3$ ($c = 0.75$, H_2O)) was comparable with the reported natural (2*S*,3*R*)-capreomycidine diflavianate,[22] thus demonstrating their same stereochemistry. We note that OPA-derivatized **3** eluted from the HPLC with the same retention time as the previously discussed VioD reaction product peak (Figure 2).

Table 1. *NMR spectra of **3** as the monoacetic acid salt in D_2O.*

Position	^1H, δ (J [Hz])	^{13}C δ and HMQC	^1H,^1H gCOSY	gHMBC
1		172.9		
2	3.81, d (4.5)	57.4	H3	C1, C3, C4
3	4.03, br s	49.8	H2, H4a, H4b	
4a	1.97, m	22.4	H4b, H3a, H2, H5a, H5b	C2, C3, C5
4b	2.13, m	22.4	H4a, H3a, H2, H5a, H5b	C2, C3, C5
5a	3.40, m	36.7	H5b, H4a, H4b	C3, C4, C6
5b	3.45, m	36.7	H5a, H4a, H4b	C3, C4, C6
6		154.8		
CH$_3$COO$^-$	1.93, s	23.6		CH$_3$*COO*$^-$
CH$_3$*COO*$^-$		181.8		

We have presented the complete in vitro reconstitution of the **3** biosynthetic pathway. The first enzyme, VioC, is an unusual αKG-dependent non-heme iron dioxygenase that catalyzes the stereospecific hydroxylation of the C3 of **4** to generate **5** (Scheme 2). This product is subsequently recognized by a novel PLP-dependent enzyme, VioD, which catalyzes the replacement of the C3 hydroxyl of **5** with the guanido group of **5** (Scheme 2). This intramolecular cyclization of the side chain of an amino acid by a PLP-dependent enzyme is unprecedented in enzymology. This study establishes the enzymatic steps needed for the biosynthesis of **3**, and this two-enzyme pathway is likely to be followed during **1b**–**e** and **2a**–**d** biosynthesis. Furthermore, as we have previously noted, homologues of VioC and VioD are coded by the biosynthetic gene cluster for **6** (SttL and SttN, respectively).[9] Thus, we hypothesize the same mechanism as in the formation of **3** will be followed during formation of the streptolidine lactam moiety of the streptothricin antibiotics. This work sets the stage for the use of these enzymes for combinatorial biosynthesis or the production of enantiomerically pure **3** for semisynthetic purposes to introduce new structural diversity to natural products.

Experimental Section

Overproduction and purification of VioC and VioD. The genes coding for VioC and VioD were independently PCR amplified from cosmid pVIO-P8C8RH[9] and cloned into the overexpression vector pET28b (Novagen), and the resulting plasmids were transformed into *E. coli* BL21(DE3) for overproduction of the proteins with N-terminal hexahistidine affinity tags. Overproduction strains were grown in LB supplemented with kanamycin (50 μg mL^{-1}) in 3×1 L batches. For overproduction, medium (1 L) was inoculated with a fresh overnight culture of BL21(DE3) (10 mL) carrying either plasmid. Cultures were grown for 24 h at 25 °C, after which cells were harvested by centrifugation.

Cells overproducing VioC were resuspended in buffer A (30 mL; Tris-HCl pH 8.0 (20 mM), NaCl (300 mM), glycerol (10% *v/v*)) with imidazole (5 mM). Cells were broken by sonication, and cell debris was removed by centrifugation. The supernatant was incubated with Ni-NTA Agarose (Qiagen) resin (1 mL) at 4 °C for 1 h with gentle rocking. The resin was recovered and washed with buffer A containing imidazole (20 mM). VioC was eluted with a step gradient of buffer A plus varying concentrations of imidazole (40, 60, 100, or 250 mM). Fractions containing VioC, based on SDS-PAGE/Coomassie staining, were pooled and dialyzed against buffer B (Tris-HCl pH 8.0 (50 mM), NaCl (100 mM), glycerol (10% *v/v*)). The same protocol was followed for VioD purification, with the exception that PLP (0.1 mM) was included in all buffers. The concentration of VioC was determined spectrophotometrically at 280 nm by use of the calculated molar extinction coefficient of VioC ($47\,630\ M^{-1}\ cm^{-1}$). The concentration of VioD was determined by BCA assay (Pierce) with bovine serum albumin as a standard.

VioC and VioD assays. Reactions monitoring VioC activity contained Na phosphate pH 7.5 (0.1 M), NaCl (0.1 M), glycerol (10% *v/v*), Na ascorbate (1 mM), Na-αKG (1 mM), dithiothreitol (1 mM), $FeSO_4$ (50 μM), **4** (400 μM), and VioC (1 μM). After the desired time of reaction, a sample (25 μL) was removed and added to OPA (Pierce; 25 μL) to derivatize all free primary amines.[23] Derivatized reactants and products were separated by HPLC with a Vydac C18 small-pore column at a flow rate of $1\ mL\,min^{-1}$. The following solvents were used: solvent A—double distilled H_2O and 0.1% trifluoroacetic acid (TFA); solvent B—acetonitrile and 0.1% TFA. The profile for separation was 5 min isocratic development at A/B (91:9%); 20 min linear gradient from A/B (91:9%) to A/B (70:30%). The elution of OPA-derivatized product was monitored at A_{340}.

Reactions for monitoring VioD activity contained Na-phosphate pH 7.5 (0.1 M), NaCl (50 mM), glycerol (5% *v/v*), PLP (0.1 mM), **5** (2 mM), and VioD ($0.25\ mg\,mL^{-1}$). Termination of the reaction by OPA-derivatization and analysis by HPLC were performed as described for VioC.

Preparative isolation of 5. A VioC reaction (50 mL) contained Na phosphate pH 7.5 (0.1 M), NaCl (0.1 M), glycerol (10% *v/v*), Na ascorbate (10 mM), Na-αKG (10 mM), dithiothreitol (1 mM), $FeSO_4$ (50 μM), **4** (2 mM), and VioC (5 μM). Progression of the reaction was monitored by using the OPA-derivatization and HPLC analysis described above. Once complete conversion of **4** to **5** was observed, the pH of the solution was adjusted to 2.5 with HCl, and the precipitated protein was removed by centrifugation. The supernatant was passed through a Dowex 50WX8-100 (H^+ form) equilibrated with ddH_2O. The column was washed with ddH_2O, and **5** was eluted with a step gradient of 1, 2, 3, 4, and 5 N NH_4OH. All fractions were flash frozen in liquid N_2 and lyophilized. Eluted product was resuspended in ddH_2O, and the pH was neutralized with HCl. Fractions containing **5** were determined by OPA-derivatization and HPLC analysis. **5** was precipitated from solution by the addition of ethanol.

Preparative isolation of 3. A reaction mixture (5 mL) contained Na phosphate pH 7.5 (0.1 M), NaCl (50 mM), glycerol (5% *v/v*), PLP (0.1 mM), **5** (40 mM), and VioD ($0.3\ mg\,mL^{-1}$). The conversion of **5** to **3** was monitored by OPA-derivitization and HPLC analysis as discussed above. Once complete conversion was observed, the protein was removed by passage through a microcon 3 spin column. The reaction mixture was then passed through a Dowex 50WX2-100 (H^+ form) column equilibrated with ddH_2O. The column was washed with ddH_2O, and **3** was eluted with a step gradient of 1, 2, 3, 4, and 5 N NH_4OH. All fractions were flash frozen in liquid N_2 and lyophilized. Eluted product was resuspended in ddH_2O, the sample was neutralized with HCl, and fractions containing **3** were determined by OPA-derivatization and HPLC analysis. Acid **3** was finally purified by low-pressure silica gel chromatography by elution with *n*-butanol/acetic acid/water (4:1:1) as the monoacetic acid salt.

^{1}H and ^{13}C NMR analysis. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 500 MHz instruments operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. ^{1}H,^{1}H gCOSY, HMQC, and gHMBC were performed by using standard Varian pulse sequences.

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