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Highly Polar Spiro-Isoxazolines from the Sponge *Aplysina fulva*

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Abstract

Two new highly polar brominated spiroisoxazolines, araplysillin N^9-sulfamate (**1**) and an *N*-[5*S*, 10*R*)-7,9-dibromo-10-hydroxy-8-methoxy-1-oxa-2-azaspiro[4.5]deca-2,6,8-triene-3-carboxy]-4-aminobutanoic acid (**2**), were isolated from a sample of *Aplysina fulva* collected in the Florida Keys. The absolute stereostructures of the new compounds were determined from analysis of MS, ^{1}H and ^{13}C NMR and CD spectroscopy. Compound **2** provides a structural clue that may unify the biosynthesis of brominated spiroisoxazolines.

Secondary metabolites from sponges of the Order Verongidae[1] are typically highly oxidized compounds derived from bromotyrosine. Modified alkaloids derived from 3′,5′-dibromotyrosine (Figure 1, ***i***, R = H) have been found from various Verongid genera[2] – mainly *Aplysina*, *Psammaplysilla*, *Pseudoceratina* and *Verongia*[3] – that are widely distributed throughout Mediterranean, Pacific and Atlantic waters.[4] Heterocycles based on brominated spiro-isoxazolines (Figure 1, ***iii***, R=H, (5*S*,10*R*)-7,9-dibromo-10-hydroxy-8-methoxy-1-oxa-2-azaspiro[4.5]deca-2,6,8-triene-3-carboxamide) are common natural products arising from bromotyrosine secondary metabolism.[5] Since the first example of a spiroisoxazoline was reported by the Minale group from two Mediterranean species, *Aplysina aerophoba* and *Verongia thiona*,[6] over 25 spiroisoxazoline analogs have been described.[4]

Recently, we reported geographic variability of the diastereomeric compositions of fistularin-3 and 11-*epi*-fistularin-3 in *Aplysina* species collected from Brazil and the USA (Florida Keys) and *Agelas* from Australia (the Great Barrier Reef).[7] We have extended these investigations and now report two new polar water-soluble spiroisoxazolines – the sulfamate **1** and carboxylic acid **2**. Both compounds occur in very low concentrations in the most polar fractions derived from column chromatography of methanol-soluble components of the sponge extract. Compound **2** is a lower homolog of purpuroceratic acid **(5)**,[8] reported by Kijoa and coworkers, and lacks the unusual aryl *C*-methyl group of the latter. Compound **1** is the *N*-sulfato derivative of the known compound araplysillin-1 (**3**) and is formally the decarboxylation product of the *N*-sulfato α-aminoacid, ianthesine D (**5**), reported by Okamoto et al. from *Ianthella* sp.[9] Carboxylic acids **2** and **5** may provide a “missing link” that unifies the biosynthesis of several spiroisoxazoline alkaloids from Verongid sponges.

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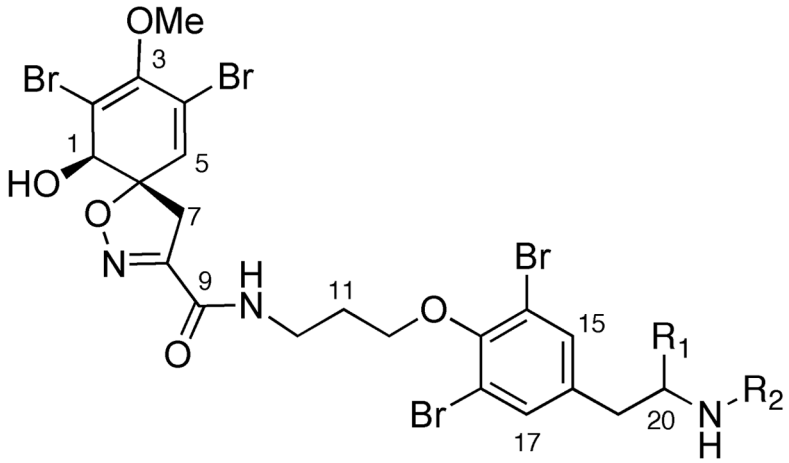
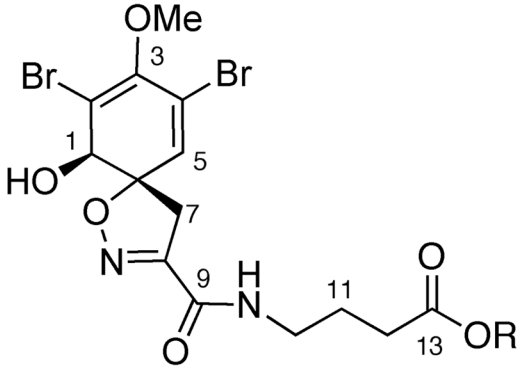
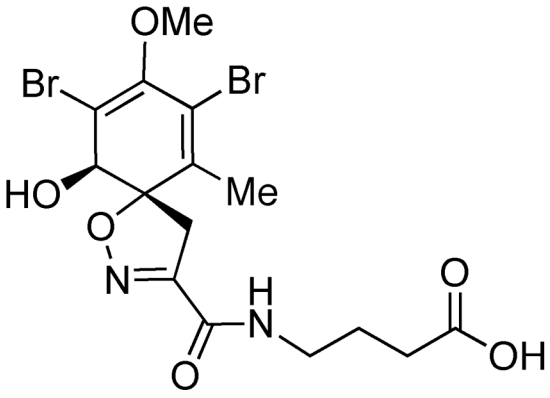


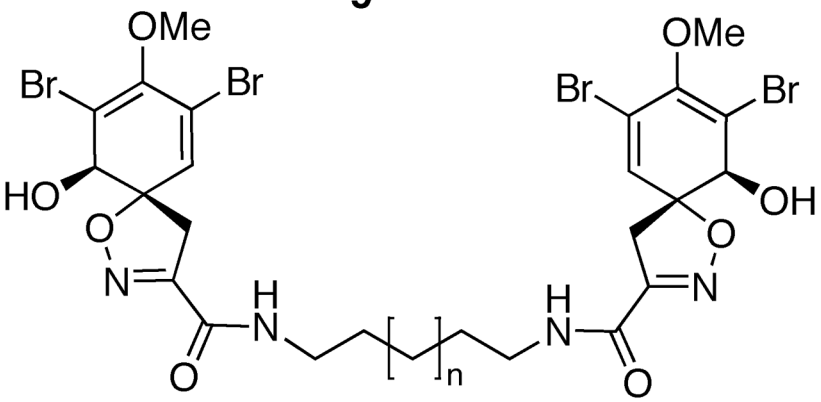
1 R = H, $R_2 = SO_3Na$
3 R = H $R_2 = H$
4 R = COOH, $R_2 = SO_3Na$



2 R = H
2a R = Me



5



6a n = 0
6b n = 1

Moderately polar fractions obtained from silica chromatography of the $CHCl_3$-MeOH extract of *Aplysina fulva* contained 11-hydroxyfistularin-3 and 11-epi-fistularin-3 in variable proportions as we have reported previously. The most polar fraction obtained from elution of the silica column (100% MeOH) was further separated by C_{18} reversed phase HPLC to give a 3:2 mixture of compounds **1** and **2**, respectively. Final purification of this mixture by preparative silica TLC gave pure samples of each compound **1** and **2** as colorless solids.

Compound **1** is an optically active solid, $[\alpha]_D$ +100 (*c* 0.06, MeOH) with UV activity [λ_{max}206 nm (log ε 4.70), 278 (3.84)]. The compound did not dissolve in $CHCl_3$, but was partially soluble in H_2O, MeOH, and soluble in DMSO and $CHCl_3$-MeOH. Analysis of the formula of **1** by mass spectrometry was complicated by multiple pseudomolecular ions and neutral losses. The negative ion ESIMS (*m/z* 841 $[M-H+Na_2]^+$, 794 $[M-Na]^-$ and 714 $[M-SO_3-Na]^-$) suggested a Na^+ salt of sulfate-half acid. The formula of $C_{21}H_{23}Br_4N_3O_8S$ for the neutral species, requiring ten double bond equivalents, was assigned from positive-ion MALDI HRMS (*m/z* 841.7643, $[M-H+Na_2]^+$ Δm = 3.3 mmu). The presence of only 19 distinct signals in the ^{13}C NMR spectrum suggested an element of symmetry in an aryl ring. The ^1H NMR spectrum of **1** (Table 1) indicated the presence of three vinyl and aromatic protons, comprising H-5 in the spiro-ring system (δ 6.31, s, 1H) and the two-proton signal in the second ring belonging to a symmetrical 3,5-dibromotyrosine (δ 7.41, s, 2H). The ^1H ($CDCl_3$-CD_3OD) and ^{13}C NMR (DMSO-d_6) signals – in particular, the C=N signal (δ 154.6, s) and the characteristic AB quartet for H_2-7 (δ 3.01, d, *J* = 18.3 Hz; 3.79, d, *J* = 18.3 Hz) – were consistent

with a 1-oxa-2-azaspirodecatriene-ring system as seen in the structures of aeroplysinin-1[10] fistularin-3,[12] and related compounds. NMR signals were observed (Table 1) for other units including a 1,3-disubstituted propane chain terminated with an oxygen atom and an NH(CO) group, and an 1-aryl-2-ethylamine side chain.

A database survey (MarinLit, University of Canterbury) of known Verongid sponge compounds, matched the expected formula (with the assumption of one SO_3 group) to a mono-sulfated derivative of araplysillin-1 (**3**).[11] Verification of this assignment came from ^{13}C NMR chemical shifts of **1** (Table 1) which matched closely those of **3**.[9] (Table 1) The assembled structure **1** and complete chemical shifts assignments were obtained by linking substructures from interpretation of 2D NMR data (gCOSY, gDQFCOSY, gHMBC, and gHSQC).

The SO_3 group was located in **1** as follows. Three exchangeable signals were detected (ESIMS measured in CD_3OD); these appeared in the ^1H NMR spectrum (DMSO-d_6) and were attributable to an OH group (δ 6.37 d, J = 7.5 Hz), one amide NH (δ 8.55, t, J = 5.7 Hz) and an unidentified upfield NH signal (δ 4.14, t, J = 6.7 Hz). Since all other heteroatoms in the formula of **1** were accounted for, the SO_3 group was placed on a nitrogen atom that allowed us to ascribe the latter NH signal to a sulfamate group (-$NHSO_3^-$). The NH chemical shift is consistent with those observed for other sulfamates (e.g., δ 4.91 for ianthenisin C[10]).

Compound **2**, $[\alpha]_D$ +140 (*c* 0.04, MeOH), also showed an isotope pattern for the MS parent ions consistent with the presence of two Br atoms. The molecular mass of **2** was inferred as 466 amu from low-resolution positive- and negative-ion ESIMS (*m/z* 489 $[M+Na]^+$, 465 $[M-H]^-$), but the formula $C_{14}H_{16}Br_2N_2O_6$ could only be confirmed by MALDI HRMS (*m/z* 488.9297 $[M+Na]^+$, Δm = 2.4 mmu). and required seven double bond equivalents. Unlike **1**, the appearance of 14 distinct signals in the ^{13}C NMR spectrum of **2** (Table 2) showed lack of symmetry. The ^1H NMR spectrum of compound **2** (Table 2) was much simplified compared to that of **1**, but retained signals due to the spiroisoxazoline unit and a 1,3-disubstituted propane side chain. The ^{13}C NMR spectrum of **2** showed two C=O ^{13}C NMR signals; one corresponding to a free carboxylic acid (δ 181.5 s) and a second due to the α-oximimo amide group **(**δ 158.9 s). Treatment of **2** with diazomethane gave the corresponding methyl ester **2a** with a new ^1H NMR signal (CD_3OD) due to a second OMe group (δ 3.66, s, 3H). Carboxylic acid **2** most closely resembles purpuroceratic acid B (**5**) with the only difference being the absence of a methyl group at C-5.[5]

The relative configurations of **1** and **2** were addressed using ^{13}C NMR, ROESY, and comparisons of their circular dichroism (CD) spectra with those of known spiroisoxazolines. Since the ^{13}C NMR chemical shifts of the 1-oxa-2-azaspirodecatriene-ring systems in **1** and **2** were essentially identical, we could assume the relative stereochemistry was the same in both and assign them by analysis of **1**. The ROESY spectrum (500 mS) of **1** (DMSO-d_6) showed dipolar coupling between the C-1 OH and the *proximal* diastereotopic proton of the C-7 methylene group (δ 3.62, d, J = 18.0 Hz)[12] that places both groups *syn* to each other. The absolute configuration of **1** and **2** were assigned by circular dichroism (CD). The CD spectra of **1** and **2** were very similar [CD MeOH, **1**: λ_{max} 244 (Δε +8.3), 285 (+8.0). **2**: λ_{max} 243 (Δε +7.0), 289 (+5.2)] and showed two prominent positive Cotton effects (CE) that were of the same sign and similar magnitudes to those of (1*R*,6*S*)-spiroisoxazolines, particularly aerothionin (**6a**).[6,13] Thus, the absolute configurations are as depicted in structures **1** and **2**.

Compounds **2** and **5** contain a structural motif – a spiroisoxazoline carboxyl group *N*-acylated to 4-aminobutanoic acid – that shows a trend that may help explain their biosynthesis. We propose each dibromospiroisoxazoline derives from a *dipeptide* comprised of an *N*-terminal 3,′5′-dibromotyrosine and another common α-amino acid (Figure 1, ***i*** R = amino acid) that has undergone decarboxylation to the corresponding amine either before or after peptide bond

formation (Figure 2). For example, if the 1,4-diaminobutane (putrescine) linker in **6a** derives from ornithine (or lysine, in the case of homoaerothionin, **6b**[14]), it would appear the 4-aminobutanoic acid unit in **2** derives from glutamic acid. In contrast, the linker in **1**, **3**, **4**, fistularin-3[12] and most other spiroisoxazolines is 3-amino-1-propanol, which does not have an obvious biogenesis unless it is considered as the decarboxylation product of homoserine, an uncommon amino acid that is an intermediate of one pathway to *S*-adenosyl methionine (SAM). Since the opposite end of the 3-amino-1-propanol linker in fistularin-3 is *O*-alkylated to the phenolic oxygen of a 3′,5′-dibromotyramine (DBT) unit, it is conceivable that the immediate precursor to the fistularin-3 linker is SAM which participates in an aberrant S_N2 type alkylation of DBT phenoxide at the more substituted *S*-CH_2 carbon (C-3) of the sulfonium ion instead of the *S*-Me carbon. After decarboxylation, the resultant amine **7** participates in amide bond formation with a spiroisoxazoline unit ***ii*** that is derived separately by 'normal' *O*-methylation of DBT with SAM and arene oxidation (Figure 1). A putative 'C^3-alanyl-methyltransferase' might catalyze the former transformation and rationalize the unusual *N*,*O*-substituted C_3 unit that links two of three DBT-derived groups in high-molecular mass bromotyrosine natural products from Verongid sponges.

Insufficient quantities of **1** and **2** were available to assess their antifungal activity against *Cryptococcus neoformans* or *Candida albicans.* Further investigations are pending to identify antifungal active principles in *Aplysina fulva* and other sponges from the Bahamas.

Experimental Section

General Experimental Procedures

Optical rotations were measured on a JASCO DIP370 or P1020 polarimeter. UV spectra were recorded on a Hewlett Packard 8452A single-beam spectrometer and CD spectra were measured using a JASCO 810 spectropolarimeter. IR spectra were recorded on Mattson Galaxy 3000 FTIR instrument ESIMS was measured using a Finnigan LCQ Deca mass spectrometer and high-resolution MALDI FTMS spectra were provided by the University of California, Riverside, MS laboratory. ^{1}H NMR and 2D NMR spectra were recorded on a Bruker 600 MHz DRX-600 equipped with a 5 mm cryoprobe and ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer. Chemical shifts were referenced to CD_3OD (δ_H = 3.31 ppm; δ_C = 49.0 ppm), and DMSO (δ_H = 2.50 ppm; δ_C = 39.5 ppm). Solvents used were of HPLC grade.

Animal Material

Samples of *Aplysina fulva* (95-03-025, total ~1.4 kg) were collected by hand using scuba from Dry Rocks near Key Largo, Florida in 1995 and immediately stored at −20 °C until needed. Voucher specimens are archived in the Department of Chemistry and Biochemistry, UC San Diego.

Extraction and Isolation

A $CHCl_3$-MeOH-soluble fraction (3.14 g) of the MeOH extract of the sponge was separated by silica flash chromatography and eluted using a solvent gradient (1.5–100% MeOH-$CHCl_3$) to give an early-eluting fraction (10% MeOH-$CHCl_3$) containing fistularin-3,[12] 11-*epi*-fistularin-3[15] and 11-oxoaerothionin[16] as previously described.[7] The crude fraction eluting with 100% MeOH was submitted to further purification by reversed-phase HPLC (Dynamax C_{18}, 5 μm, 10 × 250 mm, 3:1 H_2O-CH_3CN, 4.0 mL/min) to a single peak comprising a 3:2 mixture of **1** and **2** (4.8 mg, 0.0017% wet wt) respectively. Further purification of 3 mg of this mixture by silica TLC (4:21 MeOH-$CHCl_3$) provided **1** (1.2 mg) and **2** (0.7 mg) as white solids.

1. $[\alpha]_D^{23}$ +100 (*c* 0.06, MeOH); UV (MeOH) λ_{max} 206 nm (log ε 4.70), 278 (3.84); CD (MeOH) λ 244 nm (Δε +8.3), 285 (+8.0); IR (neat) ν_{max} 3288, 2939, 2864, 1664, 1593, 1544, 1458, 1310, 1218, 1044, 990, 933, 866, 739 cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (125 MHz), see Table 1; ESIMS *m/z* 841 $[M-H+Na_2]^+$, 794 $[M-Na]^-$, 714 $[M-SO_3-Na]^-$; MALDI HRMS *m/z* 841.7643 $[M-H+Na_2]^+$, calcd for $C_{21}H_{22}N_3O_8Na_2S^{81}Br_4$ 841.7616.
2. $[\alpha]_D^{23}$ +140 (*c* 0.04, MeOH); UV (MeOH) λ_{max} 223 nm (log ε 3.93), 281 (3.63); CD (MeOH) λ 243 nm (Δε +7.0), 289 (+5.2); IR (neat) ν_{max} 3321, 2959, 2930, 2850, 1666, 1581, 1407, 1310, 1271, 1218, 1048, 990, 921, 767, 739, 703 cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (125 MHz), see Table 2; ESIMS *m/z* 489 $[M+Na]^+$, 465 $[M-H]^-$; MALDI HRMS *m/z* 488.9297 $[M+Na]^+$, calcd for $C_{14}H_{16}N_2O_6Na^{79}Br_2$ 488.9273.

Methyl Ester of Carboxylic Acid 2. 2a

A solution of **2** (600 μg) in MeOH (~0.5 mL) at 0 °C was treated with an excess of ethereal solution CH_2N_2 (~0.2 M) and allowed to warm to rt over 20 min. The mixture was concentrated and separated on a pencil column (silica, 15:85 MeOH-CH_2Cl_2) to give **2a** as a colorless solid (310 μg). ^1H NMR (CD_3OD) δ 3.66 (s, 3H, OMe), 3.72 (s, 3H, OMe); HREIMS *m/z* 479.9542 $[M]^+$ calcd for $C_{15}H_{18}O_6N_2{}^{79}Br_2$ 479.9526.

Acknowledgements

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References and Notes

1. Class, Demospongia; Subclass, Ceractinomorph; Order, Verongidae. Verongid sponges are easily identified in the field: upon tissue damage and exposure to air, their characteristic yellow pigmentation undergoes rapid aerial oxidation to blue, purple and finally black pigments.
2. Sponges the families Aplysinidae, Aplysinellidae, Ianthellidae, and Pseudoceratinidae are responsible for >90% of brominated compounds from Verongida.
3. The species "*Verongia*" is considered now by taxonomists to be synonymous with *Aplysina*. HooperJNAWiedenmayerFWellsAZoological Catalogue of AustraliaCSIROMelbourne199412
4. Masuno, MN. Ph.D. Thesis. Chapter 1. University of California, Davis; 2006. Synthesis and Biological Evaluation of Brominated Marine Natural Product Analogs that Modulate the Skeletal Muscle FKBP12/Calcium Channel Complex.
5. It is believed the spiro-isoxazolines arise from oxidation of the α-amino group of a 4′-methoxy-3′,5′-dibromotyrosine residue to a ketoxime that undergoes nucleophilic attack upon a putative arene epoxide (Figure 1, e.g., ii, arising from further oxidation of the phenyl ring to give the heterocyclic ring and a secondary alcohol. Anderson RJ, Faulkner DJ. Tetrahedron Lett 1973;14:1175–1178. although the order of reactions are speculative and identities of the responsible enzymes are presently unknown
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13. McMillan JA, Paul IC, Goo YM, Rinehart KL, Krueger WC, Pschigoda LM. Tetrahedron Lett 1981;22:39–42. The Cotton effects of the skewed diene in the CD spectrum of aerothionin (6a) were as follows: CD (MeOH) λ_{max} 284 ($\Delta\varepsilon$ +21.3), 245 (+23.7) (after conversion, $[\theta] = 3300\Delta\varepsilon$). Since 6a is a C_2 symmetrical dimer, we estimated the following comparison values corresponding to the dichroism of one diene unit, assuming no interaction between the two chromophores: CD (MeOH) λ_{max} 284 ($\Delta\varepsilon$ +10.7), 245 (+11.8.)
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16. Acosta AL, Rodriguez AD. J Nat Prod 1992;55:1007–1012. [PubMed: 1402952]

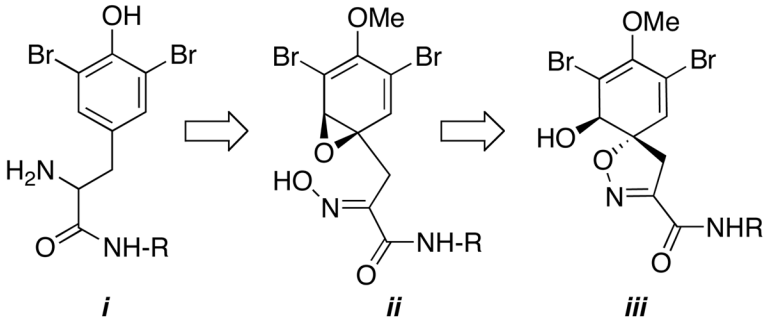

Figure 1.
Putative biosynthesis of spiroisoxazoline carboxamide ***iii*** (R = H, (5*S*,10*R*)-7,9-dibromo-10-hydroxy-8-methoxy-1-oxa-2-azaspiro[4.5]deca-2,6,8-triene-3-carboxamide) from a 3′5′-dibromotyrosine residue.

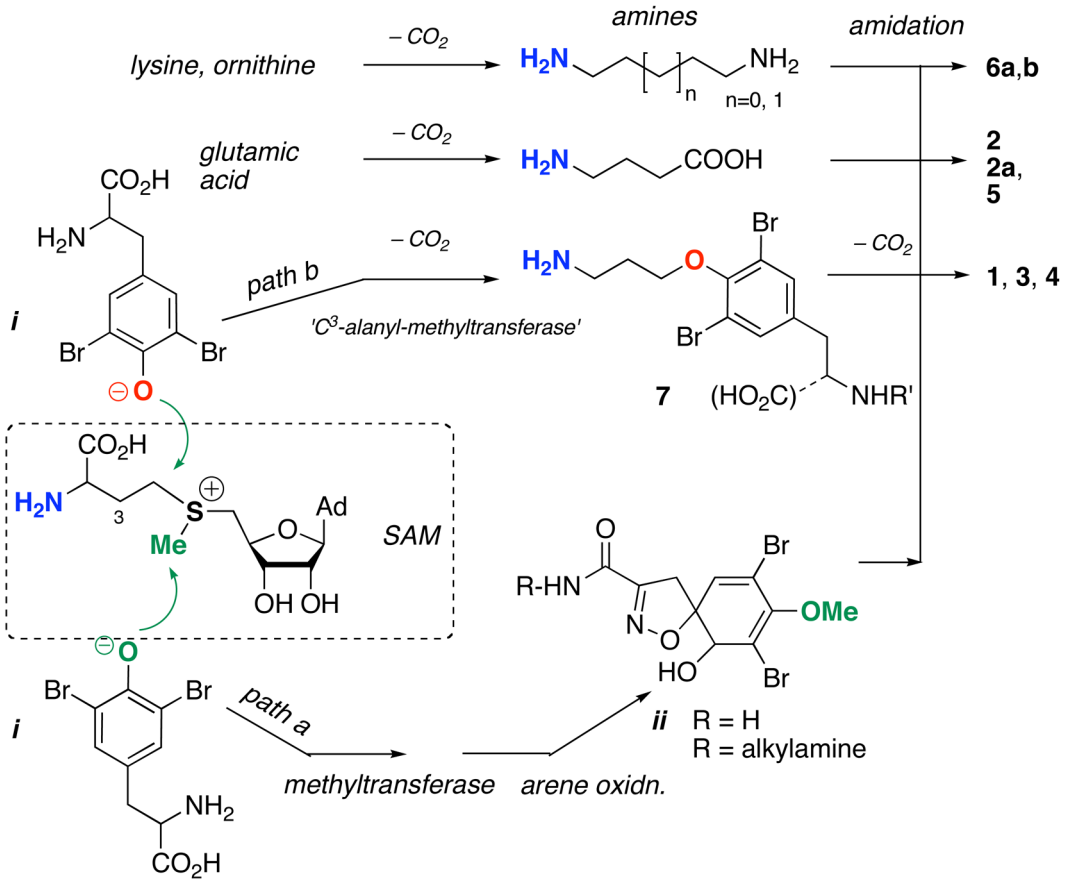

Figure 2.

Proposed unified biosynthesis of **1**–**6** and aberrant S_N2 substitution of *S*-adenosylmethionine (SAM) by 3′5′-dibromotyrosine phenoxide (path b) by hypothetical ‘C_3-alanyl-methyltransferase’.

Table 1

NMR Data for Compound **1** (600 MHz)

no.	CD_3OD-$CDCl_3$ (2:1)[a] δ_C[b]	δ_H [mult., *J* (Hz)]	HMBC (H→C)	DQF-COSY	DMSO-d_6 δ_C[b]	δ_H [mult., *J* (Hz)]	HMBC (H→C)	DQFCOSY
1	74.6 (CH)	4.16 s	3,4,5,6		73.6	3.91 d (7.5)	3,4,5,6	OH
2	122.2 (C)[d]				120.8			
3	148.5 (C)				147.3[c]			
4	113.9 (C)[d]				113.1			
5	131.4 (CH)	6.31 s	1,2,3,4,6,7		131.2	6.58 s	1,2,3	
6	92.1 (C)				90.2			
7	39.7 (CH_2)	3.02 d(18.3) 3.82 d(18.3)	1,5,6,8		39.3[c]	3.21 d (18.6) 3.62 d (18.6)	1,5,6,8	
8	154.6 (C)				154.3[c]			
9	160.6 (C)				158.9			
10	37.7 (CH_2)	3.61 t (6.4)	9,11,12	11	36.2	3.39 td (6.6, 5.7)	9,11,12	11, CONH
11	29.9 (CH_2)	2.09 pent (6.4)	10, 12	10, 12	29.4	1.98 p (6.6)	10,12	10,12
12	71.6 (CH_2)	4.05 t (6.4)	10,11,13	11	71.2	3.95 t (6.6)	10,11	11
13	151.8 (C)				150.8[c]			
14, 18	118.5 (C)				117.0			
15, 17	133.7 (CH)	7.41 s	13,14,18,19		133.0	7.49 s	13,14,18,19	
16	139.6 (C)				140.6			
19	35.3 (CH_2)	2.79 t (7.3)	15,16,17,20	20	33.7	2.67 t (6.7)	15,16,17,20	20
20	45.4 (CH_2)	3.20 t (7.3)	16,19	19	44.7	2.92 q (6.7)	16,19	19, SO_3NH
OMe	60.3 (CH_3)	3.71 s	3		59.6	3.64 s	3	
OH						6.37 d (7.5)		1
CONH						8.55 t (5.7)	9	10
SO_3NH						4.14 t (6.7)		20

[a] Run as 3:2 mixture with compound **2**.

[b] 500 MHz

[c] Assigned by HSQC and HMBC (*J* = 8 Hz) at 600 MHz

[d] Assignment based on calculated ^{13}C chemical shifts (ChemDraw Ultra).

Table 2

NMR Data for Compound **2** (600 MHz)

no.	CD_3OD-$CDCl_3$ (2:1)[a] δ_C[b]	δ_H [mult., *J* (Hz)]	HMBC (H→C)	DQF-COSY	DMSO-d_6 δ_C[b]	δ_H [mult., *J* (Hz)]	HMBC (H→C)	DQFCOSY
1	74.6 (CH)	4.15 s	3,4,5,6		73.4	3.93 s	3,4,5,6	
2	122.2 (C)[d]				120.8			
3	148.5 (C)				147.0			
4	113.9 (C)[d]				113.3			
5	131.4 (CH)	6.30 s	1,2,3, 4,6,7		131.2	6.56 s	1,2,3,4,7	
6	91.9 (C)				90.1			
7	39.8 (CH_2)	3.01 d (18.0) 3.79 d (18.0)	1,5,6,8		39.7[c]	3.22 d (17.7) 3.63 d (17.7)	1,5,6,8	
8	154.7 (C)				154.8			
9	160.6 (C)				158.9			
10	40.2 (CH_2)	3.30 t (6.9)		11	39.4[c]	3.14 t (6.6)	9,11	11
11	26.1 (CH_2)	1.81 p (6.9)	10,12,13	10, 12	24.5	1.65 p (6.6)	10,12,13	10,12
12	35.9 (CH_2)	2.21 t (6.9)	10,11,13	11	34.5[c]	2.09 bt (6.6)	10,11,13	11
13	181.5 (C)				174.7[c]			
OMe	60.3 (CH_3)	3.71 s	3		59.6	3.63 s	3	
OH								
CONH								
COOH								

[a] Run as 2:3 mixture with compound **1**.

[b] 500 MHz.

[c] Assigned by HSQC and HMBC (*J* = 8 Hz) at 600 MHz

[d] Assignment based on calculated ^{13}C chemical shifts (ChemDraw Ultra)