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De Novo Synthesis of Benzosceptrin C and Nagelamide H from 7-^{15}N-Oroidin: Implications for Pyrrole−Aminoimidazole Alkaloid Biosynthesis

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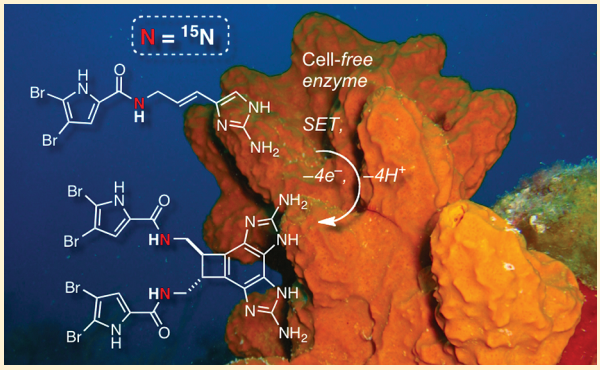
Supporting Information

ABSTRACT: De novo synthesis of the natural products benzosceptrin C (**7**) and nagelamide H (**8**) was achieved using cell-free enzyme preparations from the marine sponges *Agelas sceptrum* and *Stylissa caribica* employing synthetic 7-^{15}N-oroidin. These studies provide direct experimental evidence to support the long-standing, but untested, hypothesis that oroidin is a precursor to more complex pyrrole−aminoimidazole alkaloids, such as the sceptrins, benzosceptrins, and nagelamides. In addition, a new nagelamide, didebromonagelamide A (**5b**), was isolated from *S. caribica*, representing the first report of a nagelamide-like compound from the Caribbean.



Pyrrole−aminoimidazole alkaloids (PAIs) from marine sponges, including oroidin (**1c**),[1,2] stevensine (**4**),[3] sceptrin (**2a**),[4] and ageliferin (**3a**)[5] (Figure 1), show a broad spectrum of biological activities including antifeedant[6] and medicinal properties.[7] Prevailing hypotheses suggest that the structural complexity of PAIs, illustrated by the structure of palau'amine,[8] belies a simple biogenesis: PAIs can be explained if one assumes they arise from modular reactions of simple precursors: clathrodin, hymenidin, or oroidin (**1a**−**c**).[9]

A recent report by Genta-Jouve and co-workers substantiates the origin of oroidin (**1c**) from lysine and proline,[10] but the biosynthesis of higher-order PAIs from **1a**−**c** is somewhat more contentious. Despite several proposed hypotheses for conversion of **1a**−**c** to sceptrins (**3a**−**c**), ageliferin (**4**), benzosceptrin (**7**), and other PAIs,[11] no direct experimental evidence has been presented to support any of them. Using cell-free enzyme preparations (CFP) of *Stylissa caribica*, we recently demonstrated conversion of the synthetic, non-natural oroidin derivative dichloroclathrodin (**1d**) into dimeric non-natural PAIs. We termed this biomimetic transformation—enzymatic conversion of *non*-natural substrates to yield natural product analogues—'metabiosynthesis' and provided additional experimental evidence to support the contention that C−C bond formation is mediated by metallo-oxidoreductases.[12] Here, we report the de novo synthesis of ^{15}N-labeled benzosceptrin C (**7**)[13,14] and nagelamide H (**8**)[15] from 7-^{15}N-oroidin[16] (Figure 2) by CFPs prepared from the PAI-producing Caribbean sponges, *Agelas sceptrum* and *Stylissa caribica*, an unequivocal demonstration of the biosynthetic origin of these natural products.

A. sceptrum and *S. caribica* were each collected by hand using scuba (−20 to −30 m, Bahamas) and converted to CFPs. Incubation of 7-^{15}N-oroidin, prepared as previously described,[16] with a buffered aqueous CFP (0.1 M sodium phosphate buffer, pH 7.4) obtained from either *A. sceptrum* or *S. caribica* resulted in rapid conversion (within 30 min) into two major products: **7** and **8**. The mass of compound **7**, $C_{22}H_{18}Br_4N_8{}^{15}N_2O_2$ (HR-ESI-TOFMS m/z 772.8358 $[M + H]^+$) was 2 amu higher than that of the tetrabrominated natural product benzosceptrin C, and the ^{1}H NMR spectrum for **7** was identical to that of the natural product.[13,14] The mass spectrum of compound **8**, $C_{24}H_{25}Br_4N_9{}^{15}N_2O_5S$ (HR-ESI-TOFMS m/z 897.8502, $[M + H]^+$) showed an isotopic pattern consistent with Br_4 and was 2 amu higher than that of the natural product nagelamide H.[15] The ^{1}H NMR spectrum for **8** (see Supporting Information) was almost identical to the reported natural product, except for the presence of additional $^1J_{H,N}$ coupling (J = 90 Hz (^{15}NH−7), J = 96 Hz (^{15}NH−7′)) expected from the site-specific ^{15}N-labeling.[17]

The benzosceptrins and nagelamides have been reported from several *Agelas* spp. collected in the Pacific Ocean[13,15] and Mediterranean Sea,[21] but have not been described from Caribbean sponges. A survey of the minor constituents of an

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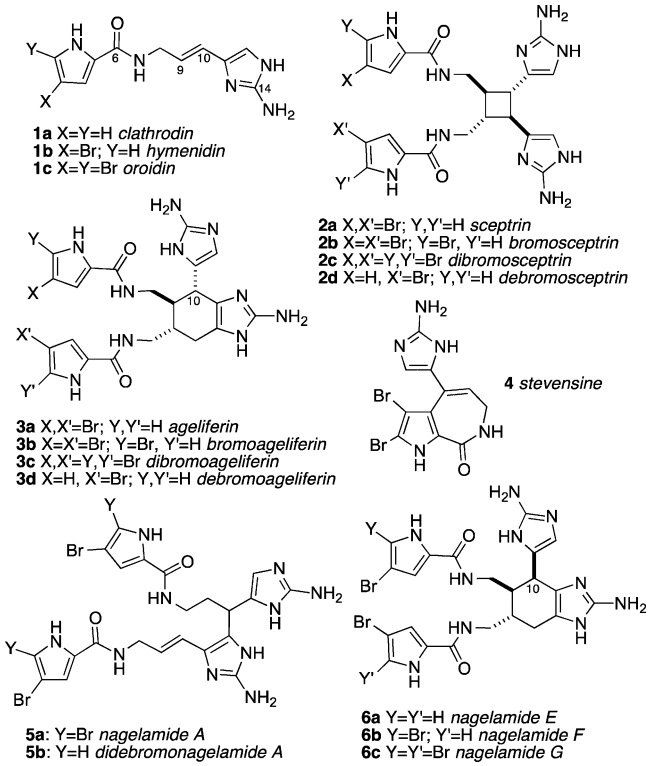

Figure 1. Select pyrrole–aminoimidazole alkaloids isolated from marine sponges *Agelas* spp. and *Stylissa caribica* and synthetic dichloroclathrodin (**1d**). For clarity, PAIs are depicted as free bases and discrete tautomers.

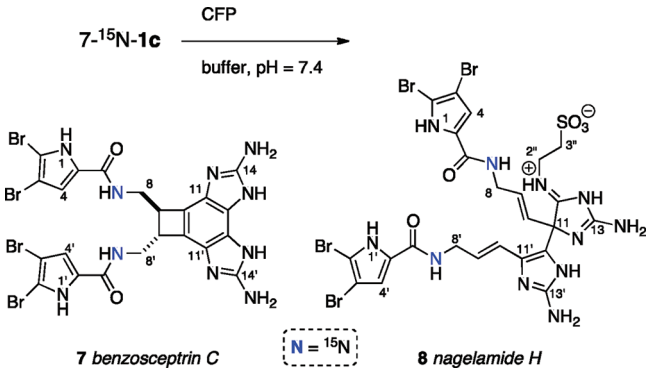

Figure 2. Conversion of 7-^{15}N-**1c** to $^{15}N_2$-labeled benzosceptrin C (**7**) and nagelamide H (**8**) using a cell-free enzyme preparation of *Agelas sceptrum*.

extract of *S. caribica*, collected in the Bahamas, by LCMS led to the identification of a new compound **5b** (Figure 1). The mass spectrum of **5b** displayed an isotope pattern for the pseudomolecular ion ($[M + H]^+$ *m/z* 619/621/623, 1:2:1) isomeric with sceptrin (**2a**) and ageliferin (**3a**); however, analysis of the ^{1}H NMR spectrum (see Supporting Information) showed the final degree of unsaturation was due to a double bond rather than a ring system. Observation of two ^{1}H NMR vinyl proton signals (δ_H 6.36, d, J = 16 Hz and δ_H 5.74, dt, J = 16.0, 6.0 Hz) verified the retention of one trans disubstituted double bond of an oroidin unit (δ_H 6.30, d, J = 16 Hz and δ_H 5.87, dt, J = 16.0, 6.0 Hz). An assessment of structures of reported PAIs showed the latter spin systems and carbon skeleton found only in nagelamides.[15] Similar ^{1}H NMR chemical shifts and spin systems were observed in **5b** and nagelamide A (**5a**), and the presence of two additional aromatic protons (δ_H 6.95 (H-2), δ_H 6.96 (H-2′)) was accounted for by the replacement of Br at C-2/2′ by H. Thus, **5b** was assigned as didebromonagelamide A.

Several species of *Agelas* and *S. caribica* from the Bahamas were analyzed by LCMS in order to ascertain the variability of composition of the major PAIs. Oroidin (**1c**) was present in all sponges (Table 1), with the exception of *A. sceptrum*, at variable concentrations (0.8–15% w/w of the *n*-BuOH extract). The

Table 1. Relative Concentrations of Oroidin (1c), Sceptrin (2a), Ageliferin (3a), and Stevensine (4) in Caribbean Sponges *Agelas* spp. and *Stylissa caribica* (% w/w)

	PAI (%)[b]			
species[a]	oroidin (**1c**)	sceptrin (**2a**)	ageliferin (**3a**)	stevensine (**4**)
A. conifera	0.8	4.0		
A. dispar	15			
A. sceptrum		52	5.0	
S. caribica[c]	0.6			5.2
S. caribica[d]	1.4			53
S. caribica[e]	7.6			28

[a]Sponges were collected by hand using scuba (−20 to −30 m). [b]w/w of *n*-BuOH fraction, containing the entire PAI constituents. Less abundant higher-order PAIs are not shown. [c]Sweetings Cay, Bahamas (26°33.420′ N, 77°53.177′ W) in 2011. [d]Little San Salvador, Bahamas (24°35.170′ N, 75°58.483′ W) in 2011. [e]Little San Salvador, Bahamas (24°35.118′ N, 75°58.419′ W) in 2008.

major PAI in *S. caribica* extracts was stevensine (**4**, up to 53%), and sceptrin (**2a**) comprised the major PAI (52%) in *A. sceptrum*, while the isomeric ageliferin (**3a**) was present at an order of magnitude lower concentration (5%). These results reveal variability in the biosynthesis of the likely first-formed PAIs from **1c**. Lack of detection of **1c** in *A. sceptrum* is consistent with a low steady-state concentration of this intermediate and rapid conversion to **2c**, **3c**, and higher-order PAIs under homeostatic conditions. The finding that ^{15}N-oroidin is converted into **7** and **8** as the major products by CFPs of either *A. sceptrum* or *S. caribica*, despite the observation that neither is the major PAI in the sponges, points to a high enzymatic activity in the CFPs that is dysregulated with respect to intact cells. It is interesting to compare two parallel observations. Sceptrin (**2a**)—formally, a redox-neutral dimerization product of **1b**—is not formed from **1b** under cell-free enzyme conditions employed in these experiments; neither is it a substrate for formation of **7**. Second, cell-free enzymatic transformations of oroidin-type precursors lead to products with conversion efficiencies and single C–C bond dimerization motifs with only slight dependence on halogen content: unnatural dichloroclathrodin (**1d**) is transformed into "tetrachloronagelamide H",[1] while **1c** gives rise to both nagelamide A and nagelamide H analogues.[12]

A parsimonious interpretation of these data is that sceptrin and the benzosceptrin family of PAIs share a common precursor, **1a–c**, but their biosynthetic pathways are channelled into different oxidative regimes, likely diverging at a common reactive intermediate. Our hypothesis—transformation of **1a–c** to higher-order PAIs is governed by enzymatic single-electron transfer (SET)—is supported by cell-free enzymatic transformations.[12] Oxidation of **1c** to a radical cation ***i*** (Figure 3) that partitions into bifurcating pathways, mediated by SET, gives rise to different motifs: dimerization (redox-neutral, **2c**

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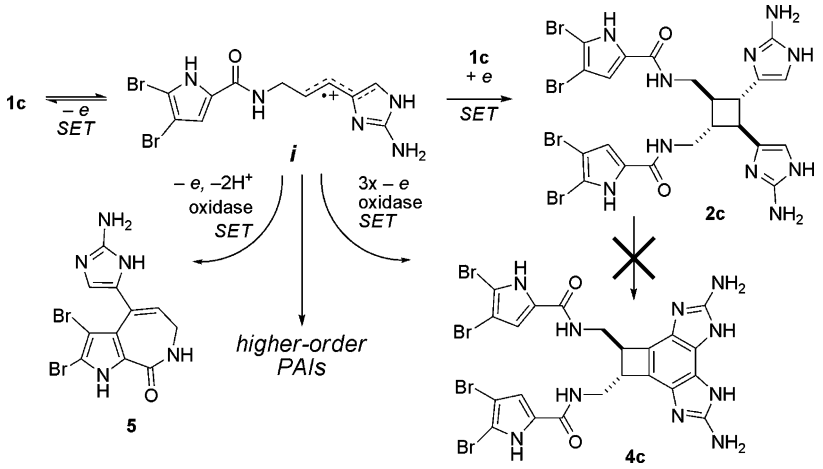

Figure 3. Proposed biosynthetic pathways by single-electron transfer (SET) to dibromosceptrin (**2c**), stevensine (**4**), benzosceptrin C (**7**), and higher-order PAIs in *Agelas* spp. and *Stylissa caribica*.

and **3c**), oxidative cyclization with removal of two electrons (**4**), and other C−C or C−N bond-forming reaction products involving removal of up to a total of four electrons (**7**), and higher-order PAIs.[12] In the absence of evidence, it is premature to speculate on the involvement of symbiotic or associated bacteria in the biosynthesis of PAI natural products, but it is more pragmatic to consider these alkaloids as products of whole sponge−microbial community assemblages.

^{15}N-Labeled compounds **7** and **8** displayed low optical activity ($[\alpha]_D$ −3.2 and −1.0, respectively; lit. −5.0[13] and ∼0[15]), similar to the low reported optical activities of the respective natural products. The new natural product **5b** also showed very low specific rotation ($[\alpha]_D$ ∼0), consistent with other nagelamides dimerized through bonds at C10−C15′ or C11−C15′.[15] Although optical purity was not reported for **7** or the nagelamides, it is possible that CFPs are dysregulated with respect to intact cells, leading to lower optical purity.[18] We hypothesize that C−C coupling in **2a**, **7**, and perhaps other PAIs occurs through SET mechanisms, likely mediated by cytochrome P_{450} enzymes analogous to bimolecular phenolic coupling observed in plant secondary metabolism.[12] Asymmetric induction in oxidative bimolecular phenolic coupling catalyzed by plant oxido-reductases has been shown to require an auxiliary or dirigent protein,[19] polypeptides that lack catalytic activity but exert asymmetric control. Lowered optical activity in products of cell-free transformations may be due to disruption of dirigent proteins or other ordered subcellular components required for PAI biosynthesis.

Samples of 7-^{15}N-oroidin were rapidly oxidized by CFPs of different PAI-producing sponges (*Agelas* spp. or *S. caribica*) to the same dimeric compounds **7** and **8**, suggesting a level of enzymatic plasticity in C−C bond construction. Nevertheless, oxidative C−C bond formation appears to be a catalytic feature exclusive to PAI-producing sponges; CFPs prepared from co-occurring sponges that do not produce PAIs failed to oxidize **1c**.[12] Transformation of **1c** to **7** is a net four-electron oxidation, with respect to the starting material, while **2a** and **3a** are redox-neutral products (Figure 3). The properties of the enzymes responsible for formation of **2a**, **7**, and **8**, including their cofactors or electron carrier dependence, were briefly investigated. Addition of electron donor−acceptor pairs known to be compatible with cytochromes, such as ferrocyanide−ferricyanide ($[Fe(II)(CN)_6]^{4-}$ and $[Fe(III)(CN)_6]^{3-}$), showed little effect on the rates of conversion or product distributions (Supporting Information). Dialysis of buffered, solubilized CFPs revealed that at least one enzyme responsible for catalytic conversion of **1c** to **7** and **8** resided exclusively in the retentate with a molecular mass of at least 10 kDa. Other features of the oxidative dimerization reactions, including measurements of oxidation potentials of **1a−c** and purification and characterization of the sponge PAI-oxidoreductases, are the subjects of ongoing investigations in our laboratory.

In conclusion, we describe didebromonagelamide A (**5b**), the first example of a C−C coupled nagelamide from a Caribbean sponge, *Stylissa caribica*, and experimental evidence that confirms the previously untested hypothesis that **1a−c** are precursors to complex dimeric PAI natural products by cell-free conversion of 7-^{15}N-oroidin (**1c**) into 7,7′-$^{15}N_2$-benzosceptrin C (**7**) and 7,7′-$^{15}N_2$-nagelamide H (**8**).

■ EXPERIMENTAL SECTION

General Experimental Procedures. General experimental procedures are described elsewhere.[20]

Cell-Free Enzyme Preparation. Sponges were collected by hand with scuba (−20 to −30 m) in the Bahamas and immediately frozen at −20 °C. Frozen sponge, within two weeks of collection, was divided into approximate cubes (∼1 cm^3) with a razor blade and combined with crushed dry ice (50 mL) and acetone (100 mL). The mixture was homogenized with a domestic high-speed hand-held blender (200 W) until a smooth, viscous paste was obtained. The mixture was immediately filtered through a prechilled Büchner funnel fitted with filter paper (Whatman #1), and the residue resubmitted to the homogenization process (2×) to obtain an orange-brown, coarse, wet powder, which was stored at −80 °C until further required. Independent assay showed that ∼70% of enzymatic activity was lost after storage at −20 °C for 9 months.

De Novo Synthesis of Benzosceptrin C (7) and Nagelamide H (8). *A. sceptrum* CFP was conditioned in 0.1 M sodium phosphate buffer, pH 7.4, over ice with gentle stirring for 15 min. The heterogeneous mixture was filtered through cheesecloth, followed by centrifugation for 5 min (1220*g*). The homogeneous supernatant was removed and then used immediately. 7-^{15}N-Oroidin (38 mg, 0.097 mmol; final substrate concentration of 10 μM) was taken up in CH_3CN (2 mL) and added dropwise to the CFP (8 mL of 0.4 mg/mL protein, Bradford assay), with vigorous stirring at 30 °C. The heterogeneous mixture was allowed to stir for 60 min, diluted with H_2O (12 mL), then acidified with acetic acid (4% v/v) and stirred for 5 min. The mixture was partitioned against *n*-BuOH (30 mL × 2), and

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the organic layer concentrated under reduced pressure and purified by two rounds of reversed-phase HPLC, first using a gradient of CH_3CN with aqueous 0.075% TFA (Luna phenyl-hexyl, 250 × 10 mm, 5 μm) and then 35:75 CH_3CN–aqueous 0.07% TFA (Synergi Hydro-RP, 250 × 10 mm, 4 μm), to yield 5.1 mg of **7** (6.9% isolated yield) and 1.0 mg of **8** (1.1% isolated yield).

Didebromonagelamide A (5b): pale yellow oil; $[\alpha]^{25}_D$ ~0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.32), 270 (4.38); FTIR (ATR, ZnSe) ν_{max} 3317 (br), 1685, 1527, 1442, 1200, 1142, 841, 802, 730 cm^{-1}; ^1H NMR data, see Supporting Information; HRESIMS $[M + H]^+$ *m*/*z* 619.0520 (calcd for $C_{22}H_{25}Br_2N_{10}O_2$, 619.0523).

7,7′-$^{15}N_2$-Benzosceptrin C (7): white solid; $[\alpha]^{25}_D$ −3.2 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.33) 276 (4.21); FTIR (ATR, ZnSe) ν_{max} 3304 (br), 1678, 1520, 1435, 1200, 1141, 848, 802, 730 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 3.47 (H-8a/8a′, dd, *J* = 14, 9.2 Hz), 3.76 (H-9/9, m), 3.97 (H-8b/8b′, dd, *J* = 14, 4.0 Hz), 6.59 (H-4/4′, s); HRESIMS $[M + H]^+$ *m*/*z* 772.8358 (calcd for $C_{22}H_{19}Br_4N_8{}^{15}N_2O_2$, 772.8361).

7,7′-$^{15}N_2$-Nagelamide H (8): pale yellow oil; $[\alpha]^{25}_D$ −1.0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 278 (4.30); FTIR (ATR, ZnSe) ν_{max} 3408 (br), 1685, 1632, 1605, 1200, 1129, 1025 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 2.67 (H-3a″, m), 2.73 (H-3b″, m), 3.54 (H-2″, m), 3.82 (H-8a′, m), 3.88 (H_2-8, m), 3.97 (H-8b′, m), 5.70 (H-9′, dt, *J* = 15.7, 6.1 Hz), 5.76 (H-9, dt *J* = 15.5, 4.8 Hz), 6.04 (H-10, d, *J* = 15.5 Hz), 6.25 (H-10′, d, *J* = 15.7 Hz), 6.96 (H-4′, s), 6.97 (H-4, s), 8.33/8.49 (^{15}NH-7′, t, *J* = 5.7 Hz; $^1J_{H,N}$ = 96 Hz), 8.38/8.53 (^{15}NH-7, t, *J* = 5.7 Hz; $^1J_{H,N}$ = 90 Hz); HRESIMS $[M + H]^+$ *m*/*z* 897.8502 (calcd for $C_{24}H_{26}Br_4N_9{}^{15}N_2O_5S$, 897.8508).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental details and NMR data for compounds **5b**, **7**, and **8** are 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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(17) Taurine, $NH_2(CH_2)_2SO_3H$, a common osmolyte of marine invertebrates that was incorporated into the structure of **8**, apparently originates from entrained components of the cell-free preparation.

(18) Benzosceptrin C (**7**) was reported independently by two groups (refs 13 and 14) as the TFA salt (Kubota and co-workers, ref 13: $[\alpha]_D$ −5.0 (*c* 0.5 in MeOH) and formate salt (Tilvi and co-workers, ref 14: $[\alpha]_D$ −22.7 (*c* 0.13 in MeOH)). In the present work, the specific rotation of ^{15}N-**7**·TFA was found to be also levorotatory ($[\alpha]_D$ −3.2 (*c* 0.5 in MeOH)). Assuming Kubota's sample of **7**·TFA is optically pure, our sample would appear to be ~64% ee. As it is known that solvent and counterion strongly affect the $[\alpha]_D$ of PAIs, and the optical purity of natural **7** is unknown, assumptions of the level of enzymatic asymmetric induction in PAI biosynthesis by cell-free systems can be only speculative at this time.

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