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Pyrrole-Aminoimidazole Alkaloid ‘Metabiosynthesis’ with Marine Sponges *Agelas conifera* and *Stylissa caribica*

Dr. E. Paige Stout,

Department of Chemistry and Biochemistry and Skaggs School of Pharmacy and Pharmaceutical Science, University of California, San Diego 9500 Gilman Drive, La Jolla, CA 92093 (USA)

Dr. Yong-Gang Wang,

Department of Chemistry, Texas A&M University P.O. Box 30012, College Station, TX 77842 (USA)

Dr. Daniel Romo [Prof.], and

Department of Chemistry, Texas A&M University P.O. Box 30012, College Station, TX 77842 (USA)

Dr. Tadeusz F. Molinski* [Prof.]

Department of Chemistry and Biochemistry and Skaggs School of Pharmacy and Pharmaceutical Science, University of California, San Diego 9500 Gilman Drive, La Jolla, CA 92093 (USA)

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Pyrrole-aminoimidazole alkaloids (PAIs, Figure 1),^[1] the characteristic class of compounds found in marine sponges of the genre *Agelas*, *Stylissa*, *Phakellia*, *Axinella* and *Hymeniacidon*, have drawn contemporary interest due to their complex structures and examples with potent biological activities.^[2]

Sceptrin (**1a**),^[3] an intriguing dimeric optically active C_2 -symmetric cyclobutane, exhibits a broad range of biological activities,^[4] including antimicrobial^[4a] and antimuscarinic^[4b] properties, binding to MreB protein in *E. coli*,^[4c] and inhibition of cell motility in a variety of cancer cell lines.^[4d] It is widely accepted that the biosynthesis of complex PAIs begins with three simple building-block PAIs; hymenidin (**3a**),^[5] oroidin (**3b**)^[6] and clathrocin (**3c**),^[7] which are produced naturally in certain sponges. Detailed field experiments support the contention that oroidin (**3b**) and related molecules, longamide and dispacamide, most likely serve as the primary chemical defenses of *Stylissa* and *Agelas* sponges.^[8] Sceptrin (**1a**) is formally derived by [2+2] cycloaddition of **3a**, and benzosceptrins A-C (**4a-c**)^[9] are cyclized analogs of **1**. The potent antitumor agents, agelastatins A (**2a**) and B (**2b**) can be envisioned as a product of enzyme-mediated cyclization-oxidation of **3a,b**. The total

*tmolinski@ucsd.edu.

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synthesis of several PAIs, including **3b**,^[10] **1a**^[11] and **2**,^[12] have been reported, culminating in the elegant synthesis of palau'amine (**5**) by Baran.^[13]

Various schemes have been advanced to explain the biosynthesis of **1a**,^[3] **5**,^[14] and related PAIs.^[15] Most proposals rationalize carbon-carbon bond formation with mechanisms based on the electrophile-nucleophile duality inherent in enamine-imine tautomerism of conjugated 2-aminopyrroles, or concerted electrocyclic reactions that even claim to explain the order of appearance of intermediates.^[11] While accounting for the general assembly of PAIs, to date, all two-electron 'arrow pushing' schemes fail to address a fundamental discrepancy: no biosynthetic *experimental evidence* has been advanced to support any of the proposed schemes.

Here, we report the first experimental evidence for the biosynthesis of PAIs by sponges with enzyme-catalyzed conversion of an oroidin analog **3d** (dichloroclathrocin) to chlorinated versions of known benzosceptrins^[13] and nagelamides. Further, we provide the first evidence of conversion of oroidin (**3b**) to two known PAIs, benzosceptrin C (**4c**) and nagelamide H (**7a**), strong evidence that oroidin is the precursor to these natural products. These successful 'metabiosynthesis'^[17] experiments employ cell-free enzyme extracts obtained from marine sponges, *Stylissa caribica*, *Agelas conifera*, and *A. sceptrum*, carry out C–C bond formation of PAIs through an oxidative mechanism likely involving single-electron transfer (SET) and radical couplings.

Many reported PAI structures share the same carbon-nitrogen framework, but differ only in the number of Br atoms in the pyrrole ring (e.g., **3a**,^[3] **b,c**,^[18] and **2a,b**^[5]). This suggests that the late-stage enzymatic steps in the biosynthesis of PAIs from **3a-c** may be indifferent to halogenation in the pyrrole ring. We exploited this feature to clearly differentiate biosynthesis of PAIs under cell-free conditions using a non-natural chlorinated precursor, dichloroclathrocin (**3d**), prepared from the known 4,5-dichloropyrrol-2-yl trichloromethyl ketone^[19] using a strategy we previously developed for ¹⁵N-oroidin.^[10c]

Live sponges, *Stylissa caribica*, *Agelas conifera*, and *A. sceptrum* were freshly collected by hand using scuba (–80 ft, Bahamas) and immediately converted to cell-free acetone powders (*sc*-, *ac*-, and *as*-CFP respectively) that contained active enzymes. An aqueous-organic suspension of *sc*-CFP (0.2 mg/mL protein, Bradford assay) in 1:9 CH₃CN–0.1M Na-phosphate buffer, pH 7-9) rapidly consumed **3d** (~90% within 30 min, Table 1) and generated several new chlorinated compounds including two with molecular masses about twice that of **3d**, and an unstable compound **6**. The two major products (Figure 1 and Scheme 1; **4d**, [M–H][–] *m/z* 593.13; **7d**, [M–H][–] *m/z* 718.21) displayed pseudo-molecular ions with isotopic compositions corresponding to Cl₄. Improved conversion of **3d** was realized by quenching the reaction mixture with bisulfite (100 mM, NaHSO₃ aq), conditions that produced a different major product **8** along with **7d**. Scaled-up enzyme-catalyzed preparative reactions with **3d** provided sufficient quantities of products (~1.0–1.5 mg) to allow complete structure elucidation of **7d** and **8** by NMR and MS.

Compound **8**, C₂₂H₂₀Cl₄N₁₀O₅S (HR-APCI-TOFMS *m/z* 675.0015, [M–H][–]) showed a new red-shifted chromophore in its UV-vis spectrum (MeOH, λ_{max} 366 nm, ε 4000), in addition to the expected band from dichloropyrrol-2-carboxamide (λ_{max} 274 nm, ε 13600), and strong fluorescence (λ_{ex} 366 nm; λ_{em} 450). The ¹H NMR spectrum of **8** (600 MHz, 1.7 mm microcryoprobe, DMSO-*d*₆) showed two relatively unchanged pyrrole NH signals (δ 12.60, s; 12.62, s) suggesting two intact 2,3-dichloropyrrole-2-carboxamide groups. The vinyl protons from the 1,2-disubstituted alkene of **3d** were absent and replaced by four contiguously coupled CH signals (COSY) assigned to a 1,2,3,4-tetrasubstituted cyclobutane ring, reminiscent of **1a** but lacking symmetry. Interpretation of one-bond sp³ heteronuclear

coupling constants, $^1J_{\text{CH}}$, which reflect ring strain,^[20] confirmed the four-membered ring (coupled HSQC of **8**: $^1J_{\text{CH}}$ 136 Hz (C9), 134 (C9'), 148 (C10), 138 (C10')); c.f. **1a**, $^1J_{\text{CH}}$ 135 Hz (C9/9'), 136 (C10/10')). Interpretation of COSY and HMBC data allowed connection of the partial structures (see Supporting Information), including key HMBC correlations from both H9' (δ 1.78) and H10' (δ 3.35) to C11' (δ 86.4) and from H10 (δ 3.58) to C12 (δ 116.0). NOESY data were used to assign the relative configuration of the cyclobutane ring. NOEs observed between H9 (δ 2.54), H10, and H10' supported a *cis* ring fusion, while correlations between both H₂-8 protons (δ 2.78, 3.00) and H9' and between both H₂-8' protons (δ 3.17, 3.25) and H9 supported a *trans* orientation for the dichloropyrrol-2-carboxamide side chains, consistent with known cyclobutane-containing PAI natural products.^[3]

Comparing the formulas of **6** and **8**, it is apparent that the latter is the bisulfite addition product of the former captured from a reactive intermediate in the reaction pathway (Table 1, entries 3 vs. 8). Treatment of **8** with alkali (NaOH, aq. 23 °C) resulted in elimination of H₂SO₃ to give the postulated intermediate **i** (Scheme 1), with loss of both the red-shifted chromophore and its associated fluorescence, and transformation into **4d**, the Cl₄-derivative of benzosceptrin A (**4a**).^[9] The ¹H NMR spectrum of **4d** is almost identical to that of the natural product benzosceptrin B (**4b**). We observed that sceptrin (**1a**), itself, is not a viable substrate for the enzyme-catalyzed oxidation; exposure of **1a** to *sc*-CFP produced no **4b** or **7a** and returned only starting material which suggests that sceptrin is not an intermediate in the biosynthesis of benzosceptrins, nor is it isomerized under these conditions to ageliferin.

The second, more hydrophobic product **7d** has a formula C₂₄H₂₄Cl₄N₁₁O₅S (HR-APCI-TOFMS *m/z* 718.0439, [M-H][−]) corresponding to four-electron oxidative dimerization of dichloroclathrocin (**3d**) and addition of the elements of taurine, NH₂(CH₂)₂SO₃H.²¹ Analysis of NMR data (800 MHz, *d*₆-DMSO; ¹H NMR, COSY, HSQC, HMBC) revealed the structure of **7d** as the chlorinated analog of nagelamide H (**7a**).^[22]

Finally, natural oroidin (**3b**) was also converted to known PAI natural products under similar conditions. Cell-free enzyme preparations of *A. sceptrum*, which contains **1a** but lacks **3b** at detectable concentrations (LCMS), rapidly oxidized **3b** to give the known natural products benzosceptrin C (**4c**) and nagelamide H (**7a**) (Table 1, entry 17 and Supporting Information). This is the first experimental evidence of formation of complex PAIs from the widely-presumed precursor oroidin.^[23]

As dimerization of hymenidin (**3a**) to benzosceptrin B (**4b**) is a net four-electron oxidation (formal loss of 2H₂), we propose that all conversions of **3** to **1,2,5-8**, involve discrete enzyme-catalyzed reactions, possibly involving one or more oxido-reductases,^[24] as supported by the following lines of evidence. Optimal conversion was found at pH 7 (Table 1, entries 1-7). Boiled *sc*-CFP (100 °C) resulted in loss of activity and no conversion of **3d** (Table 1, entry 14). Similarly, activity was greatly attenuated when incubation of **3d** was carried out under an atmosphere of CO (1 atm, Table 1, entries 10,11) or in the presence of fluoride ion (10 mM, KF aq, Table 1, entries 12,13), conditions known to inhibit cytochrome oxidases. Addition of H₂O₂ (50 μM) to the enzyme incubation made no change, however incubation of **3d** with *sc*-CFP under anaerobic conditions also led to significantly reduced yields of **4d** and **7d** (Table 1, entry 15). Collectively, these data suggest that O₂ is the terminal oxidant. Ultracentrifugation of the crude enzyme preparation (10 kDa MWCO) resulted in retention of all activity (conversion of **3d** into **4d/7d**) in the fraction >10 kDa, (see Figure S3); results that support enzyme-mediated conversion of **3d**.

The observed conversions of dichloroclathrocin (**3d**) appear to be paired to oxido-reductases associated with *Stylissa* and *Agelas* sponges. Replacement of sponge-CFPs with horseradish

peroxidase (Sigma) gave no **4d** or **7d** and only produced complex mixtures of unidentifiable products. Furthermore, 9,10-dihydrodichlorocathrocin (**3e**, Table 1, entry 18), prepared in a similar manner to **3d**, does not undergo oxidation under the reaction conditions, consistent with requirements for a π -electron-rich donor-substrate and stabilization of radical-cation intermediates through delocalization.

The enzyme-catalyzed dimerization of dichlorocathrocin (**3d**) is specifically linked to PAI-producing sponges. Cell-free enzyme preparations of several PAI-producing *Agelas* sponges all converted **3d** to **4d/7d**, while incubation of **3d** similarly prepared extracts of five co-occurring sponges that do not produce PAIs failed to generate **4d/7d** or oxidize **3d** at all (Table S1).

We detected appreciable concentrations of **3b** in both *A. conifera* and *S. caribica*, but not in *A. sceptrum* (Figure S1). Neither *S. caribica* or *A. conifera* from the Caribbean appear to contain natural benzosceptrins (**4a-c**); these natural products were described only from sponges collected in the Mediterranean Sea and Pacific Ocean. Yet, remarkably, the chlorinated analogs of the latter natural products are rapidly formed when **3d** is exposed to cell-free preparations of the sponges *A. conifera* and *S. caribica* from the Caribbean Sea, revealing the permissive nature of the sponge oxido-reductases. Cell-free enzyme preparations of both sponges unleash competent enzymes that catalyze oxidative couplings and give rise to the same products **4d** and **7d-8** found in sponge of the same genus from different geographic locations. Disruption of the living sponge tissue and cellular structure, apparently, liberates enzymes that retain their oxidative capacity to carry out oxidative biosynthesis of PAIs, albeit without the level of control that presumably operates within intact sponge cells.

We hypothesize benzosceptrins, nagelamides, and **6-8**, arise from sequential SET that result in four-electron oxidation of **3a-c**. By association, it is also likely, that biosynthesis of many other PAIs are mediated by orchestrated enzymatic SET (Scheme 2) that initiate formation resonance-stabilized radical-cation intermediates and subsequent reactions.^[25] We propose that the biosynthesis of PAIs proceeds through intermediates that partition between at least two discrete, enzyme-mediated pathways (Scheme 2) leading to oxidized products **4d**, **6** and **8** or redox-neutral products (e.g., sceptrin **1a**) with respect to the starting materials. In the case of **1a**, SET from the π -rich conjugated vinyl-2-amino-imidazole of **3a** to the metal center of the oxido-reductase generates a radical-cation (*i*) that adds to a neutral molecule of **3a**, with loss of H^+ to form the first C-C bond of the pre-cyclobutane radical intermediate (*ii*) followed by cyclization to *iii* (Scheme 2, *path a*). Subsequent SET reduction by an electron carrier provides **1a** with net-zero electron transfer. Thus, **1a** is formed through a 'cryptic' oxidative mechanism. Compounds **4d**, **6** and **8** arise from a different *path b*, possibly through cationic intermediate *iv*, and nucleophilic capture (H_2O or HSO_3^-) with net 4-electron oxidation. Elimination of HX gives **4d** after tautomerism (see Scheme 1).^[26] The nagelamide H analog **7d** may emerge from further oxidation at the imidazole ring of *ii* (hydroxylation/tautomerism, *not shown*) and nucleophilic capture of an incipient 2-iminoimidazolidinone by taurine.

Even numbers of SET oxidation steps could, conceivably, give rise to the even-electron cationic species, including acyliminium ions that have been implicated in the biosynthesis of agelastatins,^[12b] phakellins, palauamine and other PAIs.^[15] High-energy radicalcation intermediates would better explain the formation of the ring-strained PAI alkaloids; e.g., cyclobutanes (**1,4,6** and **8**) and *trans*-bicyclo[3.3.0]octane (**5**) as the enthalpic cost is paid by reduction of the terminal oxidant, O_2 . An important feature of the SET hypothesis is its compatibility with many late-stage two-electron C-C bond-forming reactions currently favored in PAI biosynthetic proposals. An additional attractive feature of odd-electron

pathways from **3** is accessibility to reactive intermediates (radical) with sufficiently high free energy, to bypass the kinetic barriers that disfavor ionic or concerted mechanisms, (e.g., thermal [2+2] cycloaddition or cycloreversion^[27]), without the need to invoke unlikely excited states or violations of orbital symmetry rules.^[26]

The evidence we present (Table 1, entries 10-13) strongly argues for a metalloenzyme catalyzed C–C bond formation in PAI biosynthesis. The modular nature of PAI biosynthesis is now succinctly explained by single-electron processes, not unlike the modular biosynthesis of lignans that also proceed through radical phenolic couplings catalyzed by metalloenzymes. Fe-porphyrin oxidoreductases are known to carry out lignan biosynthesis,^[28] for example conversion of coniferyl alcohol to pineol, with dioxygen (O₂) as terminal oxidant. We favor the latter in PAI biosynthesis, although we cannot rule out the possibility that other metalloenzymes, for example laccases, are involved.

Several details of the enzyme-mediated formation of PAIs remain to be elucidated; for example, the mechanism by which optical activity is induced. Compounds **4d**, **7d**, and **8** appear to have lower optical activity than the natural products,^[29] however, as in lignan biosynthesis, asymmetry may be induced by the action of dirigent proteins^[30] that are coupled with oxidases to control stereochemistry, a property that may be disrupted in cell-free preparations. The sub-cellular location, structural-ordering and compartmentalization of the biosynthetic enzymes, and additional oxidative steps required to generate higher order PAIs including palau'amine and 'tetrameric' members – and purification of these oxidoreductase(s) – and other aspects of SET oxidation in the biosynthesis of PAIs are the subjects of ongoing investigations in our laboratories.

In conclusion, we have demonstrated cell-free enzymatic conversion of oroidin (**3b**) and an oroidin-like precursor **3d** to natural products benzosceptrin C (**4c**), nagelamide H (**7a**), and their tetrachloro-analogs (**4d**) and (**7d**), respectively. The results implicate oxidoreductase-like activity in the biosynthesis of PAIs in marine sponges through single-electron transfer, and we suggest an SET pathway that may explain the origin of sceptrin (**1a**) and related oroidin-derived dimeric PAIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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lives in subdued light conditions, at depths no less than –25 m. In any case, failure of photochemical dimerization of **3b** to **1a** under laboratory conditions is likely due to other factors.

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Figure 1. Representative PAIs from sponges *Agelas* spp. and *Stylissa*, and unnatural chlorinated analogs (**3d,e**, **4d**).

Scheme 1.

Structures of nagelamide H (**7a**) and chlorinated products **6**, **7d**, **8** obtained from cell-free metabiosynthesis of **3d** and base promoted elimination of **8** to **4d**.

Scheme 2.

Proposed mechanisms for enzyme-catalyzed SET of **3d** and cyclization to **1a** (*path a*) or **6/8** (*path b*).

Table 1

Enzyme-catalyzed reactions of **3d** with cell-free preparations from *A. conifera* (*ac*), *A. sceptrum* (*as*), and *S. caribica* (*sc*).^[a]

Entry	Substrate/ enzyme ^[a]		% Conversion ^[b]			Rate, R $\mu\text{Ms}^{-1}[c]$	pH, conditions
			4d	7d	8		
1	3d	<i>sc</i>	0	0	–	–	5
2	3d	<i>sc</i>	0	0	–	–	6
3	3d	<i>sc</i>	52	21	–	10.3	7
4	3d	<i>ac</i>	19	13	–	10.2	7
5	3d	<i>as</i>	21	10	–	10.2	7
6	3d	<i>sc</i>	37	60	–	10.9	8
7	3d	<i>sc</i>	30	55	–	10.9	9
8	3d	<i>sc</i>	0	13	50	10.3	7 ^[d]
9	3d	<i>ac</i>	5	15	19	5.22	7 ^[d]
10	3d	<i>sc</i>	13	2	–	6.88	7, CO ^[e]
11	3d	<i>ac</i>	0.4	0.1	–	0.88	7, CO ^[e]
12	3d	<i>sc</i>	7	1	–	3.00	7, F ^[f]
13	3d	<i>ac</i>	1.2	1.7	–	6.56	7, F ^[f]
14	3d	<i>sc</i>	0	0	–	–	7, heat ^[g]
15	3d	<i>sc</i>	3	5	–	2.44	8, Ar ^[h]
16	1a	<i>sc</i>	0	0	–	–	7
17	3b	<i>as</i>	72 ^[i]	60 ^[i]	–	11.0	7
18	3e	<i>sc</i>	0	0	–	–	7

^[a] [**3d**]=20 mM in 1:4 CH₃CN–0.1M sodium phosphate buffer, 0.2 mg/mL total protein (Bradford assay) of cell-free enzyme preparation (CFP) from either *S. caribica* (*sc*), *A. conifera* (*ac*), or *A. sceptrum* (*as*) (for preparation, see Supporting Information).

^[b] Determined by LCMS (UV).

^[c] R is an estimated initial rate of consumption of **3d**, assuming substrate-saturated conditions.

^[d] NaHSO₃ aq added during workup (final [HSO₃[–]]=0.1 M).

^[e] 1 atm, 15 min pre-saturation.

^[f] KF (aq, 10 mM), 15 min pre-incubation.

^[g] CFP was boiled (100 °C) for 30 min prior to addition of substrate.

^[h] 1 atm, anaerobic conditions were achieved by purging substrate solution and CFP with Ar for 15 min prior to addition;

^[i] Observed products were tetrabrominated natural products **4c** and **7a**, respectively.