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Electron Transfer

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

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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 *Hymeniacedon*, have drawn contemporary interest because of their complex structures and, in some cases, potent biological activities.[2]

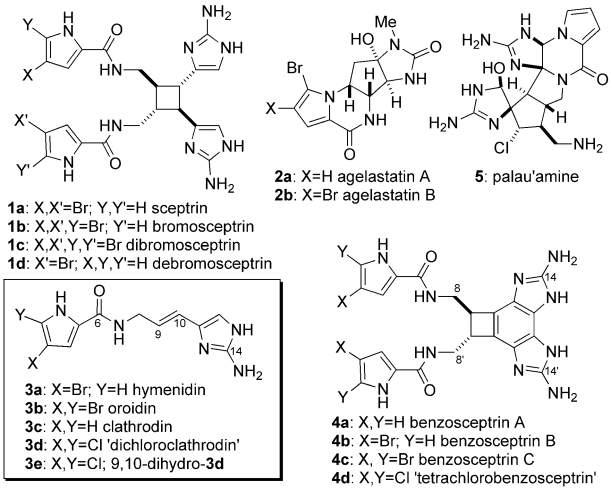

Figure 1. Representative PAIs from sponges *Agelas* spp. and *Stylissa*, and the unnatural chlorinated analogues **3d**,**e**, and **4d**.

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 clathrodin (**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 analogues of **1**. The potent antitumor agents, agelastatins A (**2a**) and B (**2b**) can be envisioned as a product of the enzyme-mediated cyclization/oxidation of **3a**,**b**. The total synthesis of several PAIs, including **3b**,[10] **1a**,[11] and **2**,[12] have been reported, thus culminating in the elegant synthesis of palau'amine (**5**) by Baran and co-workers.[13]

Various schemes have been advanced to explain the biosynthesis of **1a**,[3] **5**,[14] and related PAIs.[15,16] 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.

Herein we report the first experimental evidence for the biosynthesis of PAIs by sponges with enzyme-catalyzed conversion of the oroidin analogue **3d** (dichloroclathrodin) into chlorinated versions of known benzosceptrins[13] and nagelamides. Further, we provide the first evidence of conversion of **3b** into two known PAIs, benzosceptrin C (**4c**) and nagelamide H (**7a**), which is strong evidence that oroidin is the precusor to these natural products. These successful "metabiosynthesis"[17] experiments employ cell-free enzyme extracts obtained from the marine sponges *Stylissa caribica*, *Agelas conifera*, and *A. sceptrum*, and 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] **3b**,**c**,[18] and **2a**,**b**[5]), thus suggesting that the late-stage enzymatic steps in the biosynthesis of PAIs for **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, dichloroclathrodin (**3d**),

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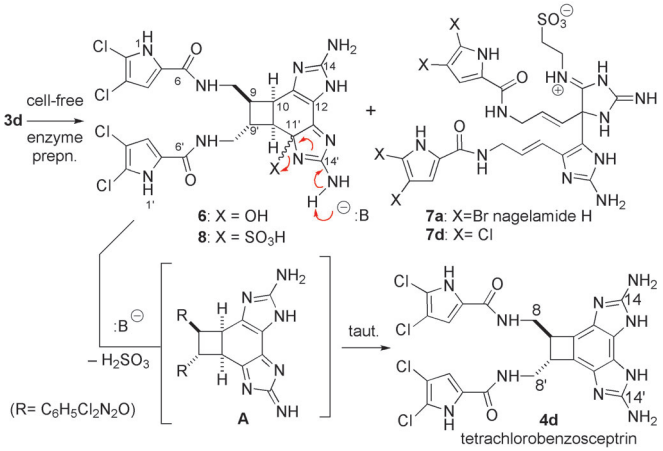
which was prepared from the known 4,5-dichloropyrrol-2-yl trichloromethyl ketone[19] using a strategy we previously developed for ^{15}N-oroidin.[10c]

Live sponges, *Stylissa caribica*, *Agelas conifera*, and *A. sceptrum* were freshly collected by hand using scuba (−80 ft, Bahamas) and immediately converted into cell-free preparations (*sc*-, *ac*-, and *as*-CFP respectively) which contained active enzymes. An aqueous-organic suspension of *sc*-CFP (0.2 mg mL^{-1} protein, Bradford assay) in 1:9 CH_3CN/0.1 M Na-phosphate buffer (pH 7–9) rapidly consumed **3d** (ca. 90% within 30 min; Table 1) and generated several new chlorinated compounds including two with molecular masses about twice that of **3d**, as well as 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 pseudomolecular ions with isotopic compositions corresponding to Cl_4. Improved conversion of **3d** was realized by quenching the reaction mixture with bisulfite (100 mM, $NaHSO_3$ 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 (ca. 1.0–1.5 mg) to allow complete structure elucidation of **7d** and **8** by NMR spectroscopy and MS.

Table 1: Enzyme-catalyzed reactions of PAIs with cell-free preparations from *A. conifera* (*ac*), *A. sceptrum* (*as*), and *S. caribica* (*sc*).[a]

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

[a] For entries 1—15: [**3d**] = 17 mM in 1:11 CH_3CN/0.1 M sodium phosphate buffer, 0.2 mg mL^{-1} total protein (Bradford assay) of cell-free preparation (CFP) from either *S.caribica* (*sc*), *A. conifera* (*ac*), or *A. sceptrum* (*as*). For further details and the conditions for entries 16–18, see the Supporting Information. [b] Determined by LCMS (UV). [c] *R* is an estimated initial rate of consumption of substrate, assuming substrate-saturated conditions. [d] $NaHSO_3$ aq. added during workup (final [HSO_3^-] = 0.1 M). [e] Run at 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] Run at 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.



Scheme 1. Structures of nagelamide H (**7a**) and the chlorinated products **6**, **7d**, and **8** obtained from cell-free metabiosynthesis of **3d** and base-promoted elimination of **8** to **4d**.

Compound **8**, $C_{22}H_{20}Cl_4N_{10}O_5S$ (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 ^{1}H NMR spectrum of **8** (600 MHz, 1.7 mm microcryoprobe, [D_6]DMSO) showed two relatively unchanged pyrrole NH signals (δ = 12.60, s; 12.62, s), thus 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 sp^3 heteronuclear coupling constants, $^1J_{CH}$, which reflect ring strain,[20] confirmed the four-membered ring [coupled HSQC of **8**: $^1J_{CH}$ 136 Hz (C9), 134 (C9′), 148 (C10), 138 (C10′); see **1a**: $^1J_{CH}$ 135 Hz (C9/9′), 136 (C10/10′)]. Interpretation of COSY and HMBC data allowed connection of the partial structures (see the 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 H8 protons (δ = 2.78, 3.00) and H9′ and between both H8′ protons (δ = 3.17, 3.25) and H9 supported a *trans* orientation for the dichloropyrrol-2-carboxamide side chains, all of which is consistent with known cyclobutane-containing PAI natural products.[3]

By 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 versus 8). Treatment of **8** with base (aq. NaOH, aq. 23 °C) resulted in elimination of H_2SO_3 to give the postulated intermediate **A** (Scheme 1), with loss of both the red-shifted chromophore and its associated fluorescence, and transformation into **4d**, the Cl_4-derivative of benzosceptrin A (**4a**; Figure 1).[9] The ^{1}H 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 reaction conditions into ageliferin.[21]

The second, more hydrophobic product **7d** has the formula $C_{24}H_{24}Cl_4N_{11}O_5S$ (HR-APCI-TOFMS *m*/*z* 718.0439, $[M-H]^-$), which corresponds to four-electron oxidative dimerization of **3d** and addition of the elements of taurine, $NH_2(CH_2)_2SO_3H$.[22] Analysis of the NMR data (800 MHz, $[D_6]$DMSO; ^{1}H NMR, COSY, HSQC, HMBC) revealed the structure of **7d** as the chlorinated analogue of **7a**.[23]

Finally, natural **3b** was also converted into known PAI natural products under similar reaction conditions. Cell-free preparations of *A. sceptrum*, which contains **1a** but lacks **3b**, **4c**, and **7a** at detectable concentrations (LCMS), rapidly oxidized **3b** to give the known natural products **4c** and **7a** (Table 1, entry 17 and see the Supporting Information). This is the first experimental evidence of formation of complex PAIs from the widely presumed precursor oroidin.[24]

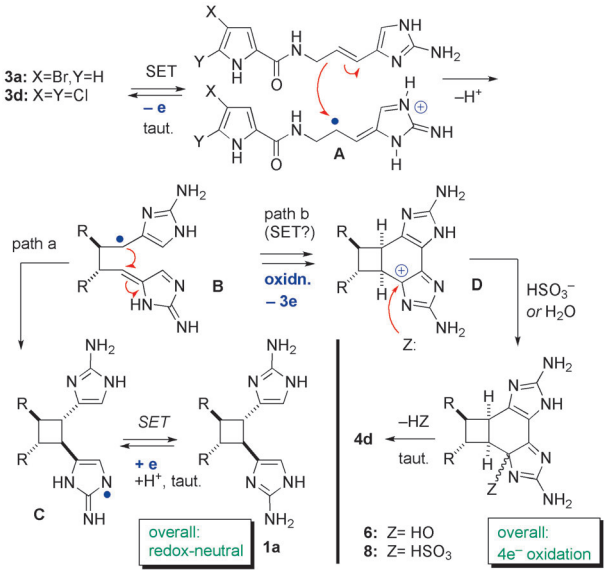
As dimerization of **3a** into **4b** is a net four-electron oxidation (formal loss of $2H_2$), we propose that all conversions of **3** into **1**,**2**, and **5**–**8** involve discrete enzyme-catalyzed reactions, which possibly involve one or more oxido reductases[25] as supported by the following lines of evidence. Optimal conversion was found at pH $\geq$ 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 and 11) or in the presence of aqueous fluoride (10 mM, KF aq., Table 1, entries 12 and 13), conditions known to inhibit cytochrome oxidases. Addition of H_2O_2 (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 O_2 is the terminal oxidant. Ultracentrifugation of the CFP (10 kDa molecular weight cut-off) resulted in retention of all activity (conversion of **3d** into **4d**/**7d**) in the fraction containing species of molecular weight greater than 10 kDa (see Figure S3 in the Supporting Information); results that support enzyme-mediated conversion of **3d**.

The observed conversions of **3d** appear to be paired to oxido reductases associated with *Stylissa* and *Agelas* sponges. Replacement of sponge-CFPs with horseradish peroxidase (Sigma) gave neither **4d** nor **7d** and only produced complex mixtures of unidentifiable products. Furthermore, 9,10-dihydro-dichloroclathrodin (**3e**; Table 1, entry 18), prepared in a similar manner to **3d**, does not undergo oxidation under the reaction conditions, which is consistent with requirements for a π-electron-rich donor substrate and stabilization of radical cation intermediates through delocalization.

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

We detected appreciable concentrations of **3b** in both *A. conifera* and *S. caribica*, but not in *A. sceptrum* (see Figure S1 in the Supporting Information). Neither *S. caribica* nor *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 analogues 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, thus revealing the permissive nature of the sponge oxido reductases. Cell-free preparations of both sponges unleash competent enzymes which catalyze oxidative couplings and give rise to the same products, **4d** and **7d**, 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 SETs which result in the four-electron oxidation of **3a**–**c**. By association, it is also likely, that biosynthesis of many other PAIs are mediated by orchestrated enzymatic SETs (Scheme 2) which initiate formation of resonance-stabilized radical cation intermediates and subsequent reactions.[26] We propose that the biosynthesis of PAIs proceeds through intermediates that partition between at least two discrete enzyme-mediated pathways (Scheme 2), thus leading to oxidized products **4d**, **6**, and **8** or redox neutral products (e.g., **1a**) with respect to the starting materials. In the case of **1a**, SET from the π-rich conjugated vinyl-2-aminoimidazole of **3a** to the metal center of the oxido reductase generates the radical cation **A** which adds to



Scheme 2. Proposed mechanisms for enzyme-catalyzed SET of **3d** and cyclization to either **1a** (path a) or **6**/**8** (path b).

a neutral molecule of **3a** with loss of H^+ to form the first C–C bond of the pre-cyclobutane radical intermediate **B** followed by cyclization to **C** (Scheme 2, path a). Subsequent SET reduction by an electron carrier provides **1a** with a net-zero electron transfer. Thus, **1a** is formed through a cryptic oxidative mechanism. Compounds **4d**, **6**, and **8** arise from path b, possibly through the cationic intermediate **D**, and nucleophilic capture (H_2O or HSO_3^-) with a net four-electron oxidation. Elimination of HX gives **4d** after tautomerism (Scheme 1).[27] The nagelamide H analogue **7d** may emerge from additional oxidation at the imidazole ring of **B** (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, palaua′mine, and other PAIs.[15] High-energy radical cation intermediates would better explain the formation of the ring-strained PAI alkaloids, for example, the cyclobutanes **1**, **4**, **6**, and **8** and the *trans*-bicyclo[3.3.0]octane **5**, because 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) having sufficiently high free energy to bypass the kinetic barriers that disfavor ionic or concerted mechanisms (e.g., thermal [2+2] cycloaddition or cycloreversion[21]) 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 which also proceeds through radical phenolic couplings catalyzed by metalloenzymes. Fe-porphyrin oxidoreductases are known to carry out lignan biosynthesis,[28] for example conversion of coniferyl alcohol into pineol, with dioxygen (O_2) as the 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] which are coupled with oxidases to control stereochemistry, a property that may be disrupted in cell-free preparations. The subcellular 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 the oroidin-like precursor **3d** into the natural the products benzosceptrin C (**4c**) and nagelamide H (**7a**), and their tetrachloro analogues **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 which may explain the origin of sceptrin (**1a**) and related oroidin-derived dimeric PAIs.

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[17] The term metabiosynthesis is coined here to define cell-free, enzymatic reactions conducted on non-natural substrates to give natural product analogues.

[18] a) M. Köck, M. Z. Assmann, *Z. Naturforsch. C* **2002**, *57*, 157 – 160; b) P. A. Keifer, R. E. Schwartz, M. E. S. Koker, R. G. Hughes, Jr., D. Rittschof, K. L. Rinehart, *J. Org. Chem.* **1991**, *56*, 2965 – 2975.

[19] P. Bélanger, *Tetrahedron Lett.* **1979**, *20*, 2505 – 2508.

[20] D. S. Dalisay, B. I. Morinaka, C. K. Skepper, T. F. Molinski, *J. Am. Chem. Soc.* **2009**, *131*, 7552 – 7553.

[21] Baran and co-workers propose that **1a** is biosynthetically transformed into the isomeric ageliferin by thermal cyclobutane ring opening of **1a** into a diradical which re-closes to the cyclohexene, see: B. H. Northrop, D. P. O'Malley, A. L. Zografos, P. S. Baran, K. N. Houk, *Angew. Chem.* **2006**, *118*, 4232 – 4236; *Angew. Chem. Int. Ed.* **2006**, *45*, 4126 – 4130. Al-Mourabit and co-workers favor an alternative even-electron pathway from **3b** to **4b**. See Ref. [9].

[22] Taurine is an osmolyte commonly found in sponges in high concentrations and, evidently, entrained in our CFP preparations.

[23] T. Endo, M. Tsuda, T. Okada, S. Mitsuhashi, H. Shima, K. Kikuchi, Y. Mikami, J. Fromont, J. Kobayashi, *J. Nat. Prod.* **2004**, *67*, 1262 – 1267.

[24] Redox-neutral conversion of **3b** into dibromosceptrin (**1c**) was not detected in this experiment, the result is possibly due to dysregulation of electron donor/acceptors in disrupted cell-free preparations. High conversions of **3b**/**d** into benzosceptrins (**4c**/**d**) and nagelamides H (**7a**/**d**) were also achieved with CFPs from other PAI-producing Caribbean sponges (see Table S1 in the Supporting Information).

[25] Peroxidase-promoted radical pairings generate C–C bonds without incorporation of oxygen: a) T. M. Kutchan, W. M. Chou, *Plant J.* **1998**, *15*, 289 – 300; b) K. A. Nielsen, B. L. Lindberg-Møller in *Cytochrome P450* (Ed.: P. R. Ortiz de Montellano), Kluwer, Dordrecht, **2005**, pp. 553 – 574.

[26] There is no a priori reason to assume the involvement of radical cation intermediates over radical anions except for abundant precedence of peroxidases in C–C coupling reactions, relatively low oxidation potential of vinyl-2-aminoimidazoles, and the experimental evidence presented herein. An efficient photochemical catalytic redox-neutral synthesis of cyclobutanes by radical anion coupling has been demonstrated. J. Du, T. P. Yoon, *J. Am. Chem. Soc.* **2009**, *131*, 14604 – 14605.

[27] For example, photochemical [2+2] cycloaddition of **3b**, which requires an S_1 excited state and intersystem crossing to T_1, is unlikely in *Stylissa caribica*, a sponge that biosynthesizes **1b** but lives in subdued light conditions, at depths no less than −25 m. In any case, failure of photochemical dimerization of **3b** into **1a** under laboratory conditions is likely due to other factors.

[28] W. Bao, D. M. O'Malley, R. R. Sederoff, *Science* **1993**, *260*, 672 – 674. We thank an anonymous reviewer for this analogy.

[29] Enantio-heterogeneity of PAIs is not unexpected. For example, both optical antipodes of the natural product, (+)-dibromophakellin, have been reported. See: a) G. Sharma, B. Magdoff-Fairchild, *J. Org. Chem.* **1977**, *42*, 4118 – 4124; b) G. de Nanteuil, A. Ahond, J. Guilhem, C. Poupat, E. Tran Huu Dau, P. Potier, M. Pusset, J. Pusset, P. Laboute, *Tetrahedron* **1985**, *41*, 6019 – 6033.

[30] L. B. Davin, H.-B. Wang, A. L. Crowell, D. L. Bedgar, D. M. Martin, S. Sarkanen, N. G. Lewis, *Science* **1997**, *275*, 362 – 366.

Communications

Electron Transfer

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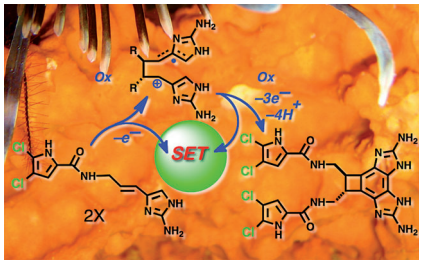
Pyrrole Aminoimidazole Alkaloid Metabiosynthesis with Marine Sponges *Agelas conifera* and *Stylissa caribica*



Game-SET-Match: Pyrrole aminoimidazole alkaloids (PAIs) are metabiosynthesized from chlorinated analogues of oroidin by cell-free enzyme preparations from PAI-producing sponges. Evidence and implications for the biosynthesis of PAIs include putative single-electron transfers (SETs) that promote C—C bond-forming reactions of precursors.