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Ypaoamide, a New Broadly Acting Feeding Deterrent from the Marine Cyanobacterium *Lyngbya majuscula*.

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Abstract: Ypaoamide, a new herbivore antifeedent metabolite, was isolated from the extract of a mixed cyanobacteria assemblage which was composed of *Schizothrix calcicola* and *Lyngbya majuscula*. The structure was determined spectroscopically by interpretation of 2D-NMR experiments, including HMBC and NOESY, and by comparison with model compounds. Isolated cells of the *L. majuscula* produced ypaoamide in laboratory culture.

Ypao Beach, a popular tourist site on Guam, experienced a temporary closure in May 1994 due to a simultaneous blue-green algal bloom (originally mistaken for an effluent spill) and a massive die off of pelagic larval rabbitfishes (*Siganus argenteus* and *S. spinus*). This microbial assemblage was composed primarily of the marine cyanobacteria *Schizothrix calcicola* with sparsely distributed strands of *Lyngbya majuscula*.[1]

Large floating masses of algal material (1.17 kg dry marc) were collected and stored at -20° until extracted with CH_2Cl_2/MeOH (1:1). The crude extract strongly deterred feeding by both the yellow-banded parrotfish (*Scarus schlegeli*) and the urchin (*Echinometra mathaei*), two common reef herbivores found on Guam.[2] 2D-TLC analysis of the extracts showed the presence of a UV-active, yellow-charring (H_2SO_4, heat) relatively non-polar secondary metabolite. A 4.9 g portion of the crude extract (20.53 g, dark oil) was fractionated by silica gel vacuum chromatography with a gradient from hexanes to EtOAc to MeOH. Parrotfish feeding deterrent fractions eluting with 25% and 50% (v/v) MeOH/EtOAc were combined (960.6 mg), separated by Sephadex LH-20 chromatography (50% (v/v) EtOAc in MeOH), and further purified by silica gel vacuum chromatography (gradient from hexanes to EtOAc to MeOH). Pure ypaoamide[3] (**1**, 767 mg, 0.27% of dry mass) was obtained from chromatographic fractions eluting with 50% to 67% (v/v) EtOAc/Hexanes.

Analysis of **1** by ^{13}C NMR and High resolution FABMS (457.2688; calc. for 457.2693; MNBA + PEG 400) provided a molecular formula for $[M+H]^+$ of $C_{26}H_{37}N_2O_5$ (calc. for 10° unsaturation). Compound **1** was optically active $[\alpha]_D^{25} = +197°$ (c = 1.0, $CHCl_3$). Examination of the IR (neat, υ = 3650-3100, 2952, 1725, 1722, 1714, 1660, 1651 cm^{-1}), UV (λ_{max} 222, 270 nm; log ϵ 4.2, 4.1; MeOH), and NMR data (Table 1) revealed the presence of a phenolic or enolic hydroxyl group, a *t*-butyl group, a 1,4-disubstituted benzylic moiety, and two α,β-unsaturated amides. Four major spin systems (Figure 1), a $^1H_9$ singlet (**e**) and a methoxy $^1H_3$ singlet could be assigned by ^1H-^1H COSY (Table 1).

^1H-^{13}C HMBC spectra were obtained in both $CDCl_3$ and D_6-benzene (Table 1) in order to facilitate attachment of the spin-systems. Specifically, 2 and 3-bond couplings from C6, C7, and C11 to H5a and H5b allowed for the attachment of the 1,4-disubstituted benzene moiety **d** to spin-system **a** (Figure 1). The C1′′ carbonyl showed $^2J_{CH}$ couplings to H5′a, H5′b, C1′′-N$\underline{H}$, $H_2$2′′, and $H_2$3′′ providing the linkage between partial structures **b** and **c**. The *t*-butyl group (**e**) was attached to position 5′′ of partial structure **c** as indicated by $^3J_{CH}$ couplings between C6′′ and $H_2$4′′, between C6′′ and the methyl groups $H_3$7′′-$H_3$9′′ (**e**), and a $^3J_{CH}$ coupling between C7′′-C9′′ (**e**) and H5′′ (**c**). Therefore, the one remaining degree of unsaturation was in the form of a ring. A nitrogen joined the two open ends of partial structure **a** to form an α,β-unsaturated-γ-lactam imide (IR υ = 1725, 1714 cm^{-1} stretches), as in the *L. majuscula* metabolites malyngamide A,[4] the

pukelemides,[5,6] majusculamide D,[7] and microcolins A and B,[8] providing a linkage from partial structures **a** to **b**. ^{13}C NMR chemical shifts of the γ-substituted α, β-unsaturated-γ-lactam imide of **1** (Table 1) are analogous to those of the similarly substituted ring systems in the microcolins (microcolin A: C1, 169.8 ppm; C2, 125.3; C3, 154.1).[8] An additional J_{CH} = 5 Hz optimized ^1H-^{13}C HMBC spectrum (Table 1) confirmed the positions of the allylic methoxy and phenolic hydroxyl groups, thus defining the structure of ypaoamide as **1**. The C2′-C3′ olefinic geometry was assigned as *E* based upon a strong nOe observed between H-2′ and the allylic methoxy group by NOESY. Stereochemistry at C4 was not assigned.

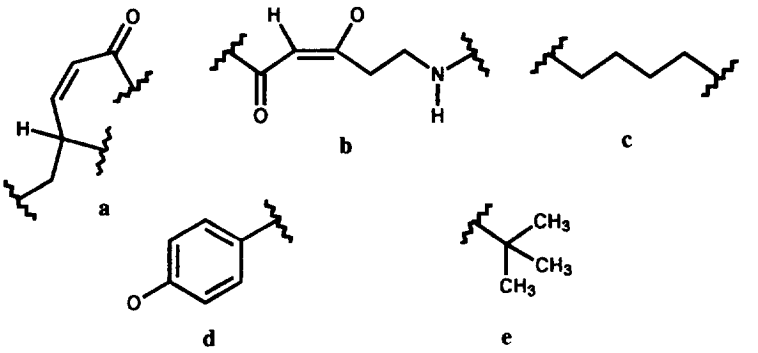
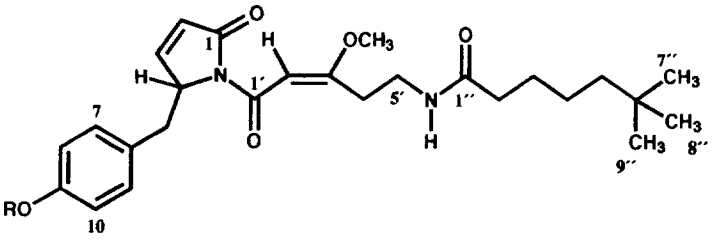


Figure 1. Partial structures **a** through **e**.

Acetylation of **1** (25.6 mg in 1:1 (v/v) Ac_2O/pyridine, 4.8 hr) formed the synthetic acetate derivative **2**.[9] Compound **2** yielded a molecular formula of $C_{28}H_{39}N_2O_6$ for $[M+H]^+$ by HR-FABMS (499.2808; calc. for 499.2798). Its ^1H NMR spectrum differed from that of **1** in the conspicuous absence of the phenolic hydroxyl resonance (δ 7.0 ppm), which was replaced by a three proton acetate methyl singlet, and significant changes in chemical shifts of the benzylic protons.[7]



In order to identify which organism produces **1** we initiated cultures of both cyanobacteria. The *L. majuscula* cells survived and reproduced, although slowly, under laboratory culture conditions. Three cultures of *L. majuscula* were analyzed separately. Several strands (1-3 mm) of each *L. majuscula* culture were extracted with CH_2Cl_2/MeOH (1:1, 3x), dried under N_2, dissolved in 10% (v/v) EtOAc in hexanes, filtered, and were subjected to GC-EIMS analysis. All three contained a peak with the same GC retention time as a standard sample of **1** (t_R = 7.55 min) and produced mass spectral fragmentation patterns essentially identical to that produced by **1**.[10] The relative abundance of **1** produced by each culture was highly variable. We therefore believe *L. majuscula*, the minor cyanobacterial strain found in the assemblage, to be the actual source of **1**.

Table 1. NMR Data for Compound **1**.[a]

C#	^{13}C δ	^{13}H δ	^{1}H-^{1}H COSY[b]	^{1}H-^{13}C HMBC[c]
1	170.25	---		
2	126.44	6.01 dd 6.0, 1.6	H3	C1, C3, C4
3	151.51	7.16 dd 6.0, 2.0	H4	C1, C2, C4, C5
4	63.16	4.97 dm 9.1	H5a, H5b	C2, C3, C5
5	37.13	a) 2.72 dd 13.4, 9.1	H5b	C3, C4, C6, C7 & C11
	---	b) 3.47 dd 13.4, 5.4		C3, C4, C6, C7 & C11
6	127.05	---		
7&11	130.49	6.98 dt 8.6, 2 (2H)	H8 & H10	C6, C7 & C11, C8, C9,
8&10	115.53	6.78 dt 8.6, 2 (2H)	C9-OH (weak)	C6, C7 & C11, C8 & C10, C9
9	155.37	---		
-OH	---	7.00 to 8.66[d] bm		(C8 & C10, C9)[e]
1′	166.24	---		
2′	95.02	6.71 s	H4a′	C3′, C4′
3′	175.47	---		
4′a	32.36	a) 2.88 ddd 13.3, 7.1, 4.9	H4′b, H5′a, H5′b	C2′, C3′, C5′
b	---	b) 3.04 ddd 13.3, 8.3, 4.9		C2′, C3′, C5′
5′a	38.24	a) 3.54 m	H5′b, C1′′-NH	C3′, C4′, (C1′′)[f]
b	---	b) 3.58 m		C3′, C4′, (C1′′)[f]
1′′	173.64	---		
2′′	36.89	2.18 t 7.7 (2H)	H3′′	C1′′, C3′′, C4′′
3′′	26.56	1.57 p 7.6 (2H)	H4′′	C1′′, C2′′, C4′′, C5′′
4′′	24.27	1.20 m (2H)	H5′′	C2′′, C3′′, C5′′, C6′′
5′′	43.81	1.10 m (2H)		C3′′, C4′′, C6′′, C7′′-C9′′
6′′	30.14	---		
7′′-9′′	29.26	0.79 s (9H)		C5′′, C6′′
-OCH_3	56.10	3.74 s (3H)		C3′
1-N	---	---		
1′′-NH	---	6.91 to 7.16[d] bt 4.0		(C4′′)[e]

a) Data reported for **1** in $CDCl_3$, except where specifically noted. NMR spectra of **1** recorded on 11.75-T instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C; ^{1}H NMR data for **2** recorded at 300 MHz. Data presented as δ in ppm, multiplicity, J in Hz. ^{1}H spectra referenced to the residual $CHCl_3$ (7.24 ppm) or residual benzene (7.15 ppm). ^{13}C chemical shifts are referenced to the center peaks of the solvents ($CDCl_3$, 77.0 ppm and D_6-benzene, 128.0 ppm). Assignments based on ^{1}H-^{13}C HMQC spectra. b) ^{1}H-^{1}H COSY data presented in non-redundant format from top to bottom. c) ^{1}H-^{13}C HMBC optimized for J_{CH} = 7 Hz, except where specifically noted. d) sample concentration dependent δ. e) ^{1}H-^{13}C HMBC optimized for J_{CH} = 5 Hz. f) ^{1}H-^{13}C HMBC data reported for **1** in D_6-benzene.

Ypaoamide is a structurally novel cyanobacterial metabolite. However, structural similarities between **1** and the malyngamides,[4] pukelelamides,[5,6] majusculamide D,[7] and microcolins A and B,[8] suggest that common biosynthetic pathways may be employed by different chemotypes of *L. majuscula*. While methylated valine derived *t*-butyl amino acids are relatively common among marine natural products,[11,12] the unusual *t*-butyl lipid side chain of **1** has little biosynthetic precedent other than in antillatoxin, an ichthyotoxic cyclic lipopeptide from a Curaçao strain of *L. majuscula*.[13]

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References and Notes

1. Voucher specimen on file with Prof. William H. Gerwick's herbarium (Oregon State University College of Pharmacy).
2. Nagle, D.G. and Paul, V.J. "Chemical Defense of a Tropical Marine Cyanobacteria Bloom" Manuscript in preparation (1996).
3. Compound **1** is named to reflect the site of collection (Ypao Beach: "I-pow.")
4. Cardellina, J.H. II; Marner, F-J.; Moore, R.E. *J. Am. Chem. Soc.* **1979**, *101*, 240-242.
5. Simmons, C.J.; Marner, F-J.; Cardellina, J.H. II; Moore, R.E.; Seff, C. *Tetrahedron Lett.* **1979**, *22*, 2003-2006.
6. Cardellina, J.H. II; Moore, R.E. *Tetrahedron Lett*, **1979**, *22*, 2007-2010.
7. Moore, R.E.; Michael, E. *Phytochemistry* **1988**, *27*, 3101-3103.
8. Koehn, F.E.; Longley, R.E.; Reed, J.K. *J. Nat. Prod.* **1992**, *55*, 613-619.
9. Acetylated ypaoamide **2**: ^{1}H NMR ($CDCl_3$) δ 7.18 ppm (2H; bd; J = 9 Hz; H7 & H11), 7.16 (1H; dd; J = 6.0, 1.6 Hz), 7.02 (2H; bd; J = 9 Hz; H8 & H10), 6.8 (1H; m, 1''-N<u>H</u>), 6.71 (1H; s; H2'), 6.01 (1H; dd, J = 6.0, 1.6 Hz; H2), 4.97 (1H; bd, J = 9 Hz; H4), 3.75 (3H; s; -OC<u>H</u>$_3$), 3.6 (3H; m; H5b & $H_2$5'), 3.0 (1H; ddd; J = 13, 8, 5 Hz; H4b), 2.9 (1H; ddd; J = 13, 7, 5 Hz, H4a), 2.75 (1H; dd; J = 13, 9 Hz; H5a), 2.28 (3H; s; -OCOC<u>H</u>$_3$), 2.18 (2H; t; J = 8 Hz; H2''), 1.57 (2H; p; J = 8 Hz; H3''), 1.2 (2H; m; H4''), 1.1 (2H; m; H5''), 0.79 (3H; s; H7''-H9'').
10. Hewlett Packard 5890 Series II gas chromatograph and a 5971 mass selective detector. 12.5 m of HP Ultra-1, 70° C for 2.0 min, 70-270° at 30° per min, then isothermal for 5 min. Ypaoamide (**1**): GC-EIMS (70 eV), t_R = 7.55 min, *m/z* 267 (6), 252 (11), 182 (99), 169 (19), 154 (5), 140 (8), 128 (100), 127 (53), 126 (47), 112 (18), 99 (22). Cultured *L. majuscula* extract compound: GC-EIMS retention time and spectrum were identical with that of authentic standard.
11. Ryu, G.; Matsunaga, S.; Fusetani, N. *Tetrahedron* **1994**, *50*, 13409-13416.
12. Talpir, R.; Benayahu, Y.; Kashman, Y.; Pannall, L.; Schleyer, M. *Tetrahedron Lett.* **1994**, *35*, 4453-4456.
13. Orjala, J.; Nagle, D.G.; Hsu, V.L.; Gerwick, W.H. *J. Am. Chem. Soc.* **1995**, *117*, 8281-8282.

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