ORGANIC LETTERS

2008
Vol. 10, No. 16
3501−3504

Cladoniamides A−G, Tryptophan-Derived Alkaloids Produced in Culture by *Streptomyces uncialis*

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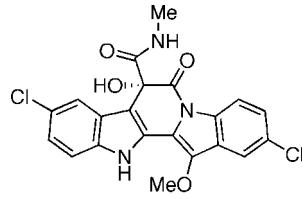
Received June 5, 2008

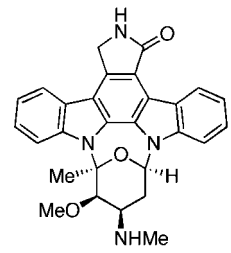
ABSTRACT



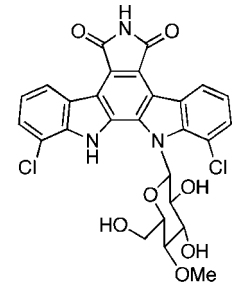
Cladoniamide G

Cladoniamides A−G (3−9) have been isolated from cultures of *Streptomyces uncialis*, and their structures have been elucidated by a combination of spectroscopic analysis and an X-ray diffraction analysis of cladoniamide A (3). The cladoniamides have unprecedented rearranged and degraded alkaloid skeletons with putative biogenetic origins from indolocarbazole precursors. Cladoniamide G (9) is cytotoxic to MCF-7 cells in vitro at 10 μg/mL.

The indolocarbazole alkaloids are a family of natural products isolated from marine invertebrates and cultures of diverse microorganisms.[1] Staurosporine (**1**) and rebeccamycin (**2**), two important members of this family, are potent inhibitors of protein kinases and topoisomerase-1, respectively. The biological activities of **1** and **2** have made them high-profile lead compounds for the development of anticancer drugs, and several analogues have entered clinical trials. Investigations of the genes and enzymes involved in the biosynthesis of indolocarbazoles has facilitated expresssion of the rebeccamycin biosynthetic pathway in *E. coli* and the production of novel analogues via genetic engineering.[2]



1 Staurosporine



2 Rebeccamycin

Recently, we reported the isolation of the new enediyne antibiotic uncialamycin from cultures of the novel actinomycete *Streptomyces uncialis* found on the surface of the lichen *Cladonia uncialis* collected near Pitt River, British Columbia.[3] Further investigation of extracts from cultures of *S. uncialis* has resulted in the isolation of the new alkaloids cladoniamides A−G (**3**−**9**). The alkaloid skeletons of the

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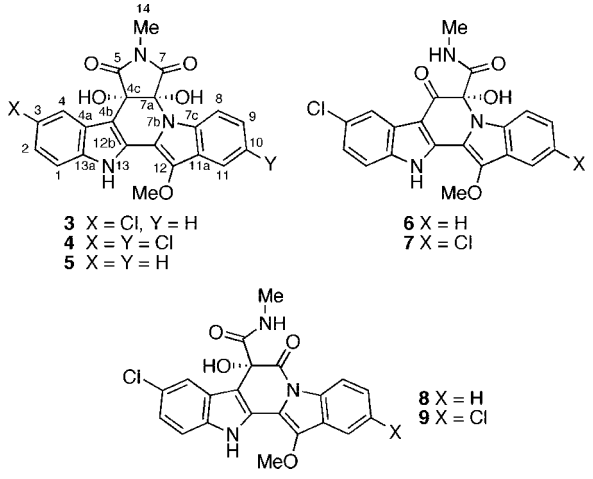
(1) Sánchez, C.; Méndez, C.; Salas, J. A. *Nat. Prod. Rep.* **2006**, *23*, 1007–1045.

10.1021/ol801274c CCC: $40.75
Published on Web 07/23/2008

cladoniamides, which are unprecedented in the scientific literature, appear to be derived by rearrangement and degradation of indolocarbazole precursors. Cladoniamide G (**9**) shows significant in vitro cytotoxicity against human breast cancer MCF-7 cells. Details of the structure elucidation of the cladoniamides and a proposed biogenesis for the new alkaloid skeletons are presented below.

Production cultures of *S. uncialis* were grown as lawns on solid agar (ISP4) at 30 °C. The cells and media from the solid agar cultures were jointly extracted repeatedly with EtOAc followed by concentration of the combined EtOAc extracts in vacuo to give a gummy purple residue that was partitioned between EtOAc and H_2O. Fractionation of the EtOAc-soluble material using sequential application of open column step-gradient reversed-phase chromatography, Sephadex LH20 chromatography, and reversed-phase HPLC gave pure samples of cladoniamides A−G (**3**−**9**) (see the Supporting Information).

Cladoniamide A (**3**) was obtained as optically active light green crystals (MeOH, mp 242−245 °C) that gave a $[M + Na]^+$ ion at *m*/*z* 460.0659 in the HRESIMS consistent with a molecular formula of $C_{22}H_{16}N_3O_5Cl$ (calcd for $C_{22}H_{16}N_3O_5ClNa$, 460.0676). The ^{13}C NMR spectrum recorded for cladoniamide A (**3**) in DMSO-d_6 showed 22 well-resolved resonances in agreement with the HREISMS measurement. HSQC correlations identified 13 protons attached to carbon (2 × CH_3; 7 × CH), and three resonances in the ^1H NMR spectrum (δ 7.19, bs, OH-4c; 8.17, bs, OH-7a; 11.62, s, NH-13) that showed no HSQC correlations to carbon were assigned to exchangable protons accounting for all 16 hydrogen atoms required by the molecular formula. The three nitrogen atoms in cladoniamide A (**3**) were observed at δ −221.8 (N-6), −254.0 (N-13), and −260.2 (N-7b) in a ^{15}N gLRHMQC experiment.



A methyl resonance at δ 2.88 (Me-14) showed correlations in the HMBC spectrum to carbon resonances at δ 171.5 (C7) and 174.5 (C-5), assigned to amide carbonyls, and a correlation in the ^{15}N LRHMQC spectrum to a nitrogen resonance at δ −221.8 (N-6). The exchangeable proton resonance at δ 7.19 (OH-4c) showed an HMBC correlation to the amide carbonyl resonance at δ 174.5 and to quaternary carbon resonances at δ 75.1 (C-4c) and 87.4 (C-7a). A second exchangeable proton resonance at δ 8.17 (OH-7a) showed HMBC correlations to the same two quaternary carbon resonances (δ 75.1 and 87.4) and to the amide carbonyl resonance at 171.5 (C-7). This set of HMBC and HMQC correlations revealed the presence of a 3,4-disubstituted-3,4-dihydroxy-*N*-methylsuccinimide fragment **A** in cladoniamide A (**3**) (Figure 1).

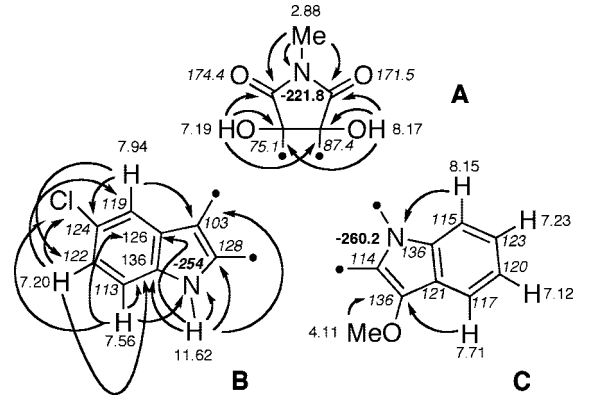


Figure 1. Selected $^1H/^{13}C$ HMBC and $^1H/^{15}N$ gLRHMQC correlations observed for cladoniamide A (**3**) in DMSO-d_6.

COSY correlations identified an isolated three-proton spin system (δ 7.20, dd, J = 2.0 Hz, 8.6 Hz, H-2; 7.56, d, J = 8.6 Hz, H-1; 7.94, d, J = 2.0 Hz, H-4) that could be assigned to a 1,2,4-trisubstituted benzene ring. The benzene resonance at δ 7.56 (H-1) and the exchangeable proton resonance at δ 11.62 (NH-13) both showed HMQC correlations into the ^{15}N resonance at δ −254.0 (N-13), and there was a ROESY correlation between δ 7.56 (H-1) and 11.62 (NH-13), suggesting that an NH was the substituent ortho to the benzene proton. HMBC correlations were observed between the NH resonance (δ 11.62) and quaternary carbon resonances at δ 103.6 (C-4b), 126.2 (C-4a), 128.3 (C-12b), and 136.7 (C-13a). The carbon resonances at δ 126.2 and 136.7 were assigned to carbons in the benzene ring on the basis of their HMBC correlations to the proton resonances at δ 7.56 (H-1) and 7.20 (H-2), respectively. An HMBC correlation between the proton resonance at δ 7.94 (H-4) and the carbon resonance at δ 103.6 (C-4b) indicated that this carbon was the subsituent ortho to the proton, and this connectivity combined with observed HMBC correlations between NH-13 (δ 11.62) and both of the carbon resonances at δ 103.6

(2) (a) Howard-Jones, A. R.; Walsh, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 12289–1229. (b) Sánchez, C.; Méndez, C.; Salas, J. A. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 560–568. (c) Sánchez, C.; Zhu, L.; Braña, A. F.; Salas, A. P.; Rohr, J.; Méndez, C.; Salas, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 461–466. (d) Hyun, C.-G.; Bililign, T.; Liao, J.; Thorson, J. S. *ChemBioChem* **2003**, *4*, 114–117. (e) Howard-Jones, A. R.; Walsh, C. T. *Biochemistry* **2005**, *44*, 15652–15663. (f) Zhang, C.; Albermann, C.; Fu, X.; Peters, N. R.; Chisholm, J. D.; Zhang, G.; Gilbert, E. J.; Wang, P. G.; Van Vranken, D. L.; Thorson, J. S. *ChemBioChem* **2006**, *7*, 795–804.

(3) Davies, J.; Wang, H.; Taylor, T.; Warabi, K.; Huang, X.-H.; Andersen, R. J. *Org. Lett.* **2005**, *7*, 5233–5236.

and 128.3 were consistent with a 2,3,5-trisubstituted indole fragment **B** in cladoniamide A (**3**) (Figure 1).

A second isolated four-proton spin system (δ 7.71, bd, J = 7.9 Hz, H-11; 7.12, t, J = 7.9 Hz, H-10; 7.23, t, J = 7.9 Hz, H-9; 8.15, bd, J = 7.9 Hz, H-8) could be assigned to a 1,2-disubsituted benzene ring on the basis of COSY correlations and multiplet patterns. HMBC correlations between both the benzene ring proton resonance at δ 7.71 (H-11) and the methyl singlet resonance at δ 4.11 and a carbon resonance at δ 136.7 (C-12), in conjunaction with a ROESY correlation between δ 7.71 (H-11) and 4.11 (OMe), revealed that one of the two substituents on the benzene ring was an sp^2-hybridized carbon attached to an methyl ether. A long-range ^{15}N HMQC correlation observed between the proton resonance at δ 8.15 (H-8) and a nitrogen resonance at δ −260.2 (N-7b) showed that the second benzene ring substituent was a nonprotonated nitrogen atom. The single remaining carbon in cladoniamide A, with a chemical shift of δ 114.5 (C-12a), was tentatively linked to the nitrogen atom and the methyl ether bearing an sp^2 carbon to give a 3-methoxyindole fragment **C** (Figure 1).

An HMBC correlation observed between the OH-4c resonance at δ 7.19 and the carbon resonance at δ 103.6 (C-4b) showed that fragments **A** and **B** were linked via C-3 of the indole residue **B** and one of the hydroxylated carbons on the succinimide residue **A**. The second hydroxylated succinimide carbon had a chemcial shift of δ 87.4 that was consistent with an aminal functionality, indicating that the indole fragment **C** was linked to the second hydroxylated succinimde carbon in fragment **A** via a C−N bond. The final site of unsaturation required by the molecular formula of **3** could be satisfied by linking the C-2 positions of the indole fragments **B** and **C** to form a six-membered ring, and attachment of the chlorine atom at C-3 of fragment **A** gave the constitution shown in **3** for cladoniamide A.

Since there was no direct NMR evidence for several of the connectivities in the proposed constitution **3**, a single-crystal X-ray diffraction analysis was carried out on cladoniamide A in order to confirm the constitution and elucidate its absolute congfiguration. Data were collected using Mo Kα radiation and were processed without merging Friedel pairs. The presence of the Cl atom allowed the unambiguous assignment of the absolute configuration on the basis of the refined Flack parameter value (−0.05(7)).[4] The ORTEP drawing for cladoniamide A (**3**) shown in Figure 2 revealed the 4c*S*,7a*R* absolute configuration.

Cladoniamide B (**4**) was isolated as an optically active pale mauve glass that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 494.0291 appropriate for a molecular formula of $C_{22}H_{15}N_3O_5Cl_2$ (calcd for $C_{22}H_{15}N_3O_5Cl_2Na$, 494.0286), which differed from the formula of cladoniamide A (**3**) by the addition of one chlorine atom and the loss of one hydrogen atom. Analysis of the 1D and 2D NMR data obtained for cladoniamide B (**4**) (Supporting Information) showed that it was the C-10 chloro analogue of cladoniamide A. Cladoniamide C (**5**) was also isolated as an optically active

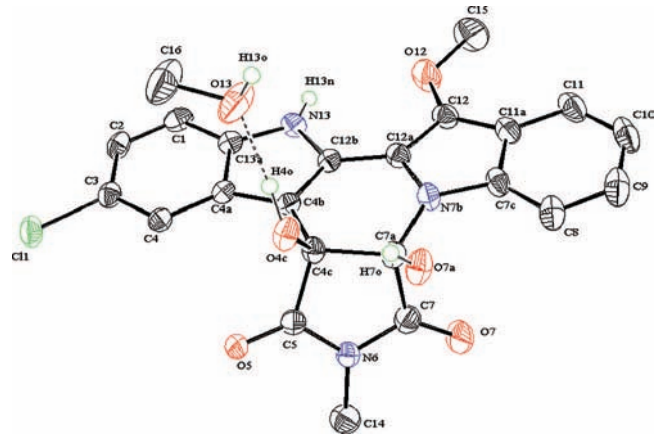


Figure 2. ORTEP drawing of cladoniamide A (**3**).

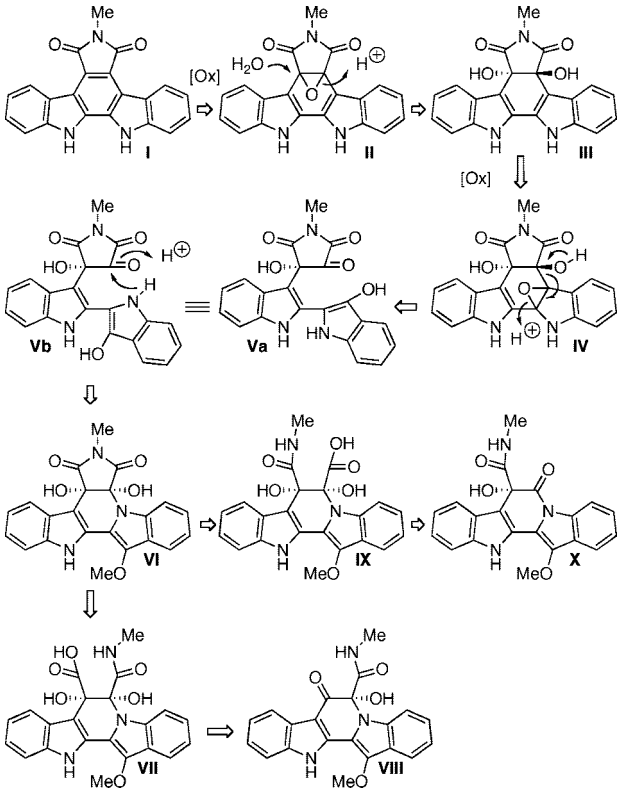
pale mauve glass that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 426.1057 consistent with a molecular formula of $C_{22}H_{17}N_3O_5$ (calcd for $C_{22}H_{17}N_3O_5Na$, 426.1066). Comparison of the NMR data obtained for cladoniamide C with the NMR data for cladoniamide A (Supporting Information) showed that **5** was simply the nonchlorinated analogue of **3**.

Cladoniamide D (**6**) was isolated as an optically active orange powder that gave a $[M + Na]^+$ ion in the ESIHRMS at *m*/*z* 432.0709 appropriate for a molecular formula of $C_{21}H_{16}N_3O_4Cl$ (calcd for $C_{21}H_{16}N_3O_4ClNa$, 432.0727), differing from the molecular formula of cladoniamide A (**3**) simply by the loss of CO. A methyl resonance at δ 2.64 (d, J = 4.9 Hz, Me-14) showed a COSY correlation to a resonance at δ 8.62 (q, J = 4.9 Hz, NH-6), a LRHMQC correlation to a nitrogen resonance at δ −277.2 (N-6), and an HMBC correlation to a carbon resonance at δ 167.6 (C-7) in the 2D NMR spectra of **6**, consistent with an *N*-methylamide fragment. HMBC correlations were observed between a deshielded resonance at δ 8.20, assigned to an OH (7a), and the amide carbonyl resonance at δ 167.6 (C-7) as well as additional carbon resonances at δ 86.4 (C-7a) and 184.7 (C-4c). This set of HMBC correlations was consistent with the presence in cladoniamide D of the C-7a hemiaminal functionality found in cladoniamide A (**3**), which was now bonded to the carbonyl of the *N*-methylamide fragment (C-7) and a conjugated ketone (C-4c). The rest of the 1D and 2D NMR data obtained for cladoniamide D (Supporting Information) was in complete agreement with the assigned structure **6**, in which the dihydroxysuccimide moiety in cladoniamide A (**3**) has been modified by excision of the C-5 carbonyl and transformation of C-4c to a ketone. Cladoniamide E (**7**), also isolated as an optically active orange powder that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 466.0329 (calcd for $C_{21}H_{15}N_3O_4Cl_2Na$, 466.0337), was routinely shown to be the 3,10-dichloro analogue of **6** via analysis of its 1D and 2D NMR data (Supporting Information).

Cladoniamide F (**8**), obtained as an optically active orange powder, gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z*

(4) Flack, H. D. *Acta Crystallogr., Sect. A* **1983**, *39*, 876–881.

Scheme 1. Proposed Biogenesis for the Cladoniamides



432.0713 (calcd for $C_{21}H_{16}N_3O_4ClNa$, 432.0727) in agreement with a molecular formula of $C_{21}H_{16}N_3O_4Cl$, making it isomeric with cladoniamide D (**6**). The 2D NMR data recorded for **8** contained a methyl resonance at δ 2.65 (d, J = 4.9 Hz, Me-14) that showed a COSY correlation to a resonance at δ 8.69 (q, J = 4.9 Hz, NH-6), a LRHMQC correlation to a nitrogen resonance at δ −278.8 (N-6), and an HMBC correlation to a carbon resonance at δ 169.9 (C-5) consistent with a *N*-methylamide fragment. A resonance at δ 7.16, assigned to an OH (4c), showed HMBC correlations to the amide carbonyl resonance at δ 169.9 (C-5) and to additional carbon resonances at δ 76.2 (C-4c) and 168.8 (C-7a). This set of HMBC correlations, and the rest of the 2D NMR data (Supporting Information), were consistent with the assigned structure **8** for cladoniamide F, in which the dihydroxysuccimide moiety in cladoniamide A (**3**) has been altered by excision of the C-7 carbonyl and conversion of C-7a to an amide carbonyl.

Cladoniamide G (**9**) was obtained as an optically active pale yellow amophous solid that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 466.0331 (calcd for $C_{21}H_{15}N_3O_4Cl_2Na$, 466.0337). Anaylsis of the 1D and 2D NMR data recorded for cladoniamide G (**9**) (Supporting Information) showed that it was a 3,10-dichloro analogue of **8**.

The three alkaloid skeletons represented by cladoniamides A (**3**), D (**6**), and F (**8**) are without precedent in natural products described in the peer-reviewed scientific literature. A compound related to cladoniamide A has been reported in the patent literature, but there is no spectroscopic support for the assigned structure.[5] The biogenetic proposal in Scheme 1 suggests that the cladoniamides are derived via rearrangement and degradation of tryptophan-derived indolocarbazole precursors **I**.[1,2] A key step in the biogenetic proposal is fragmentation of the epoxide **IV** to give **V**, which can recyclize via hemiaminal formation to give the skeleton **VI** found in cladoniamides A−C. Hydrolysis of the *N*-methylsuccinimide linkages in **VI** to give the α-hydroxycarboxylic acid intermediates **VII** or **IX**, followed by oxidative decarboxylation, leads to the degraded skeletons **VIII** and **X** of cladionamides D−G.

Cladoniamide G (**9**) was cytotoxic to human breast cancer MCF-7 cells in vitro at 10 μg/mL, while none of the other cladoniamides were active at concentrations <50 μg/mL. The cladoniamides are new rearranged and degraded structural variations on the indolocarbazole alkaloid theme. They represent novel aglycons for chemical, enzymatic, or genetic engineering mediated glycosylation approaches to generating new staurosporine/rebeccamycin analogues with potential anticancer activities.[2]

Acknowledgment. Financial support was provided by NCIC (R.J.A., M.R.), NSERC (R.J.A., J.E.D.), and CIHR (J.E.D.). The authors thank Karen Lu and Ivan Villanueva (Department of Microbiology and Immunology) for rRNA sequencing and characterization of the producing strain.

Supporting Information Available: Experimental details, tables of NMR asignments, and 1D and 2D NMR spectra for cladoniamides A−G (**3**−**9**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) Nakase, K.; Nakajima, S.; Hiriyama, M.; Kondo, H.; Kojiri, K.; Suda, H. JP 2000178274.