Revisiting the Sponge Sources, Stereostructure, and Biological Activity of Cyclocinamide A

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The dramatic biogeographical variations in the secondary metabolites from *Psammocinia* aff. *bulbosa* have complicated our efforts to reisolate the two most cytotoxic of its metabolites, (+)-psymberin and (+)-cyclocinamide A. Reported now are the results of a new study that demonstrates our ability to repeatedly isolate these two compounds through targeted collection efforts. Additional study of the new sample of (+)-cyclocinamide A has enabled finalizing its biological activity and absolute stereochemistry as 4*S*, 7*S*, 11*S*, 14*S*.

In the early 1990s we began a campaign to survey the chemical ecology of *Psammocinia* aff. *bulbosa* communities from Papua New Guinea. It is now clear that a side-by-side chemical and taxonomic examination is obligatory to differentiate among populations of *Psammocinia* (Dictyoceratida, Irciniidae), *Cacospongia* (Dictyoceratida, Thorectidae), and *Ircinia* (Dictyoceratida, Irciniidae),[1] having very similar morphologies. Recognizing this critical step made it possible to engage in a meaningful synopsis of the chemical variations observed for a small library of *P*. aff. *bulbosa* specimens. Reported now are the final results of a project whose goals were to (a) obtain fresh sponge specimens containing both psymberin and cyclocinamide A in order to facilitate ongoing molecular genetics studies on their biosynthetic pathways,[2] (b) complete the full absolute chirality assignment for cyclocinamide A,[3,4] and (c) accumulate additional biological activity data for cyclocinamide A.[3]

Outlined in Table 1 are an astonishing variety of biosynthetic products that we have observed to date from six distinct collections whose voucher material has each been identified as *P*. aff. *bulbosa*. The major constituents of specimens I−V range from sesterterpenes (variabilin[5]), polyketides (preswinholide A[6] and swinholide A[6,7]), a cyano-sponge cyclic hexapeptide (psymbamide A[7]), and a halogenated hexapeptide (cyclocinamide A[3]) to a NRPS-PKS mixed biogenetic derived compound (psymberin[8]). By contrast, meroterpenes (cacospongin B[9] and chromarol D[9]) usually observed from sponges of *Cacospongia* genus were isolated from sample VI. Though samples V and VI were obtained from adjacent sites, the chemical profiles of their major constituents clearly showed that the biosynthetic machinery of these two sponge collections was distinct. These plus the other dramatic biogeographical variations shown in Table 1 complicated our efforts to reisolate the two most structurally unique of these metabolites, psymberin and cyclocinamide A.

Before proceeding, it is important to briefly review the historical record highlighted in Figure 1 pertaining to absolute stereochemical assignments for psymberin and cyclocinamide A. The 5*S*, 8*S*, 9*S*, 11*R*, 13*R*, 15*S*, 16*R*, 17*R* structure we originally proposed for (+)-psymberin[8] (**1a**) was reaffirmed from two independent total syntheses of **1c**.[10,11] The synthetic products also provided the basis for the 4*S* designation at the single unassigned carbon and verified that (+)-psymberin (**1a**) we isolated from *P*. aff. *bulbosa* was identical to (+)-ircinistatin A[12] (**1b**) reported by Pettit from *Ircinia* cf. *ramosa*. Four additional diastereomers were also created during these synthesis projects, and the most important included **1d** and **1e**.[10,11,13] The stereostructure of (+)-cyclocinamide A (**2a**), which we described in 1997, included firm assignments at 7*S* and 14*S*, but the 4*S* and 11*S* designations were speculative.[3] In 1998, a total synthesis by Grieco afforded the 4*R*, 7*S*, 11*R*, 14*S* structure **2b** (no [α] reported), whose ^{1}H NMR spectra was similar to but not identical to that of **2a**.[4] Rather fascinating is the report of (+)-4*S*,7*R*,11*S*,14*R*-cyclocinamide B[14] (**3**) by Ireland from a Fijian sponge *Corticium* sp. (Homoslerophida, Plakinidae), which would be enantiomeric to **2b** if the C-36 substituents were identical.

The depletion of both sponge material and authentic samples of psymberin and cyclocinamide A in our repository necessitated a re-collection effort. Our plan to obtain taxa containing both psymberin and cyclocinamide A was formulated on the basis of the clarifying chemotype patterns shown in Table 1. The best Papua New Guinea collection site for the new samples appeared to be in the Amphlett vicinity of 9°43′ S: 150°50′ E to 9°14′ S, 150°46′ E. Thus, in May 2007, re-collections were conducted at two locations, yielding (a) sample VII (Milne Bay 10°16′ S, 150°47′ E (coll. no. 07202, 76 g dry weight)); and (b) sample VIII (Amphlett 9°14′ S, 150°46′ E (coll. no. 07208, 21 g dry weight)). Parallel extraction of both collections followed by LC-MS analyses of the resultant crude mixtures indicated the presence of major metabolites as follows. Sample VII: swinholide A[15] and preswinolide A[6] but no psymberin. Sample VIII: (+)-psymberin (**1a**) (MW 609, $[\alpha]^{27}_D$ +23.4, 3.2 mg, 1.5 × 10^{-2}% of the dry weight), microgram quantities of two psymberin analogues of MW 595 and MW 625 previously observed,[7] and (+)-cyclocinamide A (**2a**) (MW 772, $[\alpha]^{27}_D$ +32, 0.5 mg, 2.4 × 10^{-3}% of the dry weight). Although additional work on sample VII was discontinued, the preceding results reaffirmed the expectations shown in Table 1 pertaining to the best collection sites for **1a**- and **2a**-containing samples. Once these outcomes had been obtained, portions of sponge VIII were shuttled to the Piel group at the University of Bonn for molecular genetics experiments paralleling those completed for pederin,[16] and findings will be published in the near future.

The isolation work on sample VIII proceeded in a multistep fashion. The dichloromethane (coded XFD) and methanol (coded XFM) fractions showed potent activity in the soft agar colony-forming disk diffusion assay (DDA).[3] However, due to the paucity of the biological material, a traditional round of prefractionation was forgone. Both these extract fractions were directly subjected to semipreparative HPLC. The new sample of (+)-psymberin (**1a**) was obtained from the XFD and XFM fractions, while (+)-cyclocinamide A (**2a**) came from the XFM fraction. The identities of both compounds were confirmed from ^{1}H and ^{13}C NMR data and included in the case of **2a** the additional diagnostic ESITOFMS BrCl pseudomolecular ion cluster at $[M + Na]^+$ m/z 772.[3] In the

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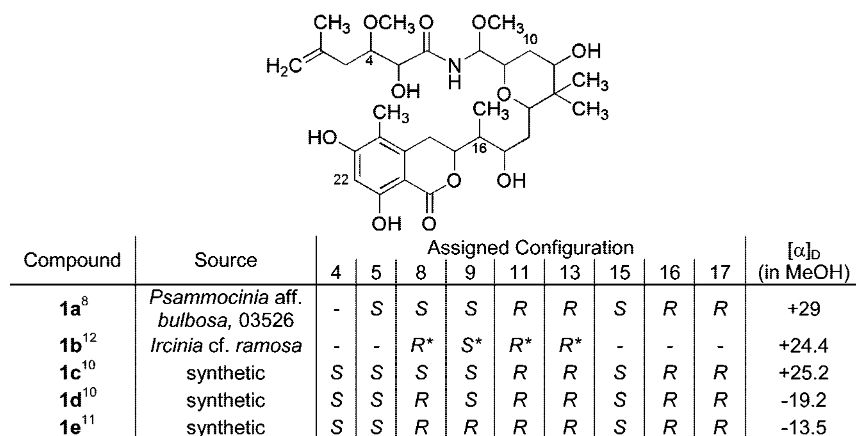
Table 1. Varying Morphotypes of *Psammocinia* aff. *bulbosa* from Papua New Guinea Correlated with Biogeographical Variations in their Constituents (underwater pictures of all samples have been previously published)[1,7]

specimen type	coll. no.	location	coordinates	major constituents
I	93145[7]	Normanby	9°43′ S, 150°47′ E	(+)-cyclocinamide A (−)-variabilin
IIa, IIb	02315[7]	Amphlett, Milne Bay	9°43′ S, 150°44′ E 10°13′ S, 150°52′ E	(+)-psymberin (+)-swinholide A
III	03526[1]	Amphlett	9°37′ S, 150°57′ E to 9°14′ S, 150°46′ E	(+)-psymberin
IV	05411[1]	Milne Bay to Pilkinton Reef	10°41′ S, 152°50′ E 11°19′ S, 154°16′ E 11°26′ S, 154°24′ E	(−)-preswinholide A (+)-swinholide A
V	06121[7]	Milne Bay	9°58′ S, 150°57′ E 10°15′ S, 150°40′ E	(−)-preswinholide A (−)-psymbamide A
VI	06122[7]	Milne Bay	9°58′ S, 150°57′ E 10°15′ S, 150°40′ E	(+)-chromarol D (−)-cacospongin B

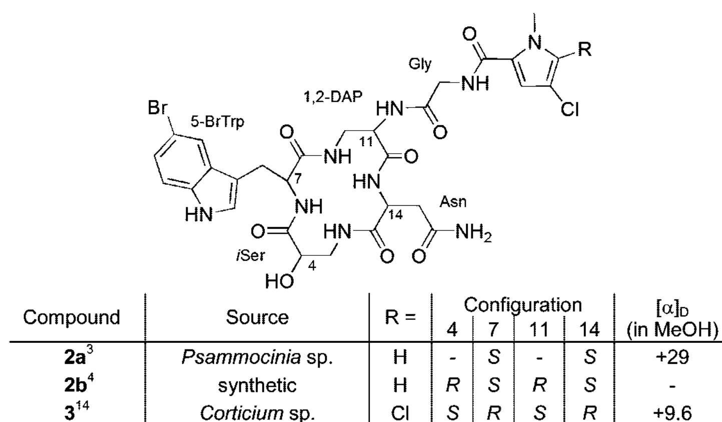
original structure elucidation of **2a**, the *i*Ser OH ^{1}H NMR shift was reported at δ_H 3.27 from water suppression data, but further inspection of the published ^{1}H NMR spectra of **2a** reveals that in either DMSO-d_6 or DMSO-d_6/benzene-d_6 there are exchangeable proton signals at δ_H 6.05 and 6.15, respectively; however, these are not detected under CD_3OD conditions. The δ_H 6.01 (d, $^3J = 5$ Hz) *i*Ser OH signal was clearly evident in the new sample of **2a** and consistent with the observation that in (+)-cyclocinamide B (**3**) the *i*Ser OH occurs at δ_H 6.00 (d, $^3J = 4$ Hz).[14]

Marfey's method was employed to establish the absolute configuration of the chiral carbons in the new sample, leading to the structure assigned as 4*S*,7*S*,11*S*,14*S*-cyclocinamide A (**2a**). This began by treating the acid hydrolysate of **2a** with L-FDAA (*N*-α-(2,4-dinitro-5-flourophenyl)-L-alanine amide)[17] followed by HPLC analysis. The reference complexes were prepared by a similar derivatization employing standard amino acids. The HPLC conditions gave profiles in which each L-amino acid−FDAA complex eluted before the D-amino acid analogue, except for the β-amino acid *i*Ser, whose L/D elution order was reversed.[18] Acceptable retention time differences were observed for all HPLC runs. Thus, the comparative analysis by LC-MS of the derivatized hydrolysates obtained from **2a** versus that of the standard amino acids was the basis for the all L-amino acid configuration assigned.

The solid tumor selectivity of (+)-cyclocinamide A (**2a**), originally reported as selective against colon-38 versus L1210 cells in the DDA,[3] has stimulated sustained interest in this unusual cyclic tetrapeptide core structure possessing the unique dipeptide side chain. The Grieco total synthesis of **2b** was relevant as a next step; unfortunately the small amount of material obtained was insufficient for further in vitro evaluation. Another development was that Ireland found (+)-cyclocinamide B (**3**) to be inactive against HCT-116 cells.[14] Our further evaluation of **2a** revealed nonpotent, modest



Compound	Source	4	5	8	9	11	13	15	16	17	[α]D (in MeOH)
		Assigned Configuration									
1a[8]	*Psammocinia* aff. *bulbosa*, 03526	-	*S*	*S*	*S*	*R*	*R*	*S*	*R*	*R*	+29
1b[12]	*Ircinia* cf. *ramosa*	-	-	*R**	*S**	*R**	*R**	-	-	-	+24.4
1c[10]	synthetic	*S*	*S*	*S*	*S*	*R*	*R*	*S*	*R*	*R*	+25.2
1d[10]	synthetic	*S*	*S*	*R*	*S*	*R*	*R*	*S*	*R*	*R*	-19.2
1e[11]	synthetic	*S*	*S*	*R*	*R*	*R*	*R*	*S*	*R*	*R*	-13.5



Compound	Source	R =	4	7	11	14	[α]D (in MeOH)
			Configuration				
2a[3]	*Psammocinia* sp.	H	-	*S*	-	*S*	+29
2b[4]	synthetic	H	*R*	*S*	*R*	*S*	-
3[14]	*Corticium* sp.	Cl	*S*	*R*	*S*	*R*	+9.6

Figure 1. Psymberin (**1a**)/ircinistatin A (**1b**) and cyclocinamide (**2**, **3**) compounds.

solid tumor selectivity with inhibition zone sizes (90 μg/disk) against HCT-125 and murine CFU-GM of 26 and 16 mm, respectively, in the DDA, and the IC_{50} against HCT-116 was >10 μg/mL. Thus, the previously encouraging solid tumor selectivity for **2a** against colon-38 was not seen against HCT cell lines for both **2a** and **3**.

The results presented above have added a new dimension to the chemical ecology of *P*. aff. *bulbosa* sponges. First, neither **1a** nor **2a** can be considered as a marker compound for *Psammocinia* because of their occurrence in other unrelated sponges. Second, a specific population of *P*. aff. *bulbosa* has now been shown to repeatedly be a source of **1a** and **2a**, meaning that further examination of the microbial communities associated with these distinct populations could be rewarding.[22] Third, adjacent populations of material identified as *P*. aff. *bulbosa* (see samples V and VI, Table 1) have been shown to vary considerably in their terpene versus their PKS- and NRPS-type major metabolites. Finally, our re-examination of the bioactivity of **2a** offers additional clarifying data. Future efforts to prepare libraries of compounds based on the cyclocinamide framework both with and without the BrTrp moiety could be warranted.[19]

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR experiments were run in Shigemi tubes on a Varian UNITY-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C, respectively) using standard pulse sequences with residual solvent protons and carbons as references (DMSO-d_6: 2.50 and 39.51 ppm for ^{1}H and ^{13}C, respectively). Mass measurements were obtained on a Mariner benchtop ESITOFMS. Crude extractions were obtained using an accelerated solvent extractor (1500 psi, 100 °C). HPLC was performed with Phenomenex Luna reversed-phase semipreparative (10 mm × 250 mm) and Altima reversed-phase analytical (5 mm × 250 mm) C18 5 μm columns.

Collection and Identification. The sponge samples were collected in May 2007 using scuba at depths of 30−60 ft. coll. no. 07202 (sample VII) was collected from Milne Bay, Papua New Guinea (GPS = 10°15.830′ S, 150°46.191′ E), and coll. no. 07208 (sample VIII) was collected in the Amphlett region of Papua New Guinea (GPS = 9°14.280′ S, 150°46.946′ E). The collected specimen were globular in morphology with a small apical oscule. The gray-colored ectosome is composed of a neat reticulation of foreign material. The cream-colored endosome is a loose network of cored primary fibers with sparse cored secondary fibers. The fine collagen filaments form a dense network in the endosome. Both collections were identified as a *Psammocinia* aff. *bulbosa* (Bergquist, 1995) by Dr. N. J. de Voogd. Voucher samples have been deposited at UCSC and Naturalis (RMNH); UCSC coll. no. 07202 = RMNH Por. 2980; UCSC coll. no. 07208 = RMNH Por. 2982. Topside and underwater photographs are also available from the Crews laboratory.

Extraction and Isolation. Biological material was handled using our standard laboratory protocol.[1] Crude extracts of the dried sponge (21 g dry weight) were obtained employing an accelerated solvent extractor (ASE) to yield hexanes (XFH, 344.8 mg), CH_2Cl_2 (XFD, 214.6 mg), and CH_3OH (XFM, 839.1 mg) fractions. By LCMS, both the XFD and XFM showed trace amounts of (+)-psymberin (**1a**). The XFD was fractionated using semipreparative RP-HPLC (25% aqueous to 100% CH_3CN with 0.1% formic acid over 30 min) to afford 1.7 mg of pure **1a** in fraction H17. The XFM was fractionated in a similar manner to yield 1.5 mg of **1a** in fraction H7. Fraction XFM-H5 contained (+)-cyclocinamide A (**2a**), which was purified using analytical RP-HPLC (isocratic 30% aqueous CH_3CN with 0.1% formic acid) to yield 0.5 mg of pure compound.

Hydrolysis and L-FDAA Derivatization of (+)-Cyclocinamide A (2a). (+)-Cyclocinamide A (**2a**) (0.25 mg) was hydrolyzed with 1 mL of 6 N HCl (0.1% phenol w/v)[20] for 4 h in a sealed, thick-walled vial at 110 °C. The hydrolysate was evaporated under nitrogen and then derivatized by treatment first with 100 μL of 1 M $NaHCO_3$ and second with 50 μL of *N*-α-(2,4-dinitro-5-flourophenyl)-L-alaninamide (L-FDAA) solution (10 mg/mL in acetone).[17] The reaction mixture was heated to 80 °C for 30 min, cooled to room temperature, and finally quenched with 50 μL of 2 N HCl. CH_3CN (300 μL) was then added for subsequent LC-MS analysis. Each standard amino acid (0.5 mg) was derivatized under the same procedure with L-FDAA. For each reaction product, a linear gradient of 10% to 50% CH_3CN (0.1 M NH_4OAc, pH = 6) over 60 min was used to separate the derivatized products by LC-MS (Phenomenex Luna C_{18} column, 5 mm × 250 mm, UV 340 nm, flow rate 1 mL/min).[14] Retention times of the derivatized hydrolysate were monitored by mass and UV (340 nm) and compared to that of the derivatized commercially available standard amino acids. The HPLC of co-injected 2:1 derivatized standard amino acid−FDAA diastereomer complexes gave profiles for DAP and Asp in which the L-amino acid−FDAA complex eluted before the D-amino acid analogue, while the β-amino acid, *i*Ser,[18] demonstrated a D to L elution order.[23] As a racemic mixture of BrTrp was used for derivatization, we assumed the standard elution order to be L versus D elution. This was identical to that observed by Ireland[14] using similar HPLC analysis conditions. Finally, the optical properties of the new sample of **2a** ($[\alpha]^{27}_D$ +32, in MeOH) match that of our original compound ($[\alpha]^{27}_D$ +29, in MeOH)[3] in which L-BrTrp was established. Retention times for standard amino acid−FDAA derivatives: L-Asp 17.3 min, D-Asp 20.0 min, D-*i*Ser 30.6 min, L-*i*Ser 31.3 min, L-DAP 49.1 min, L-5-BrTrp 49.8 min, D-DAP 50.1 min, D-5-BrTrp 53.2 min. Retention times for the FDAA hydrolysate derivatives of **2a**: L-Asp 17.3 min, L-*i*Ser 31.4 min, L-DAP 49.2 min, L-5-BrTrp 50.0 min.

Biological Assays. The disk diffusion soft agar colony-forming assay (DDA)[21] and IC_{50} determinations[7] were performed as previously described.

(+)-Psymberin (1a). Spectroscopic data in accordance with published data.[8]

(+)-Cyclocinamide A (2a). Spectroscopic data in accordance with published data.[3]

Swinholide A. Spectroscopic data in accordance with published data.[15]

Preswinholide A. Spectroscopic data in accordance with published data.[6]

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Supporting Information Available: Sponge photographs and isolation scheme. This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) This L to D elution order for the *i*Ser−FDAA complex is opposite of that reported under the HPLC conditions for the derivitized hydrolysates for cyclocinamide B.[14] Due to the hydrophilic nature of the *i*Ser side chain, this elution order can change with pH variance.

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