Asperazine, a Selective Cytotoxic Alkaloid from a Sponge-Derived Culture of *Aspergillus niger*

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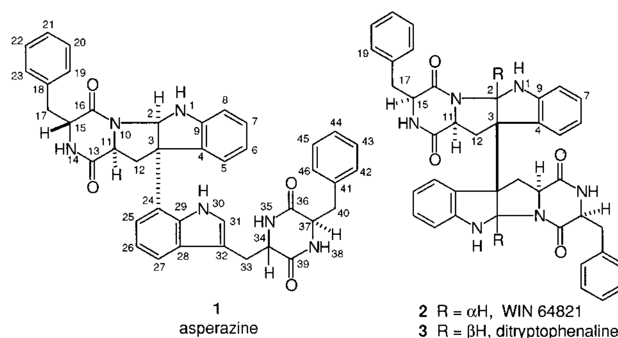
Chemical investigations of fungi derived from marine sources[1] have lagged behind those of terrestrial fungi.[2] Recently, it has been recognized that fungi can be isolated from marine substrates which should accelerate the rate of research in this area.[3] In this context we have discovered how to regularly obtain such microorganisms from sponge endosomes. Furthermore, the saltwater culture of these isolates appears to be a rich source of novel metabolites.[4] Recent reviews have summarized the very small numbers of metabolites derived from cultured fungi obtained from other marine substrates.[1a−d] An interesting emerging theme is that marine-derived fungi appear to be a good source of complex diketopiperazines.[5] In this report we describe the structure of asperazine (**1**),[6] an unusual unsymmetrical diketopiperazine dimer, obtained by saltwater culture of *Aspergillus niger* derived from a Caribbean *Hyrtios* sponge.

The process to obtain asperazine was begun with the collection by SCUBA of a small portion of *Hyrtios proteus* from the Dry Tortugas National Park, Florida. On shore, the sponge was used to initiate a fungal culture on salt water corn meal agar plates containing antibiotics. Large scale liquid cultures (made up with filtered Monterey Bay sea water) were subsequently prepared.[7] The fungal mycelium was separated from the broth, and parallel extractions of the broth and mycelium were performed.[7] Eventually, 5.8 mg of **1** was obtained as an amorphous white powder after HPLC purification of the broth extract. Interpretation of the ^{1}H and ^{13}C NMR data including DEPT 135 and 2D NMR spectra especially HMBC revealed the amino acid nature of **1**. Four amide carbonyl ^{13}C resonances were suggested by signals between δ 168−170. Also, four amino acid α-CH groups were identified by $^{13}C/^{1}H$ NMR peaks[6] (Figure S1, Table S1) δ 60.5/4.12, 57.8/3.32, 57.1/3.59, and 56.4/3.51. When the NMR data, which indicated a partial formula of $C_{40}H_{31}N_4O_4$, was combined with the HRFAB mass spectrum (m/z = 665.2889, $[M + H]^+$, Δ 1.3 mmu of calcd for $C_{40}H_{36}N_6O_4$), it was apparent that **1** was potentially related to isomeric terrestrial fungal natural products WIN 64821, $C_{40}H_{36}N_6O_4$ (**2**)[8] and ditryptophenaline, $C_{40}H_{36}N_6O_4$ (**3**).[9]



The structural similarities and differences between **1** and **2**[8] or **3**[9] were rapidly pinpointed by a side-by-side comparison of their respective NMR data (Table S1). The diasteromeric, symmetrical dimers **2** and **3**, both incorporating *S*-phenylalanine and *S*-tryptophan, have NMR spectra displaying only 18 signals for the 40 carbons present in the each. By comparison, there were 34 distinct ^{13}C NMR signals observed for **1**, and this included two extra sp^2 carbons not present in **2**. There were also five NH protons seen by ^{1}H NMR for **1** versus the four NH protons in **2** and **3**. These observations plus the list of substructures that were assembled meant that **1** contained an extra double bond and one less ring in comparison to the known isomers. The HMBC plus $^{1}H-^{1}H$ COSY NMR data were consistent with two phenylalanine subunits and one tryptophan unit having analogous cyclization patterns to **2** or **3**, but it was clear that the second tryptophan of **1** was quite different. Inter-

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(4) Examples include: chlorocarolides [(a) Abrell, L. M.; Borgeson, B.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 2331−2334], chloriolins [(b) Cheng, X.-C.; Varoglu, M.; Abrell, L.; Crews, P.; Lobkovsky, E.; Clardy, J. *J. Org. Chem.* **1994**, *59*, 6344−6348], nectriapyrones [(c) Abrell, L. M.; Cheng, X.-C.; Crews, P. *Tetrahedron Lett.* **1994**, *35*, 9159−9160], secocurvalarin [(d) Abrell, L. M.; Borgeson, B. M.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 8983−8984], trichohrazin [(e) Kobayashi, M.; Uehara, H.; Matsunami, K.; Aoki, S.; Kitagawa, I. *Tetrahedron Lett.* **1993**, *34*, 7925−7928].

(5) Examples include: marine-derived leptosins [(a) Takahashi, C.; Takai, Y.; Kimura, Y.; Numata, A.; Shigematsu, N.; Ito, Y.; Matsumura, E.; Tanaka, H. *Phytochemistry* **1995**, *38*, 155−158. (b) Takahashi, C.; Minoura, K.; Yamada, T.; Numata, A.; Kushida, K.; Shingu, T.; Hagishita, S.; Nakai, H.; Sato, T.; Harada, H. *Tetrahedron* **1995**, *51*, 3483−3498] which strongly resemble terrestrial fungal compounds: chaetocin A [(c) Hauser, D.; Weber, H. P.; Sigg, H. P. *Helv. Chim. Acta.* **1970**, *53*, 1061−1073], verticillin A [(d) Minato, H; Matsumoto, M.; Katayama, T. *J. Chem. Soc. Perk. Trans. I* **1973**, 1819−1825] and the melinacidins III and IV from both a marine [(e) Furuya, K.; Okudaira, M.; Shindo, T.; Sato, A. *Sankyo Kenkyusho Nempo* **1985**, *37*, 140−142] and a terrestrial fungi [(f) Argoudelis, A. D.; Mizsak, S. A. *J. Antibiot.* **1977**, *30*, 468].

(6) Asperazine (**1**) white powdery flakes, $[\alpha]_D = +53°$ (c, 0.2, CH_3OH); IR (film) 3448, 3365, 2931, 1681, 1673, 1667, 1651, 1444, 1314, 1093, 743, 700 cm^{-1}; λ_{max} = 300, 285, 225 nm; CD nm (mdeg) 210 (−3.3), 220 (−−0), 230 (+8.5), 290 (+6.0); LRFABMS, positive ion, m/z (relative intensity) 665.3 ($[M + H]^+$, 60), 409 (100); HRFABMS 665.2889 $[M + H]^+ = C_{40}H_{37}N_6O_4$ (Δ 1.3 mmu of calcd); NMR assignments in Table S1 and Figure 1 are based on $^{1}H-^{1}H$ COSY, HMQC, HMBC, NOESY and difference NOE data: $^{13}C-^{1}H$ COSY NMR data 500/125 MHz (CD_3CN).

(7) Details appear in the Experimental Section (Supporting Information).

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(9) (a) Springer, J. P.; Buchi, G.; Kobbe, B.; Demain, A. L.; Clardy, J. *Tetrahedron Lett.* **1977**, *28*, 2403−2406. (b) Maes, C. M.; Potgieter, M.; Steyn, P. S. *J. Chem. Soc. Perkin Trans. 1* **1986**, 861−866.

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pretation of the selected HMBC correlations shown in Figure S1 including those from H-2 to C3, C4, C9, C12, C24 and additional two and three bond HMBC correlations from H12/12′ to C2, C3, C4, C13, and C24 confirmed the bond envisioned between C3 and C24.

The substitution pattern of the aryl ring containing C24 was determined by using the well resolved ^{1}H NMR signal of H27 (Figure S1) as an anchor point. A strong three-bond HMBC correlation from H27 to C32 was used to place C27 conclusively beside C28. It was next necessary to clarify whether H27 was part of a two- or three-proton proton spin system. This was accomplished by HMQC−TOCSY correlations observed from H27 to C26 and from C26 to H25. The NMR assignments for this ring were completed by placing the remaining quaternary carbon C24 adjacent to C25. At this point the collective NMR data allowed the gross structure of **1** to be assembled as shown.

Attention was next shifted to defining the relative and absolute stereochemical features of **1**. Difference NOE and NOESY experiments were used to establish the relative stereochemistry of H2, H11, and C24. A NOESY correlation from the indole NH30 to H2 and a 1D difference NOE from H2 to H11, placed H2, H11 and C24 in close proximity. The multiplet patterns of the H11, H15, H34, and H37 were examined next to decide the relative stereochemistry of the diketopiperazine rings. In **2**,[8] **3**,[9] and other proline-containing diketopiperazines,[10] a $^5J_{HH} \cong 1$ Hz has been observed between the syn α protons of the diketopiperazine rings. This small J was not found between H11 and H15, nor between H34 and H37. In particular, spectral simulation and additional spin decoupling experiments revealed that there were no ^5J couplings between these protons. Alternatively, the $^5J_{HH} \cong 1$ Hz was observed between H11 and H15 in **2**. The lack of an observable $^5J \geq 1$ Hz between H11 and H15 in **1** suggests an anti configuration for these two atoms. Unfortunately, the relative stereochemistry of H34 and H37 could not be determined based on ^{1}H NMR data, because this ^5J coupling might not be a reliable indication of stereochemistry.[11]

Hydrolysis of **1** and chiral TLC of the free amino acids[12] provided data to assign the absolute configuration of the phenylalanine residues. Subjecting 0.1 mg of **1** to hydrolysis followed by normal phase TLC showed phenylalanine was the only free amino acid present, as *R*-phenylalanine by chiral TLC, $R_f = 0.43$ (standard *R*-phenylalanine $R_f = 0.44$; standard *S*-phenylalanine $R_f = 0.55$ in 80/20/20 $CH_3CN/H_2O/CH_3OH$). This allowed the *R* configuration to be specified at C15 and C37. Based on the conclusions above, additional stereocenters could be assigned as 11*S*, 2*R*, 3*S*. Unfortunately, neither the CD[9] spectrum nor the lack of a coupling between H34 and H37 provided clear evidence of the absolute configuration of C34, which is tentatively assigned here as *S* based on a biogenetic analogy to the stereochemistry at C11/C15.

The bioactivity properties of **1** were explored, and it displayed an unusual profile of cytotoxicity. While asperazine did not exhibit activity in antibacterial (*Bacillus subtilis*) or antifungal assays (*Candida albicans*), it showed significant leukemia selective cytotoxicity in the Corbett−Valeriote soft agar disk diffusion assay.[13] Interesting selective activity was observed for **1** at 50 μg/disk in the primary *in vitro* assay which employs human leukemia murine colon 38 and human colon H116 or CX1 cell lines. The zone sizes observed in the primary assay[13b,14] are as follows [μg/disk: L1210/C38/H116 or CX1] **1** 50: 400/40/300 versus cytosine arabinoside (standard chemotherapeutic agent) 2.5: 910/610/250 which indicates excellent leukemia selectivity.[14] Follow-up in the secondary assay[13b] [μg/disk: L1210/CFU-GM] gives zone sizes of **1** 50: 450/50 versus cytosine arabinoside 0.5: >1000/45. This constitutes significant differential cytotoxicity and means that **1** is leukemia selective. Unfortunately, further follow-up via *in vivo* experiments was thwarted due to lack of material.

The structure of **1** is unique and continues to build on the theme that some marine-derived fungi elaborate complex diketopiperazines. The highly modified tryptophan units of **1**, though similar to those of **2** and **3**, differ in a fundamental way. The presence of the *R*-phenylalanine in **1** is unprecedented when compared to the structures of **2** and **3**. Interestingly, in a directed biosynthesis experiment using *R* amino acid precursors for the generation of WIN 64821 (**2**) analogs there was no incorporation of either *R*-phenylalanine or *R*-tryptophan. The linkage of the two subunits through the aryl ring of tryptophan in **1** contrasts to the situation in other diketopiperazines dimers.[5] One such example is the terrestrial fungal product chaetocin[5c] which is a diketopiperazine dimer through the tryptophan units. The other closest analogs to the linkage of the subunits of **1** through an aryl carbon are the plant secondary metabolites hodgkinsine,[15] psychotridine,[15] and the quadrigemines.[15] Finally, the novel structural features of asperazine add an important new facet to the growing field of fungal natural products derived from saltwater culture.

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Supporting Information Available: Table S1, Experimental Section, Figure S1, and spectral data (S2−S11) for asperazine (13 pages).

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(14) A zone differential of 250 (= 6.5 mm) units or greater is the basis for denoting an agent as either solid tumor or leukemia selective compound. A 1-log dilution produces a 330 unit zone change on average. Thus, a 250 unit differential represents approximately an eight fold change in sensitivity.[13c] In past experience with over 20,000 purified organic compounds, about 28 were leukemia selective.[13b]

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