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SPIROQUINAZOLINE, A NOVEL SUBSTANCE P INHIBITOR WITH A NEW CARBON SKELETON, ISOLATED FROM *ASPERGILLUS FLAVIPES*

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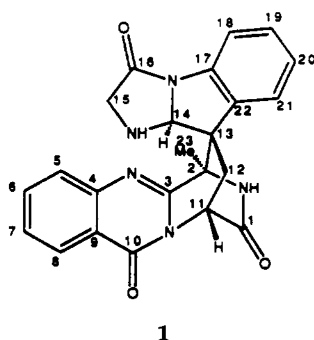
ABSTRACT.—A novel substance P inhibitor, spiroquinazoline [**1**], was isolated from the fungus *Aspergillus flavipes*, which was originally obtained from soil. The structure of **1** was determined by analysis of spectroscopic data and **1** was shown to contain a new carbon skeleton containing a spiro-carbon center. Also isolated from the same culture extract were the new natural product, benzodiazepinedione [**3**], and the known compounds, acyl aszonalenin [**4**], *N*-benzoyl-L-phenylalaninol, and seven diketopiperazines.

Substance P (SP), a member of the neurokinin family and the endogenous ligand for the neurokinin-1 (NK1) receptor, has been implicated in a number of physiological activities and is believed to play an important role in the perception of painful stimuli and in inflammatory response (1–3). Recently we reported the discovery of a novel cyclic peptide, isolated from a culture broth, which is a potent and competitive antagonist to substance P at the human NK1 receptor (4). In our search for related compounds from the same *Aspergillus flavipes* fungal culture extract we have now isolated a nonpeptide substance P inhibitor, namely spiroquinazoline [**1**], which has a new carbon skeleton. Spiroquinazoline is structurally related to fumiquinazoline C [**2**], which was isolated from the marine fungus *A. fumigatus* (5), and also to the fiscalins, substance P inhibitors isolated from the fungus *Neosartorya fischeri* (6). From *A. flavipes* we have also isolated the new natural product, benzodiazepinedione [**3**], although this was inactive in our substance P binding assay. Herein we report the isolation and structure determination of **1** and **3**, and also report the isolation of the known compounds acyl aszonalenin [**4**], *N*-benzoyl-L-phenylalaninol, cyclo(L-phe-L-pro), cyclo(L-phe-D-pro), cyclo(L-leu-L-pro), cyclo(L-tyr-L-pro), cyclo(L-tyr-D-pro), cyclo(L-val-L-pro), and cyclo(L-val-D-pro), from the same culture broth. Of the known compounds, only acyl aszonalenin [**4**] (7) is a substance P-binding inhibitor at the human NK1 receptor.

RESULTS AND DISCUSSION

In a search for SP inhibitors from the fungus *Aspergillus flavipes* we isolated spiroquinazoline [**1**], benzodiazepinedione [**3**], acyl aszonalenin [**4**], *N*-benzoyl-L-phenylalaninol, and seven diketopiperazines. The structure of **1** was determined by spectroscopic analysis, the structure of **3** was confirmed by synthesis, and the structures



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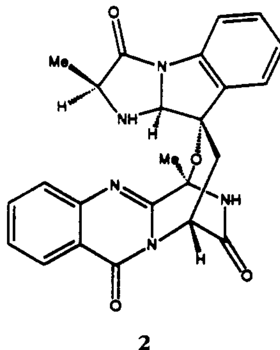

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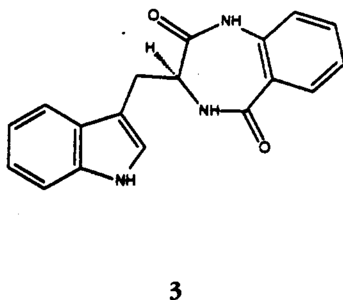

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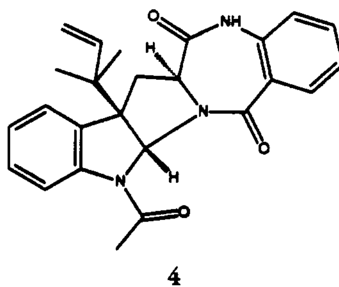

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of the known natural products were established by comparison with literature data (7–9), and synthesis.

The uv spectrum of **1** indicated the presence of a quinazoline ring (5). The molecular formula of **1** was established as $C_{23}H_{19}N_5O_3$ by hrfabms. The ^{1}H-nmr spectrum of **1** (Table 1) indicated the presence of two 1,2-disubstituted benzenes, two methylenes, two methines, a tertiary methyl, and two exchangeable NH protons. The ^{13}C-nmr spectrum and a DEPT experiment showed eight aromatic methine, two other methine, two methylene, one methyl, and ten non-protonated carbons. Direct and long-range COSY experiments established the presence of two four-proton aromatic spin systems, the coupling of a methine proton at 5.67 ppm (H-11) to methylene protons at 1.88 and 3.26 ppm (H-12a and H-12b), and the long-range coupling of a methine proton singlet at 5.47 ppm (H-14) to isolated methylene protons at 3.49 and 3.83 ppm (H-15).

TABLE 1. ^{1}H- and ^{13}C-Nmr Spectroscopic Data for Spiroquinazoline [**1**].[a]

Position	^{1}H (Hz)	^{13}C
1		171.31 s
2		61.76 s
3		152.62 s
4		146.71 s
5	7.70 d (8.0)	127.87 d
6	7.76 dt (7.6, 1.4)	134.66 d
7	7.52 m	127.67 d
8	8.30 dd (8.0, 1.0)	127.24 d
9		120.57 s
10		158.93 s
11	5.67 s	52.05 d
12a	1.88 d (14.0)	33.27 t
	3.26 dd (14.1, 4.2)	
13		56.36 s
14	5.47 s	81.13 d
15a	3.49 dd (15.8, 1.3)	52.84 t
15b	3.83 d (15.8)	
16		170.05 s
17		138.00 s
18	7.55 m	116.18 d
19	7.33 t (7.7)	129.37 d
20	7.16 t (7.6)	126.42 d
21	7.51 m	124.04 d
22		135.65 s
23	1.74 s	14.97 q
NH	1.60 br s	
NH	8.09 br s	

[a]Values in ppm, relative to δ_C=77.0 and δ_H=7.25 for $CDCl_3$.

After assigning the carbon chemical shifts for protonated carbons with the aid of an HMQC experiment, from an HMBC experiment we obtained long-range hydrogen to carbon connectivities to determine the carbon skeleton of **1**. Long-range coupling from the aromatic methine at 8.30 ppm (H-8) to a carbonyl carbon at 158.93 ppm (C-10) was observed. Further long-range couplings from the methyl group at 1.74 ppm (H-23) to carbons at 61.76 (C-2) and 152.62 ppm (C-3), from H-11 to both C-3 and a carbonyl at 171.31 (C-1), and from H-12 to C-1 and C-11, established the quinazoline ring structure shown in Figure 1 (partial structure **A**).

Long-range couplings from H-15 to the methine carbon at 81.13 ppm (C-14) and the carbonyl carbon at 170.05 ppm (C-16), and long-range couplings from both H-14 and the aromatic proton H-21 to the quarternary carbon at 56.36 ppm (C-13), established a second moiety (partial structure **B**, Figure 1). Long-range connectivities from H-12 to C-1, C-11, C-13, and C-14 established that the two partial structures are linked through methylene-14. Further long-range connectivities from H-14 to C-2 and from H-23 to C-13 established that a carbon-carbon bond exists between C-2 and C-13, defining the structure of **1** (Figure 1).

The relative stereochemistry of **1** was defined by the observation of an nOe from H-14 to H-23, but not to H-12. Two possible diastereoisomers exist for **1**. The first diastereoisomer is that drawn as structure **1**, and the second has the opposite chirality at C-14. Drawing and minimizing these structures using Macromodel (10) clearly established that an H-14 to H-23 nOe should be observed for the isomer drawn as structure **1**, but not for its diastereoisomer. The absolute stereochemistry of **1** remains undefined and the structure drawn is based on the likely biosynthesis from natural L-tryptophan.

The structure of **1** is related to that of fumiquinazoline C [**2**], which was isolated

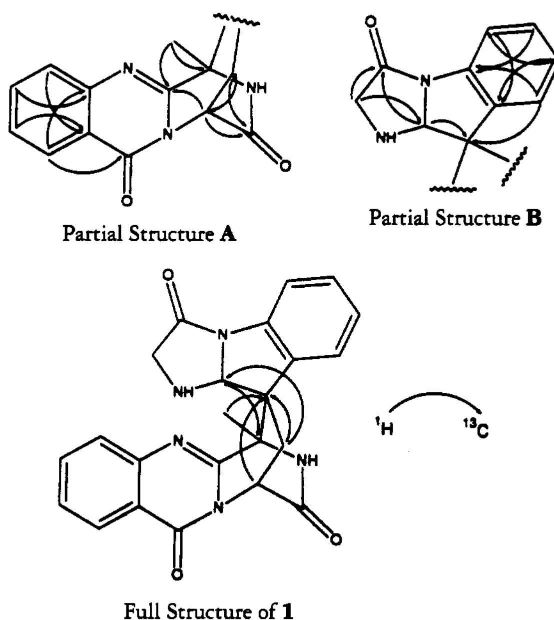

FIGURE 1. Partial Structures **A** and **B** were Derived from Spectroscopic Data and their Linkage Positions Established from Hydrogen to Carbon Multiple-bond Connectivities, Obtained from an HMBC Experiment for Spiroquinazoline [**1**].

from a fungus isolated from saltfish (5). The absolute stereochemistry of **2** was determined by crystal structure and isolation of L-alanine. Compounds **1** and **2** have the same relative stereochemistry and probably the same absolute stereochemistry, although the absolute stereochemistry of **1** could not be established by optical rotation comparison because of the presence of a chiral center at C-15 in **2**. The most significant difference in the structures of **1** and **2** is the presence of a carbon-carbon bond from C-2 to C-13 in **1**, so that **1** has a new carbon skeleton with a spiro center at C-13. No substance P inhibition data were reported for **2**. However, the recently reported compounds, fiscalins A, B and C, bearing similar structures to **2**, inhibit substance P binding to human astrocytoma cells (6).

Together with **1**, we isolated the known compound acyl aszonalenin [**4**] (7), which we found to be active in our substance P binding assay. Compound **4** was previously synthesized via, and is probably biosynthetically derived from, the benzodiazepinedione [**3**], although this compound has never been previously isolated as a natural product. We now report the isolation of **3** together with **1** and **4** from *A. flavipes*. The structure of **3** was initially determined from spectroscopic data and the absolute configuration confirmed from synthesis. Benzodiazepinedione [**3**] was synthesized by reacting isatoic anhydride with L-tryptophan in triethylamine and H_2O. The synthetic compound was purified using Si gel flash chromatography. Spectroscopic data for synthetic **3**, including optical rotation and circular dichroism, were identical with those obtained for the natural product. Also from the extract of *A. flavipes* we isolated the known compound *N*-benzoyl-L-phenylalaninol and seven diketopiperazines. The structures of these compounds were determined spectroscopically by comparison with literature data and authentic samples (8,9).

Spiroquinazoline [**1**] shows inhibition of [^{3}H]-SP binding to human astrocytoma cells with an inhibitory concentration (K_i) of 95 μM. The K_i of **4** in this assay was 170 μM. The remaining compounds were all inactive at 200 μM.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—One- and two-dimensional nmr spectra were recorded on a Bruker AMX 360 spectrometer. Chemical shifts are reported as δ values in ppm using the solvent as reference. Circular dichroism (cd) curves were measured on a Jasco J-600 spectropolarimeter. Ultraviolet (uv) spectra were recorded on a Shimadzu UV160U spectrophotometer. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. Ir spectra were recorded on a Nicolet IBM IR/3X spectrometer. Ms were performed on a Finnigan MAT TSQ 70 mass spectrometer and high-resolution ms were obtained from M-Scan (Malvern) using a VG analytical ZAB 2-SE high-field mass spectrometer. Photodiode array hplc was performed on a Waters system including 990 photodiode array detection, 510 pump, 715 ultra-WISP, automated gradient controller, and Powermate 386/20.

ISOLATION OF SPIROQUINAZOLINE [1], BENZODIAZEPINEDIONE [3], ACYL ASZONALENIN [4] AND *N*-BENZOYL-L-PHENYLALANINOL.—After cultivation of *Aspergillus flavipes* for 6 days the whole culture (2.75 liters) was extracted with EtOAc (2×1 liter). (The producing fungus, strain SC 230, has been preserved as part of the Sterling microbial culture collection.) The dried EtOAc extract (2.26 g) was then solvent partitioned using hexane-EtOAc-MeOH-H_2O (5:6:5:3). The lower layer (497 mg) was further separated using repetitive reversed-phase hplc with a H_2O/MeCN solvent system, yielding [**1**] (16 mg), [**3**] (8.5 mg), [**4**] (33 mg) and *N*-benzoyl-L-phenylalaninol (5.9 mg).

Spiroquinazoline [**1**] was isolated as a yellow solid. Uv, λ max (ε) (MeOH) 211.8 (22530), 225.0 (20620), 264.8 (7010), 277.0 (6350), 303.0 (2440), 315.0 (1980) nm, λ min (ε) 271.6 (5920), 298.0 (2070), 310.6 (1690) nm; ir, ν max 3235, 3065, 2930, 1710, 1640, 1620, 1485, 1470, 1390, 1335, 1310, 1180, 775, 760, 735, 420 cm^{-1}; ^{1}H- and ^{13}C-nmr spectra, see Table 1; hrfabms *m/z* $[M+H]^+$ 414.1590 ($C_{23}H_{20}N_5O_3$ requires 414.1566); cd, [θ] (nm) −59570 (210.0), 9980 (225.4), −38750 (239.0), 7690 (302.2) (MeOH); $[\alpha]_D$ −2.5° (*c*=0.7, MeOH), mp 166–168° (uncorrected).

Benzodiazepinedione [**3**] was isolated as a white solid. Uv, λ max (ε) (MeOH) 216.6 (57400), 282.0 (6460), 289.6 (5950) nm, λ min (ε) 266.0 (4760), 286.8 (5220) nm; ir, ν max 3460, 2990, 1660, 1485, 1455, 1420, 1408, 760, 745, 430 cm^{-1}; ^{1}H nmr (d_4-MeOH) δ 7.08 (dd, *J*=8.1 and 1.2 Hz, H-3), 7.48 (dt,

J=1.6, and 7.3 Hz, H-4), 7.19 (dt, J=1.2 and 7.5 Hz, H-5), 7.75 (dd, 7.9 and 1.6 Hz, H-6), 4.07 (dd, J=8.5 and 5.1 Hz, H-9), 3.12 (dd, J=15.1 and 8.8 Hz, H-10a), 3.38 (dd, J=15.1 and 5.1 Hz, H-10b), 7.11 (d, J=2.2 Hz, H-12), 7.28 (dd, J=8.1 and 1.0 Hz, H-14), 7.03 (dt, J=1.3 and 8.2 Hz, H-15), 6.92 (dt, J=1.1 and 8.1 Hz, H-16), 7.41 (d, J=7.8 Hz, H-17); ^{13}C nmr (d_4-MeOH) δ 173.74 (C-1), 138.12 (C-2), 122.50 (C-3), 135.16 (C-4), 125.82 (C-5), 131.70 (C-6), 128.43 (C-7), 170.97 (C-8), 54.64 (C-9), 25.01 (C-10), 110.60 (C-11), 124.84 (C-12), 138.12 (C-13), 112.37 (C-14), 122.31 (C-15), 118.86 (C-16), 119.87 (C-17), 127.13 (C-18); hrfabms *m/z* [M+H]$^+$ 306.1234 ($C_{18}H_{16}N_3O_2$ requires 306.1243); cd, [θ] (nm) 30990 (213.2), −96260 (228.8), 125420 (249.6), −16780 (289.6) (MeOH); [α]D=+138.8° (c=0.5, MeOH); mp 236–240° (uncorr.).

Acyl aszonalenin [**4**] was isolated as a yellow solid. Spectroscopic data were consistent with those previously published (6).

N-Benzoyl-L-phenylalaninol was isolated as a yellow solid. Spectroscopic data were consistent with those previously published (7). Uv, λ max (ε) (MeOH) 207.4 (8630), 273.0 (870) nm; ^{1}H nmr (d_4-MeOH) δ 7.71 (d, J=7.4 Hz, H-2, H-6), 7.41 (t, J=7.3 Hz, H-3, H-5), 7.49 (t, J=7.1 Hz, H-4), 4.34 (m, H-8), 3.64 (d, J=5.2 Hz, H-9), 2.86 (dd, J=13.6 and 8.4 Hz, H-10a), 3.02 (dd, J=13.6 and 6.1 Hz, H-10b), 7.26 (m, H-12, H-16), 7.24 (m, H-13, H-15), 7.17 (m, H-14); ^{13}C nmr (d_4-MeOH) δ 136.04 (C-1), 128.26 (C-2, C-6), 129.42 (C-3, C-5), 132.47 (C-4), 170.35 (C-7), 54.94 (C-8), 64.31 (C-9), 38.00 (C-10), 139.97 (C-11), 130.34 (C-12, C-16), 129.35 (C-13, C-15), 127.33 (C-14); fabms *m/z* MH$^+$ 256; [α]D=−62.4° (c=0.55, MeOH); cd, [θ] (nm) −37750 (224.8), −5860 (272.0) (MeOH).

SYNTHESIS OF BENZODIAZEPINEDIONE [**3**].—A mixture of finely ground isatoic anhydride (3.26 g), L-tryptophan (4.08 g), triethylamine (3.0 ml) and H_2O (20 ml) was stirred at room temperature for 5 h. Volatile material was then removed *in vacuo* (80° maximum), glacial HOAc (40 ml) was added and the solution refluxed for 8 h. The solvent was then removed *in vacuo* and the residue partitioned between H_2O and EtOAc. The organic layer was washed with aqueous $NaHCO_3$ followed by H_2O (2×), then dried with Na_2SO_4 and the solvent removed *in vacuo*. Silica flash chromatography yielded [**3**] as a white solid (4.8 g, 94%).

ISOLATION OF DIKETOPIPERAZINES.—The dried EtOAc extract (400 mg) from the producing culture was solvent partitioned as before, using hexane-EtOAc-MeOH-H_2O (5:6:5:3). Solvent was removed from the lower polar layer and the residue subjected to C18 flash cc using an MeCN/H_2O gradient. The first fraction (70 mg), eluting with 20:80 MeCN/H_2O, contained a series of diketopiperazines. Purification by repetitive C8 and C18 hplc gave the purified diketopiperazines, cyclo(L-phe-L-pro) (2.0 mg), cyclo(L-phe-D-pro) (3.8 mg), cyclo(L-leu-L-pro) (3.8 mg), cyclo(L-tyr-L-pro) (7.0 mg), cyclo(L-tyr-D-pro) (7.5 mg), cyclo(L-val-L-pro) (3.5 mg) and cyclo(L-val-D-pro) (2.3 mg). Structures of these diketopiperazines were confirmed by spectroscopic comparison with commercially available or synthesized material.

DIKETOPIPERAZINE SYNTHESIS.—The following general method illustrated for cyclo(L-val-L-pro) was also used to synthesize cyclo(L-leu-L-pro) and cyclo(L-tyr-L-pro). Cyclo(L-phe-L-pro) was purchased from Chemalog.

The dipeptide (L-val-L-pro, 1 g) was dissolved in MeOH (30 ml) and concentrated HCl (3 ml) and the solution refluxed overnight. The MeOH was removed *in vacuo*, $CHCl_3$ (50 ml) added, and then NaOH (6 N) added to pH 9. After partitioning, the aqueous layer was extracted with $CHCl_3$ (2×) and the combined organic layer washed with saturated aqueous Na_2CO_3, dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue was dissolved in HCO_2H (5 ml) and this was then removed *in vacuo*. The residue was then dissolved in *t*-BuOH (20 ml) and toluene (10 ml) and this solution heated to reflux with no condenser. After 15 min a condenser was added and the solution refluxed overnight. Removal of the solvent *in vacuo* gave cyclo(L-val-L-pro) as a white solid (0.9 g, 95%).

CONVERSION OF CYCLO(L-PHE-L-PRO) TO CYCLO(L-PHE-D-PRO), CYCLO(L-TYR-L-PRO) TO CYCLO(L-TYR-D-PRO) AND CYCLO(L-VAL-L-PRO) TO CYCLO(L-VAL-D-PRO).—The following general procedure illustrated for the conversion of cyclo(L-val-L-pro) to cyclo(L-val-D-pro) was also used in the conversion of cyclo(L-tyr-L-pro) to cyclo(L-tyr-D-pro) and cyclo(L-phe-L-pro) to cyclo(L-phe-D-pro).

Cyclo(L-val-L-pro) (50 mg) was dissolved in NaOH (0.1 N, 10 ml) and the solution left for 4 h at room temperature. The solution was extracted with EtOAc (3×) and the organic layer washed with dilute HCl followed by H_2O. Drying over Na_2SO_4 followed by solvent removal *in vacuo* afforded cyclo(L-val-D-pro) as a white solid (45 mg, 90%).

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