FISCALINS: NEW SUBSTANCE P INHIBITORS PRODUCED BY THE FUNGUS *Neosartorya fischeri*

TAXONOMY, FERMENTATION, STRUCTURES, AND BIOLOGICAL PROPERTIES

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Three new compounds, named fiscalins A, B, and C, were found in culture broth produced by a *Neosartorya fischeri*. These compounds inhibit the binding of radiolabeled substance P ligand to the human neurokinin (NK-1) receptor, with *Ki* values of 57, 174, and 68 μM, respectively.

Detailed spectroscopic and amino acid analyses led to the elucidation of structures for the three fiscalins. The structures contain an indolyl moiety linked to an athranilic acid derived tricyclic system. The absolute configuration of fiscalin A was determined by X-ray crystallography and chiral amino acid analysis. The presence of fiscalins was detected directly in crude cellular extracts using LC-MS methods.

Substance P (SP) is an undecapeptide belonging to a family of related peptides called neurokinins, which act as neurotransmitters and neuromodulators. Cell surface receptors for the neurokinins have been classified into three subtypes, based upon affinities for selective ligands.[1] SP is a potent agonist, and believed to be the endogenous ligand for the neurokinin-1 (NK-1) receptor subtype. SP induces a variety of physiological responses such as salivation, vasodilation, smooth muscle contraction, and is thought to be involved in pain transmission and inflammatory response.[2] Therefore, selective antagonists of SP might prove to be novel analgesics or anti-inflammatory agents.

In order to evaluate the pharmacologic activities of SP antagonists, bioavailable and metabolically stable compounds are required. Earlier work on SP antagonists generally focused on modifications of the SP peptide, such as described in a recent review.[3] More recently, several nonpeptide SP antagonists have been reported, from synthetic[4~7] or microbial[8] sources, and a microbial cyclic peptide.[9,10] These compounds, which varied in structural features, potency and receptor specificities, exhibited activity in various cellular, tissue or *in vivo* systems, furthering our understanding of the therapeutic potential of SP antagonists.

In our search for novel nonpeptide SP antagonists, we wanted to detect compounds active against a NK-1 receptor of human origin. We therefore screened microbial broth extracts for the ability to inhibit radiolabeled SP binding to human astrocytoma U373M6 intact cells, which are a convenient source of the NK-1 receptor subtype. One strain of the fungus, *Neosartorya fischeri*, produced three new compounds, fiscalins A, B, and C (Fig. 1), which exhibit moderate inhibition of SP binding (*Ki*=57, 174, and 68 μM, respectively). X-ray crystallography, chiral amino acid analysis, and spectroscopy were used to determine the structure of fiscalin A. Structure determination of all three metabolites and their production and biochemical properties are presented herein.

Results

Taxonomy and Fermentation of Fungal Culture SC4

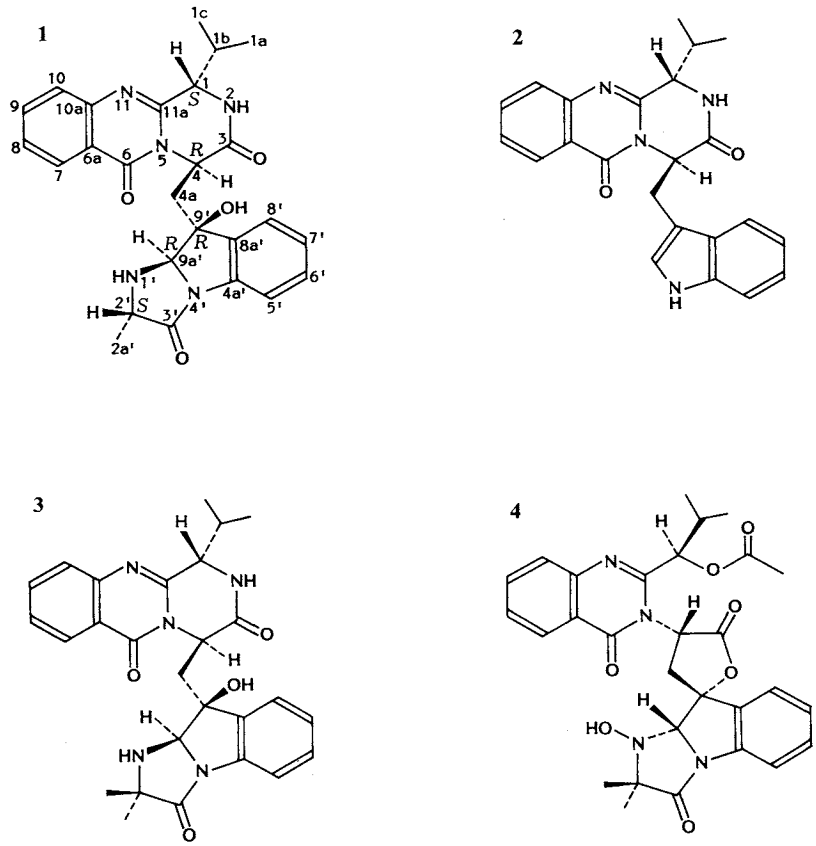
A fungal culture was isolated from a plant rhizosphere collected near the We Fung Chi Cascade region of Taiwan. The culture is preserved as SC4 in the Sterling Winthrop Biologicals Collection, Collegeville, PA, U.S.A. Taxonomic examination has determined SC4 to be *Neosartorya fischeri*, based on the following morphologic descriptions, after 12 days growth on Czapek-Dox agar. (1) Teleomorph (sexual) stage keys as *Neosartorya fischeri*, with mycelial wall several layers thick, white-cream in color, $9\sim15\,\mu$m asci, with eight uncolored ascospores, $5\sim8\,\mu$m in length and $4\sim6\,\mu$m in width. (2) Anamorph (asexual) stage keys as *Aspergillus fischerianus*, with conidial heads radiate to columnar, aspergilla uniseiate, conidia smooth to finely rough, spherical to ellipsoidal, $2.5\sim3\,\mu$m by $2\sim3\,\mu$m.

Fermentation was carried out at 26°C in baffled 250-ml Erlenmeyer flasks containing 30 ml production medium for six days. Fiscalin production and cell growth peaked after six days, although production was evident up to day nine (data not shown). The major portion of fiscalins was recovered by ethyl acetate extraction of harvested cell mass.

Isolation Fiscalins

Ethyl acetate extract of the cell mass from culture SC4 was defatted with hexane. An enriched bioactive

Fig. 1. Structures of fiscalins A (**1**), B (**2**), C (**3**), and tryptoquivaline (**4**).



fraction was obtained after flash silica gel chromatography eluting with $CHCl_3$-methanol (1:1), which was further purified by semi-preparative reverse phase HPLC to give the major component, fiscalin A (**1**), and fiscalins B (**2**) and C (**3**) (Fig. 1). All three compounds were obtained as white amorphous solids, and **1** was recrystallized from dichloromethane-methanol (1:1) to provide colorless orthorhombic crystals, mp 242~243°C, $[\alpha]_D$ −169° (*c* 0.5, MeOH).

Spectroscopic Properties and Structures of Fiscalins A, B, and C

In the mass spectrum of fiscalin A (**1**) a molecular formula of $C_{26}H_{27}N_5O_4$ was suggested by the molecular ion $[M]^+$ at 473 daltons and was confirmed by high resolution mass spectral data (measured 473.2032; calculated 473.2063). The presence of a tricyclic substituted quinazolone system was deduced from ^1H NMR, COSY and UV spectral data (λ_{max}=226, 278, 306 nm). The IR spectrum contained bands characteristic of NH (3378~3279 cm^{-1}) and amide (1689 cm^{-1}) functions. Examination of the ^1H NMR spectrum of **1** revealed two sets of four adjacent sp2 olefinic proton signals representing two *ortho* disubstituted aromatic rings. The ^1H NMR spectrum of **1** also provided evidence for an indole ring with substitution across the C-9′~C-9a′ double bond. COSY cross peaks, starting from the 9a′-H methine proton signal (4.93 ppm), revealed an alanine unit with the alpha amino nitrogen linked to the C-9a′ carbon of the indole. The carboxyl end of the alanine could therefore be linked to the N-4′ nitrogen through an amide bond. The 2′-H proton was observed as a multiplet and collapsed to a quartet upon D_2O exchange of the N-1′-H proton, whereupon the signal

Table 1. ^1H NMR assignments of **1**, **2**, and **3**.

H	^1H Chemical shifts (ppm)		
	1	**2**	**3**
1	4.41	2.70	4.56
2	8.43	5.97	6.12
4	5.31	5.63	5.82
7	7.99	8.33	8.30
8	7.26	7.49	7.51
9	7.55	7.74	7.79
10	7.43	7.53	7.68
1a	0.94	0.61	0.95
1b	2.85	2.60	3.16
1c	0.68	0.62	1.18
4a	2.51, 2.68	3.69, 3.59	2.47, 2.69
1′	3.14		
2′	3.47		
2a′	0.82		1.39
2b′			1.49
4′		8.21	
5′	7.17	7.38	7.51
6′	6.88	6.88	7.09
7′	7.07	7.08	7.32
8′	7.14	7.25	7.32
9a′	4.93	6.57	5.25

Solvents: $CDCl_3$ for **2** and **3**; $CDCl_3+CD_3OD$ or DMSO-d_6 for **1**.

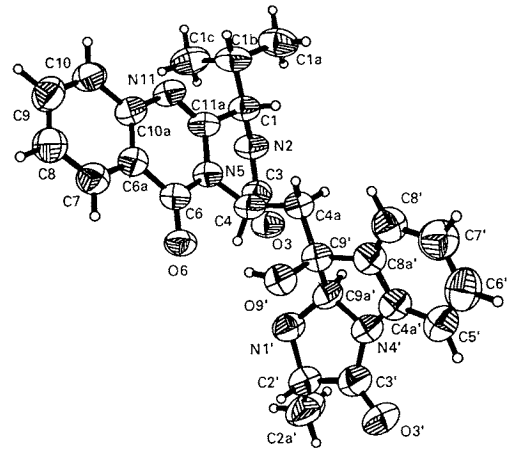
Table 2. ^{13}C NMR assignments of **1**, **2**, and **3**.

C	^{13}C Chemical shifts (ppm)		
	1	**2**	**3**
1	60.4	58.4	48.6
3	172.4	170.2	170.8
4	54.4	57.1	51.8
6	163.9	161.6	161.3
6a	122.2	120.7	120.9
7	128.4	127.4	127.7
8	129.1	127.7	128.0
9	136.8	135.3	135.5
10	129.2	127.7	128.1
10a	149.4	147.7	147.5
11a	152.2	151.0	149.8
1a	20.0	19.0	19.8
1b	31.2	29.8	29.0
1c	16.5	14.9	15.6
4a	40.1	27.6	40.0
2′	62.9		65.4
2a′	18.0		25.4
2b′			26.4
3′	177.0		175.9
4a′	139.1	127.8	138.0
5′	117.3	119.2	116.3
6′	127.5	120.5	125.8
7′	132.2	123.1	130.8
8′	126.4	111.6	124.7
8a′	139.2	136.6	138.5
9′	76.3	109.7	74.5
9a′	84.4	124.2	78.9

Solvents: $CDCl_3$ for **2** and **3**; $CDCl_3+CD_3OD$ or DMSO-d_6 for **1**.

for 9a'-H (4.93 ppm) sharpened to a broad singlet. Three methyl signals were accounted for: 1c (0.68 ppm) and 1a (0.94 ppm) were coupled to 1b-H (2.85 ppm); 2'-CH_3 (0.82 ppm) was coupled to 2'-H (3.47 ppm). ^{13}C NMR assignments were made on the basis of 2D HETCOR and DEPT experiments. Three carboxyl signals, 163.9 ppm, 172.4 ppm, and 177.0 ppm were attributed to amide carbons. Proton and carbon assignments are summarized in Tables 1 and 2.

Fig. 2. Computer generated perspective view of fiscalin A (**1**), with atomic numbering.



From X-ray crystallographic analysis of **1** (Fig. 2), the alanine unit was confirmed to be linked to the C-9a' and the N-4' through the alpha nitrogen and the carbonyl carbon of the alanine unit, respectively. As a result, the relative configuration of **1** was established. Alanine was the major amino acid detected in the acid hydrolysate. Chiral amino acid analysis of **1** using MARFEY's reagent[11] determined the alanine unit of **1** to be L-alanine. Therefore, the absolute configuration of **1** is C-2'*S*, C-9a'*R*, C-9'*R*, C-4*R*, and C-1*S*.

Fiscalin B (**2**) gave a $[M+H]^+$ at 387 daltons, suggesting a molecular formula of $C_{23}H_{22}N_4O_2$ (HR-MS, 386.1743). The composition of **2** was revealed by the two major mass fragments of *m/z* 257.1158 ($C_{14}H_{15}N_3O_2$) and *m/z* 130.0649 (C_9H_8N). Since the UV spectrum of **2** suggested the presence of quinazolone and indole chromophores, the indole ring structure could be assigned to the C_9H_8N unit and the quinazolone chromophore was associated with the $C_{14}H_{15}N_3O_2$ unit.

In the ^1H NMR spectrum of **2**, signals for the following subunits were observed: $(CH_3)_2CH-CH$, $-CH_2-CH$, two D_2O exchangeable protons, and the protons of two *ortho* substituted aromatic rings (thus accounting for the eight aromatic protons). Since two carbonyl carbon signals (170.2 and 161.6 ppm) were observed in the ^{13}C NMR of **2**, the two D_2O exchangeable proton signals (8.22 and 6.01 ppm) were assigned to the NH protons. The assignments were further supported by the IR absorption bands at 1685 cm^{-1} (C=O), and 3400 and 3279 cm^{-1} (NH).

In the COSY spectrum of **2**, coupling between the vinylic proton at 6.57 ppm and the N–H proton at 8.21 ppm led to the assignment of the two signals to protons at C-9a' and N-4' of the indole ring, respectively. Allylic coupling between the methylene proton signals (3.59 and 3.69 ppm) and the C-9a' vinylic proton signals resulted in the placement of the CH_2–CH-group at C-9' of the indole ring. Since long range coupling between the N–H proton at 8.21 ppm and a broad aromatic proton doublet at 7.25 ppm was observed, the signals observed at 7.25 ppm (d), 7.08 ppm (t), 6.88 ppm (t), and 7.38 ppm (d) could be assigned unambiguously to the aromatic ring of the indole unit. The other N–H proton signal at 5.97 ppm was shown to be coupled to the methine signal at 2.70 ppm which in turn was coupled to the isopropyl methine signal at 2.60 ppm. As a result, a partial structure, $(CH_3)_2CH-CH-NH-$, was established. The remaining aromatic proton signals resonating at 7.53 ppm (d), 7.74 ppm (t), 7.49 ppm (t), and 8.33 ppm (d) thus belong to the *ortho* substituted aromatic ring and part of the quinazolone moiety (Table 1).

Through a one-bond HETCOR experiment, all the proton linked carbons in **2** were assigned (Table 2). The methine signal at 5.63 ppm (C-4) and the methylene signals at 3.69 and 3.59 ppm (C-4a) were

shown to have two and three bond long range couplings with the C-3 carbonyl carbon signal at 170.2 ppm. As a result, the linkage between the C-3 carbonyl to C-4 carbon was established. Similarly, a two bond coupling between the proton signal at 2.70 ppm (C-1) and the sp2 carbon signal at 151.0 ppm, and a three bond coupling between the aromatic proton signal at 8.33 ppm (C-7) and the carbonyl carbon signal at 161.6 ppm led to the assignment of the C-6 carbonyl and C-11a carbons. Based on the long range HETCOR spectral data, all the carbon signals were unambiguously assigned (Table 2).

A quinazolone unit can be formed by putting the two remaining nitrogen atoms of **2** at positions 5 and 11; one linked to the carbon signal at 147.7 ppm (C-10a) and the other linked to the carbonyl carbon at 161.6 ppm (C-6) and then combining with the sp2 carbon at 151.0 ppm (C-11a). Finally, the observed three bond coupling between the methine proton signal at 2.70 ppm (C-1) and the carbonyl carbon signal at 170.2 ppm (C-3) of the tryptophan unit supported the linkage between the C-3 carbonyl carbon with the NH at position 2. The remaining CH unit should therefore be linked to the N atom of the quinazolone unit. This is further supported by the observed three bond coupling between the methine proton at 5.63 ppm (4-H) and the carbonyl carbon at 161.6 ppm (C-6) of the quinazolone unit. As a result the structure of **2** was established.

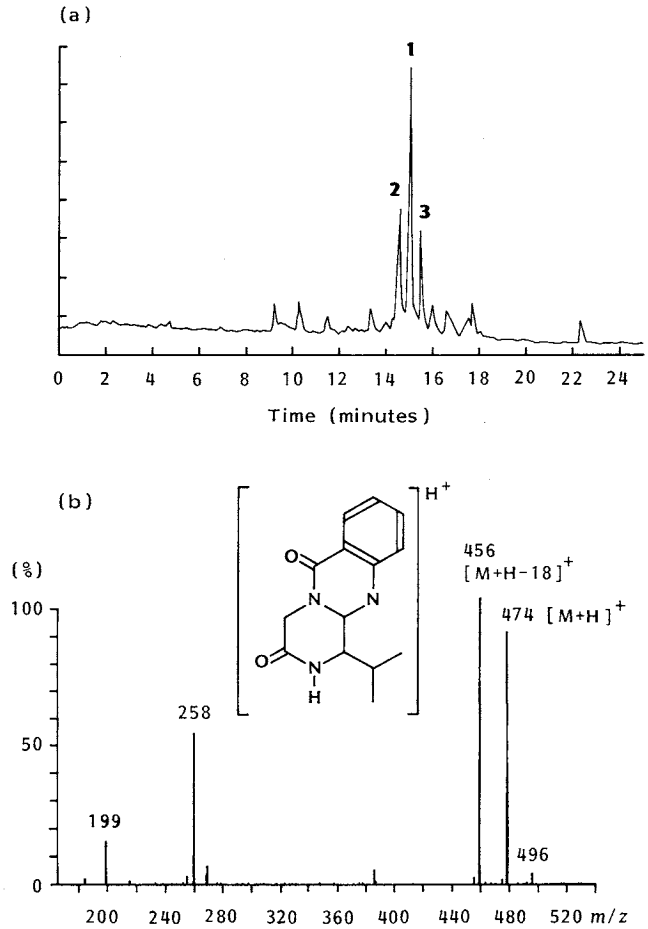
Structural similarity among **1**, **2**, and **3** was revealed by comparison of the spectral data of these three compounds (Tables 1 and 2). In the high resolution mass spectrum of fiscalin C (**3**) a $[M+H]^+$ ion was observed at *m/z* 488.2318, indicating a molecular formula of $C_{27}H_{29}N_5O_4$. In the ^1H NMR spectrum of **3**, four methyl signals (0.95, 1.18, 1.39, 1.49 ppm) were observed, as compared to three methyl signals (0.68, 0.94, 0.82 ppm) for structure **1** (Table 1).

The presence of two methyl singlets (1.39 and 1.49 ppm) for a $(CH_3)_2C$ in ^1H NMR spectrum of **3** in place of a methyl doublet and a methine multiplet (0.82 and 3.47 ppm, respectively) corresponding to the CH_3CH unit at C-2′ of **1** indicated an alpha amino-isobutyric acid substitution at the C-9a′ and N-4′ positions of the indole moiety of **3**. The presence of alpha amino-isobutyric acid in **3** was confirmed by amino acid analysis of the acid hydrolysate of **3**, resulting in the structure for fiscalin C. Based on comparison of spectral assignments to **1**, the proton and carbon signals of **3** were assigned as shown in Tables 1 and 2.

The CD spectra of **2** and **3** are virtually superimposable on that of **1**. The CD maxima are as follows: λ_{max} nm ($\Delta\varepsilon$) 228 (−44.4), 307 (−3.9) for compound **1**, λ_{max} nm ($\Delta\varepsilon$) 228 (−62.1), 307 (−9.5) for compound **2**, and λ_{max} nm ($\Delta\varepsilon$) 228 (−44.6), 307 (−5.0) for compound **3**. Hence, the absolute configuration of **2** and **3** should be identical to that of **1**, allowing the assignment of the chiralities in **2** and **3**.

Using a reverse phase HPLC column and a MeOH-H_2O gradient elution, all three compounds in a crude ethyl acetate extract of SC4 cell mass could be base line resolved. By including a slow flow of NH_4OAc post column to aid sample ionization, thermospray LC-MS was performed. Compounds **1**, **2**, and **3** in the crude extract appeared as peaks in the total ion current chromatogram, as shown (Fig. 3). Mass spectra obtained from these peaks gave molecular ions corresponding to **2** (386 daltons), **1** (473 daltons), and **3** (487 daltons). In addition, the common mass fragment at *m/z* 258 was observed in the thermospray mass spectra of these three compounds, as represented by analysis of compound **1**, shown in Fig. 3b. The appearance of this mass fragment at *m/z* 258 together with the characteristic UV spectrum obtained from the photodiode array HPLC provide a powerful method for detection of this class of compounds in crude fermentation extracts.

Fig. 3. LC-MS analysis of crude ethyl acetate extract of harvested SC4 cells: a) total ion current chromatogram; b) thermospray MS of **1**, including characteristic *m/z* 258 mass fragment.



Biological Activity of Fiscalins

Fiscalins A, B, and C inhibit the binding of ^{125}I-Bolton-Hunter-substance P to human astrocytoma U-373 MG intact cells, with *Ki* values of 57, 174, and 68 μM, respectively. The similar inhibitory activity of **1** and **3** might be predicted by the close structural similarity of these two compounds. In contrast, **2** was somewhat less active than **1** and **3**. Structurally, **2** differs by having an intact indole ring. This difference in substitution at the C-9′, C-9a′, and N-4′ of the indole moiety of this class of compound appears to have an influence on activity.

Conclusion

Fiscalins A, B, and C are new inhibitors of binding of SP to human NK-1 receptors. The tremorgenic tryptoquivaline mycotoxins, *e.g.* **4**, isolated from *Aspergillus clavatus*[12] and *Aspergillus fumigatus*,[13,14] have similar chemical composition to the fiscalins. In addition, at the conclusion of our study, we became aware of a recent report on the structurally similar fumiquinazolines.[15] These compounds were isolated from an *Aspergillus fumigatus*, and were reported to be cytotoxic agents. The fumiquinazolines and the fiscalins are composed of two structural units, an indolyl moiety and a tricyclic unit presumably derived

from athranilic acid. They are structurally different from the tryptoquivalines by having a differently substituted quinazolone ring, the absence of the spiro-gamma-lactone ring and the opposite absolute stereochemistry. Thus, these compounds may be regarded as a novel class.

Experimental

Fermentation of SC4

Culture SC4 was propagated on LCSB agar slants for 10 days at 27°C. Spore suspensions (1×10^5 spores/ml) were obtained by washing the creamy-white spores from slants with a cryopreservation solution of 10% glycerol and 5% lactose. One ml of frozen spores was used to inoculate a 250 ml baffled Erlenmeyer flask containing 30 ml seed medium, composed of glucose 2%, Pharmamedia (Traders) 1.5%, yeast extract (DIFCO) 0.5%, ammonium sulfate 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. Flasks were incubated for two days at 26°C on a rotary shaker (New Brunswick Scientific) at 220 rpm. One ml of the vegetative seed culture was transferred into 250 ml baffled Erlenmeyer flasks containing 30 ml of production medium, composed of glycerol 2%, dextrin (DIFCO) 2%, Bacto-Soytone (DIFCO) 1%, yeast extract (DIFCO) 0.3%, ammonium sulfate 0.2%, and $CaCO_3$ 0.2%, pH 7.0. The fermentation was carried out at 26°C with shaking at 220 rpm. Fiscalin production and cell growth peaked after 6 days. The pH dropped from 7.1 to 5.0 as production increased. The majority of fiscalins was associated with the cell mass.

Isolation of Fiscalins A, B, and C

Harvested cells obtained from 1 liter of broth were extracted with ethyl acetate to yield 0.8 g of brown residue. After redissolving in a mixture of methanol and dichloromethane (1 : 1), the sample was subjected to flash silica gel chromatography with stepwise elution using chloroform, chloroform - methanol (1 : 1), and finally methanol. Bioactive eluents were collected from the chloroform - methanol fraction, and dried *in vacuo* to yield 450 mg of white powder. After rinsing with methanol, the white solids were redissolved in a mixture of dichloromethane - methanol (1 : 9) and then subjected to semi-preparative HPLC using a YMC reverse phase column (C-18, S-5, 120 A, A323) and isocratic elution with 86% methanol and 14% water at 1 ml/minute. Eluents were collected to yield: **1** (10 mg), **2** (8 mg), and **3** (5 mg).

Analysis of the Chirality of the Alanine Unit in Fiscalin A

Fiscalin A (**1**) (3 mg), was refluxed with 6 N HCl for 1 hour. The reaction mixture was dried at 40°C under a stream of nitrogen. To the dried residue, 200 μl of 1% (w/v) solution of MARFEY's reagent[11] in acetone and 40 μl of 1% $NaHCO_3$ were added. After heating at 40°C for 1 hour the reaction mixture was cooled to room temperature and then treated with 20 μl of 2 N HCl. The solution was filtered through glass wool and the filtrate was subjected to HPLC analysis using a Nova-Pak C18 column (3.9 mm × 1.5 cm, Waters) and a mixture of triethylamine phosphate buffer (pH 3.5) and acetonitrile (7 : 5) at a flow rate of 0.6 ml/minute.

Spectroscopic Measurements

^1H NMR experiments (COSY, decoupling experiments), and ^{13}C NMR experiments (BBD, SFORD, DEPT, HETCOR, Long Range HETCOR) were performed on a Varian Gemini 300. Mass spectrometry (CI and HRMS) was performed by MSCAN, Inc. (West Chester, PA). IR spectra were obtained in KBr with a Nicolet FT/IR spectrophotometer. UV and CD spectra were determined in methanol with a Hewlett Packard Photodiode Array UV spectrophotometer and a JASCO J-600 spectropolarimeter, respectively.

X-Ray Crystallography

A crystal of approximate dimensions 0.06 × 0.12 × 0.36 mm was mounted on a Siemens R3m/V diffractometer. The crystallographic data were obtained using CuK_α radiation monochromated by a highly oriented graphite crystal. The crystal structure was determined with direct methods and refined by full-matrix least squares approximations with a Siemens SHELXTL PLUS system. Crystal data and atomic coordinates are available as supplementary material.

LC-MS of Ethyl Acetate Extract of SC4

Defatted extract of SC4 cells (2 mg/ml in MeOH, 5 μl injection) was directly analyzed by thermospray LC/MS. A Nova-Pak C-18 15 cm column (Waters) with a linear solvent gradient from 100% A (30% methanol in water) to 100% B (methanol) was run in 20 minutes. Flow rate was adjusted to 1.5 ml/minute. Ammonium acetate (0.5 M in water) at a flow rate of 0.5 ml/minute was added post column. The HPLC was monitored at 220 nm. HP 5988A thermospray unit was connected at the HPLC outlet. The source temperature of the mass spectrometer was 350°C, with scanning from 145 to 800 amu/second.

^{125}I-Bolton-Hunter-Substance P Binding Assay

The ^{125}I-BH-SP binding assay was performed using a Beckman BIOMEK 1000 Workstation on intact U-373 MG cells (90~100% confluent) in 96-well plates, as described.[16] ^{125}I-BH-SP (2,000 Ci/mmol) was obtained from Amersham. Following the reaction, cells were detached from the plates by the addition of 140 μl of 0.2% Triton X-100 containing 1 mg/ml BSA, and 100 μl was counted in a Packard Cobra gamma counter.

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