WIN 64821, A NOVEL NEUROKININ ANTAGONIST PRODUCED BY AN *Aspergillus* sp.

I. FERMENTATION AND ISOLATION

DAVID M. SEDLOCK,*[,1] COLIN J. BARROW,[1] JAMES E. BROWNELL,[1] ANDERSON HONG,[2] AMANDA M. GILLUM[1] and DAVID R. HOUCK[1]

[1] Sterling Winthrop Pharmaceuticals Research Division, 1250 S. Collegeville Rd., Collegeville, PA 19426-0900, U.S.A.
[2] Panlabs Taiwan, Ltd., No. 158, Li Teh Road, Peitou, Taipei, Taiwan, R.O.C.

(Received for publication October 1, 1993)

WIN 64821, a nonpeptide neurokinin antagonist, was isolated from a strain of *Aspergillus* sp., SC319. The compound was produced in different fermentation media with greatest yields observed when the culture was grown in a synthetic medium supplemented with L-tryptophan and L-phenylalanine. After 6 days fermentation, yields greater than 600 mg/liter were obtained. Two analogs of WIN 64821 were also identified in the culture extracts and subsequently tested for biological activity. WIN 64821 was the most potent compound isolated from this culture and exhibited activity as a substance P-binding inhibitor with submicromolar potency against the human neurokinin 1 receptor.

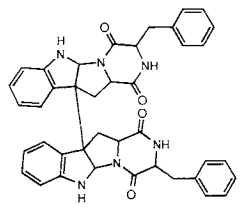
The tachykinins, or neurokinins, are a family of undecapeptides that share the common COOH-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2 (where X is Phe, Tyr, Val or Ile). Research has shown the existence of three distinct mammalian tachykinins, termed substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Receptors for these ligands have been classified into three subtypes termed NK1, NK2 and NK3, respectively.

While many selective, peptide agonists for neurokinin receptors have been characterized,[1] very few peptide[2] or nonpeptide[3,4] antagonists have been described. Recent reports have detailed the presence of neurokinin binding antagonists isolated from microbial sources. A cyclic depsipeptide with activity against both NK1 and NK2 receptors has been described.[5] In addition, nonpeptide inhibitors, termed fiscalins, with moderate NK1 binding activity have also been described.[6] There has also been a report of a tetracyclic compound, anthrotainin, with NK1 activity;[7] however, this compound was found to be a noncompetitive SP antagonist.

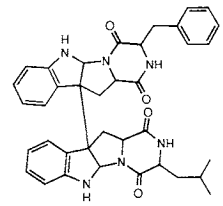
While screening for antagonists of SP, we observed an extract from a freshly isolated soil fungal culture to possess activity as an inhibitor of SP binding in a rat submaxillary gland preparation. Isolation of the active component from this extract yielded a nonpeptide compound, WIN 64821, structurally identified as a dimerized Trp-Phe condensate[8] (**1**, Fig. 1). The isolated compound possessed structural similarities to the verticillins[9] and chaetocins[10,11] and is a close analog of ditryptophenaline.[12,13]

Here we report the isolation procedure for **1**, the taxonomy and fermentation characteristics of the producing organism, and the bioanalytical profile of extracts generated from shake flask and fermenter cultures. The biological activity profile and biosynthesis of WIN 64821 and analogs are reported in accompanying papers.[14,15]

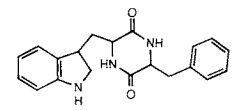
Fig. 1. WIN 64821 (**1**) and related metabolites (**2** and **3**) isolated from *Aspergillus* sp. SC319.



WIN 64821
1



WIN 64745
2



Cyclo(L-Trp-L-Phe)
3

Materials and Methods

Microorganism

The producing fungus, strain SC319 (ATCC 74177), was isolated from a soil sample obtained from Lung Tien, Taiwan.

Fermentation

Seed cultures for inoculation of production media were typically prepared as follows: a single vial of frozen spore suspension was used to inoculate a 250-ml shake flask with 30 ml of medium containing, in % w/v: glucose 2.0, Pharmamedia (Traders Protein) 1.5, yeast extract 0.5, $(NH_4)_2SO_4$ 0.3, $CaCO_3$ 0.4, $ZnSO_4 \cdot H_2O$ 0.003. The final spore concentration was approximately 10^6 spores/ml. The flask was incubated at 27°C and aerated at 210 rpm (Braun BS-4 shaking incubator) for 24 hours. The entire contents of the flask were transferred to a 2.8-liter Fernbach flask containing 500 ml of glycerol-beef extract (GBE) transfer medium, containing (% w/v): glycerol 3.0, beef extract 3.0, tryptophan 0.1, $CaCO_3$ 0.3, K_2HPO_4 0.05, $MgSO_4 \cdot 7H_2O$ 0.05, KCl 0.03. The culture was incubated for 48 hours as above at which point a 10% v/v transfer was made to the production medium: either GBE medium or a synthetic medium (Syn-2) which was composed of (% w/v): glycerol 6.0, NH_4Cl 0.15, sodium glutamate 0.75, KCl 0.05, K_2HPO_4 0.01, $MgSO_4 \cdot 7H_2O$ 0.05, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot H_2O$ 0.01, Dow Chemical P2000 1.0. Characteristics of an effective second stage transfer culture at 48 hours were pH 7.5~7.7 and homogenous, mycelial morphology (pellet and pigment formation were not apparent).

Production of **1** was examined in 250-ml shake flasks containing 30 ml medium and in 5.0-liter bench-top fermentors (BioFlo III, New Brunswick Scientific). Experiments performed in 250-ml flasks were incubated at 27°C at 210 rpm. Fermentors were incubated at 27°C, agitated at 400 rpm, and sparged with air at 2.5 liters/minute; in some cases automatic adjustment of the impeller rotation rate was used to maintain the dissolved oxygen within 50 to 80% saturation. In fermentors operated under pH control, 6 M sulfuric acid was added as required. Glycerol concentrations in the fermentors were determined using Boehringer Mannheim test kit No. 148270. Packed cell volume (PCV) was used to estimate cell mass by centrifuging 15 ml of whole culture broth for 10 minutes at 3,500 rpm and estimating the ratio of cell volume to total volume (% PCV = cells volume/total volume × 100).

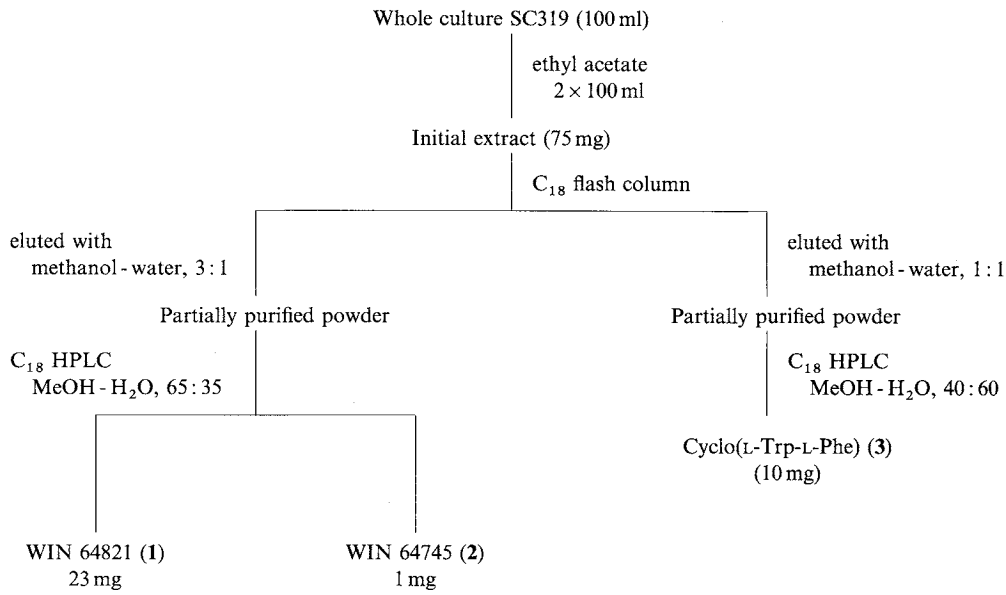
Analytical Chromatography

Samples were prepared for chromatography by first performing an ethyl acetate extraction of whole culture. An equal volume of ethyl acetate was combined with whole culture, vortexed for 1 minute then centrifuged for 10 minutes at 3,500 rpm. The ethyl acetate layer was removed and dried using a SpeedVac Sample Concentrator (Savant). The residue was reconstituted in isopropanol to a concentration 100-time the original culture volume and 10 μl was injected into the HPLC system.

A Waters 600E multisolvent delivery system with a model 990 photodiode array detector was used to analyze the samples. A Waters C_{18} Nova-pak 8 × 10 cartridge was developed with 54% methanol in water at a flow rate of 2 ml/minute.

A calibration curve of concentration *vs.* peak area was generated by injecting methanol or isopropanol

Fig. 2. Isolation procedure for compounds **1**, **2**, and **3**.



solutions of authentic **1** at various concentrations. The detector response for **1** at 242 nm was linear from 50 to 5,000 ng.

Isolation

The isolation of **1** was carried out using a bioassay directed fractionation scheme (Fig. 2), monitoring the inhibitory activity against ^{125}I labeled Bolton-Hunter (BH)-substance P binding to human U-373 MG astrocytoma cells. After cultivation of the producing organism for 48 hours by the procedure described above, the whole culture (100 ml) was extracted with ethyl acetate (100 ml × 2). The extract (75 mg) was then adsorbed onto C_{18} reverse-phased silica gel (1 g, 40 μm particle) and this was placed at the top of a C_{18} flash column packed with the same material (40 g). The column was eluted with a gradient, from 90 : 10 water - methanol to 100% methanol. The biological activity concentrated in a single fraction eluting with 3 : 1 methanol - water. This fraction contained both **1** and the related compound WIN 64745 (**2**; Fig. 1). An earlier fraction, eluting with 1 : 1 methanol - water, contained cyclo(L-Trp-L-Phe) (**3**; Fig. 1). Separation of **1** and **2** was achieved using preparative HPLC on an ODS C-18 reverse-phased silica gel column (YMC-Pack, 15 × 250 mm; YMC Co., Ltd) with 65 : 35 methanol - water and a 3 ml/minute flow. **1** (24 mg) and **2** (1 mg) were both obtained as white solids.

Bioassay Experiments

Human U-373 MG astrocytoma cells (ATCC No. HTB 17) were cultured in Minimal Essential Medium (MEM) replacing the medium twice weekly. Frozen cell stocks were prepared by centrifuging trypsinized cells in MEM (250 × *g* for 10 minutes), removing the medium, and adding medium containing 7.5% DMSO at 4°C (1.2 ml/75 cm^2 flask) to the pelleted cells. The cells (1.2 ml) were stored at −80°C and were rapidly thawed at 37°C prior to use. Sterile, Corning 96-well plates were pretreated at room temperature (RT) with 0.25 ml poly-D-lysine (0.05 g/ml in phosphate buffered saline [PBS]) and incubated for 40 minutes at RT. The poly-D-lysine was then removed and each well was washed twice with 0.25 ml PBS. To each well was added 0.2 ml of thawed cells from above stock such that the final density was $1.4 \times 10^4 \sim 1.9 \times 10^4$ cells/well. The culture was incubated for 2 days in an atmosphere of 95% air - 5% CO_2 and the resulting monolayer used for binding experiments.

The binding assay was performed on intact cells (90 ~ 100% confluent) in 96-well Corning plates. The

medium was first removed and the cells were washed three times with 0.25 ml binding buffer (BB) which consisted of 25 mM HEPES, 5.8 mM KCl, 2.2 mM KH_2PO_4, 115 mM NaCl, 0.6 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1% bovine serum albumin (BSA), 5 mM glucose, 1 μM thiorphan, and 0.04 g bacitracin, pH 7.40 at room temperature. The binding assay mixture (0.1 ml) consisted of 60 μl BB, 0.1 nM ^{125}I-BH-SP (20 ml of 0.5 nM in BB), and 20 μl BB with test compound or 0.1 μM substance P (final concentration) to define nonspecific binding. The plates were incubated at 25°C for 90 minutes. The buffer containing unbound ^{125}I-BH-SP was removed and the cells were washed 4× with 0.25 ml ice-cold BB. The cells were detached from the plates by the addition of 0.14 ml of 0.2% Triton X-100 in H_2O with 1 mg/ml BSA and 0.1 ml was counted in a Packard Cobra gamma counter.

Results and Discussion

Culture Taxonomy

Taxonomic investigation of SC319, performed by Angela Belt (The Biotic Network, Sonora, CA) identified the strain as an *Aspergillus* sp. based on colony and cellular morphology observed after 10~21 days growth at 26~28°C on malt extract and CZAPEK's agar. The colonies were blue-green with white margins and became a darker teal gray with age. The culture produced distinct conidiophores which were erect, long (greater than 60 μm), up to 4 μm wide, and smooth. The conidiophores contained terminal vesicles which were up to 5 μm long and 5.5 μm wide and were present without subterminal constriction. Phialides, which were uniseriate and 7~10 μm long, radiated in a smooth and uniform pattern from the vesicles. There were no foot cells observed. The conidia were globose, approximately 4 μm in diameter and finely roughened. Metulae were not observed on CZAPEK's medium. In addition, no ascomata, hülle cells, or sclerotia were observed during the three week incubation period.

Fermentation

The kinetics of growth and WIN 64821 production by SC319 were examined initially in the GBE medium. The production of **1** in typical shake flask fermentations stabilized to about 370 μg/ml by 144 hours incubation (Table 1). At this time, the fermentation broth pH had increased from an initial pH of 7 to pH 8.5, and a soluble reddish pigment had been produced. The concentration of **1** in the broth did not significantly change in experiments incubated longer than 144 hours (Table 1).

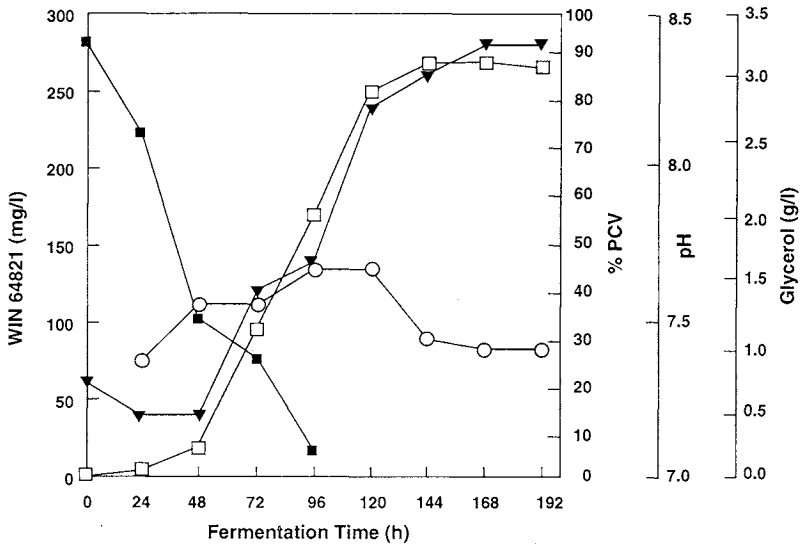
The progress of culture growth (estimated by packed cell volume, PCV) and product formation in 5-liter benchtop fermentors with GBE medium (Fig. 3) were similar to the results in shake flasks. The PCV increased from less than 5% at inoculation to 35~40% after 48 hours, at which time the level of **1** was observed to be less than 50 μg/ml. Within those first 48 hours, the glycerol concentration decreased by 60%. Following the initial two days, the rate of product formation increased and the culture morphology changed from a very fine, highly branched, off-white colored mycelium to a more dense, darker colored mycelium accompanied by the formation of a reddish, soluble pigment. PCV stabilized by 72 hours and began to decrease after 120 hours; the greatest rate of **1** formation, and a shift in pH from pH 7 to approximately pH 8.5, were observed between 3 and 5 days. About 24 hours after the glycerol was exausted at day 4, the biosynthesis of **1** began to

Table 1. WIN 64821 production in GBE medium (shake flask).

Fermentation time (hours)	Harvest pH	WIN 64821 (μg/ml)
48	6.4	47
72	6.4	103
96	7.2	157
120	7.9	267
144	8.3	376
168	8.5	370

Fig. 3. Fermentation data for the culture grown in GBE medium in a 5-liter fermentor with dissolved O_2 controlled between 50% and 80%.

WIN 64821 □, glycerol ■, pH ▼, % packed cell volume (PCV) ○.



wane such that there was no net production after day 6; the final titers were typically about 300 μg/ml. Operating fermentors under pH controlled conditions (maintaining at pH 7.5) did not significantly influence production of **1** in this medium.

Table 2. Effect of phenylalanine and tryptophan on WIN 64821 production in Syn-2 medium.

Media supplement		WIN 64821 (mg/liter)
Phenylalanine (g/liter)	Tryptophan (g/liter)	
0	0	210
0	2	475
2	0	377
2	2	632

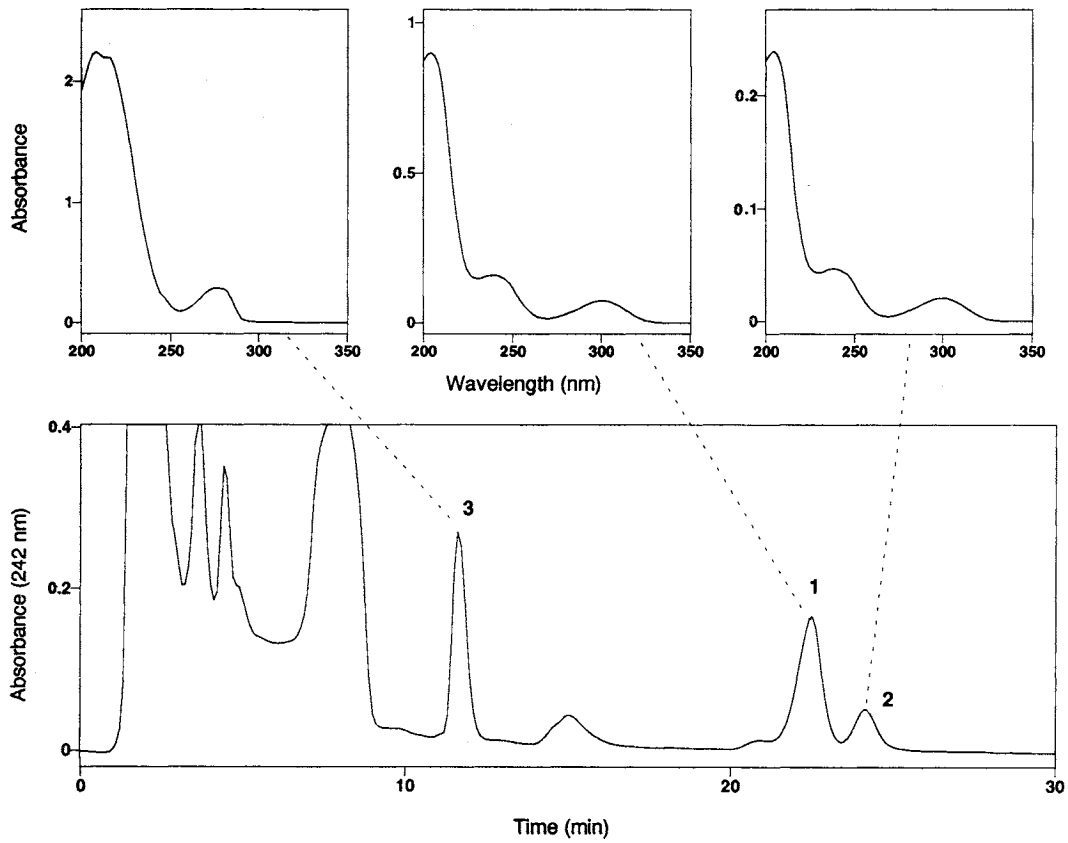
Utilizing the Syn-2 medium, originally developed for the production of asperlicin by *Aspergillus alliaceus*,[16,17] we designed a series of shake flask experiments to examine the effects of medium components and their relative concentrations on culture growth and productivity. Table 2 shows the effect of adding exogenous L-tryptophan and L-phenylalanine to the Syn-2 medium on the production of **1** in 250-ml shake flask fermentations. The addition of 2 g/liter each of L-Trp and L-Phe resulted in the production of over 600 mg/liter of **1** after 7 days. Because Phe and Trp are the likely natural precursors of **1**, addition of these amino acids to the medium probably increased the concentration of available substrates for biosynthesis. This is supported by the fact that the amount of **2** in crude extracts is increased by the addition of L-leucine to Syn-2. The chromatogram in Fig. 4 was generated from a fermentation in Syn-2 which included 100 mM L-Leu; the ratio of **1** to **2** was approximately 4:1. Without exogenous leucine, the ratio was usually closer to 25:1. Thus, selective feeding of amino acids to the culture not only increased the yield of WIN 64821 but also changed the ratio of related metabolites present in the extract.

Isolation

The high yield of **1** in the fermentation broth and the characteristic UV spectrum of the indoline chromophore enabled a clear correlation of biological activity to a single peak in the HPLC chromatogram.

Fig. 4. HPLC trace from an ethyl acetate extract of the culture grown in GBE medium containing 100 mM L-leucine.

Shown above the chromatogram are the UV spectra for the compounds eluting in the respective peaks. The numbers refer to the chemical structures shown in Fig. 1.



The distinct chromophore of **1** allowed us to use diode-array UV detection to monitor enrichment of **1** during the isolation procedure. We found that enrichment of **1** correlated to an increase in biological activity.

During the isolation of **1** we also obtained the related compounds **2** and **3**. The structure determinations of these compounds are reported elsewhere.[8] The relative HPLC retention times and UV spectra of these compounds are shown in Fig. 4. Compound **2** (24 minutes) was identified as an unsymmetrical-dimer analog of **1** in which a phenyl moiety was replaced with isopropyl. Compound **3** (11 minutes) was identified as cyclo(L-Trp-L-Phe) diketopiperazine. The existance of these related metabolites and the data from the amino acid feeding experiments support the intuitive hypothesis that the biosynthesis of **1** proceeds from amino acids in two stages: L-Trp is condensed with other amino acids to form diketopiperazines, which are then intramolecularlly cyclized at indole C-2 and intermolecularlly joined ("dimerized") at the indole C-3.

Physico-chemical Properties

The physico-chemical properties of **1** and **2** are summarized in Table 3. Both compounds were isolated as amorphous white powders. Their molecular formulae were confirmed by HRFAB-MS. The structure of **3** was confirmed by synthesis.[8] Crystallization of **1** was attempted using various solvent systems; however, crystals of sufficient quality for X-ray studies were not obtained. The structure determinations

Table 3. Physico-chemical properties of WIN 64821 (**1**) and WIN 64745 (**2**).

Compound	**1**	**2**
Nature	White powder	White powder
Melting point	203~205°C	194~196°C
Molecular formula	$C_{40}H_{36}N_6O_4$	$C_{37}H_{39}N_6O_4$
MW	664	631
HRFAB-MS $(M+H)^+$		
Found:	665.2913	631.3033
Calcd:	665.2876	631.3033
$[\alpha]_D$	+200.0° (*c* 0.15, MeOH)	+280.0° (*c* 0.012, MeOH)
UV λ_{max} (ε) in MeOH	241 nm (12,000), 301 nm (5,300)	242 nm (10,000), 301 nm (4,500)
Solubility	Acetone, DMSO	Acetone, DMSO
HPLC retention time	22 minutes	24 minutes

Table 4. The bioactivity of WIN 64821 and analogs present in SC319 culture extracts.

Compound	SP antagonist activity K_1 (μM)
WIN 64821	0.29
WIN 64745	3.9
Cyclo(L-Trp-L-Phe)	>50

of **1** and **2** relied heavily on one and two dimensional NMR spectroscopy and mass spectrometry. The absolute configurations of **1** and **2** were determined by a combination of amino acid analysis, MARFEY's derivatization, circular dichroism, and NOE analysis.

Biological Activity

When all three of these compounds were examined for bioactivity in the human astrocytoma model, we observed a marked difference in their relative potencies (Table 4). The modification from a phenyl to isopropyl moiety, which describes the chemical difference between **1** and **2**, resulted in more than a 10-fold reduction in activity. The monomeric cyclo(L-Trp-L-Phe) compound was completely inactive. The full biological profile of WIN 64821 as reported in the accompanying paper[14] documents this compound as a functional competitive inhibitor both of substance P and NKA at the NK1 and NK2 receptors, respectively. The bioactivity of these compounds was also compared to ditryptophenaline, a closely related compound with opposite stereochemistry at the indoline bridge and methyl substitutions at the secondary amines.[12,13] The compound was reisolated in our laboratories from a producing *Aspergillus* culture obtained from A. DEMAIN, MIT, and submitted for bioassay. Ditryptophenaline did exhibit some activity, though about 40-fold weaker than **1**.[8]

The discovery of WIN 64821 as a potent nonpeptide neurokinin antagonist from microbial sources might offer a unique research or therapeutic tool for the ultimate alleviation of nociceptive or inflammatory responses. The fact that WIN 64821 exhibited activity at both the NK1 and NK2 receptors supports this conclusion as both sites have been implicated in the cascade of signal events leading to pain and/or inflammation.[18,19]

Acknowledgments

The authors wish to thank KEN APPELL for his generous support conducting the bioassay experiments and to ABE CIMIJOTTI and JANET LEE for their assistance with the fermentation studies.

References

1) REGOLI, D.; G. DRAPEAU, S. DION & J. COUTURE: New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. TIPS 9: 290~295, 1988
2) ENGBERG, G.; T. H. SVENSSON, S. ROSELL & K. FOLKERS: A synthetic peptide as an antagonist of substance P. Nature 293: 222~223, 1981
3) SNIDER, R. M.; J. W. CONSTANTINE, J. A. LOWE III, K. P. LONGO, W. S. LEBEL, H. A. WOODY, S. E. DROZDA, M. C. DESAI, F. J. VINICK, R. W. SPENCER & H.-J. HESS: A potent nonpeptide antagonist of the substance P (NK1) receptor. Science 251: 435~437, 1991

4) GARRET, C.; A. CARRUETTE, V. FARDIN, S. MOUSSAOUI, J.-F. PEYRONEL, J.-C. BLANCHARD & P. M. LADURON: Pharmacological properties of a potent and selective nonpeptide substance P antagonist. Proc. Natl. Acad. Sci. U.S.A. 88: 10208～10212, 1991
5) HAYASHI, K.; M. HASHIMOTO, N. SHIGEMATSU, M. NISHIKAWA, M. EZAKI, M. YAMASHITA, S. KIYOTO, M. OKUHARA, M. KOHSAKA & H. IMANAKA: WS9326A, a novel tachykinin antagonist isolated from *Streptomyces violaceusniger* No. 9326. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1055～1063, 1992
6) WONG, S.-M.; L. L. MUSZA, G. C. KYDD, R. KULLNIG, A. M. GILLUM & R. COOPER: Fiscalins: New substance P inhibitors produced by the fungus *Neosartorya fischeri*. Taxonomy, fermentation, structures, and biological properties. J. Antibiotics 46: 545～553, 1993
7) WONG, S.-M.; R. KULLNIG, J. DEDINAS, K. C. APPELL, G. C. KYDD, A. M. GILLUM, R. COOPER & R. MOORE: Anthrotainin, an inhibitor of substance P binding produced by *Gliocladium catenulatum*. J. Antibiotics 46: 214～221, 1993
8) BARROW, C. J.; P. CAI, J. K. SNYDER, D. M. SEDLOCK, H. H. SUN & R. COOPER: WIN 64821, a new competivive antagonist to substance P, isolated from an *Aspergillus* species: structure determination and solution conformation. J. Org. Chem. 58: 6016～6021, 1993
9) MINATO, H.; M. MATSUMOTO & T. KATAYAMA: Studies on the metabolites of *Verticillium* sp.; structures of verticillins A, B, and C. J. Chem. Soc. Perkin Trans. I 1973: 1819～1825, 1973
10) SAITO, T.; Y. SUZUKI, K. KOYAMA, S. NATORI, Y. IITAKA & T. KINOSHITA: Chetracin A and chaetocins B and C, three new epipolythiodioxopiperazines from *Chaetomium* spp. Chem. Pharm. Bull. 36: 1942～1956, 1988
11) SEKITA, S.; K. YOSHIHIRA & S. NATORI; Mycotoxin production by *Chaetomium* spp. Can. J. Microbiol. 27: 766～772, 1981
12) SPRINGER, J. P.; G. BÜCHI, B. KOBBE, A. L. DEMAIN & J. CLARDY: The structure of ditryptophenaline—a new metabolite of *Aspergillus flavus*. Tetrahedron Lett. 1977: 2403～2406, 1977
13) NAKAGAWA, M.; H. SUGUMI, S. KODATO & T. HINO: Oxidative dimerization of Nb-acyltryptophans total synthesis and absolute configuration of ditryptophenaline. Tetrahedron Lett. 22: 5323～5326, 1981
14) OLEYNEK, J. J.; D. M. SEDLOCK, C. J. BARROW, K. C. APPELL, F. CASIANO, D. HAYCOCK, S. J. WARD, P. KAPLITA & A. M. GILLUM: WIN 64821, a novel neurokinin antagonist produced by an *Aspergillus* sp. II. Biological activity. J. Antibiotics 47: 399～410, 1994
15) POPP, J. L.; L. L. MUSZA, C. J. BARROW, P. J. RUDEWICZ & D. R. HOUCK: WIN 64821, a novel neurokinin antagonist produced by an *Aspergillus* sp. III. Biosynthetic analogs. J. Antibiotics 47: 411～419, 1994
16) HOUCK, D. R.; J. ONDEYKA, D. ZINK, E. INAMINE, M. A. GOETZ & O. D. HENSENS: On the biosynthesis of asperilicin and the directed biosynthesis of analogs in *Aspergillus alliaceus*. J. Antibiotics 41: 882～891, 1988
17) MONAGHAN, R. L.; E. ARCURI, E. E. BAKER, B. C. BUCKLAND, R. L. GREASHAM, D. R. HOUCK, E. D. IHNEN, E. S. INAMINE, J. J. KING, E. LESNIAK, P. S. MASUREKAR, C. A. SCHULMAN, B. SINGLETON & M. A. GOETZ: History of yield improvements in the production of asperlicin by *Aspergillus alliaceus*. J. Ind. Microbiol. 4: 97～104, 1989
18) FLEETWOOD-WALKER, S. M.; R. MITCHELL, P. J. HOPE, N. EL-YASSIR, V. MOLONY & C. M. BLADON: The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat. Brain Res. 519: 169～182, 1990
19) FLEETWOOD-WALKER, S. M.; P. J. HOPE, R. M. C. PARKER & R. MITCHELL: Effects of antagonists selective for neurokinin-1 and neurokinin-2 receptors on somatosensory responses of rat dorsal horn neurones. Br. J. Pharmacol. 104: 472, 1991