# WIN 66306, A NEW NEUROKININ ANTAGONIST PRODUCED BY AN *Aspergillus* species: FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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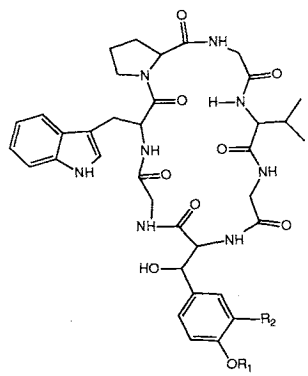
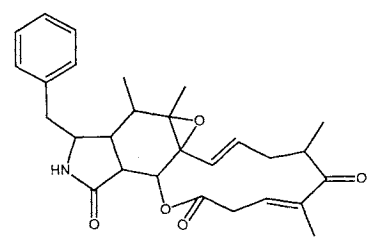
WIN 66306 (**1a**), a cyclic peptide containing a novel amino acid, was isolated as a neurokinin antagonist from an *Aspergillus* species, labelled SC230. Conditions that maximized the production of **1a** were developed, leading also to production of the related compound WIN 68577 (**2**) and rosellichalasin (**3**). Both **2** and **3** were more active in the rat NK1 than in the human NK1 receptor binding assay, while **1a** was more active at the human receptor with an inhibitor affinity constant of 7 $\mu$M.

The tachykinins are a family of undecapeptides that share the common *C*-terminal amino acid sequence Phe-X-Gly-Leu-Met-$NH_2$. Substance P (SP) is the best studied member of this family, and believed to be the endogenous ligand for the NK1 receptor subtype[1]. Other tachykinins are neurokinin A and neurokinin B, which are the endogenous ligands for the NK2 and NK3 receptor subtypes, respectively. Substance P and neurokinin A are widely distributed in the central and peripheral nervous systems and are believed to play important roles in pain stimuli and inflammatory response[2,3]. Recently several selective SP antagonists, or mixed SP/NKA antagonists, have been discovered[4~7] including some from microbial sources[8~10].

In the course of screening for inhibitors of SP binding to the NK1 receptor we discovered WIN 66306 (**1a**), which is biologically active at both the human NK1 and NK2 receptor subtypes. Also isolated from the same culture broth was the related compound WIN 68577 (**2**), and the known compound

Fig. 1. Chemical structures of WIN 66306 (**1a**), WIN 67689 (**1b**), WIN 68577 (**2**) and rosellichalasin (**3**).





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**1a** $R_1$=H $R_2$=$CH_2CH$=$C(CH_3)_2$
**1b** $R_1$=$CH_3$ $R_2$=$CH_2CH$=$C(CH_3)_2$
**2** $R_1$=H $R_2$=H

rosellichalasin[11]. Here we report the fermentation conditions for the culture (SC230), and the isolation, physico-chemical properties, and biological activity of **1a**, **1b**, **2** and **3**. The structure determinations of **1a** and **2**, and the procedure for conversion of **1a** to **1b**, are published elsewhere[12].

## Materials and Methods

### Microorganism

The producing fungus, strain SC230, was isolated from a soil sample obtained from a mesic prairie in Iowa. The culture has been preserved as part of the Sterling microbial culture collection.

### Fermentation

Seed cultures for inoculation of production media were typically prepared as follows: one ml of frozen stock, originally prepared by washing fresh culture plates and storing the spore suspension at −70°C, was added to 30 ml of seed medium in baffled flasks. The seed medium consisted of 2% glucose, 1.5% Pharmamedia (Traders Protein), 0.5% yeast extract, 0.3% ammonium sulfate, 0.4% calcium carbonate, and 0.003% zinc sulfate monohydrate. The culture was incubated at 27°C for 2 days at 220 rpm in a New Brunswick Psychrotherm shaker. A one ml volume of culture was transferred to 30 ml of production medium, in 250 ml nonbaffled flasks, to generate a second stage seed.

Scale up to 500 ml volume: Medium (1) was a glucose-starch medium. This medium contained 1% glucose, 3% soluble starch, 0.5% casitone, 0.3% beef extract, 0.3% N-Z Amine YTT, 0.05% potassium phosphate (monobasic), 0.05% magnesium sulfate (hepta hydro), 0.03% potassium chloride and 0.3% calcium carbonate. Medium (2) was a corn starch-casitone based medium. This medium consisted of 2% soluble starch, 2% casitone, 0.5% yeast extract, 0.05% potassium phosphate (monobasic), 0.05% magnesium sulfate heptahydrate, 0.03% potassium chloride, and 0.3% calcium carbonate. The Fernbach cultures were incubated for 6 days at 27°C in Braun BS4 shakers at 220 rpm. Experiments were conducted both in baffled and nonbaffled Fernbach flasks.

### Isolation

After cultivation of the producing organism for 6 days the whole culture (1 liter) was extracted with ethyl acetate (1 liter, 2×). The dried ethyl acetate extract (1.2 g) was then solvent partitioned using hexane - ethyl acetate - methanol - water (5 : 6 : 5 : 3). Both the upper organic layer (790 mg) and the aqueous layer (405 mg) showed activity in the NK1 binding assay.

After solvent removal, the organic layer was preadsorbed onto silica gel (1 g, 40 $\mu$M particle size) and packed on top of a flash column packed with the same material (40 g). The column was eluted with a gradient, from 40 : 60 ethyl acetate - hexane to 100% ethyl acetate. The biological activity concentrated in a single fraction eluting with 70 : 30 ethyl acetate - hexane, which was further purified using preparative HPLC on an ODS C-18 reverse-phased silica gel column (YMC-Pack, 15 × 250 mm; YMC Co., Ltd) with an isocratic 65 : 35 acetonitrile - water solvent system and a 3 ml/minute flow rate. Rosellichalasin (**3**) was obtained as a pure compound (58 mg).

After solvent removal the aqueous layer was dissolved in a minimum of solvent (1 ml 70 : 30 methanol - water) and subjected to centrifugal countercurrent chromatography (CCCC) using a chloroform - methanol - water (4 : 4 : 3), lower phase as stationary phase, solvent system. The biologically active fractions were combined and further purified using reverse-phase HPLC, as above, with an isocratic acetonitrile - water 35 : 65 solvent system, giving WIN 66306 (**1a**) (23 mg) and WIN 68577 (**2**) as pure compounds.

### Biological Assays

The NK1 binding assays were performed by measuring the ability of samples to compete with [$^3$H]SP binding to either rat submaxillary gland membranes, guinea pig submaxillary gland membranes, or human U-373 MG astrocytoma cells (ATCC HTB 17). The NK2 binding assay was performed by measuring the ability of samples to compete with [$^{125}$I]NKA binding to human urinary bladder membranes. The procedures used were the same as those previously published[13].

## Results

### Culture Taxonomy

Taxonomic investigation of SC230 (performed by ANGELA BELT, Blue Sky Research Service, Sonora, CA) identified the culture as an *Aspergillus* species, based on colony and cellular morphology observed after 10~21 days growth at 26~28°C on Czapeks and malt extract agar. These conditions generated white colonies which gave rise to loosely columnar, persistently white conidial heads. Metulae were present and the conidiophores were smooth walled with yellow to light brown pigment. Under the culture conditions used to produce material for isolation the culture exhibited isolated, colonies with brownish orange spores on the surface. The mycelial color was yellowish white and the culture produced a brown soluble pigment. No ascomata, hulle cells, or sclerotia were observed during the three week incubation period.

### Fermentation

Production of the bioactive components was originally scaled up to a 500 ml volume in 2.8-liter Fernbach flasks, using a glucose-starch medium. HPLC analysis of the culture extracts grown in the original glucose-starch screening medium revealed two regions of activity using a guinea pig NK1 binding assay (Fig. 2). Activity appeared to be associated with a peak at 15 minutes (**1a**) and another at 22 minutes (**3**). When assayed in the human NK1 binding assay, however, the 15 minute compound was approximately ten-fold more potent that the 22 minute compound. Because of this high activity at the human receptor we developed fermentation conditions which would maximize the production of the 15 minute compound.

Some initial media development pilot studies indicated that the formulation of a soluble starch-casitone based medium maximized production of the 15 minute component, and under these conditions a second more polar bioactive component was detected at 11 minutes (**2**) (Fig. 3). However, this more polar compound was more active at the rat, than at the human, NK1 receptor.

The HPLC profile was reproducible in both baffled and nonbaffled flasks with nonbaffled flasks ultimately being chosen because of the detection of higher titers. After scale-up and isolation of the active components calibration tables were set up and it was determined that **1a** was produced in yields of

Fig. 2. HPLC profile and associated bioactivity of SC230 extract in glucose-starch medium.

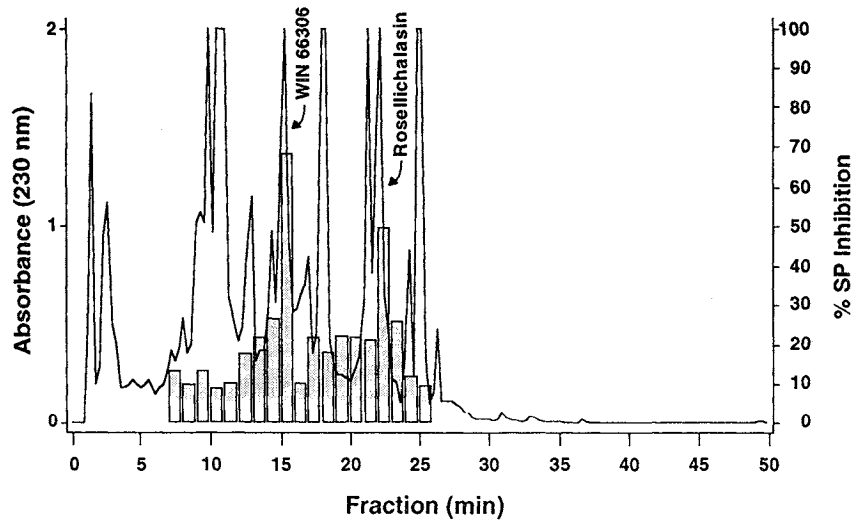


Fig. 3. HPLC profile and associated bioactivity of SC230 extract in starch-casitone medium.

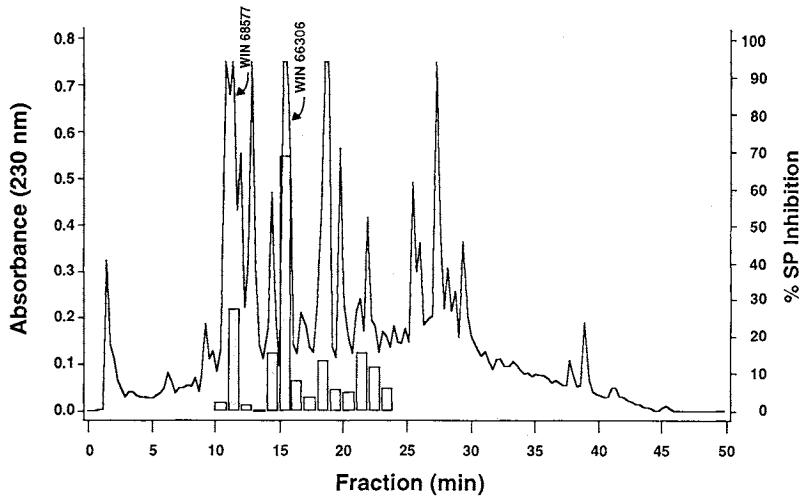
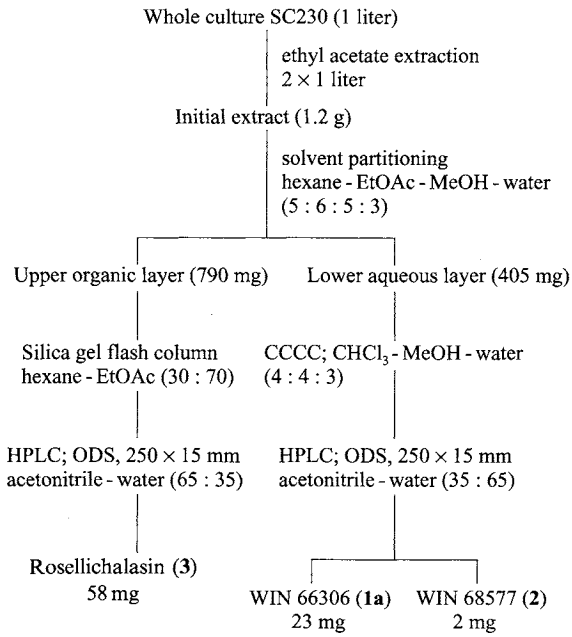



Fig. 4. Isolation scheme for compounds WIN 66306 (**1a**), WIN 68577 (**2**) and rosellichalasin (**3**).




approximately 2 mg per liter.

## Isolation of WIN 66306 (**1a**), WIN 68577 (**2**) and Rosellichalsin (**3**)

The isolation of **1a**, **2** and **3** was carried out using a bioassay directed isolation scheme (Fig. 4), monitoring the inhibitory activity against $^{125}I$ labeled BH-substance P binding to human U-373 MG astrocytoma cells. Because the biological activity of the extract was clearly associated with two well separated regions on HPLC chromatography, we assumed that there was a significant difference in the polarity of these compounds. We thus decided to develop a rapid, large-scale solvent partitioning method

to separate these two activity regions as a first isolation step. Guided by bioassay and HPLC analysis we found that the system hexane-ethyl acetate-methanol-water, 5:6:5:3, cleanly separated these two regions of activity.

The active component that partitioned into the upper organic phase was isolated using silica gel flash chromatography followed by reverse-phase HPLC. This compound was identified as the known compound rosellichalasin (**3**) by comparison with literature spectroscopic data[11]. Rosellichalasin, because of its abundance, accounted for the majority of the activity observed in the original extract. Using CCCC to isolate the active components from the lower polar layer gave two bioactivity enriched fractions, each containing a single component of greater than 80% purity. Final purification using reverse-phased HPLC gave, as pure compounds, WIN 66306 (**1a**) and WIN 68577 (**2**). The isolation scheme for these compounds is shown in Fig. 4.

Table 1. Physico-chemical properties of WIN 66306 (**1a**) and WIN 68577 (**2**).

| Compound | **1a** | **2** |
|---|---|---|
| Nature | White powder | White powder |
| Melting point | 160~164°C | 185~190°C |
| Molecular formula | $C_{41}H_{53}N_8O_9$ | $C_{36}H_{45}N_8O_9$ |
| MW | 800 | 732 |
| HRFAB-MS ($MH^+$) | | |
| Found: | 801.3903 | 733.3337 |
| Calculated: | 801.3870 | 733.3252 |
| $[\alpha]_D$ | +30.1° (*c* 0.98 MeOH) | +35.5° (*c* 0.05, MeOH) |
| UV $\lambda_{max}(\varepsilon)$ in MeOH | 224.0 nm (48,400), 281.0 (8,400), 289.5 (6,200) | 223.5 (46,500), 280.5 (8,050), 289.0 (5,800) |
| Solubility | Methanol, DMSO | Methanol, DMSO |

## Physico-chemical Properties

The physico-chemical properties of **1a**, **1b** and **2** are summarized in Table 1 and the structures shown in Fig. 1. Compounds **1a** and **2** were isolated as amorphous white powders. Compound **1b** was produced by methylation of **1a**[12]. All molecular formulae were confirmed by HRFAB-MS. Crystallizations of **1a** and **1b** were attempted using various solvent systems, but no crystals suitable for X-ray analysis were obtained. The structure determination of **1a** and **2** was carried out using extensive spectroscopic and chemical methods[12]. The structure of **3** was confirmed by comparison of its UV, MS, $^1H$ and $^{13}C$ NMR spectral data with those published for rosellichalasin[11].

## Biological Activity of WIN 66306 (**1a**), WIN 67689 (**1b**), WIN 68577 (**2**) and Rosellichalasin (**3**)

Compounds **1a** and **1b** are active at both the human NK1 and NK2 receptors, with **1b** being the most potent (*Ki* of 0.12±0.03 mM at the human NK1 receptor)[12]. Compound **1a** is approximately 3 times more active at the human NK1 receptor than at the rat NK1 receptor (*Ki* of 21.0 $\mu$M in rat). In contrast, compounds **2** and **3** are more active at the rat NK1 receptor than at the human NK1 receptor, with *Ki* values of 24.0 $\mu$M and 40.0 $\mu$M in rat, respectively, and 100 $\mu$M or greater in human. Structure-bioactivity results at the human receptors for these compounds, and other analogs, are discussed elsewhere[12]. Both **1a** and **1b** appear to be competitive antagonists of SP at both the guinea pig and human NK1 receptors[12].

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