WS9326A, A NOVEL TACHYKININ ANTAGONIST ISOLATED FROM *Streptomyces violaceusniger* No. 9326

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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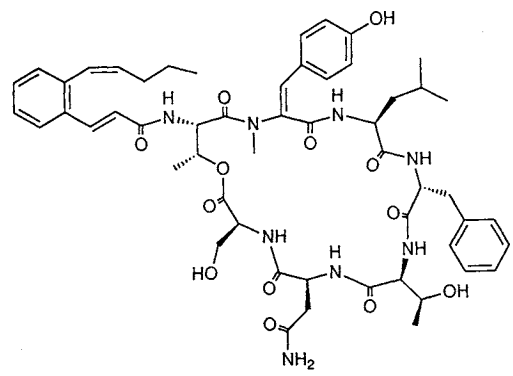
Data from several studies suggest that tachykinins may play an important role in the pathophysiology of airway diseases, especially asthma. Our aim is to discover tachykinin antagonists which exhibit therapeutically useful anti-asthmatic activity. In our search for activities inhibiting the binding of [^{3}H]substance P to guinea-pig lung membrane preparations, we have found that the fermentation product, WS9326A, isolated from *Streptomyces violaceusniger*, is a potent tachykinin receptor antagonist.

The tachykinins and their receptors represent a complex system involving at least three related peptides, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), and three different receptor types, which have been recently named NK-1, NK-2 and NK-3, respectively[1]. Members of the tachykinin family share the common *C*-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X is an aromatic or hydrophobic amino acid.

A possible role of tachykinins in asthma has been suggested recently[2~5]. The working hypothesis is that mechanical and chemical stimulation of the sensory airway nerve endings causes antidromic release of tachykinins such as SP and NKA from axon collaterals, and that this leads to smooth muscle contraction, mucus secretion, vasodilation and protein extravasation[2]. In order to confirm this hypothesis, the availability of a potent and selective tachykinin antagonist is essential. A number of SP antagonists have been prepared by chemical modification of SP. However, these SP analogues possess relatively low potency, and some of them retain agonist activity and can stimulate histamine-release from mast cells. Our aim is to discover tachykinin receptor antagonists which exhibit therapeutically useful anti-asthmatic activity. In our search for activities inhibiting the binding of [^{3}H]substance P ([^{3}H]SP) to guinea-pig lung membrane preparations, we found that the fermentation product WS9326A, isolated from *Streptomyces violaceusniger*, is a potent tachykinin receptor

Fig. 1. Chemical structure of WS9326A.



antagonist. In this paper, we describe a taxonomic study on strain No. 9326, fermentation and isolation procedures, and some chemical and biological properties of WS9326A. As shown in Fig. 1, WS9326A is cyclic heptapeptide lactone. Determination of the chemical structure and total synthesis of WS9326A will be published elsewhere[6,7].

Materials and Methods

Taxonomic Studies

Strain No. 9326 was isolated from a soil sample obtained from Suwa City, Nagano Prefecture, Japan. The methods described by SHIRLING and GOTTLIEB[8] were employed for the taxonomic study. Morphological observations were made using light and electron microscopes from cultures grown at 30°C for 14 days on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. Cultural characteristics were observed on the media described by SHIRLING and GOTTLIEB[8], and WAKSMAN[9]. Incubation was carried out at 30°C for 21 days. The color names used in this study were taken from the Methuen Handbook of Colour[10]. Wall analysis was performed by the methods of BECKER *et al.*[11], and YAMAGUCHI[12]. The temperature range for growth was determined on yeast extract - malt extract agar using a temperature gradient incubator (Advantec Toyo Co., Ltd.). Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[13].

Fermentation

A loopful of *Streptomyces violaceusniger* No. 9326 on mature slant culture was transferred into twenty five 500-ml Erlenmeyer flasks each containing 160 ml of sterile seed medium composed of soluble starch 1%, sucrose 1%, glucose 1%, Pharmamedia (Traders Protein) 1%, peptone 0.5%, soybean meal 0.5% and $CaCO_3$ 0.2%. The medium was adjusted to pH 6.5 prior to addition of $CaCO_3$. These flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 3 days at 30°C.

The resultant seed culture was used to inoculate 160 liters of seed medium containing soluble starch 1%, sucrose 1%, glucose 1%, Pharmamedia 1%, peptone 0.5%, soybean meal 0.5%, $CaCO_3$ 0.2%, Adekanol LG-109 0.07% (deforming agent, Asahi Denka Co.) and Silicone KM-70 0.05% (defoaming agent, Shin-etsu Chemical Co.) in a 500-liter stainless steel jar-fermenter which had been sterilized at 120°C for 30 minutes in advance. The fermentation was carried out at 30°C for 1 day under aeration of 160 liters/minute, back pressure 1.0 kg/cm^2 and agitation speed 200 rpm.

The resultant seed cultured broth (60 liters) was inoculated into 3,000 liters of production medium containing sucrose 5%, soybean meal 1%, chicken meat meal 1%, $CaCO_3$ 0.2%, Adekanol LG-109 0.07% and Silicone KM-70 0.05% in a 4,000-liter stainless steel fermenter which had been sterilized at 120°C for 30 minutes in advance. The fermentation was carried out at 30°C for 4 days under aeration of 3,000 liters/minute, back pressure 1.0 kg/cm^2 and agitation speed 100 rpm.

The progress of fermentation was monitored by high performance liquid chromatography (HPLC) using an Hitachi Model 655 pump. A steel column (4.00 mm diameter, 250 mm length) packed with LiChrosorb RP-18 (E. Merck) was used at a flow rate of 1.0 ml/minute. The mobile phase was an aqueous solution of 45% acetonitrile. The sample for the HPLC assay was prepared as follows; an equal volume of acetone was added to a broth with vigorous stirring and the mixture was allowed to stand for 1 hour and then centrifuged. Five μl of the supernatant was injected *via* the injector of the Hitachi Model 655 HPLC.

Substance P Receptor Assay

(a) Crude Membranes Preparation: Male Hartley guinea-pigs (600 g) were used as the tissue source for membrane preparations. The trachea and lungs were removed, and stored at −80°C until use. These tissues (150 g) were thawed and minced with scissors and then homogenized with a Polytron (Brinkmann PT-20, maximal speed for 3 × 10 seconds) in 100 ml buffer (0.25 M sucrose, 10 mM Tris-HCl, 0.1 mM EDTA). The homogenates were centrifuged at 900 × g for 10 minutes at 4°C. The supernatant, containing the plasma membrane fraction, was centrifuged at 100,000 × g for 60 minutes at 4°C, and the resultant pellets were referred to as crude membrane fractions. The pellets were resuspended in 25 ml of binding assay buffer (50 mM Tris-HCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 2 μg/ml chymostatin, 4 μg/ml leupeptin and 40 μg/ml bacitracin), and homogenized with a teflon homogenizer and centrifuged at 14,000 × g for 20 minutes. The

pellets were stored at −80°C until use.

(b) [^{3}H]SP Binding Assay[14]: [^{3}H]SP (New England Nuclear) binding to guinea-pig lung membranes was determined in a mixture (final volume 250 μl) containing 50 mM Tris-HCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 2 μg/ml chymostatin, 4 μg/ml leupeptin and 40 μg/ml bacitracin (binding assay buffer), [^{3}H]SP (final 1×10^{-9} M) and 50 μl of membranes for 30 minutes at 4°C. After incubation, the mixture were filtered through a glass-fiber GF/B filter (pretreatment with 0.1% polyethyleneimine for 3 hours prior to use) using a cell harvester (Brandel M-24S). The filters were then washed ten times with a total of 5 ml of the washing buffer (50 mM Tris-HCl, pH 7.5) at 0°C. The radioactivity was counted in 3 ml of scintillation cocktail (Aquasol-2, New England Nuclear) in Packard sintillation counter (Packard TRI-CARB 4530). Specific binding was defined as total binding minus nonspecific binding in the presence of an excess (10^{-6} M) of unlabeled SP.

Antimicrobial Activity

The antimicrobial activity of WS9326A was determined by a serial broth dilution method in bouillon medium for bacteria and SABOURAUD's medium for fungi and yeasts. The antimicrobial activity was observed after overnight incubation at 37°C for bacteria and 48~72 hours incubation at 28°C for a filamentous fungus and a yeast.

Results

Taxonomy of the Producing Strain

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed spiral chains of spores with 10 to 30 spores per chain. The spores had a smooth surface and were oval in shape with a size of 0.6~0.8 × 0.8~1.3 μm. No sclerotic granules, sporangia or zoospores were observed (Fig. 2).

The results of cultural characteristics are shown in Table 1. The aerial mycelium was gray to brownish gray. Part of colony became black and moist, and exhibited hygroscopic characteristics on most agar media. On the reverse side, the growth was yellowish brown, brown and dark brown. Reverse mycelial pigment was not pH sensitive. Melanoid pigments were not produced in Tryptone - yeast extract broth, peptone - yeast extract - iron agar and tyrosine agar. Other soluble pigments were not produced.

Analysis of whole cell hydrolysates of strain No. 9326 showed the presence of LL-diaminopimelic acid.

Physiological properties and utilization of carbon sources of strain No. 9326 are shown in Tables 2 and 3, respectively.

The morphology and chemical characteristics of strain No. 9326 permitted a clear assignment of the organism to the genus *Streptomyces*. Strain No. 9326 was compared with *Streptomyces* species described in BERGEY's Manual[15], SHIRLING's ISP reports[16~18], the other species listed on "Approved lists of bacterial names"[19], and the species described in other references[20,21]. As a result, it was found that strain No. 9326 closely resembled *Streptomyces violaceusniger* with a little difference about reverse side color of growth[22].

Reverse side color of strain No. 9326 was

Fig. 2. Scanning electron micrograph of spore chain of strain No. 9326 on inorganic salts - starch agar.

Scale: 5 μm.

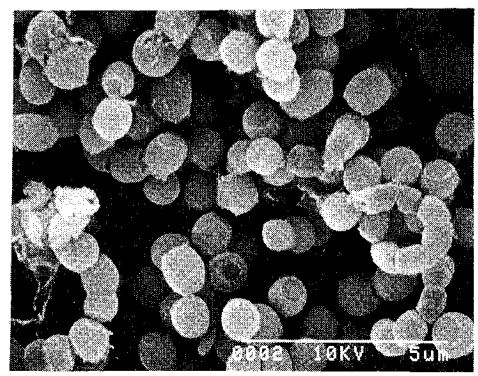


Table 1. Cultural characteristics of strain No. 9326.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar	G: Good A: Abundant, brownish gray (6E2) R: Dark brown (7F6) S: None	Glucose-asparagine agar	G: Good A: Moderate, brownish gray (19E2) R: Brown (6F4) S: None
Oatmeal agar	G: Good A: Moderate, dark brown (7E3) R: Brownish gray (7F2) S: None	Nutrient agar	G: Moderate A: Moderate, brownish gray (9E2) R: Grayish brown (5E3) to yellowish brown (6F4) S: None
Inorganic salts-starch agar	G: Good A: Abundant, brownish gray (7E2) R: Yellowish brown (5E6) S: None	BENNET agar	G: Poor A: Poor, dark brown (6F4) R: Dark brown (6F4) S: None
Glycerol-asparagine agar	G: Good A: Abundant, grayish violet (19E3) R: Brown (6E4) S: None	Sucrose-nitrate agar	G: Poor A: None R: Grayish brown (6E3) S: None
Peptone-yeast extract-iron agar	G: Good A: Thin, grayish white (1B1) R: Yellowish brown (5D6) S: None		

Abbreviation: G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2. Physiological properties of strain No. 9326.

Conditions	Characteristics
Temperature range for growth	11°C~47°C
Optimum temperature range for growth	29°C~31°C
Gelatin liquefaction	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Starch hydrolysis	Positive
Production of melanoid pigments	Negative
Decomposition of cellulose	Negative

Table 3. Carbon utilization of strain No. 9326.

Compound	Growth
D-Glucose	+
Sucrose	+
D-Xylose	+
D-Fructose	+
L-Rhamnose	+
Raffinose	+
L-Arabinose	+
Inositol	+
Mannitol	+

+: Utilization.

yellowish brown to dark brown, but that of *S. violaceusniger* NRRL B-1478 was grayish yellow or dark grayish green. It is considered that this difference is too small to regard strain No. 9326 as a different species. Strain No. 9326 was therefore identified as a strain of *Streptomyces violaceusniger*.

A lyophilized sample of the *Streptomyces violaceusniger* No. 9326 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM BP-1667 (deposited date: 20th, January, 1988).

Production of WS9326A

Fig. 3 presents the data from a typical 4,000-liter fermentation and gives information regarding WS9326A production, pH and packed mycelium volume. WS9326A production began at 24 hours, and

Fig. 3. Time course of fermentation.

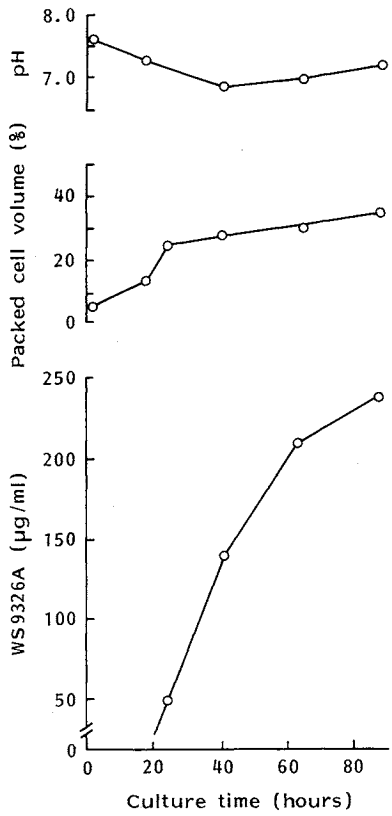


Fig. 4. Isolation procedure of WS9326A.

Fermentation broth (2,650 liters)
|
Mycelial cake
|
EtOAc extract (1,600 liters)
|
Carbon column (200 liters)
| 1) washed with EtOAc (120 liters)
| 2) eluted with EtOAc - MeOH (5 : 1) (980 liters)
| 3) concentrated (45 liters)
Precipitates with *n*-hexane (120 liters)
| 1) washed with *n*-hexane
| 2) eluted with MeOH (20 liters)
Residue (500 g)
| silica gel column (70 liters)
| eluted with CH_2Cl_2 - MeOH (8 : 1)
Active fraction
| precipitated with CH_3CN
Powder (190 g)

the maximum production was observed after 4 days of cultivation and the yields were 240 μg/ml.

Isolation and Purification

A flow diagram of the isolation procedure described below is shown in Fig. 4. The culture broth thus obtained was filtered with the aid of diatomaceous earth (15 kg; Perlite Topko No. 34, Showa Chemical Industry Co., Ltd.). The mycelial cake was extracted with ethyl acetate (1,600 liters) and the extract was filtered. The filtrate (1,400 liters) was absorbed on an activated carbon column (200 liters; Shirasagi KL, Takeda Pharmaceutical Co., Ltd.). After washing the column with 120 liters of ethyl acetate, the active fractions were eluted with 980 liters of ethyl acetate - methanol (5 : 1). The eluate was concentrated to 45 liters under reduced pressure. *n*-Hexane (120 liters) was added to the resultant solution with stirring. The mixture was allowed to stand at room temperature for 1 hour and then filtered with the aid of Silika No. 600 (3 kg; Chuo Silica Co., Ltd.). The cake thus obtained was washed with *n*-hexane (15 liters) and the desired substances were eluted with methanol (20 liters).

The eluate was concentrated to dryness under reduced pressure. The residue (500 g) was dissolved with 4 liters of methanol - acetic acid - dichloromethane (1 : 1 : 2) and applied to a column of silica gel (Kieselgel 60, 70~230 mesh, 70 liters). The column was developed with 0.5 liter of methanol - acetic acid - dichloromethane (1 : 1 : 2) and dichloromethane (25 liters). The active substances were eluted with dichloromethane - methanol (8 : 1). The active fractions were combined and concentrated under reduced pressure. The residue was dissolved with methanol (1 liter). Acetonitrile (9 liters) was added to the resultant solution with stirring. The mixture was allowed to stand at room temperature for 1 hour and the resultant precipitate was collected by filtration. This precipitation step was repeated three times. The combined

precipitates thus obtained were washed with acetonitrile (1 liter) and dried to give a white powder (190 g) of WS9326A.

Physico-chemical Properties

The physico-chemical properties of WS9326A are summarized in Table 4. WS9326A is soluble in methanol, ethanol and DMSO. It is slightly soluble in acetone and ethyl acetate and insoluble in hexane. The color reactions of WS9326A are as follows: positive to iodine vapor, cerium sulfate and sulfuric acid though negative to ninhydrin.

The ^{1}H NMR spectrum of WS9326A is shown in Fig. 5. ^{13}C NMR spectrum of WS9326A is shown in Fig. 6. The IR spectrum of WS9326A is shown in Fig. 7. The absorptions at 1730 and 1650 cm^{-1} in the IR sepctrum indicated the presence of ester and amide function in the molecule. Structural elucidation and total synthesis of WS9326A will be published elsewhere[6,7].

Table 4. Physico-chemical properties of WS9326A.

Appearance	Colorless powder
Melting point	187~190°C
$[\alpha]_D^{23}$	−84° (*c* 1.0, MeOH), +50° (*c* 1.0, DMSO)
UV λ_{max}^{MeOH} nm	280 (ε 34,700)
Molecular formula	$C_{54}H_{68}N_8O_{13}$
HR-FABMS Calcd:	1,037.4984 $(M+H)^+$
Found:	1,037.4980 $(M+H)^+$
IR ν_{max} (KBr) cm^{-1}	3300, 1730, 1650, 1510
Elemental analysis	
Calcd for $C_{54}H_{68}N_8O_{13}\cdot 2H_2O$:	C 60.43, H 6.76, N 10.44
Found:	C 60.48, H 6.61, N 10.32
Solubility	
Soluble:	MeOH, EtOH
Slightly soluble:	Acetone, ethyl acetate
Insoluble:	H_2O, $CHCl_3$
TLC (Rf)	Silica gel
	$CHCl_3$-MeOH (5:1) 0.38
	RP-18 80% MeOH 0.46

Biological Properties

The biological activity of WS9326A was evaluated in a SP binding assay using guinea-pig lung membranes. WS9326A inhibited [^{3}H]SP binding to guinea-pig lung membranes in a dose-dependent manner with an IC_{50} value of

Fig. 5. ^{1}H NMR spectrum of WS9326A in CD_3OD.

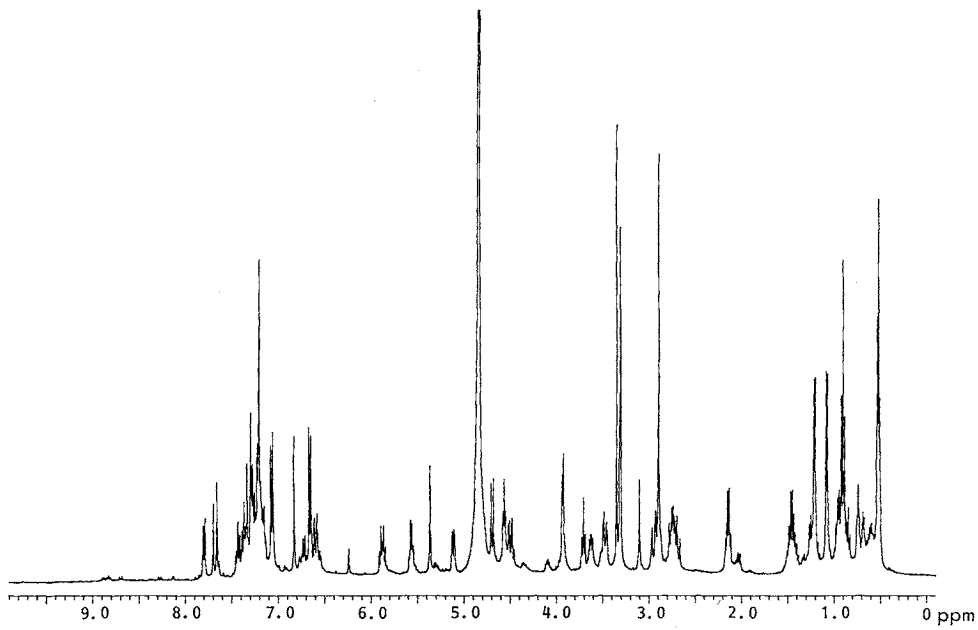


Fig. 6. ^{13}C NMR spectrum of WS9326A in CD_3OD.

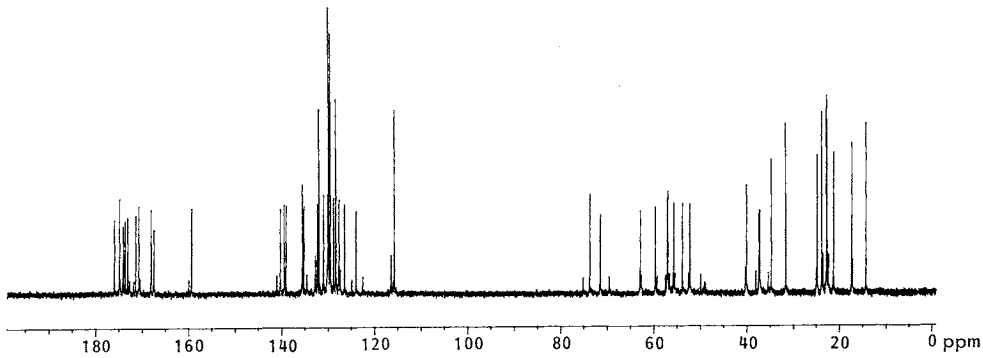


Fig. 7. IR spectrum of WS9326A (KBr).

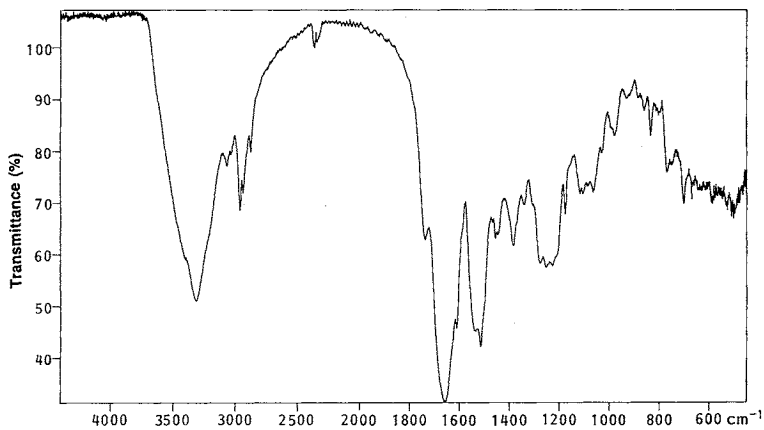
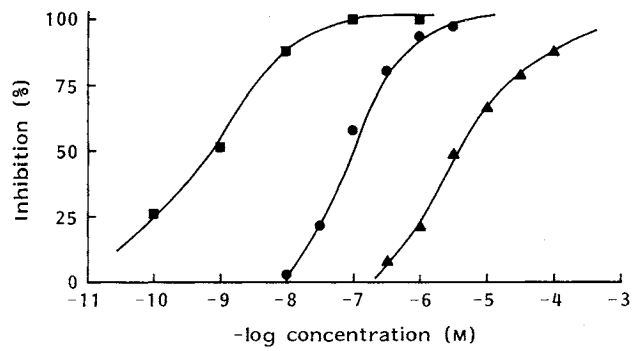


Fig. 8. Effect of WS9326A and FK224 on the binding of substance P to guinea-pig lung membranes.

[^{3}H]Substance P concentration of 1.0×10^{-9} M. ■ substance P, $IC_{50}=7.0\times10^{-9}$ M. ● FK224, $IC_{50}=1.0\times10^{-7}$ M. ▲ WS9326A, $IC_{50}=3.6\times10^{-6}$ M.



3.6×10^{-6} M. Antimicrobial activities of WS9326A were evaluated by serial broth dilution method. WS9326A shows weak antimicrobial activity against *Staphylococcus aureus* 209P JC-1 (MIC 1,000 μg/ml). It has no antibiotic activity against *Escherichia coli* NIHJ JC-2, *Pseudomonas aeruginosa* NCTC 10490, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris*, *Candida albicans*, or *Aspergillus fumigatus* IFO 5840 at 1,000 μg/ml.

Discussion

Data from several studies suggest that SP and NKA may play an important role in the pathophysiology of airway diseases, especially asthma. NKA is a more potent *in vitro* and *in vivo* bronchoconstrictor than SP in both animals and humans. This suggests that NK-2 receptors mediate the bronchoconstriction action of neurokinins. Both NK-1 and NK-2 receptors are present in guinea-pig and human airways, and we will demonstrate that WS9326A is a dual NK-1, NK-2 receptor antagonist in other papers[23,24].

Thus, WS9326A is considered to be the first microbially produced tachykinin receptor antagonist.

Our final goal is to identify tachykinin (neurokinins) receptor antagonists which exhibit therapeutically useful anti-asthmatic activity. While determining the structure of WS9326A, we obtained tetrahydro-WS9326A (FK224) which exhibits more potent biological activity than WS9326A. The catalytic hydrogenation of WS9326A produces FK224. Pharmacological studies showed that FK224 was about ten times more active than WS9326A both *in vitro* (Fig. 8) and *in vivo*, and it also act as a dual NK-1 and NK-2 tachykinin receptor antagonist[23].

Acknowledgments

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