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Xanthofulvin, a Novel Semaphorin Inhibitor Produced by a Strain of *Penicillium*

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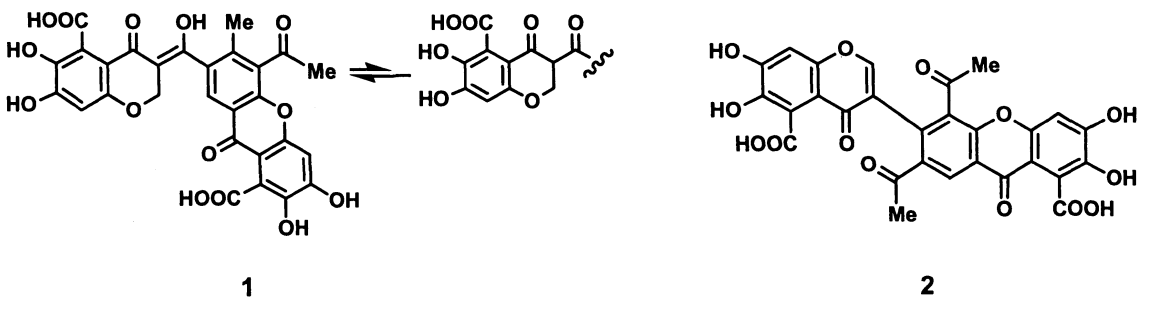
A new semaphorin inhibitor xanthofulvin was isolated from the cultured broth of a fungus *Penicillium* sp. SPF-3059 along with a known compound vinaxanthone by solvent extraction and bioassay-guided fractionation. The tautomeric structure of xanthofulvin was determined by spectroscopic analyses. The two compounds exhibited significant semaphorin inhibitory activity with IC_{50} values of 0.09 and 0.1 μg/ml, respectively, in semaphorin3A-induced growth cone collapse assay using cultured chick dorsal root ganglia neurons.

Semaphorins are a family of soluble and membrane-bound proteins that have been shown to function as regulatory factors during neuronal development of the central and peripheral nervous systems. Most semaphorins act as a chemorepellent on a wide variety of axons, thus they are thought to be repulsive guidance cues to axons[1]. To date more than 20 semaphorins have been identified and classified into 8 classes[2]. Semaphorin 3A (Sema3A), a soluble and the prototype semaphorin, has been shown to cause collapse of neurite growth cones, resulting in inhibition of neuronal outgrowth *in vitro* and *in vivo*[3,4]. In the mature mammalian central nervous system, expression of semaphorins elevates after neuronal injury[5]. Once injured, axons can hardly regenerate in the presence of these repulsive proteins. Therefore we consider that inhibitors of semaphorins may be of potential use as drugs for the treatment of traumatic neuronal injury.

In search for semaphorin inhibitors from microorganisms, we isolated two active compounds from the cultured broth of a fungal strain SPF-3059. The compounds were identified as a new compound xanthofulvin (**1**) and previously reported vinaxanthone[6,7] (**2**) (Fig. 1). Although both compounds were first isolated in the early 1990s, the former has not yet been reported in publication. This is the first report describing xanthofulvin in detail. In this paper we describe the taxonomy of the

Fig. 1. Structures of xanthofulvin (**1**) and vinaxanthone (**2**).



1 **2**

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producing organism, fermentation, isolation, structure elucidation of xanthofulvin and semaphorin inhibitory activity of these two compounds.

Materials and Methods

General

UV spectra were recorded on a Hitachi U-2000 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. FAB-MS spectra were obtained on a JEOL JMS-SX102A spectrometer. NMR spectra were recorded on a JEOL JNM α-500 spectrometer and the chemical shifts are given in ppm referenced to DMSO-d_6 as 2.49 ppm (^{1}H) and 39.5 ppm (^{13}C).

Microorganism

The fungal strain SPF-3059 was isolated from a soil sample collected in Osaka, Japan. The strain has been deposited at the International Patent Organism Depositary, the National Institute of Advanced Industrial Science and Technology, Japan under the accession number FERM P-17766.

Taxonomy

Taxonomic studies of the strain SPF-3059 were done according to the method of PITT[8]. For the evaluation of cultural characteristics, Czapek yeast extract agar (CYA, K_2HPO_4 0.1%, yeast extract 0.5%, sucrose 3%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, agar 1.5%), malt extract agar (MEA, malt extract 2%, peptone 0.1%, glucose 2%, agar 1.5%), 25% glycerol nitrate agar (G25N, K_2HPO_4 0.075%, yeast extract 0.37%, glycerol 25%, $NaNO_3$ 0.23%, KCl 0.038%, $MgSO_4 \cdot 7H_2O$ 0.038%, $FeSO_4 \cdot 7H_2O$ 0.0008%, agar 1.2%), yeast extract - soluble starch agar (yeast extract 0.2%, soluble starch 1%, agar 1.5%), potato dextrose agar (PDA, Nihon Pharmaceutical) and ISP medium No. 3 (Nihon Pharmaceutical) were used. Fine morphological structures were observed using a Hitachi S-800 scanning electron microscope. Color names were determined by using the Color Tone Manual[9].

Fermentation

A slant culture of the strain SPF-3059 was inoculated into a 500-ml Sakaguchi flask containing 75 ml of liquid medium consisted of glucose 2%, sucrose 5%, cottonseed flour 2%, $NaNO_3$ 0.1%, L-histidine 0.1%, K_2HPO_4 0.05%, KCl 0.07%, $MgSO_4 \cdot 7H_2O$ 0.0014%, pH 7.0, and cultured for 5 days at 27°C with reciprocal shaking at 130 rpm. An aliquot of 6 ml of this seed culture was transferred into 2-liter Sakaguchi flasks containing 300 ml of the same medium, and cultured at 27°C with reciprocal shaking at 110 rpm.

HPLC Analysis

The cultured broth (10 ml) was centrifuged at 3,000 rpm for 5 minutes, then the supernatant and the cell cake were extracted with 10 ml of formic acid - EtOAc (1 : 99), and Me_2CO, respectively. The extracts were evaporated to dryness under reduced pressure, and the residues were dissolved in 1 ml of MeOH. Samples (20 μl) were analyzed by reversed-phase HPLC using a Wakopak Wakosil-II5C18RS column (4.6×150 mm) with 1% aqueous formic acid - MeOH (80 : 20 to 30 : 70 in 77 minutes) at a flow rate of 1.3 ml/minute, and detection of UV absorption at 260 nm. Compounds **1** and **2** were eluted at 37.5 and 28.0 minutes, respectively.

Preparation of Sema3A

The cDNA of mouse Sema3A[10] was cloned into pUCSRα expression vector[11] and transiently expressed in COS-7 cells using FuGene6 transfection reagent (Roche Diagnostics). The cultured conditioned medium was used as recombinant Sema3A without further purification[12].

Semaphorin Inhibitory Assay

Dorsal root ganglia (DRG) explants excised from E7 chick embryos were cultured in F12 medium supplemented with 10% fetal bovine serum (Gibco BRL) and 20 ng/ml nerve growth factor (Promega) at 37°C for 20 hours using 96-well culture plates pre-coated with poly-L-lysine and laminin. Assay samples were added to the wells 1 hour before addition of the recombinant mouse Sema3A at a final concentration of 3 U/ml to induce complete collapse of growth cones (1 U/ml of Sema3A is defined as the amount required to collapse 50% of DRG growth cones)[3]. One hour after addition of Sema3A, wells were fixed with 1% glutaraldehyde, then the numbers of collapsed and uncollapsed growth cones were counted under a light microscope. In each well at least 100 growth cones were counted.

Results

Taxonomy

Colonies of the strain SPF-3059 grown on CYA, MEA, PDA and yeast extract - soluble starch agar at 25°C for 7

Fig. 2. Scanning electron micrograph of strain SPF-3059 grown on ISP medium No. 3 at 25°C for 14 days.

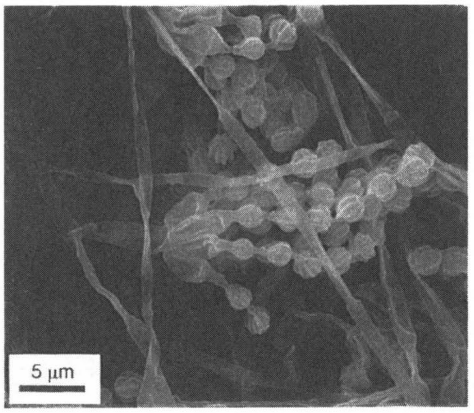
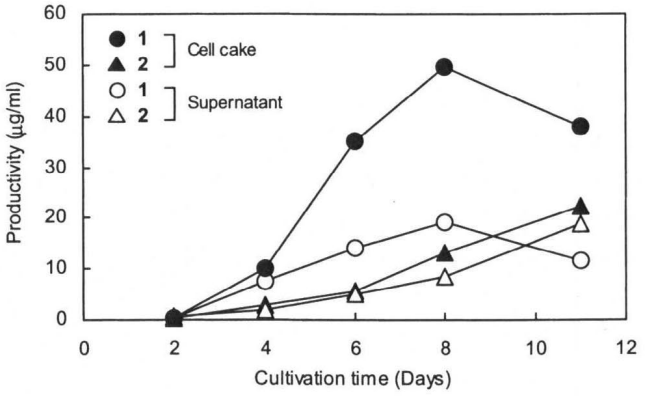


Fig. 3. Time course of production of xanthofulvin (**1**) and vinaxanthone (**2**) by *Penicillium* sp. SPF-3059.



days were white and floccose with a diameter of 9~11 mm. The reverse side color was pale yellow. Colonies on G25N were white and floccose with a diameter of 3~4 mm. The reverse side color was pale yellow. Neither soluble pigment nor sporulation was observed on these agar media even after one month of incubation. On the contrary, on ISP medium No. 3, one of actinomycete identification agar media, unexpected sporulation was observed in 7 days. Colonies grown on ISP medium No. 3 at 25°C for 14 days were beige to grayish green and floccose with a diameter of 11~12 mm. The reverse side color was beige to light grayish green due to good sporulation. Conidiophores were formed from substrate and aerial hyphae, and penicillia were monoverticillate (Fig. 2). Stipes were smooth-walled and 5~20 μm long. Phialides were ampulliform and 3~5 μm long. Conidia were globose to subglobose, 2~2.3 μm in diameter with striated surface. Teleomorph was not observed. From the above characteristics, the strain SPF-3059 was identified as a member of the genus *Penicillium* and named *Penicillium* sp. SPF-3059.

Isolation

In shaking culture, productivity of **1** reached maximum at day 8 of fermentation (Fig. 3). So the cultured broth (3 liters) was harvested at day 8 and centrifuged at 10,000 *g* for 10 minutes at 4°C. The cell cake was extracted with 3 liters of acetone and evaporated under reduced pressure. The resulting aqueous solution was extracted with 1 liter of formic acid - ethyl acetate (1 : 99). The culture supernatant was extracted with 3 liters of formic acid - ethyl acetate (1 : 99). Both acidic ethyl acetate extracts were combined and concentrated to dryness under reduced pressure to yield 10.4 g of oily material. The material was dissolved in 100 ml of methanol and applied to a column of Sephadex LH-20 (Amersham Biosciences), and the column was eluted with methanol. Active fractions (2.6 g) were collected, applied to a column of Toyopearl HW-40F (Tosoh) and eluted with methanol. Active fractions (1.6 g) were pooled and injected into preparative HPLC equipped with Wakopak Wakosil-II5C18HG-Prep columns (30×100+30×250 mm). The elution was performed with 1% aqueous formic acid - MeOH (55 : 45 to 25 : 75 in 120 minutes) at a flow rate of 20 ml/minute and detection of UV absorption at 260 nm. Two active substances were obtained and finally purified by re-chromatography using the HPLC to give **1** (64 mg) and **2** (34 mg) as yellow powders.

Structure Elucidation

The physico-chemical properties of **1** and **2** are summarized in Table 1. Similarity in these data suggested that they were structurally related. Compound **2** was identified as a known compound vinaxanthone[6] by its UV, IR, MS and ^{1}H and ^{13}C NMR spectral data. The ^{13}C-^{1}H long-range couplings of ^{2}J, ^{3}J and ^{4}J observed in the HMBC experiments supported the identity of **2** and were helpful in determining the structure of **1** (Fig. 4).

The molecular formula of **1** was established as $C_{28}H_{18}O_{14}$ by HRFAB-MS, differing from that of **2** by the addition of

Table 1. Physico-chemical properties of **1** and **2**.

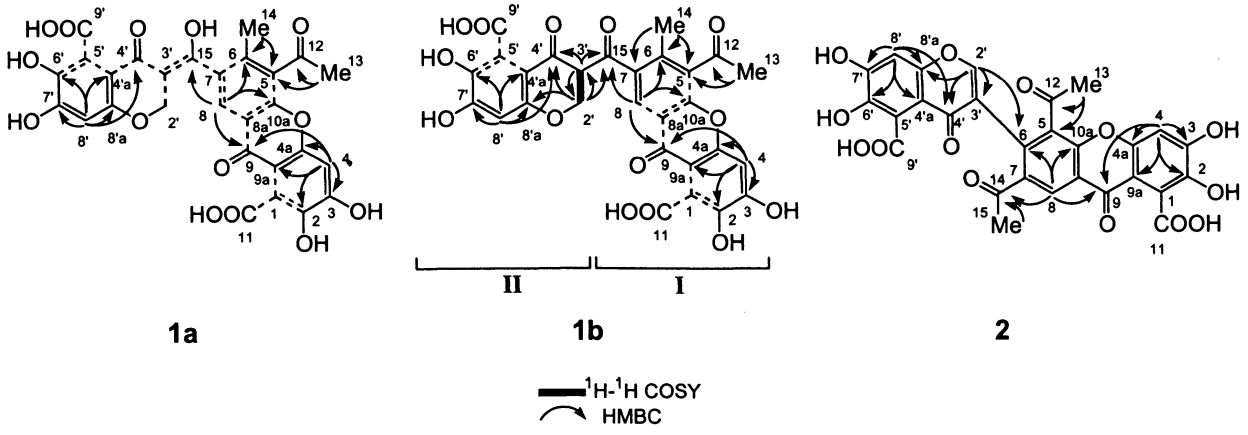
	1	**2**
Appearance	Yellow powder	Pale yellow powder
Molecular formula	$C_{28}H_{18}O_{14}$	$C_{28}H_{16}O_{14}$
FAB-MS (*m/z*)	579 $(M+H)^+$, 577 $(M-H)^-$	577 $(M+H)^+$, 575 $(M-H)^-$
HRFAB-MS (*m/z*)		
Found:	579.0772 $(M+H)^+$	577.0618 $(M+H)^+$
Calcd.:	579.0776	577.0619
UV λ_{max} nm (ε, MeOH)	241 (31,600), 315 (23,400), 365 (16,500)	229 (35,800), 284 (22,600), 322 (21,000)
IR ν_{max} (KBr) cm^{-1}	3330, 1700, 1620, 1570, 1460, 1270	3260, 1684, 1626, 1567, 1467, 1288
Solubility		
Soluble:	DMSO, Tetrahydrofuran	DMSO, Tetrahydrofuran
Slightly soluble:	MeOH	MeOH
Insoluble:	H_2O, *n*-Hexane	H_2O, *n*-Hexane

Table 2. ^1H and ^{13}C NMR chemical shifts of **1** (tautomers **1a** and **1b**) and **2** in DMSO-d_6.

No.	**1a**		**1b**		**2**	
	^{13}C	^1H (J = Hz)	^{13}C	^1H (J = Hz)	^{13}C	^1H (J = Hz)
1	120.6		120.6		119.6	
2	140.7		140.8		141.2	
3	153.8		153.8		154.5	
4	102.3	6.91 (1H, s)	102.3	6.91 (1H, s)	102.3	6.93 (1H, s)
4a	150.2		150.1		150.4	
5	132.4		132.4		133.4	
6	138.1		139.1		132.5	
7	129.9		134.9		136.2	
8	125.8	7.91 (1H, brs)	127.7	8.52 (1H, s)	126.3	8.53 (1H, s)
8a	118.5		118.2		120.3	
9	172.8		172.7		172.5	
9a	110.1		110.1		109.9	
10a	152.2		152.2		152.1	
11	167.5		167.6		167.5	
12	202.7		202.9		201.1	
13	32.4	2.69 (3H, s)	32.4	2.67 (3H, s)	32.1	2.55 (3H, s)
14	16.5	2.28 (3H, brs)	17.0	2.28 (3H, s)	199.1	
15	172.7		199.1		29.1	2.53 (3H, s)
2'	65.7	4.68 (2H, brs)	68.0	4.61 (1H, dd, 11.6, 9.2) 4.71 (1H, dd, 11.6, 4.9)	152.7	8.17 (1H, s)
3'	104.2		56.2	5.02 (1H, dd, 9.2, 4.9)	120.9	
4'	183.7		186.3		172.9	
4'a	110.1		108.8		112.4	
5'	120.6		122.2		119.8	
6'	137.6		137.6		141.7	
7'	154.5		154.5		152.7	
8'	102.3	6.40 (1H, s)	102.3	6.40 (1H, s)	102.3	6.96 (1H, s)
8a'	156.3		156.3		150.8	
9'	167.5		167.6		167.4	
3-OH		9.30[a] (1H, br s)		ND[c]		9.52[d] (1H, br s)
7'-OH		8.70[a] (1H, br s)		ND		9.52[d] (1H, br s)
2-OH		11.61[b] (1H, br s)		ND		11.53[e] (1H, br s)
6'-OH		11.22[b] (1H, br s)		ND		11.53[e] (1H, br s)
11,9'-OH		12.71 (2H, br s)		ND		12.62 (2H, br s)
15-OH		ND				

[a,b] Assignments may be interchanged. [c] Not determined because signals were too broad to determine chemical shifts. [d,e] Overlapped.

Fig. 4. ^{1}H-^{1}H COSY and HMBC correlations observed in **1a**, **1b** and **2**.



two hydrogen atoms. The UV spectrum and IR absorption bands (1620 and 1570 cm^{-1} for γ-pyrone) suggested the presence of a xanthone chromophore. The ^{1}H and ^{13}C NMR spectral data of **1** in comparison with **2** are summarized in Table 2. These NMR spectral data indicated that **1** existed as a mixture of two tautomers **1a** and **1b** in DMSO-d_6, with **1a** being the preferred form. The ratio was approximately 4 : 1 by the integration value in the ^{1}H NMR spectrum.

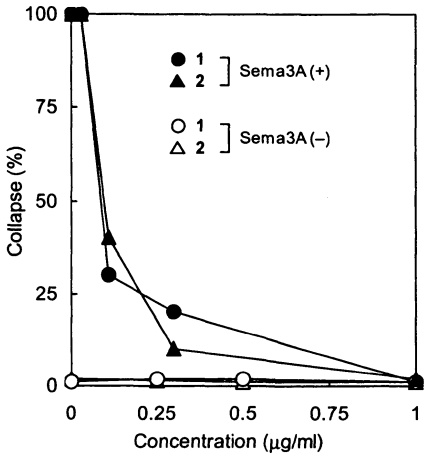
The ^{1}H and ^{13}C NMR spectra of **1b** were very similar to those of **2**, except that **1b** showed signals for a methine group (C-3′; δ_H 5.02, δ_C 56.2) and a methylene group (C-2′; δ_H 4.61, 4.71, δ_C 68.0) in place of one of the aromatic methine groups and one of the quaternary carbons observed in **2**. Analysis of the COSY and HMBC spectra of **1b** revealed two partial structures **I** and **II** (Fig. 4). The partial structure **I** was assigned on the basis of ^{1}H and ^{13}C chemical shifts and the HMBC correlations analogous to those observed for the two aromatic protons at H-4 and H-8 in **2**. The aryl methyl group (δ_H 2.28, δ_C 17.0) was located at C-6 on the basis of the HMBC correlations of H_3-14 with C-5, C-6 and C-7 and of H-8 with C-6. The HMBC correlations of H_3-13 with C-5 and C-12 and of H-8 with C-15 placed the ketone carbon (C-15; δ_C 199.1) at the *ortho* position to H-8 and the aryl methyl group at the *ortho* position to both the acetyl group and C-15. The partial structure **II** was identified through the proton spin network of H_2-2′ (δ_H 4.61, 4.71) and H-3′ (δ_H 5.02) and HMBC correlations of H-8′ with C-4′a, C-6′, C-7′, C-8′a and C-4′ (four-bond coupling) and of H_2-2′ (δ_H 4.61, 4.71) with C-3′, C-4′ and C-8′a and of H-3′ (δ_H 5.02) with C-2′ and C-4′. The cross peaks from H-2′ and H-3′ to the ketone carbon (C-15; δ_C 199.1) indicated the connection of C-3′ in the partial structure **II** to C-15 in the partial structure **I**. The assignments of remaining three quaternary carbons (C-1, C-8a and C-5′) and two carboxyl carbons (C-11 and C-9′) were established by comparing the ^{13}C NMR chemical shifts with those of **2**. Taken together, the structure of **1b** was determined as shown in Fig. 4.

The ^{1}H and ^{13}C NMR spectra and HMBC correlations of **1a** were almost identical to **1b**, but significant differences in chemical shift were observed at C-15 (**1b**: 199.1 ppm; **1a**: 172.7 ppm), C-3′ (**1b**: 56.2 ppm; **1a**: 104.2 ppm) and H-3′ (**1b**: 5.02 ppm; **1a**: absent). These data indicated that the 1,3-diketone moiety in **1b** was enolized in **1a**. The ^{13}C NMR chemical shifts for C-15 (δ_C 172.7) and C-4′ (δ_C 183.7) and the HMBC correlations of H-8 with C-15 and of H-8′ with C-4′ revealed that **1a** was enolized at C-15, not C-4′. Thus **1a** was determined to be the 15-enolized tautomer of **1b**.

Semaphorin Inhibitory Activity

To evaluate semaphorin inhibitory activity, semaphorin-induced growth cone collapse assay was employed. Compounds **1** and **2** inhibited Sema3A-induced growth cone collapse dose-dependently (Fig. 5). The IC_{50} values were 0.09 and 0.1 μg/ml, respectively. In control experiments in the absence of Sema3A, they exhibited no adverse effect on the morphology of DRG cells. These indicate that the two compounds are potent and specific semaphorin inhibitors.

Fig. 5. Inhibition of Sema3A-induced growth cone collapse by xanthofulvin (**1**) and vinaxanthone (**2**).



Discussion

We have isolated **1** and **2** from the cultured broth of *Penicillium* sp. SPF-3059 as semaphorin inhibitors. Recently several protein kinase inhibitors such as lavendustin A and olomoucine have been reported to have semaphorin inhibitory activity[13,14]. Compounds **1** and **2** are structurally unrelated to these compounds.

The structure of compound **1** was established as a new compound xanthofulvin. However, xanthofulvin was first discovered by MASUBUCHI *et al.* from a strain of *Eupenicillium* as a chitin synthase II inhibitor (IC_{50} 2.2 μM) as appeared in a patent literature[15]. Compound **2** has been previously isolated from *Penicillium* spp. as inhibitors of phospholipase C (IC_{50} 5.4~44 μM)[6], CD4-anti-Leu3a binding (IC_{50} 2 μM), CD4-MHC class II binding (IC_{50} 1 μM) and antigen-induced CD4-dependent T cell proliferation (IC_{50} 1~10 μM)[16]. Compared with these reported activities, semaphorin inhibitory activity we report here is sub-μM range and stronger by several tens-fold. Detailed evaluation of semaphorin inhibitory activity of **1** *in vitro* and *in vivo* as well as its mechanism of action will be reported elsewhere[12].

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