ORGANIC LETTERS

2002
Vol. 4, No. 17
2845−2848

Azaspirene: A Novel Angiogenesis Inhibitor Containing a 1-Oxa-7-azaspiro[4.4]non-2-ene-4,6-dione Skeleton Produced by the Fungus *Neosartorya* sp.

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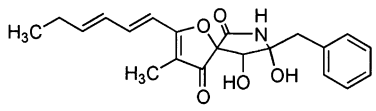
Received May 22, 2002

ABSTRACT



azaspirene

Azaspirene isolated from the fungus *Neosartorya* sp. is a novel angiogenesis inhibitor with a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton. Azaspirene inhibits the endothelial migration induced by vascular endothelial growth factor (ED_{100} = 27.1 μM).

Angiogenesis is recognized as a critical process in the growth and metastasis of tumor cells and many pathological conditions.[1] Accordingly, effective inhibition of this process should be a promising way to treat angiogenesis-related diseases, including cancer and rheumatoid arthritis. In this regard, we have commenced a screening program to identify and develop new angiogenesis inhibitors. Recently, we reported the identification of a novel angiogenesis inhibitor with a pentaketide dimer structure, epoxyquinol A, isolated from an uncharacterized fungus.[2] In the course of our continuous screening for new angiogenesis inhibitors from microbial metabolites, we discovered that the fungus *Neosartorya* sp. isolated from a soil sample produced a new angiogenesis inhibitor designated as azaspirene (**1**), as shown in Figure 1. The structure of **1** was determined by detailed NMR experiments to have a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton. Although this structural frame is similar to that of pseurotins and synerazol (**3**), **1** has unique characteristics such as the presence of a benzyl group instead of benzoyl, and three conjugated double bonds coupled to the carbonyl group and a vicinal diol. The structure of **1** is also significant with respect to the biosynthesis of this series of compounds. The total synthesis of pseurotins has never been achieved despite several reports regarding their synthetic studies.[3] The fact that **1** lacks an oxygen atom at the benzyl position suggests a more convenient route for the synthesis of this series of compounds. We report herein the isolation, structural elucidation, and biological activity of **1**.

A slant culture of the fungal strain *Neosartorya* sp. was inoculated into three 500 mL flasks containing 70 mL of

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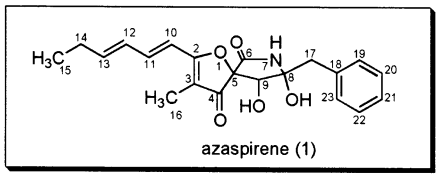
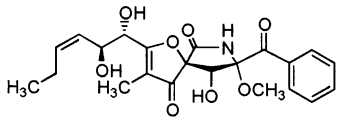
10.1021/ol020104+ CCC: $22.00
Published on Web 07/24/2002





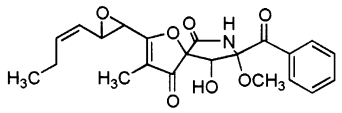


Figure 1. Structures of azaspirene (**1**), pseurotin A (**2**), and synerazol (**3**).

glucose 1%, soluble starch 2%, soybean meal 1.5%, malt extract 0.5%, vegetable extract 10%, and potato dextrose 2.5%, KH_2PO_4, $MgSO_4 \cdot 7H_2O$. The flasks were shaken on a rotary shaker (150 rpm) at 28 °C for 72 h. This preculture (210 mL) was transferred into a 30 L jar fermenter containing 15 L of the same medium. The fermentation was carried out at 28 °C, with a stirring speed of 350 rpm and an aeration rate of 10 L/min for 72 h. The culture broth (15 L) was then centrifuged to separate the supernatant and the mycelium. The mycelium was extracted with acetone and filtrated and the filtrate concentrated in vacuo to remove the acetone. The pH of the resulting water solution was adjusted to pH 7.3, extracted with ethyl acetate, and evaporated to dryness to afford a brown oil (4.5 g). This oily substance was chromatographed on a silica gel column and eluted stepwise with a mixture of $CHCl_3$−MeOH (0−50% MeOH in $CHCl_3$). The active fraction eluted with a mixture of 50:1 $CHCl_3$−MeOH was collected and concentrated in vacuo. The residue was chromatographed using reverse-phase HPLC (PEGASIL ODS, 20 i.d. × 250 mm, Senshu Scientific Co., Ltd., Tokyo) with a linear gradient solvent system of CH_3CN−water (from 20:80 to 0:100 for 30 min) at a flow rate of 9.0 mL/min to give a yellow powder. This yellow powder was recrystallized in *n*-hexanes−EtOAc to produce 83.5 mg of azaspirene (**1**).[4]

The molecular formula of azaspirene (**1**) was established to be $C_{21}H_{23}NO_5$ by high-resolution FAB-MS (*m*/*z* 370.1683 $(M + H)^+$, +2.9 mmu error), which was consistent with data from ^1H and ^{13}C NMR spectra. In the IR spectrum, absorption bands at 3570, 1735, 1715, 1705, and 1675 cm^{-1} indicated the presence of a hydroxyl, an amide, and an unsaturated carbonyl group. In the ^1H NMR spectrum measured in DMSO-d_6, three exchangeable protons were observed in the downfield region at 5.87, 6.19, and 9.52 ppm due to the presence of hydroxyl or amide protons that were quenched by the addition of D_2O. Four olefinic methine protons at 6.34 (dd, 10.5, 15.2), 6.42 (dt, 15.2, 6.2), 6.60 (d, 15.2), and 7.16 (dd, 10.5, 15.2) ppm appeared in the downfield region in addition to five aromatic protons at 7.29 (1H, dd, 1.2, 6.7), 7.34 (2H, dd, 6.7, 7.6), and 7.36 ppm (2H, dd, 1.2, 7.6). Furthermore, one oxygenated methine proton at 4.09 ppm (d, 5.4) was observed together with two methylene protons at 2.18 (2H, m), 2.94 (1H, d, 14.0), and 3.01 (1H, d, 14.0) ppm and two methyl protons at 0.99 and 1.68 ppm. The ^{13}C NMR and DEPT spectra revealed the presence of 21 carbons, which were classified into two methyls, two methylenes, one sp^3 methine, nine sp^2 methines, two sp^3 quaternary carbons, two sp^2 quaternary carbons, one oxygenated sp^2 quaternary carbon, and two carbonyl carbons. The PFG-HMQC spectrum revealed all of the one-bond $^1H-^{13}C$ connectivities (Table 1). Four partial structures A−D were established predominantly from the PFG-DQFCOSY and PFG-HMBC experiments, as shown in Figure 2. A proton spin system from 10-H to 15-H through 11-H, 12-H, 13-H, and 14-H was observed in PFG-DQFCOSY. In addition, long-range couplings in PFG-HMBC from 14-H and 15-H to C-13, 11-H and 13-H to C-12, and 11-H and 12-H to C-10 revealed a conjugated diene moiety [A]. The geometry of double bonds

Table 1. ^{13}C (125 MHz) and ^1H (500 MHz) NMR Data for Azaspirene (**1**) in DMSO-d_6

position	^{13}C (multi)[a,b]	^1H (multi, *J* (Hz))[a]
1		
2	181.46 s	
3	110.25 s	
4	198.93 s	
5	93.54 s	
6	164.80 s	
7		
8	85.36 s	
9	71.09 d	4.09 (d, 5.4)
10	115.08 d	6.60 (d, 15.2)
11	140.69 d	7.16 (dd, 10.5, 15.2)
12	128.38 d	6.34 (dd, 10.5, 15.2)
13	147.47 d	6.42 (dt, 15.2, 6.2)
14	25.59 t	2.18 (m)
15	12.61 q	0.99 (d, 7.3)
16	5.22 q	1.68 (s)
17	40.43 t	2.94 (d, 14.0)
		3.01 (d, 14.0)
18	135.80 s	
19,23	130.78 d	7.36 (dd, 1.2, 7.6)
20,22	128.13 d	7.34 (dd, 6.7, 7.6)
21	126.83 d	7.29 (dd, 1.2, 6.7)
8-OH		5.87 (br s)
9-OH		6.19 (d, 5.4)
NH		9.52 (br s)

[a] ^{13}C and ^1H NMR chemical shifts are given in parts per million with 39.50 and 2.49 ppm of DMSO as internal references, respectively. [b] Multiplicity was determined by DEPT experiment.

(4) Azaspirene (**1**) was obtained as a pale yellow powder, soluble in MeOH and DMSO but not in H_2O. The R_f value in the solvent system 40:1 $CHCl_3$−MeOH was 0.34 on a silica gel TLC (Merck 60 F_{254}): mp 166−167 °C; $[\alpha]_D^{25}$ −204.4° (*c* 0.158, MeOH); IR (KBr) ν_{max} 3570, 2975, 1735, 1715, 1705, 1675, 1610, 1410, 1135, 700 cm^{-1}; UV (MeOH) λ_{max} nm (ϵ) 231 (5900), 341 (27860).

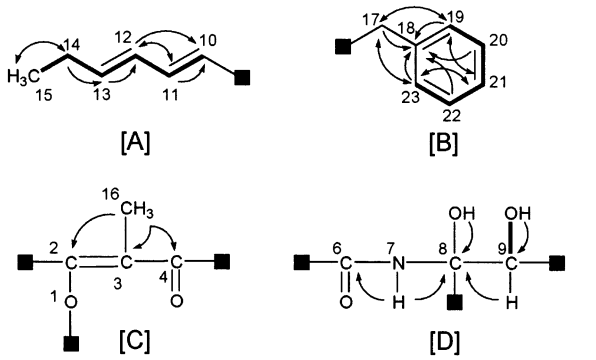


Figure 2. Partial structures [A]−[D] of azaspirene (**1**). Key correlations in PFG-DQFCOSY and PFG-HMBC spectra in azaspirene (**1**). (Bold lines show proton spin networks, and arrows show $^{1}H-^{13}C$ long-range correlations.)

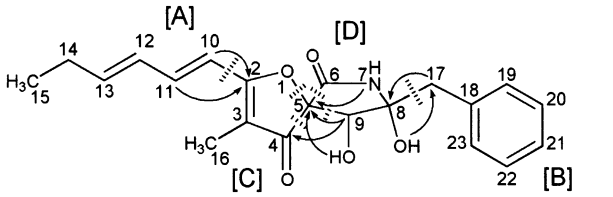


Figure 3. Connectivities among the partial structures in azaspirene (**1**). Arrows show key $^{1}H-^{13}C$ long-range correlations in PFG-HMBC.

at C-10 and C-12 was determined to be of the (*E*)-configuration on the basis of the large vicinal coupling constants ($J_{10-11} = 15.2$ and $J_{12-13} = 15.2$ Hz, respectively). This was supported by the significant NOEs between 10-H and 12-H as well as between 11-H and 13-H. The $^{1}H-^{1}H$ spin couplings among five aromatic methine protons (from 19-H to 23-H) as well as the long-range couplings from methylene protons at 2.94 and 3.01 ppm to sp^2 carbon atoms C-18, C-19, and C-23 confirmed the presence of a benzyl group [B]. The singlet methyl proton H-16 at 1.68 ppm displayed a long-range coupling to quaternary sp^2 carbons C-2 (δ 181.46) and C-3 (δ 110.25), and carbonyl carbon C-4 (δ 198.93), suggesting the presence of an α,β-unsaturated carbonyl moiety [C]. The chemical shifts of C-2 at 181.46 ppm were assigned to the oxygen-attached olefinic sp^2 carbon, and both chemical shifts at C-2 and C-3 indicated the presence of a Δ^2-furenidone-(4) system because of the strong deshielding effect of the β-atom toward the C-4 carbonyl group.[5]

As well as the structure of [A]−[C], four hydrogen, four carbon, three oxygen, and one nitrogen atoms are also present in compound **1**. Since **1** shows no basic character, the nitrogen atom should be present in an amide group. The sp^3 methine carbon at 71.09 ppm is assignable to the oxygen-attached carbon. The chemical shift of the sp^3 quaternary carbon at 85.36 ppm suggests that this carbon should link to both oxygen and nitrogen atoms. The $^{1}H-^{13}C$ couplings from a broad singlet at 5.87 ppm and a doublet at 4.09 ppm ($J = 5.4$ Hz) of exchangeable protons confirmed two free hydroxyls attached to C-8 and C-9, respectively. In addition, the chemical shift at C-8 as well as the long-range couplings from an amide proton to C-8 and the carbonyl carbon C-6 and from 9-H to C-8 established the partial structure [D]. The molecular formula of **1** indicates the index of hydrogen deficiency was 11, 9 of which account for the structure [A]−[D]. The remaining molecule was one sp^3 quaternary carbon at 93.54 ppm, and this has to be a spiro carbon in order to account for the remaining hydrogen deficiency. In fact, the chemical shift of C-5 at 93.54 ppm indicates that the oxygen-attached carbon atom. 7-NH, 9-H, and 9-OH displayed long-range coupling to C-5 (Figure 3). In addition, the long-range coupling from 9-H to C-4 established the 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton, which was supported by the carbon chemical shifts at C-2 and C-3 in the Δ^2-furenidone-(4) system. Connection between structures [A] and [C] was confirmed by the long-range couplings from 10-H and 11-H to C-2. The position of the benzyl group was deduced from the long-range coupling from 17-H to C-8 and from 8-OH to 17-C. Thus, the total structure of **1** was unambiguously established that as shown in Figure 1. In NOE experiments of **1**, NOEs were slightly observed between 9-H and 8-OH, whereas the significant NOEs were observed between 8-OH and 9-OH, suggesting a cis configuration of the vicinal hydroxyl groups.

We investigated the inhibitory effect of azaspirene (**1**) on VEGF-induced cell migration in human umbilical vein endothelial cells (HUVECs).[6] VEGF (12.5 ng/mL) supplemented in the lower chamber significantly stimulated cell migration from the top chamber to the lower chamber through the filters in a CHEMOTAXICELL chamber. As shown in Table 2, 10 μM SU5614, a well-known inhibitor of the VEGF receptor tyrosine kinase (VEGF-R2/KDR/Flk-1),[7,8] inhibited VEGF-induced cell migration. We found that

Table 2. Inhibition of VEGF-Induced Cell Migration by Azaspirene (**1**) in HUVECs

VEGF (12.5 ng/mL)	drugs	migrated cells (numbers/field)
−	none	3 ± 2
+	none	93 ± 14
+	**1** (8.1 μM)	68 ± 19
+	**1** (27.1 μM)	8 ± 11
+	**1** (81.3 μM)	7 ± 10
+	SU5614 (10 μM)	3 ± 3

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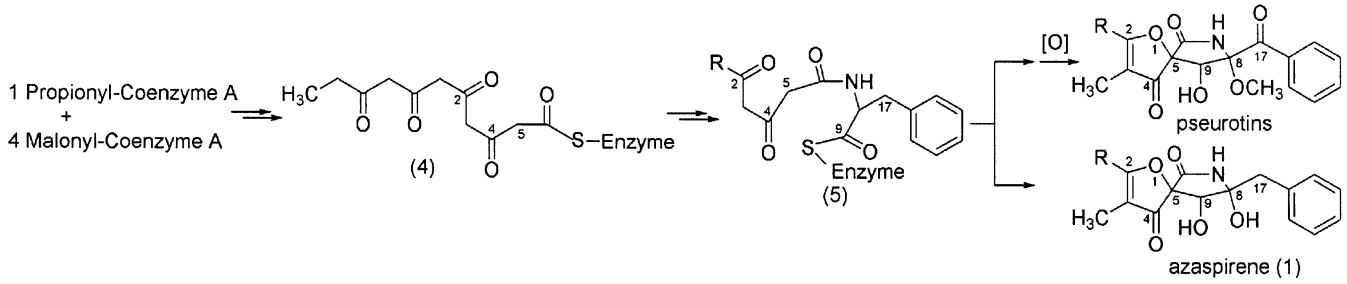
(6) Human umbilical vein endothelial cells (HUVECs) (1×10^5) suspended in Humedia-EG2 medium (KURABO, Osaka) with various concentrations of azaspirene (**1**) were added to the upper compartment of a CHEMOTAXICELL chamber (KURABO, Osaka) and incubated with

Scheme 1. Possible Biosynthetic Pathway for the Production of **1**



27.1 μM **1** completely inhibited cell migration without showing any significant cell toxicity, as estimated using a trypan blue dye exclusion assay. These results suggest that **1** is a promising small molecule angiogenesis inhibitor. Pseurotin A (**2**) and synerazol (**3**) have been reported to have neuritogenic activity in rat pheochromocytoma PC12 cells[9] and antifungal activity against *Candida albicans* and other fungi,[10] respectively. However, compounds in this class with a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton were reported to have neither the inhibitory activity in VEGF-induced intracellular signal transduction nor antiangiogenic activity.

The structure of azaspirene (**1**) is similar to pseurotin A (**2**)[9,11−13] and synerazol (**3**)[10] isolated from the fungal strains *Pseudeurotium ovalis* STOLK (*Ascomycetes*) and *Aspergillus fumigatus*, respectively. The structure of **2** including absolute stereochemistry has been determined by means of X-ray crystallographic analysis of its 12,13-dibromo derivatives.[13] The ^{13}C NMR chemical shifts consisting of 1-oxa-7-azaspiro-[4.4]non-2-ene-4,6-dione skeleton of **1** in DMSO-d_6 agree with those of **2**, indicating that **1** has a stereochemical resemblance to **2**. There have been reports that the building blocks of **2** arise from 1 unit of propionate, 4 units of malonate, 1 unit of phenylalanine, and 2 units of methionine.[14] Since the culture broth of the azaspirene (**1**)-producing strain contained pseurotin A, these compounds could share the same biosynthetic pathway. In particular, the structure of **1** with its benzyl group is the first example of this series of compounds and also indicates that oxidation at the benzyl position occurs after the incorporation of phenylalanine. A possible biosynthetic pathway from a starter unit, propionyl-coenzyme A, for the production of **1** is shown in Scheme 1. The spiro-center in compound **1** could be formed via key intermediates **4** and **5** accompanied with the incorporation of 1 unit of phenylalanine and 1 unit of methionine.

In summary, a novel angiogenesis inhibitor, azaspirene (**1**), whose structure was determined to consist of a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione moiety was isolated from the fungus *Neosartorya* sp. Detailed chemical and biological studies of **1** may lead to the development of a promising angiogenesis inhibitor with a different chemical structural frame compared that of other drugs currently under clinical trial.

Acknowledgment. This work was supported in part by a Special Grant for Promotion of Research from RIKEN and a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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HuMedia-EG2 medium containing 12.5 ng/mL of VEGF in the lower compartment for 18 h at 37 °C in a 5% CO_2 atmosphere. The filter was fixed with MeOH and stained with hematoxylin. The cells on the upper surface of the filter were removed by wiping with cotton swabs. Cells that migrated through the filter to the areas of the lower surface were counted manually under a microscope at a magnification of 100×. Values are means ± SD for triplicate samples.

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