Epoxytwinol A, a novel unique angiogenesis inhibitor with C_2 symmetry, produced by a fungus†

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We isolated a novel unique pentaketide dimer designated as epoxytwinol A from the fermentation broth of a fungus. The structure of epoxytwinol A was determined to have a new carbon skeleton with C_2 symmetry by elucidation of spectroscopic evidence. Epoxytwinol A inhibited endothelial cell migration stimulated by vascular endothelial growth factor (ED_{100} = 2.6 μM).

Vascular endothelial growth factor (VEGF) plays a central regulatory role as one of the most potent pro-angiogenic factors.[1] It regulates differentiation, migration, proliferation, capillary tube formation, and survival of endothelial cells. Thus, novel small molecules that control the VEGF-induced signal transduction pathway in endothelial cells hold great promise both as bioprobes (a biochemical tool) in the field of angiogenesis, and in drug development related to antiangiogenic therapy.[2] In our attempts to discover new inhibitors of angiogenesis from microbial metabolites,[3] we have isolated and carried out structure determination of epoxyquinols A (**1**) and B (**2**) (Fig. 1), novel unique pentaketide dimers produced by an uncharacterized fungus isolated from a soil sample.[4,5] During our continuous search for the same fungal metabolites, we have discovered a novel natural product designated as epoxytwinol A (**3**) that has a novel unique 17,19-dioxapentacyclo[8.6.2.2^{2,9}.0^{3,8}.0^{11,16}]icosa-3(8),11(16)-diene skeleton with C_2 symmetry.[6] We describe herein the isolation, spectroscopic structural elucidation, and biological properties of **3**.

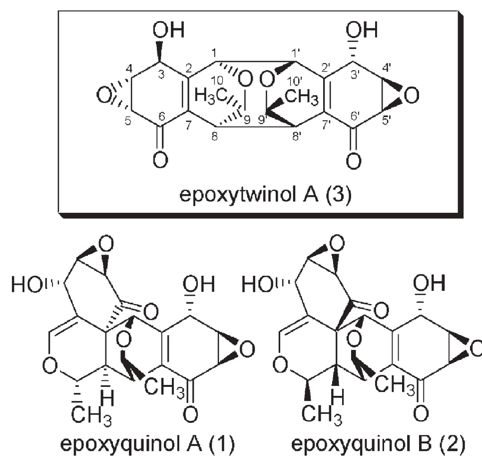

Fig. 1 Structures of epoxyquinols A (**1**) and B (**2**), and epoxytwinol A (**3**).

The producing fungal strain was cultivated in a 30-litre jar fermenter containing 18 litres of fermentation medium for 4 days at 28 °C. The broth filtrate, adjusted to pH 7.0, was extracted with the same volume of ethyl acetate. The organic extract, concentrated *in vacuo*, was applied to a silica gel column and chromatographed using 0–50% methanol in chloroform in a stepwise manner. Epoxytwinol A (**3**) was eluted with 2% methanol in chloroform, and further separated by reversed-phase HPLC. Finally, purification by thin layer chromatography, using $CHCl_3$–MeOH = 30:1 as a solvent, afforded 9 mg of **3** as colorless oil. The molecular formula of **3** was established as $C_{20}H_{20}O_8$ on the basis of high-resolution EI-MS (found: *m*/*z* 388.1173, calcd: *m*/*z* 388.1158). UV spectra showed absorption maxima (ε) at 238 (7490) and 255 (sh, 6590) nm in methanol. The IR spectra showed characteristic bands at 3450, 1670, 1620, and 1255 cm^{-1}, indicating the presence of hydroxyl and α,β-unsaturated ketone carbonyl groups.

The ^{13}C NMR spectrum of **3** in acetone-d_6 shows 10 signals, indicating the presence of a proper axis of symmetry, not a plane or point symmetry, since the compound is optically active, $[\alpha]_D^{21}$ +303.3 (*c* 0.184, in acetone). The ^{13}C NMR and DEPT spectra revealed the presence of oxygen-bearing sp^3 methine carbons (δ 53.46, 58.46, 66.20, 72.83, and 81.57), an sp^3 methine carbon (δ 39.93), sp^2 quaternary carbons (δ 132.69 and 155.59), a carbonyl carbon (δ 192.66), and a methyl carbon (δ 23.01). In the ^1H NMR spectrum measured in acetone-d_6, eight signals were observed. An exchangeable proton was observed in the downfield region at 4.36 ppm due to the presence of hydroxyl groups that were quenched by the addition of D_2O. A methyl group at 0.76 ppm (d, 6.3) was observed together with oxygenated methine protons at 3.52 (dd, 3.4, 1.0), 3.85 (dd, 3.4, 1.0), 4.19 (q, 6.3), 4.60 (br d, 9.1), and 4.79 (s) ppm as well as a methine proton at 3.21 (s) ppm. The PFG-HMQC spectrum revealed all of the one-bond ^1H–^{13}C connectivities (Table 1). The PFG-DQF-COSY spectrum confirmed the presence of two partial structures (Fig. 2-a): (i) two epoxy methine and adjacent hydroxyl methine carbons and (ii) two sequential methine protons with a terminal methyl group. The connectivities of those partial structures and the remaining sp^3 methine carbon (δ 81.57) and quaternary carbons were determined by analysis of ^1H–^{13}C long-range correlations of the PFG-HMBC spectrum (Fig. 2-b). The important long-range correlations are as follows. From the epoxy methine H-5 at 3.52 ppm and a methine proton H-8 at 3.21 ppm (β to the C-10 methyl group) to α,β-unsaturated carbonyl carbon C-6 (δ 192.66) and sp^2

† Abbreviations used: DEPT, distortionless enhancement by polarization transfer; PFG, pulse field gradient; COSY, correlated spectroscopy; DQF, double quantum filtered; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple-bond correlation; NOE, nuclear Overhauser effect.

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Table 1 ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data for epoxytwinol A (**3**) in acetone-d_6

Number	^{13}C (multiplicity)	$^{1}J_{CH}$ (Hz)^a	^{1}H (multiplicity)	J (Hz)
1, 1′	81.57 (d)	152.9	4.79 (s)	
2, 2′	155.59 (s)			
3, 3′	66.20 (d)	145.0	4.60 (br d)	9.1
4, 4′	58.46 (d)	181.5	3.85 (dd)	3.4, 1.0
5, 5′	53.46 (d)	187.5	3.52 (dd)	3.4, 1.0
6, 6′	192.66 (s)			
7, 7′	132.69 (s)			
8, 8′	39.93 (d)	136.3	3.21 (s)	
9, 9′	72.83 (d)	148.8	4.19 (q)	6.3
10, 10′	23.01 (q)	129.0	0.76 (d)	6.3
3-OH, 3′-OH			4.36 (br d)	9.1

^{a} $^{1}J_{CH}$ values were determined by the PFG-HMQC non-decoupling method.

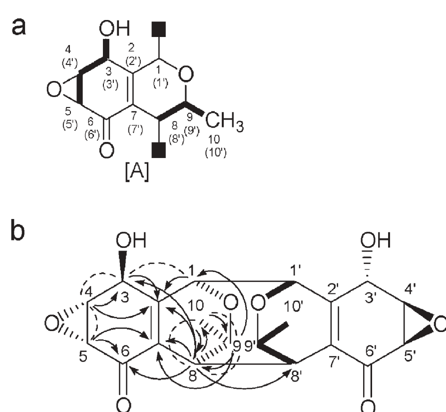


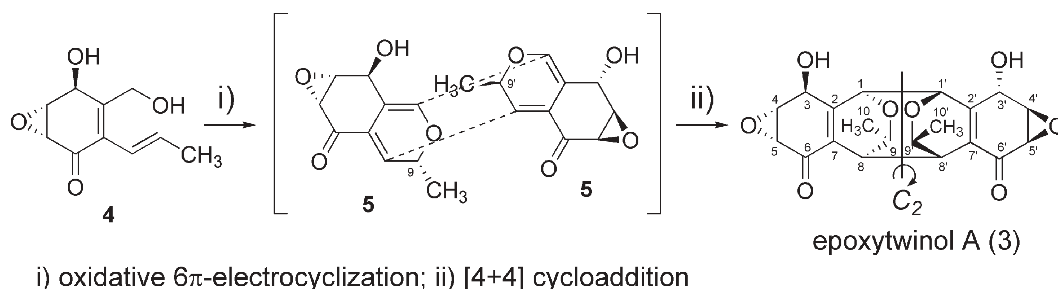
Fig. 2 (a) Partial structure [A] of epoxytwinol A (**3**). (Bold lines show significant proton spin networks in the PFG-DQF-COSY spectrum.) (b) PFG-HMBC and NOE data summary for epoxytwinol A (**3**). Data show half of the whole parts. (Arrows show ^{1}H–^{13}C long-range correlations in the PFG-HMBC spectrum, and dotted lines indicate significant NOEs.)

quaternary carbon C-7 (δ 132.69). The sp^2 carbon C-2 (δ 155.59) (β to the C-6 ketone group) has long-range correlations from H-1, H-3, H-4, and H-8 methine protons. Long-range correlations are also observed from an oxygenated methine H-9 at 4.19 ppm to C-7 and two methine carbons C-1 (δ 81.57) and C-8 (δ 39.93).

Thus, the corresponding monomer structure [A] was established as shown in Fig. 2-a. The molecular formula of **3** indicates that the index of hydrogen deficiency is 11, 5 of which account for the monomer structure [A]. Because the structure of **3** is dimeric, 10 unsaturations can be attributed to 2 units of the structure [A], leaving 1 unassigned unsaturation and indicating that epoxytwinol A (**3**) is pentacyclic. Of a few "dimeric" structures that can account for the structural features outlined above, only one, the structure of **3**, exhibits optical activity. The connectivities of C-1 and C-1′ and of C-8 and C-8′ are confirmed by the 2D non-decoupling PFG-HMQC spectrum. In this spectrum, COSY cross-peaks between H-1 and H-1′, and between H-8 and H-8′ are observed with *cis* large vicinal coupling constant values of 8.8 and 12.2 Hz, respectively. $^{2}J_{H,C}$ values between H-8 and C-8′ of 5.4 Hz are obtained from the spectrum. In the HMBC spectrum, the lack of correlation between H-1 and C-1′ is caused by the $^{2}J_{H,C}$ value of almost 0 Hz, which is confirmed by the 2D non-decoupling PFG-HMQC spectrum. The small coupling constant values of $^{3}J_{H\text{-}3,H\text{-}4}$ ($^{3}J_{H\text{-}3',H\text{-}4}$) confirm that the relative stereochemistry between hydroxyl and epoxy groups has a *trans* configuration.[4,7] Significant coupling between H-8 and H-9 in the ^{1}H NMR spectrum was not observed, indicating that the dihedral angle between H-8 and H-9 would be *ca.* 90°. Furthermore, NOE differential spectra of **3** established the relative stereochemistry (Fig. 2-b). In particular, a large NOE enhancement between H-8 and H-10, as well as between H-1 and H-3, confirmed the stereochemistry. Thus, based on these NMR data and the physico-chemical properties, the relative stereochemistry of **3** was unambiguously determined to be as shown in Fig. 1.

Epoxytwinol A (**3**) is composed of the same part that has been fused together *via* intermolecular [4+4] cycloaddition of the predicted 2*H*-pyran monomer compound **5** (Scheme 1). The precursor **5** would be generated from compound **4**, which was also isolated from the same fungal metabolites. Epoxyquinols A (**1**) and B (**2**) could be formed *via* an *endo*/*exo* Diels–Alder reaction of **5** and/or its diastereomer at the C-9 methyl group,[4,5] which is supported by the biomimetic asymmetric total synthesis of **1** and **2** *via* the oxidative dimerization of **4**.[8] There have been several reports of the intermolecular/intramolecular photo-[4+4] cycloaddition of 2-pyridone mixtures[9] or 2-pyridones with 1,3-dienes.[10] However, the reports of natural products similar to **3** with C_2 symmetry in microbial metabolites is rare. Furthermore, the synthetic methodology for [4+4] cycloaddition of polyketide compounds with 1,3-dienes has not been studied in detail. Very recently, asymmetric total synthesis of **3** has been completed, with the absolute configuration of **3** having been determined as shown in Fig. 1.[11] The mechanisms of intermolecular [4+4] cycloaddition of 2*H*-pyran monomer **5** and their derivatives are now being experimentally and theoretically investigated.

Epoxytwinol A (**3**) inhibits the human endothelial cell (HUVEC) migration induced by VEGF in a dose-dependent



Scheme 1 Possible biosynthetic pathway of epoxytwinol A (**3**).

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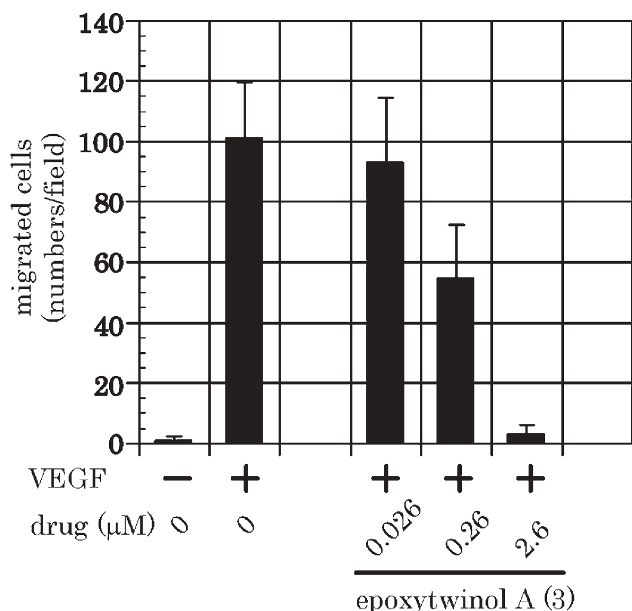

Fig. 3 Inhibitory activity by epoxytwinol A (**3**) on VEGF-induced cell migration in HUVECs.

manner, as shown in Fig. 3.[12] The ED_{100} value of **3** at 2.6 μM is more potent than that of **1** and the same as that of **2** (ED_{100} of **1** = 7.7 μM).[4,5] These results suggest that both the monomer core [A] and its bridge frame would be useful for the drug design of this series of novel angiogenesis inhibitors.

Further chemical and biological studies as well as studies of the biosynthetic pathways of **3** are now underway.

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Notes and references

1 J. Folkman, *Nat. Med.*, 1995, **1**, 27; D. Hanahan and J. Folkman, *Cell*, 1996, **86**, 353; W. Riasu, *Nature*, 1997, **386**, 671; M. Shibuya, *Cell Struct. Funct.*, 2001, **26**, 25; M. Shibuya, *Cancer Sci.*, 2003, **94**, 751.
2 N. Ferrara and K. Alitalo, *Nat. Med.*, 1999, **5**, 1359; D. Gasparini, *Drugs*, 1998, **58**, 17.
3 Y. Asami, H. Kakeya, R. Onose, A. Yoshida, H. Matsuzaki and H. Osada, *Org. Lett.*, 2002, **4**, 2845; H.-R. Ko, H. Kakeya, A. Arika, R. Onose, M. Ueki, M. Muroi, A. Takatsuki, H. Matsuzaki and H. Osada, *J. Microbiol. Biotechnol.*, 2002, **12**, 829; Y. Asami, H. Kakeya, R. Onose, Y.-H. Chang, M. Toi and H. Osada, *Tetrahedron*, 2004, **60**, 7085.
4 H. Kakeya, R. Onose, H. Koshino, A. Yoshida, K. Kobayashi, S.-I. Kageyama and H. Osada, *J. Am. Chem. Soc.*, 2002, **124**, 3496.
5 H. Kakeya, R. Onose, A. Yoshida, H. Koshino and H. Osada, *J. Antibiot.*, 2002, **55**, 829.
6 H. Osada, H. Kakeya, H. Konno and S. Kanazawa, *PCT Int. Appl.*, 2002, WO0288137. We named RKB-3564D in the PCT as epoxytwinol A (**3**).
7 T. Fex and B. Wickberg, *Acta Chem. Scand. Ser. B*, 1981, **35**, 97; R. L. Edwards, D. J. Maitland, I. J. Scowen, A. J. T. De Sousa and A. J. S. Whalley, *J. Chem. Soc., Perkin Trans 1*, 2001, 537.
8 M. Shoji, J. Yamaguchi, H. Kakeya, H. Osada and Y. Hayashi, *Angew. Chem. Int. Ed.*, 2002, **41**, 3192; C. Li, S. Bardhan, E. A. Pace, M.-C. Liang, T. D. Gilmore and J. A. Porco, Jr., *Org. Lett.*, 2002, **4**, 3267; G. Mehta and K. Islam, *Tetrahedron Lett.*, 2004, **45**, 3611; S. Kuwahara and S. Imada, *Tetrahedron Lett.*, 2005, **46**, 547.
9 E. C. Taylor and W. W. Paudler, *Tetrahedron Lett.*, 1960, **25**, 1; S. M. Sieburth, C.-H. Lin and D. Rucando, *J. Org. Chem.*, 1999, **64**, 950.
10 E. Sato, Y. Ikeda and Y. Kanaoka, *Liebigs Ann. Chem.*, 1989, 781.
11 M. Shoji, H. Imai, M. Mukaida, K. Sakai, H. Kakeya, H. Osada and Y. Hayashi, *J. Org. Chem.*, 2005, **70**, 79; C. Li and J. A. Porco, Jr., *J. Am. Chem. Soc.*, 2004, **126**, 1310.
12 VEGF-induced migration assay protocol: human umbilical vein endothelial cells (HUVEC) (1×10^5) suspended in HuMedia-EG2 medium (KURABO, Osaka) with various concentrations of **3**, were added to the upper compartment of a CHEMOTAXICELL chamber (KURABO, Osaka) and incubated with HuMedia-EG2 medium containing 12.5 ng ml^{-1} of VEGF in the lower compartment for 18 hours 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.