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NOTE

2754A: a new inhibitor of VEGFR-2 isolated from the *Streptomyces* sp. 2754

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Angiogenesis has an important role in solid tumor growth and tumor metastasis. VEGF functions as a highly specific mitogen directly involving in the process of angiogenesis, which induces endothelial cell division and proliferation and also increases vascular permeability.[1,2] VEGF performs its function through binding to special receptors. Studies have showed that VEGFR-2 is the major signal transducer for the differentiation and proliferation of endothelial cells. Consequently, inhibition of VEGFR-2 signal is an effective way to block tumor angiogenesis.[3,4] In the course of screening for VEGFR-2 inhibitor, a new compound, 2754A (**1**, Figure 1) was isolated from the *Streptomyces* sp. 2754. In this paper, we report the fermentation, isolation, physico-chemical properties, structure elucidation and bioactivities of 2754A.

A stock culture of the strain *Streptomyces* sp. 2754 was maintained on yeast and malt extract with glucose International Streptomyces Projects Medium 2 agar slant consisting of 0.4% yeast extract (Beijing Aoboxing Biotechnology, Beijing, China), 1.0% malt extract (Beijing Aoboxing Biotechnology), 0.4% glucose and 1.2% agar (pH 7.1). The stock culture was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of medium consisting of 0.5% yeast extract (Beijing Aoboxing Biotechnology), 0.5% glucose, 0.5% tryptone, 0.5% beef extract, 0.4% corn steep liquor (North China Pharmaceutical Corporation, Hebei, China), 1.0% soybean extract (Beijing Comwin Pharm-Culture, Haidian, Beijing, China), 2.0% starch and 0.4% $CaCO_3$ (pH 7.1). The flask culture was incubated on a rotary shaker (250 r.p.m.) at 28 °C for 48 h. The seed culture (5 ml) was transferred into each of the 100, 500-ml Erlenmeyer flasks containing 100 ml of the same medium. The fermentation was carried out at 28 °C for 96 h on a rotary shaker (250 r.p.m.).

The fermentation broth (10 l) was filtered and the filtrate was absorbed on a column of Amberlite XAD-5 (Amberlite XAD-5 Macroporous Resin, Sigma, St Louis, MO, USA) (1 l), after washing with 4 l of water, 3 l of 30% aqueous acetone and 3 l of 50% aqueous acetone, successively, the absorbed materials were eluted with 3 l of 80% aqueous acetone. The fraction eluted with 80% aqueous acetone showed the VEGFR-2 antagonistic activity. The active fraction was concentrated in vacuo and lyophilized to obtain a crude powder (2.8 g). It was then chromatographed on a column of Sephadex LH-20 (Pharmacia, Sweden, 1.5×100 cm), developed with methanol. The first yellow fraction showing the activity was concentrated to give a yellow material (12 mg). This material containing enriched **1** was finally purified by medium-pressure liquid chromatography (Yamazen FMI-C pump, Yamazen, Japan; column: Ultra-pack ODS, 15× 300 mm, 30 per 50 μm; mobile phase: 30% aqueous acetonitrile; flow rate: 1 ml min^{-1}) to yield **1** (1.5 mg) and **1** of 14 mg was obtained from 100 l of the fermentation broth.

Compound **1** was obtained as pale yellow amorphous powder and is soluble in methanol, acetonitrile and dimethyl sulfoxide, but insoluble in *n*-hexane and water. The other physico-chemical properties of **1** are as follows: HR-ESI-MS $(M+Na)^+$ (*m/z*) 373.0829, calcd. 373.0899 for $C_{17}H_{18}O_8$; λ_{max}^{MeOH}nm (log ε) 202 (4.13), 231 (4.35) and 345 (3.73); IR ν_{max} (KBr) cm^{-1} 3422, 2951, 1709, 1656, 1605, 1578, 1266, 1037 and 758. The direct connectivity between protons and carbons was established by the heteronuclear single quantum coherence. The ^{1}H- (nuclear magnetic resonance) NMR and ^{13}C-NMR spectral data of **1** are shown in Table 1.

Three carbonyl carbon signals (δ 198.97, 193.19 and 171.49) and six aromatic carbon signals (δ 162.21, 137.00, 132.84, 124.17, 119.33 and 115.78) were readily observed by analysis of ^{13}C-NMR and DEPT of **1**. Further analysis of the six aromatic carbon signals through heteronuclear signal quantum coherence together with ^{1}H-^{1}H COSY and HMBC (Figure 2) revealed that three quaternary carbon signals at δ 132.84 (C-5a), δ 115.78 (C-9a) and δ 162.21 (C-9), and three tertiary carbon signals at δ 119.33 (C-6), δ 137.00 (C-7) and δ 124.17 (C-8) formed a 1, 2 and 3-trisubstituted benzene ring, as proton signal at δ 7.64 (1H, t, J=7.8 Hz, 7-H) was coupled with proton signals at δ 7.57 (1H, d, J=7.8 Hz, 6-H) and δ 7.27 (1H, d, J=7.8 Hz, 8-H) in ^{1}H-^{1}H COSY. Both 8-H and 6-H were long-range correlated with C-9a, and 7-H was long-range correlated with C-9 and C-5a in HMBC.

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By tracing cross peaks from the aromatic proton (6-H) in HMBC, the carbonyl carbon of δ 193.19 (C-5) was assigned. The proton signals at δ 11.61 was also readily observed and assigned to a phenolic hydroxyl proton (9-OH), according to the chemical shift of 162.21 (C-9), a phenolic hydroxyl was substituted at C-9, and according to the chemical shift of 11.61 (9-OH), it should form an intramolecular hydrogen bond with a carbonyl group,[5,6] so another carbonyl carbon of δ 198.97 (C-10) was assigned. It led to the unambiguous assignments of NMR data in the substructure I (Figure 2).

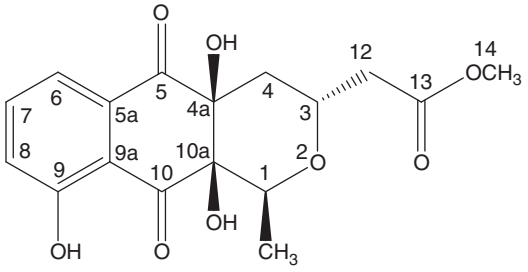

Figure 1 Structure of 2754A (**1**).

Table 1 NMR data of 2754A (1) in $CDCl_3$

Position	δ_C[a]	δ_H *(mult, J (in Hz)*[b]	*^{1}H-^{1}H COSY*	*HMBC*
1	74.47	4.32 (1H, q, J=7.2)	11	3, 4a, 11
3	62.38	4.59 (1H, m)	4, 12	
4	32.93	1.95 (1H, dd, J=2.4, 12.6)	3	4a, 10a, 12
		2.28 (1H, dd, J=15.0, 12.6)		3, 12
4a	75.95[c]			
4a-OH		6.53 (OH, s)[c]		10a[c]
5	193.19			
5a	132.84			
6	124.17	7.27 (1H, d, J=7.8)	7	5, 8, 9a
7	137.00	7.64 (1H, t, J=7.8)	8, 6	5a, 9,
8	119.33	7.57 (1H, d, J=7.8)	7	9a, 6
9	162.21			
9-OH		11.61 (OH, s)		8, 9, 9a
9a	115.78			
10	198.97			
10a	74.90[c]			
10a-OH		6.71 (OH, s)[c]		10[c]
11	14.06	1.74 (3H, d, J=7.2)	1	1, 10a
12	40.34	2.62 (1H, dd, J=15.6, 9.7)	3	4, 13
		2.66 (1H, dd, J=15.6, 3.3)	3	4, 13
13	171.49			
14	51.98	3.72 (3H, s)		13

[a]^{13}C-NMR was measured at 150 MHz.
[b]^{1}H-NMR was measured at 600 MHz.
[c]NMR data for 4a, 10a, 4a-OH and 10a-OH were measured in DMSO-d_6.

As the three-carbonyl carbons and one benzene ring in **1** accounted for seven of the nine degrees of unstauration required for the molecular formula, **1** should have another two rings. This was confirmed by tracing cross peaks in the ^{1}H-^{1}H COSY and HMBC from the two oxy-methine protons at δ 4.32(1H, q, J=7.2 Hz, 1-H), δ 4.59 (1H, m, 3-H) and the two methene protons at δ 1.95 (1H, dd, J=2.4, 12.6 Hz, 4-H), δ 2.28 (1H, dd, J=15.0, 12.6 Hz, 4-H) observed in ^{1}H-NMR. The cross peaks in ^{1}H-^{1}H COSY between the protons at 3-H and δ 2.28 (1H, dd, J=15.0, 12.6 Hz 4-H), together with the cross peaks in HMBC between 1-H and C-4a (δ 75.95), 1-H and C-3 (δ 62.38), 4-H and C-10a (δ 74.90) established the structure of the six-membered tetrahydropyrane ring. The methyl proton signals at δ 1.74 (3H, d, J=7.2 Hz, 11-CH_3) observed in ^{1}H-NMR were assigned by ^{1}H-^{1}H COSY, and long-range coupling between 11-H and C-1 (74.47), C-10a in HMBC further confirmed its substitute position in the ring. A methyl acetate side chain linked with the ring at C-3 was identified by the contiguous correlation from 12-H to 3-H in ^{1}H-^{1}H COSY and correlations from proton signals at δ 2.62 (1H, dd, J=15.6, 9.7 Hz, 12-H), δ 2.66 (1H, dd, J=15.6, 3.3 Hz, 12-H) to C-4 (δ 32.93) and C-13 (δ 171.49), δ 3.72 (3H, s, 14-H) to C-13 in HMBC. The cross peaks observed in HMBC between hydroxyl proton signals at δ 6.53 (4a-OH) and C-10a, δ 6.71 (10a-OH) and C-4a, 10a-OH and C-10, as well as 11-H and C-10a, indicated that 4a-OH and 10a-OH were linked to the tetrahydropyrane ring at C-4a and C-10a, respectively. These results indicated the presence of substructure II in **1** (Figure 2).

To satisfy nine degrees of unstauration required for the molecular formula, **1** must have another ring, and the linkage between the two substructures (I and II) was established by HMBC. The long-range coupling between 10a-OH and C-10 was observed in HMBC. The data above revealed the linkage of two substructures through 10a-C with 10-C and 4a-C with 5-C. Rotating frame overhauser effect spectroscopy (ROESY) spectrum of **1** further confirmed the result. In the ROESY experiment, the correlation peaks were not observed between 4-H (δ 1.95, 2.28) and 10a-OH, the correlation peaks were observed between 4-H (δ 1.95) and 4a-OH. Thus, the planar structure of **1** was determined.

The relative stereochemistry of **1** was determined by the analysis of ROESY (in dimethyl-d6 sulfoxide (DMSO-d_6)) spectra. In the ROESY experiment, the correlation peaks were observed between 1-H and 10a-OH, between 3-H and 11-H, 4a-OH, 4-H (δ 1.95) and between 4a-OH and 4-H (δ 1.95), 11-H. However, the correlation peaks were not observed between 10a-OH and 4-H (δ 2.28) (Figure 2). On the basis of the above results, the chemical structure of **1** was determined as shown in Figure 1. It was a new member of nanaomycins group of antibiotics and was named 2754A.

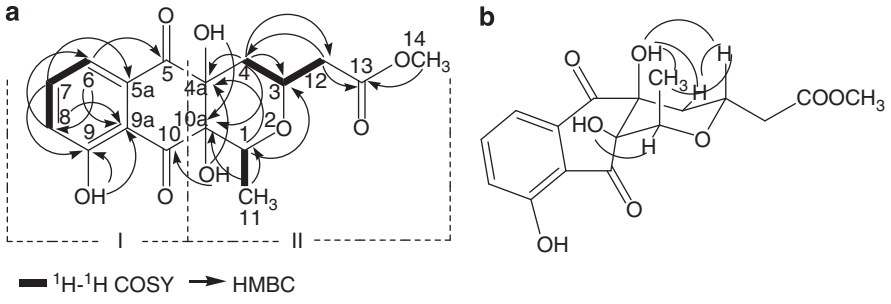

Figure 2 (**a**) Summary of ^{1}H-^{1}H COSY and selected HMBC correlations of **1**. (**b**) ROESY spectrum of **1**.

1 belongs to benzoisochromane-quinone class of antibiotics and is most closely related to nanaomycin αB produced by *Streptomyres* sp. OM-173, which possess a hydrogen rather than a hydroxyl substituent on the 4a-position of the tetrahydropyrane ring.[7] It was reported that nanaomycin-type antibiotics showed inhibitory activities against Gram-positive bacterias and fungi.[5,7–11] However, **1** has no inhibitory activity against *Candida albicans*, *Piricularia oryzae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Sarcina lutea* and *Escherichia coli* at 100 μg ml^{-1} using the paper (6 mm i.d.) disc method and does not have any cytotoxicities on the HCT-8 (human coloncancer), Bel-7402 (human hepatocarcinoma) and BGC-823 (human gastric carcinoma) cell lines at 10 μmol l^{-1} by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays,[12] but was detected for the VEGFR-2 antagonistic activity using an ELISA assay method.[13] In addition, nanaomycin αA and nanaomycin βA isolated from *Streptomyces* sp. 2754 also were detected for the VEGFR-2 antagonistic activity.

The experimental procedures were described as follows: A random co-polymer of glutamic acid and tyrosine (poly (Glu, Tyr) 4:1) (Sigma) was coated on to a 96-well immunoplate (Nunc) at 37 °C overnight (2 μg/well). After washing the plate with phosphate-buffered saline (PBS) (0.024% KH_2PO_4, 0.144% Na_2HPO_4, 0.02% KCl and 0.8% NaCl, pH 7.4) three times and blotting dry, the purified recombinant VEGFR-2-CD (the catalytic core in intracellular tyrosine kinase domain) protein was dissolved in TK buffer (50 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 1.0 mM $MnCl_2$, 0.2 mM Na_3VO_4 and 1 mM dithiothreitol). Then, 2 μl of VEGFR-2-CD (0.5 μg), 50 μl of ATP solution (12.5 μM ATP in TK buffer) and 50 μl sample (dissolved in 10% DMSO) were added to each well. After being allowed to react at 37 °C for 1 h and washing the plate five times with PBST (PBS with 0.1% Tween 20), 200 μl of 5% (w/v) bovine serum albumin in PBS was added to each well. After incubation for 1 h at 25 °C, the plate was washed five times with PBST and dried. Then, 100 μl of mouse monoclonal antibody against phosphotyrosine (PY99) (1:1000 (v/v) dilution in PBS) was added to each well, and the plate was left at 25 °C for 2 h. The plate was washed again as described above before reaction with 100 μl of secondary antibody, namely horseradish-peroxidase-conjugated horse anti-mouse polyclonal antibody IgG (H+L) (1:2000 (v/v) dilution; Zhongshan), at 25 °C for 1 h. After final washing as described above, 3, 3′, 5, 5′-tetramethylbenzidine solution (You Yi Biotechnology Company, Beijing, China) and H_2O_2 each 50 μl was added to each well, and the reaction was allowed to proceed at 25 °C for 1 h (during which the solution should turn blue), before the reaction was stopped by the addition of 100 μl of 1 M HCl. The reaction was monitored by measuring the optical density at A_{450}. DMSO (10%) was used as the negative control, The inhibition rate was calculated using the following equation:

$$\text{Inhibition rate}\,(\%) = [1 - (A_{450}/A_{450},\ \text{control})] \times 100\%.$$

The VEGFR-2 antagonistic activities of 2754A, nanaomycin αA and nanaomycin βA are shown in Figure 3.

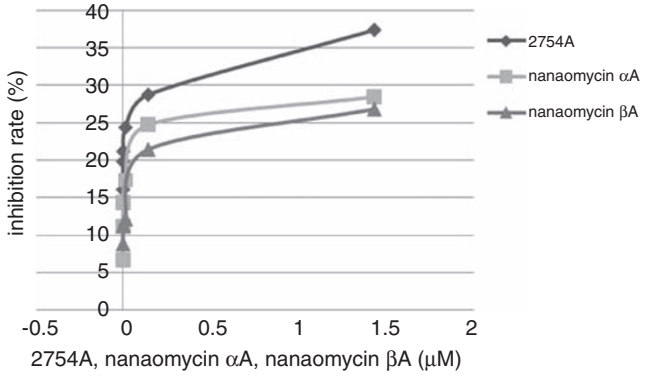

Figure 3 Inhibition of VEGFR-2/KDR by 2754A, nanaomycin αA and nanaomycin βA. The VEGFR-2 antagonistic activities were determined in an ELISA assay method, each assay point was determined in duplicate. 2754A, nanaomycin αA and nanaomycin βA were isolated from *Streptomyces* sp. 2754.

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