Sch 50673 and Sch 50676, Two Novel Antitumor Fungal Metabolites

MIN CHU*, IMBI TRUUMEES, MAHESH PATEL, CHRISTINE BLOOD, PRADIP R. DAS and MOHINDAR S. PUAR

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, U.S.A.

(Received for publication November 10, 1994)

Several biologically active keto-epoxides, containing a unique decalone with a spiro-ketal linkage through a naphthalene moiety, have been isolated from various fungi.[1~7] In the course of our continuing search for new antitumor agents, two novel minor metabolites, Sch 50673 (**1**) and Sch 50676 (**2**) were discovered from the fungus, *Nattrassia mangiferae* (ATCC 74078), in a large scale fermentation broth. This paper describes the fermentation, isolation, structure elucidation and bioactivity of these two secondary fungal metabolites.

The fungus was isolated from a substrate collected along the road between Cuilapa and Chiquimullilla, Guatemala. The area is arid and approximately 300 meters in elevation. The strain itself was isolated from dead leaves about one meter above ground[8] and identified as *N. mangiferae* which was originally characterized and described by SUTTON and DYKO.[9]

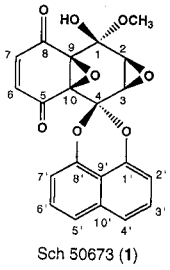
A loopful of the fungal culture was stored as a growing stock culture on potato dextrose agar slants at 4°C in sterile mineral oil. Three loopfuls of the stock culture were used to inoculate 70 ml of a germination (stage one) medium in a 250-ml Erlenmyer flask consisting of proteus peptone 0.5%, $NaCO_3$ 0.5%, KH_2PO_4 0.5%, Difco yeast extract 0.3%, cerelose 2%, soy bean grits 0.5% and Dow Corning antifoam-B 0.1% in tap water, and adjusted to pH 7.0 (±0.2). After a 7 day incubation at 24°C on a rotary shaker operating at 300 rpm, 25 ml of the stage one germination was used to inoculate 500 ml of the same germination medium in a 2-liter Erlenmyer flask. The culture was incubated as described above. After 7 days, 5 liters of stage two germination was used to inoculate 100 liters of fermentation medium consisting of neopeptone 1%, cerelose 4% and $CaCO_3$ 0.4% in tap water adjusted to pH 7.4 in a Biolafitte fermentor (LSL Biolafitte). The fermentation was run at 24°C with aeration of 25 lpm air and agitation of 350 rpm for 7 days.

The fermentation broth (100 liter) was extracted with ethyl acetate at harvest pH. The EtOAc extract was evaporated *in vacuo*. The residue was purified by silica gel flash chromatography with 2~10% EtOAc in CH_2Cl_2. Two fractions, which are active in the invasion chamber assay, were collected. The early fraction mainly contained **1**. A pure yellow crystal of **1** (100 mg) was obtained from this fraction by crystallization with MeOH - CH_2Cl_2 (1 : 1). The latter fraction was found to be a mixture of **2** and Sch 49209 (**3**) which was reported previously.[2] Further purification of the mixture was performed by semi-preparative reversed-phase HPLC (YMC-ODS 50 × 500 mm column, 60~90% linear aqueous methanol gradient in 30 minutes, 20 ml/minute flow-rate, 320 nm UV detection). Compounds **2** (50 mg) and **3** (500 mg) were precipitated with CH_2Cl_2 - MeOH (1 : 1) to obtain light-gray and white amorphous powders, respectively. The physico-chemical properties of **1** and **2** are listed in Table 1. Both compounds were soluble in $CHCl_3$ and DMSO, partially soluble in MeOH, and insoluble in hexane and H_2O.

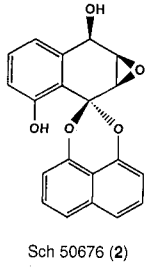
The structure determination of these two components (see Fig. 1 for structures) was based on the analysis of spectroscopic data including ^1H and ^{13}C NMR experiments (see Tables 2 and 3).

The molecular ion of **1** (*m/z* 394, M^+) was found to be a predominant peak in comparison with the protonated molecular ion $(M+H)^+$ at *m/z* 395 in FAB-MS. The molecular weight of 394 for **1** was confirmed by the observation of a $(M+Na)^+$ peak at *m/z* 417 by addition of NaCl in FAB-MS. The molecular formula of **1** was established as $C_{21}H_{14}O_3$ by analysis of high resolution FAB-MS (calcd: 394.0689 for M^+, found: 394.0681), ^1H and ^{13}C NMR spectral data. UV absorptions showed a typical profile of keto-epoxides previously reported.[2,3] IR spectroscopy revealed the existence of a hydroxyl group (br. 3459 cm^{-1}) and a quinone group (br. 1692 cm^{-1}). This was also confirmed by ^1H NMR

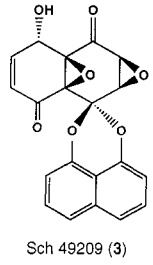
Fig. 1. Structures of **1**, **2** and **3** (relative stereochemistry).



Sch 50673 (**1**)



Sch 50676 (**2**)



Sch 49209 (**3**)

Table 1. Physico-chemical properties of Sch 50673 (**1**) and Sch 50676 (**2**).

	1	**2**
MP °C (dec)	164~166	235~238
Molecular formula	$C_{21}H_{14}O_8$	$C_{20}H_{14}O_5$
FAB-MS (*m/z*)	395 $(M+H)^+$	335 $(M+H)^+$
$[\alpha]_D^{22}$ ($CHCl_3$)	−89.8 (*c* 0.2)	−133.5 (*c* 0.2)
UV (MeOH) λ_{max} nm (ε)	225 (79,450), 298 (6,740), 313 (5,120), 327 (4,380)	227 (46,560), 299 (5,850), 313 (4,420), 328 (3,860)
IR (KBr) ν_{max} cm^{-1}	3459, 1692 (br), 1611, 1415, 1383, 1274, 1096, 1075, 1045, 819, 756	3424, 3357, 1609, 1413, 1384, 1272, 1110, 1029, 965, 820, 756

Table 2. ^{1}H NMR chemical shift assignments of Sch 50673 (**1**) and Sch 50676 (**2**)[a].

Proton	1	2
1	—	5.46 (dd, 2.7, 10.5)[c]
2	3.48 (d, 4.2)[b]	3.77 (dd, 2.7, 4.4)
3	3.57 (d, 4.2)	3.89 (d, 4.4)
5	—	—
6	6.60 (d, 15.1)	7.08 (dd, 2.4, 6.9)
7	6.64 (d, 15.1)	7.38~7.45 (m)
8	—	7.38~7.45 (m)
2′	7.59 (d, 8.0)	7.56 (d, 8.0)
3′	7.48 (t, 8.0)	7.47 (t, 8.0)
4′	7.03 (d, 8.0)	6.94 (d, 8.0)
5′	7.15 (d, 8.0)	7.16 (d, 8.0)
6′	7.52 (t, 8.0)	7.53 (t, 8.0)
7′	7.60 (d, 8.0)	7.57 (d, 8.0)
OCH_3	3.68 (s)	—
OH	5.10[d] (s)	3.51[d] (s)
Ar-OH	—	8.41[d] (br s)

[a] Recorded at 300 MHz in $CDCl_3$, chemical shifts in ppm from TMS.
[b] Multiplicity and coupling constant (Hz) in parentheses.
[c] Broad singlet after D_2O exchange.
[d] Exchangeable with D_2O.

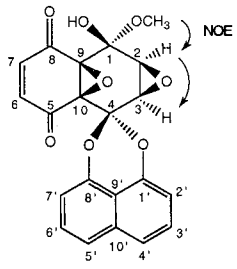
Table 3. ^{13}C NMR chemical shift assignments of **1** and **2**[a].

Carbon	1	2
1	92.58 s[b]	66.52 d
2	52.60 d	52.55 d
3	53.83 d	54.12 d
4	94.10 s	96.86 s
5	185.4 s	156.8 s
6	135.3 d	119.0 d
7	137.4 d	130.6 d
8	193.8 s	119.3 d
9	63.33 s	134.3 s
10	63.49 s	118.7 s
1′	145.2 s	147.5 s
2′	121.4 d	121.1 d
3′	127.5 d	127.6 d
4′	109.3 d	109.2 d
5′	110.0 d	110.0 d
6′	127.9 d	127.9 d
7′	121.5 d	121.2 d
8′	145.4 s	147.6 s
9′	112.1 s	113.0 s
10′	134.4 s	132.2 s
OCH_3	49.73 q	—

[a] Recorded at 75 MHz in $CDCl_3$, chemical shifts in ppm from TMS.
[b] Multiplicity was determined by DEPT data.

spectrum showing a D_2O-exchangeable singlet OH at δ 5.10 and an AB quartet of quinone double bond protons at δ 6.62, and ^{13}C NMR data showing two 1,4-quinone carbonyl carbons at δ 185.4 and 193.8 and two olefinic carbons at δ 135.3 and 137.4. Two oxygenated quaternary carbons at δ 63.33 and 63.49 were assigned as a tetra-substituted epoxide group in the middle of the decalone ring system. Two oxygenated methine carbons at δ 52.60 and 53.83 in ^{13}C NMR as well as two methine

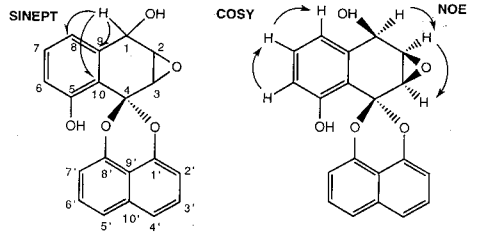
Fig. 2. NOE experiment for **1**.



proton doublets with coupling constant J=4.2 Hz at δ 3.48 and 3.57 in ^{1}H NMR indicated a *cis*-1,2 disubstituted epoxide functional group. Both ^{1}H and ^{13}C NMR displayed six proton signals at δ 7.03~7.60 and ten aromatic carbon signals at δ 109.3~145.4 indicating a 1,8-disubstituted naphthalene moiety. The assignments of these naphthalene carbons and protons were accomplished by direct comparison to the major component **3** which was fully characterized based on detailed NMR and X-ray studies.[2] Two ketal carbons were observed in the ^{13}C NMR at δ 94.10 and 92.58 representing a ketal linked to naphthalene and a methylated hemiketal, respectively. The assignment of an oxy-methyl carbon at δ 49.73 and a corresponding methoxyl proton singlet at δ 3.68 supported the structure of the hemiketal functional group. The stereochemistry of **1** was determined based on a direct correlation with the major component **3**.[2] The relative stereochemistry at the 1-position was established on the basis of an NOE experiment. The observation of an NOE signal of the proton at the 2-position by irradiation of methoxyl group suggested that both methoxyl group and the proton should be *cis* based on the assumption of the more stable conformation expecting from a molecular model. Therefore, the structure of **1** was proposed as shown in Fig. 2.

Compound **2** showed a protonated molecular ion m/z 335 $(M+H)^+$ in the FAB mass spectrum. The molecular weight, therefore, was determined as 334. The molecular formula, $C_{20}H_{14}O_5$, was deduced from the analysis of high resolution EI-MS (calcd: 334.2144, found: 334.2149), ^{1}H and ^{13}C NMR data. The UV profile of this compound was the same as for other components. However, IR spectroscopy showed the absence of absorption for a carbonyl functional group. This result was consistent with carbon NMR spectrum. ^{13}C NMR data also revealed the lack of two oxygenated quaternary carbon signals. Two aromatic carbons were observed at δ 118.7 and 134.3 instead. This evidence indicated that the oxygen of tetra-substituted epoxide was no longer present and a double bond was formed in the middle of bicyclic ring system. In addition, the observation of three aromatic methine carbon resonances and one oxy-aromatic quaternary carbon resonance at δ 119.0, 119.3, 130.6 and 156.8 respectively suggested a formation of a

Fig. 3. Some important COSY, SINEPT and NOE data for **2**.



phenol ring in the decalone unit. This assignment was also supported by observing three aromatic proton multiplets at δ 7.38~7.45 as well as a hydroxyl proton singlet (D_2O exchangeable) at δ 8.41 in the ^1H NMR spectrum. The presence of an ABC spin system for these three aromatic protons was further confirmed by a COSY experiment which indicated the correlations of proton-6, 7 and 8. As shown in Fig. 3, the regiochemistry of hydroxyl group on phenol ring was determined by SINEPT experiments. Two oxygenated methine carbons at δ 52.55 and 54.12 were assigned for a *cis* 1,2-disubstituted epoxide. It should be noted that one epoxide proton displayed as a doublet of doublets at δ 3.77 with coupling constants $J=2.7$ and 4.4 Hz in proton NMR. The evidence suggested that this proton was also coupled to an adjacent methine proton at δ 5.46. The methine proton was associated with a secondary hydroxyl group based on the result of D_2O exchange experiment. This was also consistent with the observation of oxy-methine carbon at δ 66.52. The remaining part of the molecule, a ketal linked to naphthalene moiety, was identical to **1**. The stereochemistry of secondary hydroxyl group was established by an NOE experiment. The presence of an NOE signal of proton-2 by irradiation of proton-1 suggested that these two protons are *cis* configuration.

Both compounds **1** and **2** were tested *in vitro* for inhibitory activity in the tumor cell invasion assay. In this assay, invasion of HT 1080 human fibrosarcoma cells through an artificial membrane was performed.[10] Polyvinyl pyrrolidone-free polycarbonate filters were coated with 100 μg of fibronectin for 1 hour, and subsequently with 500 μg of Matrigel. After drying for overnight, filters were rehydrated with serum-free DMEM and assembled in 48-microwell chemotaxis chambers. Compounds to be tested were placed in upper wells of the chambers with HT 1080 cells (3.5×10^4 cells/well). Chambers were incubated for 5 hours at 37°C and disassembled. Filters were fixed and stained with Diff-Quick reagents. The filters were then mounted between two glass slides, and tumor cells that have invaded the Matrigel barrier in each microwell were analyzed with a Cue-2 Image Analysis System. Degree of invasion was expressed as percent of surface area of filters covered by cells. The IC_{50} values of **1** and **2** were found to be 6.2 and 2.8 μM, respectively, in the invasion chamber assay. These compounds were $<10\%$ cytotoxic at 25 μM over the course of the assay, as determined by calcein AM fluorescence.

Acknowledgments

The authors are grateful to Mr. F. GENTILE for fermentation work, Mr. P. BARTNER and Mr. Y. H. ING for MS data, and Ms. J. CILIBERTI for cytotoxicity data.

References

1) OGISHI, H.; N. CHIBA, T. MIKAWA, T. SAKAKI, S. MIYAJI & M. SEZAKI: Novel Antibiotic MK 3018 Substance—Useful as Antimicrobial Agent. JP 01294686, Nov. 28, 1988
2) CHU, M.; I. TRUUMEES, M. G. PATEL, V. P. GULLO, M. S. PUAR & A. T. MCPHAIL: Structure of Sch 49209: A novel antitumor agent from the fungus *Nattrassia mangiferae*. J. Org. Chem. 59: 1222~1223, 1994
3) SCHLINGMANN, G.; R. R. WEST, L. MILNE, C. J. PEARCE & G. T. CARTER: Diepoxins, novel fungal metabolites with antibiotic activity. Tetrahedron Lett. 34: 7225~7228, 1993
4) CHU, M.; I. TRUUMEES, M. G. PATEL, V. P. GULLO, C. BLOOD, I. KING, J.-K. PAI & M. S. PUAR: A novel class of antitumor metabolites from the fungus *N. mangiferae*. Tetrahedron Lett. 35: 1343~1346, 1994
5) THIERGARDT, R.; P. HUG, G. RIHS & H. H. PETER: Cladospirone bisepoxide, a novel fungal metabolite structure determination. Tetrahedron Lett. 35: 1043~1046, 1994
6) CHU, M.; I. TRUUMEES, M. G. PATEL, V. P. GULLO, J.-K. PAI, P. R. DAS & M. S. PUAR: Two new phospholipase D inhibitors, Sch 49211 and Sch 49212, Produced by the Fungus *N. mangiferae*. Bio. Med. Chem. Lett. 4: 1539~1542, 1994
7) PAI, J.-K.; E. A. DOBEK-FRANK, C. BLOOD & M. CHU: Novel keto-epoxides block phospholipase D. Activation and tumor cell invasion. Anticancer Drug Design. 9: 363~372, 1994
8) The fungus was supplied by Dr. B. KATZ from MYCO search Lab
9) SUTTON, B. C. & B. J. DYKO: Revision of *Hendersonula*. Mycol. Res. 93: 466~448, 1989
10) ALBINI, A.; Y. IWOMOTO, H. K. KLEINMAN, G. R. MARTIN, S. A. AARONSON, J. M. KOZLOWSKI & R. N. MCEWAN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res. 47: 3239~3245, 1987