Pergamon

0031-9422(94)00620-2

Phytochemistry, Vol. 38, No. 3, pp. 595–597, 1995

0031-9422/95 $9.50 + 0.00

ASTEROMINE, A BIOACTIVE SECONDARY METABOLITE FROM A STRAIN OF *MYCOSPHAERELLA ASTEROMA**

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(*Received in revised form* 7 *July* 1994)

Key Word Index—*Mycosphaerella asteroma*; toxin; 6,6′-binaphtho-α-pyrone; ^{13}C NMR.

Abstract—The structure of asteromine, a new 6,6′-binaphtho-α-pyrone metabolite, has been elucidated by means of NMR spectroscopy. In bioassays, asteromine exhibits phytotoxic effects on *Cucumis sativus*, a low antibacterial and antifungal activity, and is lethal to *Artemia salina* shrimps at 10^{-4} M.

INTRODUCTION

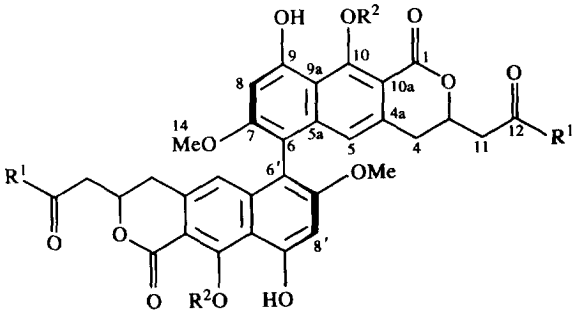
The genus *Mycosphaerella* Johanson includes many phytopathogenic species; most of them are necrotrophic pathogens either on agricultural crops or forest trees. Structural studies on the toxic principles of some species of this group have been the subject of numerous reports [1] which include our own on *M. rosigena*, *M. ligulicola*, *M. laricina*, *M. rubella* and *M. lethalis* [2].

Further investigations on a strain of *M. asteroma* obtained from *Polygonatum odoratum* led to the isolation of small amounts of a new metabolite (**1**) for which we propose the name of asteromine. In this paper, we present the determination of the structure of the compound by means of NMR studies on its methylester derivatives (**2**).

RESULTS AND DISCUSSION

The main metabolite purified from agar cultures of *M. asteroma* (CBS 266.53) is asteromine (**1**), a yellow solid, mp 210–215°; $[\alpha]_D$ −88° (MeOH; *c* 0.1). The IR spectrum (KBr) showed hydroxyl and carbonyl bands at 3400, 1740 and 1660 cm^{-1}. The molecular formula ($C_{34}H_{30}O_{14}$) was established by elemental analysis and by FABMS ($m/z = 663$ $[MH]^+$).

The structure of **1** was deduced mainly from NMR analyses carried out on its methyl derivative (**2**) which analysed for $C_{36}H_{34}O_{14}$ (FABMS $m/z = 691$ $[MH]^+$). The ^{13}C NMR spectrum of **2** (Table 1) showed 18 signals which were assigned to three methyl, two methylene, three methine and 10 quaternary carbon atoms. The ^{1}H NMR spectrum (Table 2) indicated the presence of 17 protons attributable to one aromatic hydroxyl group, two aromatic protons, three OMe groups and a



	R¹	R²
1	OH;	Me; asteromine
2	^{13}OMe;	^{15}Me
3	OMe;	H; viriditoxin

Block

–C(4)H$_2$–C(3)H–C(11)H$_2$ grouping. Moreover, the chemical shift values exhibited by H$_2$-4 and H$_2$-11, and by H-3 suggest that the corresponding carbons were linked to sp^2 carbons and to an ester oxygen, respectively.

These findings, together with the results of a series of selective low-power ^{13}C–{^{1}H} decoupling experiments, pointed to the presence in the molecule of the part structure of **2** depicted in Fig. 1. In fact, the C-9 hydroxyl proton presented ^2J and ^3J with C-9, C-8 and C-9a, and both the C-5 and C-8 protons showed ^3J with C-6 and C-9a. The allocation of the above grouping at C-4 followed from the ^2J and ^3J observed between the C-4 methylene protons and C-4a, C-5 and C-10a, while the ^2J and ^3J observed between the carbonyl C-12 carbon and H$_2$-11, H-3 and H$_3$-13 not only indicated that C-13 was part of a carbomethoxy group, but also that it was linked to C-11.

*Part 48 in the series 'Secondary Mould Metabolites'. For Part 47 see Arnone, A., Cardillo, R., Nasini, G., Meille, S. V. and Tolazzi, M. (1994) *J. Chem. Soc. Perkin Trans. I* 2165.

Table 1. ^{13}C NMR spectral data for **2** ($CDCl_3$)

C	δ_c	^1J(C, H)/Hz	^1J(C, H)/Hz
1	162.3 (162.1)* S *br s*		
3	74.0 (74.9) D *m*	151.5	
4	34.5 (34.8) T *m*	130.5	
4a	135.1 (136.7) S *dt*		3 (H-3), 5.5 (H_2-4)
5	118.8 (119.3) D *t*	162.5	4 (H_2-4)
5a	138.4 (139.3) S *br s*		
6	109.2 (110.3) S *dd*		4.5 (H-5), 5 (H-8)
7	159.9 (160.7) S *ddq*		3 (H-8), 2 (OH-9), 4 (H_3-14)
8	99.0 (99.5) D *d*	160	8 (OH-9)
9	158.0 (158.9) S *dd*		5 (H-8), 5 (OH-9)
9a	112.2 (112.8) S *ddd*		6 (H-5), 5.5 (H-8), 3.5 (OH-9)
10	162.3 (162.8) S *brq*		4 (H_3-15)
10a	108.7 (10.0) Sdt		8 (H-5), 3.5 (H_2-4)
11	39.4 (39.8) T *m*	130	
12	170.1 (170.7) S *dtq*		3.5 (H-3), 7.5 (H_2-11), 4 (H_3-13)
13	52.1 (52.0) Q *s*	147.5	
14	56.3 (56.4) Q *s*	145	
15	62.6 (64.8) Q *s*	148	

*Values in parentheses are chemical shifts in acetone-d_6.

Table 2. ^1H NMR spectral data of **2** (acetone-d_6)

H	δ_H	*J*(H, H)	Hz
3	4.84 (4.87)*	3, 4a	3.6
4a	2.90 (2.83)	3, 4b	10.6
4b	2.86 (2.83)	3, 11a	7.1
5	6.68 (6.53)	3, 11b	5.8
8	6.89 (6.86)	4a, 5	1.0
11a	2.77 (2.87)	4b, 5	1.5
11b	2.72 (2.62)	4a, 4b	16.2
13	3.64 (3.68)	11a, 11b	16.2
14	3.78 (3.79)		
15	4.22 (4.21)		
9-OH	10.19 (10.19)		

*Values in parentheses are chemical shifts in $CDCl_3$.

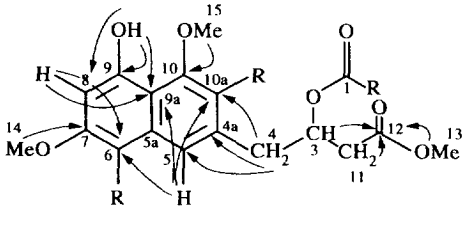


Fig. 1. Partial structure of the methyl ester of asteromine (**2**). The arrows refer to two- and three- bonds ^1H–^{13}C coupling constants selectively removed by low-power decoupling experiments.

Finally, the remaining two OMe groups were placed to the unaccounted C-7 and C-10 carbons of the naphthalene ring.

The presence in both the ^1H and ^{13}C NMR spectra of only half of the expected resonances is indicative of a symmetrical structure for **2**. Thus, C-1 must through necessity be connected to C-10a to form an α-pyrone ring and, as a consequence, the two portions of the molecule are linked via a (C-6)–(C-6′) bond.

The mutual NOEs observed between H-8 and H_3-14 (4.5 and 21%), H-8 and 9-OH (2.5 and 2.5%), 9-OH and H_3-15 (2.5 and 2.5%), and H-5 and H_2-4 (3 and 10%) gave further support to the structure of **2**.

Asteromine belongs to the restricted family of binaphtho-α-pyrone metabolites like viriditoxin (**3**), a compound toxic to mice; previous structural studies on viriditoxin [3], isolated from *Aspergillus viridinutans* and successively from several fungi [1, 4], demonstrated, erroneously, that the substance was a dimer in which the two units were linked via a (C8)-(C8′) bond. Very recently, the structure was revised [4] to a dimeric naphtho-α-pyrone C(6)-C(6′) linked. The differences between **1** and **3** can be summarized as follows: the first compound bears two OMe in place of OH groups at the C-10 and C-10′ positions and has two free carboxylic groups.

The CD spectra of **1** exhibited a strong negative, first and a positive, second Cotton curves [4]; thus, the axial chirality of the 6-6′-axis is *R*.

In the antibacterial and antifungal assays, the following microorganisms developed inhibition zones around the site of application (asteromine 100 μg disk^{-1}); diameter zone of inhibition in bracket: *Escherichia coli* (12 mm), *Bacillus subtilis* (12), *B. cereus* (12), *Ustilago maydis* (20), *Ophiostoma ulmi* (18), *Geotrichum candidum* (14).

When topically applied to detached leaves of *C. sativus*, asteromine showed phytotoxic effects, visible from

0.1 mM up under light, and from 1 mM in the dark as enlarged (5 mm) necrotic spots around application points. Controls showed only necrosis of the needle-injured cells. The same solutions, sprayed on to whole plants, caused browning of the nervations and ribs, and necrosis of the foliar edge, in comparison with control plants, but were not able to protect against infection following inoculation of the plant with the fungus *Sphaerotheca fuliginea*, the causal agent of powdery mildew of cucumber.

Moreover, asteromine exhibits a toxic effect in the *Artemia salina* shrimp assay, producing a 71% mortality at a concentration of 10^{-4} M, 33% at 10^{-5} M, 8% at 10^{-6} and 3% at 10^{-8} M.

EXPERIMENTAL

Mps uncorr.; UV: 95% EtOH; FAB-MS: 70 eV; NMR: 300.13 (^{1}H) and 75.47 (^{13}C) MHz with TMS as int. standard; Flash CC: Merck silica gel (0.040–0.063 mm); TLC: Merck HF_{254} silica gel. The purity of products was checked by TLC, NMR and MS, and deemed sufficient for the purpose of structural elucidation.

Separation and purification of asteromine (**1**). A strain of *Mycosphaerella asteroma* Lindau (CBS 266.33) supplied by the Central Bureau voor Schimmel Cultures, Baarn, was grown on malt extract–peptone–glucose–agar (MPGA, 20, 2, 20, 15 g l^{-1}) in Roux flasks (40) for 2 weeks at room temp. The agar cultures were extracted twice with EtOAc, the extract dried with Na_2SO_4 and the organic solvent evapd *in vacuo*; the crude extract (200 mg) was defatted by washing with hexane and then purified by CC on silica gel buffered with 2% H_3PO_4 and eluted with CH_2Cl_2–MeOH of increasing polarity. Asteromine (**1**) was further purified by PLC in CH_2Cl_2–MeOH–HCOOH (15:1:0.5) to obtain 10 mg of the pure compound; $R_f = 0.2$ in the same solvent.

Asteromine (**1**). Found C, 61.3; H, 4.5%; $C_{34}H_{30}O_{14}$ requires C, 61.63; H, 4.56%; UV λ_{max} nm: 220, 263, 340sh, 368sh, 380 (ε 19 470, 32 890, 6300, 8950, 8950); ^{1}H NMR (acetone-d_6): δ 2.6–3.1 (4H, *m*, H_2-4 and H_2-11), 3.63 and 3.76 (6H, *s*, H_3-14 and H_3-15), 5.02 (1H, *m*, H-3), 6.39 and 6.84 (2H, *s*, H-5 and H-8), 9.75 and 13.95 (2H, *br* signals, 9-OH and 12-OH).

Asteromine dimethyl ester (**2**). Compound **1** (50 mg) was dissolved in CH_2Cl_2–MeOH and treated with CH_2N_2–Et_2O at 0° for 10 min. Evapn of the solvent and PLC in CH_2Cl_2–MeOH (30:1) gave **2** (40 mg) as a solid mp 120–125°; $[\alpha]_D$ −289° ($CHCl_3$; *c* 0.06); CD ($CHCl_3$; *c* 25 mg ml^{-1}) 275 and 255 nm/Δε: −175.6, +108.7; FABMS *m/z* 691 $[MH]^+$, 690, 676, 660; IR ν_{max} cm^{-1}: 3350 (OH), 1745 (lactone CO), 1650 (ester CO); ^{13}C and ^{1}H NMR: Tables 1 and 2.

Biological tests. Antibacterial and antifungal activity was tested for by the agar diffusion method. Paper disks (6 mm) were soaked with 20 μl of an asteromine soln (5 mg ml^{-1} in EtOH 95%) and the solvent allowed to evaporate (100 μg disk^{-1}). The tested organisms used were *E. coli*, *B. subtilis*, *B. cereus*, *U. maydis*, *O. ulmi* and *G. candidum*. A portion (1 ml) of cell or spore suspension (10^4 ml^{-1}) was poured with 20 ml of nutrient agar Difco (bacteria) or potato dextrose agar (fungi) at 45° into Petri dishes. Phytotoxicity was first assayed by applying with a microsyringe 10 μl of asteromine soln (0.1, 1, 10 mM) to detached leaves of *C. sativus* which had been grown in a pot for 10 days in a greenhouse. Second and third enlarged leaves were utilized in the assay, and control solns were tested at the highest level of DMSO (30% in H_2O). Detached leaves were maintained in a moist chamber and exposed to continuous light (5000 lux by white cool lamp) or in the dark. Necrosis was measured after 24 hr.

A second trial for phytotoxicity was performed by spraying the solns on whole plants. After 24 hr, the effects were recorded and then the same plants were inoculated with a spore suspension of *S. fuliginea* (10^6 conidia ml^{-1}).

Toxicity tests with the 'shrimp assay' were performed in 96-well plates with 300 μl of test soln at defined concns and individuals well^{-1}. *Artemia salina* eggs were left to hatch in a brine shrimp hatcher for 24 hr in a saline soln. Individuals were transferred to 96-well plates and received asteromine in DMSO (0.3% final conc) from 10^{-4} to 10^{-8} M. The control received only diluted solvent. Mortality per cent was calcd on 360 individuals in 12 wells replicated in 2 sepd experiments.

Acknowledgements—This work was supported by Consiglio Nazionale delle Ricerche (CNR) Roma, Progetto finalizzato 'Chimica Fine II'.

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