Auranthine, a New Benzodiazepinone Metabolite of *Penicillium aurantiogriseum*

Stephanie E. Yeulet and Peter G. Mantle *
Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ
John N. Bilton, Henry S. Rzepa, and Richard N. Sheppard
Department of Chemistry, Imperial College of Science and Technology, London SW7 2AZ

A novel metabolite, auranthine, has been isolated from sporing cultures of *Penicillium aurantiogriseum* and its structure, 6,7,7a,8-tetrahydroquinazolino[3',2':1,6]pyrido[2,3-*b*][1,4]benzodiazepine-9,16-dione comprising anthranilate and glutamine moieties, has been elucidated from spectral evidence and biosynthetic reasoning.

In the course of isolating and characterising nephrotoxic[1] and other metabolites from a fungus which was originally described as *P. verrucosum var. cyclopium*[1] but which is now assigned as *P. aurantiogriseum*,[2] a new alkaloid has been found. Acetone extracts of lyophilised whole fungal cultures were resolved by column chromatography and semi-preparative reversed-phase h.p.l.c. A prominent component (2 mg/100 ml culture) was isolated and for which the name auranthine and structure (**1**) are proposed.

The pure auranthine was an amorphous white solid, insoluble in water but soluble in organic solvents, in which it had a negative specific rotation.

The 70 eV mass spectrum run on a glass probe at 200 °C exhibited the peaks shown in Table 1. Peaks at *m/z* 248, 220, 192, 124.5 and 124 are reported to be present in the mass spectrum of trypthanthrin (**2**),[3,4] with the first two losses arising by successive extrusions of carbon monoxide. Auranthine (**1**) does not show a significant ($M^+ - 28$) peak and this is in agreement with observations on the mass spectra of quinazolones[5] and benzodiazepinones.[6,7]

The distribution of doubly charged ions in the spectrum pointed to the increasing aromatic character of the generated fragments. Consequently the fragmentation of auranthine can be explained in terms of the increasing stability of the ions generated by extrusion of C_3H_3N, CH_2N, HNCO, H', two carbon monoxide molecules, and HCN from the molecular ion.

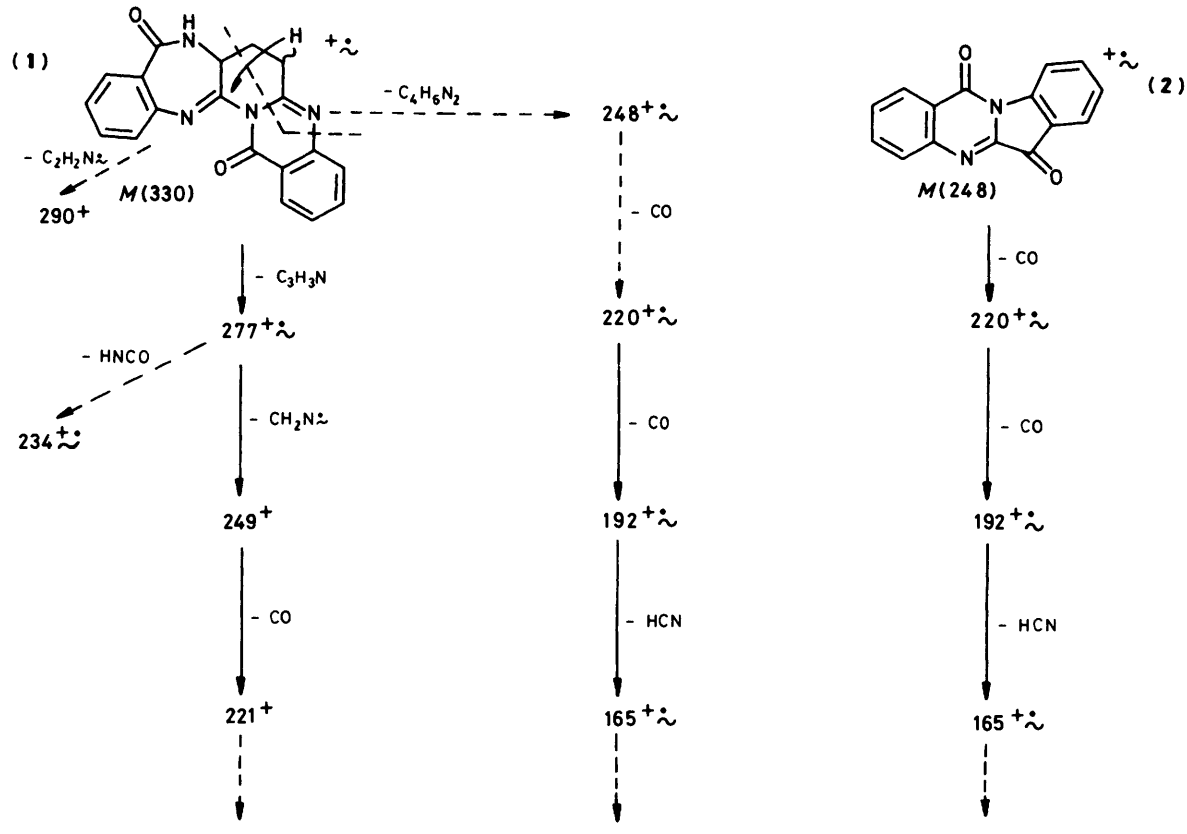
The rearrangement process involving hydrogen-atom transfer to give a loss of 53 a.m.u. from the molecular ion is an unusual feature of the spectrum and deserves further investigation. In the low-eV spectrum, peaks at *m/z* 330 and 277 are accompanied by a clearly defined metastable ion centred at 232.6 a.m.u. At slightly higher ionizing energies the peaks at *m/z* 277, 249, and 234 appear with relative abundances altered in a way consistent with the operation of the processes shown in equation (1).

$$330^+ \xrightarrow{*} 277^+ \begin{cases} \xrightarrow{*} 249^+ \\ \longrightarrow 234^+ \end{cases} \quad (1)$$

However, any metastable transition for the process $277 \longrightarrow 234$ is obscured under the broad metastable ion centred at 196.2 a.m.u. The precise mechanisms of breakdown, particularly with regard to the first two losses of C_3H_3N and CH_2N, are subject to uncertainty in the absence of labelling studies, and therefore the Scheme serves to indicate the similarity of fragmentation between tryptanthrin (**2**) and auranthine below *m/z* 249. The base peak *m/z* 249 is presumably isomeric with respect to a protonated tryptanthrin structure.

Table 1.

m/z	Rel. int. (%)	Composition	Assignment	Metastable ions (*m**)
330	53.6	$C_{19}H_{14}N_4O_2$	M^+	
290	6.5	$C_{17}H_{12}N_3O_2$	$M - CH_2CN$	
277	89.6	$C_{16}H_{11}N_3O_2$	$M - C_3H_3N$	232.6(330 ⟶ 277)
249	100	$C_{15}H_9N_2O_2$	$M - C_3H_3N - CH_2N$	223.9(277 ⟶ 249)
248	19.6	$C_{15}H_8N_2O_2$	$M - C_4H_6N_2$	
234	21.9	$C_{15}H_{10}N_2O$	$M - C_3H_3N - HNCO$	
221	9.5	$C_{14}H_9N_2O$	$M - C_3H_3N - CH_2N - CO$	196.2(249 ⟶ 221)
220	18.1	$C_{14}H_8N_2O$	$M - C_4H_6N_2 - CO$	
192	13.8	$C_{13}H_8N_2$	$M - C_4H_6N_2 - 2CO$	167.7(220 ⟶ 192)
165	5.9	$C_{12}H_7N$	$C_{13}H_8N_2 - HCN$	141.7(192 ⟶ 165)
132	12.7	C_8H_6NO		
130	19.3	C_8H_4NO		
124.5	4.7		249^{++}	
124	25.7		248^{++}	
119	11.6			
105	7.6		$C_6H_5CO^+$	
102	25.2	C_7H_4N		
92	6.7			
90	15.5			
77	20.4			
76	23.3			



Scheme.

Table 2. Coupling constants (Hz) of aromatic protons, defining two four-spin systems[a]

Spin		A′	B′	C′	D′
	Chemical shift*	8.101	7.311	7.106	6.879
A	7.938		0.582	1.560	7.907
B	7.265	0.430		8.151	1.171
C	7.007	1.659	8.231		7.219
D	6.920	7.748	1.190	7.940	

[a] Assignment of protons A—D (A′—D′) is arbitrary.
* In p.p.m. from $SiMe_4$.

Biosynthetic probing, using several ^{14}C-labelled amino acids such as are common precursors of fungal alkaloids, explored the origin of the four nitrogens of the molecule. Autoradiography of thin-layer chromatograms of extracts confirmed the significant scintillation counting evidence of the direct incorporation of L-[U-^{14}C]glutamine and [*carboxy*-^{14}C]anthranilic acid, mean percentages 0.08 and 0.30 respectively, into the alkaloid.

A ^{1}H n.m.r. spectrum reveals the presence of 8 aromatic protons, a broad doublet corresponding to an N–H proton, and resonances for five closely coupled aliphatic protons. A two-dimensional COSY spectrum showed that the aromatic protons consisted of two four-spin systems corresponding to *ortho*-disubstituted benzenes, and a closely coupled aliphatic region probably comprising an $NHCHCH_2CH_2$ fragment. With the assignment of the aromatic resonances into two four-spin systems, an iterative refinement giving the values of the chemical shifts and coupling constants was possible (Table 2). The calculated and observed spectra (Figure 1) are in excellent agreement, and the spectral parameters strongly suggest the presence of two non-equivalent anthranilate residues.

The remaining features of the structure can be assigned on the basis of the ^{13}C spectrum recorded using the *J*-modulated spin-echo technique to enable assignment of the proton multiplicities (Figure 2). This confirms the aliphatic region as comprising a CH and two CH_2 carbons, and indicates that the aromatic region contains 8 CH carbons and four quaternary carbons, corresponding to anthranilate moieties. There are also two quaternary carbons corresponding to an imine (C=N) system, and two carbonyl resonances. A fully ^{1}H-coupled ^{13}C spectrum shows the imine resonance at δ_C 145.4 to be a triplet (J ±7.2 Hz), indicating two-bond coupling to a CH_2 group, and the resonance at δ_C 153.6 to be a doublet (J ±4.0 Hz), indicative of adjacency to a CH group. The absolute values of these two-bond coupling constants are consistent with the known trend in substitution of the sp^3 carbon [*cf.* J_{CH} in H–CH(Me)–CO, ±5.7 Hz; J_{CH} in H–$C(Me)_2$–CO, ±4.1 Hz].[8] In addition, the carbonyl resonance at δ_C 160.7 is a doublet (J ±4.5 Hz), which suggests assignment of this resonance to the CO–NH group, whereas the other carbonyl resonance, at δ_C 169.7, remains a singlet.

From the spectral and biosynthetic evidence the structure (**1**), that of 6,7,7a,8-tetrahydroquinazolino[3′,2′:1,6]pyrido-[2,3-*b*][1,4]benzodiazepine-9,16-dione, and the trivial name auranthine have been assigned to the new product; the name is derived from that of the producing organism and the product's biosynthesis from anthranilic acid and, probably, glutamine.

Auranthine is a complementary addition to the small group of fungal secondary metabolites which are biosynthesised from anthranilic acid. Amongst these it is similar to cyclopenin[9] with respect to its benzodiazepine moiety, to chrysogine[10] with respect to the quinazoline moiety, and to anthglutin[11] on account of their both containing a glutamate-derived function. The closest analogy is with the novel microbial metabolite asperlicin,[12] a potent neuropeptide antagonist, the benzodiazepinone moiety of which is probably biosynthesised from two

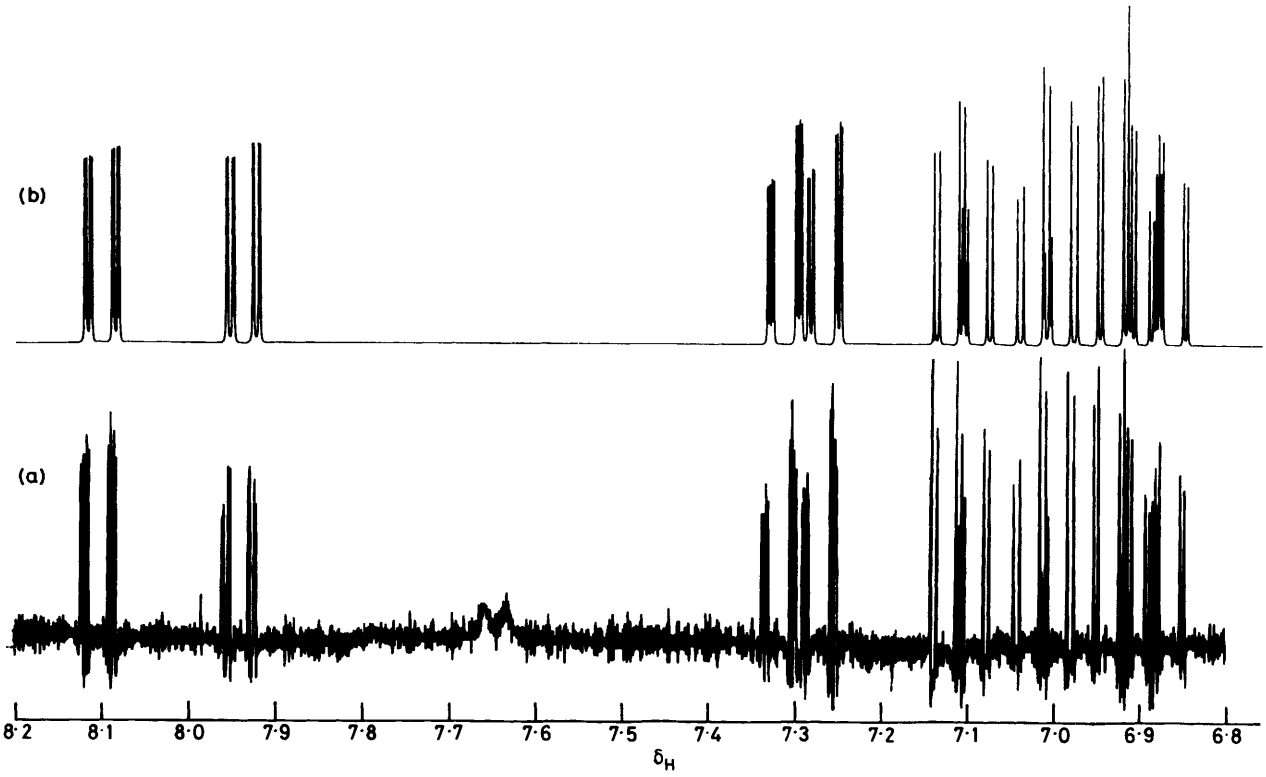


Figure 1. (a) Resolution-enhanced ^1H spectrum (250 MHz), in [$^2H_6$]benzene, of the aromatic region and (b) spectrum calculated according to the spectral parameters given in Table 2.

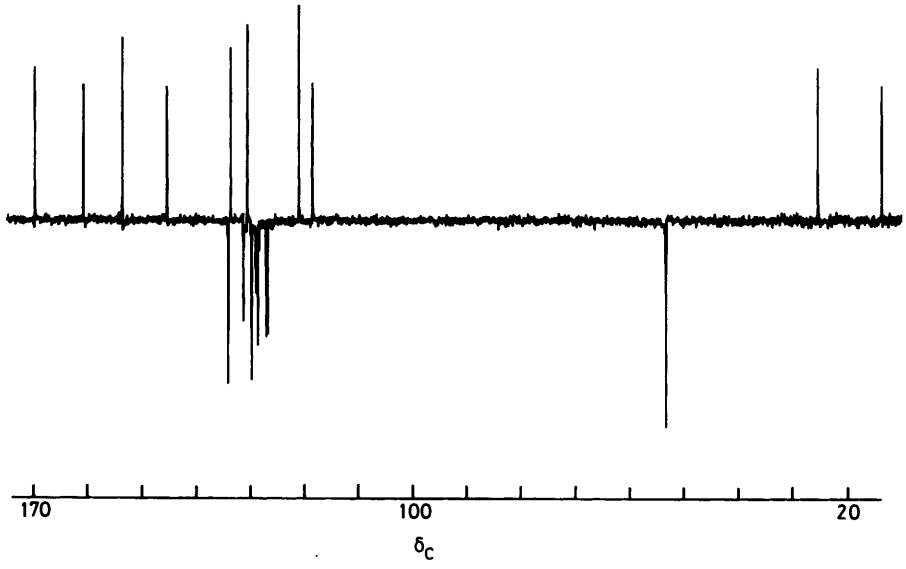


Figure 2. 62.9 MHz ^{13}C Spectrum recorded in [$^2H_6$]benzene by means of the *J*-modulated spin-echo method. Positive peaks correspond to either CH_2 or C, negative peaks to CH. Numerical values for the 19 peaks are δ_C 169.7, 160.7, 153.6, 145.4, 134.1, 133.6, 131.3, 130.6, 129.9, 129.0, 128.7, 127.1, 127.06, 126.9, 121.1, 118.6, 53.2, 25.4, and 13.6

anthranilate molecules and tryptophan. Otherwise anthranilate-derived metabolites are limited mainly to tryptanthrin the tryptoquivalines, and the questiomycins.[13]

Experimental

^1H and ^{13}C n.m.r. spectra were recorded on a Bruker WM 250 spectrometer with samples of 3 and 35 mg in [$^2H_6$]benzene (0.5 ml), respectively. Electron-impact mass spectra were obtained on a V.G. Micromass 7070E instrument; high-resolution mass data are in Table 3. I.r. spectra were run as KBr discs on a Perkin-Elmer 683 spectrophotometer, and u.v. spectra on a Varian Cary 210 scanning spectrophotometer for solutions in ethanol. All solvents were evaporated off under reduced pressure below 40 °C and any water was removed by freeze-drying. Radiolabelled compounds were purchased from Amersham International. The polarimeter was an Optical Activity Ltd., AA-1000 instrument.

Table 3. High-resolution mass data for auranthine (1)

	Observed	Requires
M^+	330.1110 $C_{19}H_{14}N_4O_2$	330.1117
	290.0925 $C_{17}H_{12}N_3O_2$	290.0930
	277.0846 $C_{16}H_{11}N_3O_2$	277.0851
	249.0666 $C_{15}H_9N_2O_2$	249.0664
	248.0592 $C_{15}H_8N_2O_2$	248.0586
	234.0799 $C_{15}H_{10}N_2O$	234.0793
	221.0719 $C_{14}H_9N_2O$	221.0715
	220.0646 $C_{14}H_8N_2O$	220.0637
	192.0688 $C_{13}H_8N_2$	192.0687
	165.0580 $C_{17}H_7N$	165.0578
	132.0452 C_8H_6NO	132.0449
	130.0290 C_8H_4NO	130.0293
	102.0347 C_7H_4N	102.0344

Culture Conditions.—*Penicillium aurantiogriseum* Dierckx (I.M.I. 180922) was grown as a sporing mycelial mat in 500 ml Erlenmeyer flasks containing Czapek Dox broth (100 ml) supplemented with yeast extract (0.5% w/v). The medium was inoculated with spore-covered bran which, floating on the surface of the liquid, ensured efficient surface growth of the fungus. Cultures were incubated at 27 °C for 14 days.

Isolation.—Whole cultures were homogenised, freeze-dried, and extracted exhaustively with acetone. The solvent was removed under reduced pressure and the residue was analysed by t.l.c. on silica plates (Polygram Sil G/UV_{254} 0.25 mm, Camlab) with chloroform–acetone (93:7 v/v) as eluant. The new alkaloid was evident (R_F 0.3) since it caused quenching of the fluorescent dye at 254 nm. Preparative separation of the crude extract was achieved on a column (0.3 m × 50 mm i.d.) of Kieselgel 60 (Merck) packed in chloroform–acetone (93:7 v/v).[14] The column was eluted with the same solvent mixture and sequential fractions (50 ml) were analysed by t.l.c. to locate the alkaloid which occurred in the 150—700 ml portion of the eluate.

Further purification of the solute was achieved by semi-preparative reversed-phase h.p.l.c. through a Beckman Ultrasphere-ODS 5 μm column (0.25 m × 10 mm i.d.) with methanol–water (2:1 v/v) as eluant at a flow rate of 4 ml min^{-1} and u.v. detection (λ 210 nm). On an analytical-scale column, auranthine (1) was found to be homogenous in a similar h.p.l.c. system.

Auranthine showed the following spectral data: $\lambda_{max.}$ (1 cm) 228 (ε 38 400 dm^3 mol^{-1} cm^{-1}), 268 (8 790), 280 (7 470), 310 (3 760), and 322 nm (3 850); $\nu_{max.}$ 3 450m, 3 175s, 1 675s, 1 615s, 1 595s, 1 450w, 1 380s, and 1 250w cm^{-1}; $[\alpha]_D^{25}$ (1% w/v in EtOH) −164°.

Auranthine forms a crystalline hydrochloride: fine needles, m.p. >300 °C.

Biosynthesis.—All putative precursors examined were ^{14}C-labelled, and each was added to separate cultures of the organism after biomass had been formed, *i.e.* after 7, 9, and 13 days incubation (total 25 μCi). Acetone extracts were resolved by t.l.c., and incorporation of the radiolabel into the alkaloid was examined qualitatively by autoradiography (Fuji, RX-Xray film), and quantitatively by scintillation counting of aliquots purified by h.p.l.c.

Acknowledgements

We thank S.E.R.C. for a case award (to S. E. Y.)

References

1 J. M. Barnes, R. L. Carter, G. C. Peristianis, P. K. C. Austwick, F. V. Flynn, and W. N. Aldridge, *Lancet*, 1977, **289**, 671.
2 J. I. Pitt, 'The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*,' Academic Press, London, 1979, p. 353.
3 M. Brufani, W. Fedeli, F. Mazza, A. Gerhard, and W. Keller-Schierlein, *Experientia*, 1971, **27**, 1249.
4 E. Fiedler, H.-P. Fiedler, A. Gerhard, W. Keller-Schierlein, W. A. König, and H. Zahner, *Arch. Microbiol.*, 1976, **107**, 249.
5 S. C. Pakrashi, J. Bhattacharyya, L. F. Johnson, and H. Budzikiewicz, *Tetrahedron*, 1963, **19**, 1011.
6 H. Budzikiewicz and N. Mohr, *Org. Mass Spectrom.*, 1981, **16**, 329.
7 M. McCamish and J. D. White, *Org. Mass Spectrom.*, 1970, **4**, 241.
8 D. F. Ewing, *Annu. Rep. NMR Spectrosc.*, 1975, 389.
9 Y. S. Mohammed and M. Luckner, *Tetrahedron Lett.*, 1963, 1953.
10 H. Hikino, S. Nabetani, and T. Takemoto, *Yakugaku Zasshi*, 1973, **93**, 619 (*Chem. Abstr.*, 1973, **79**, 40922).
11 S. Minato, *Arch. Biochem. Biophys.*, 1979, **192**, 235.
12 R. S. L. Chang, V. J. Lotti, R. L. Monaghan, J. Birnbaum, E. O. Stapley, M. A. Goetz, G. Albers-Schonberg, A. A. Patchett, J. M. Liesch, O. D. Hensens, and J. P. Springer, *Science*, 1985, **230**, 177.
13 W. B. Turner and D. C. Aldridge, 'Fungal metabolites II,' Academic Press, London, 1983, pp 397 and 434.
14 W. C. Still, M. Kahn, and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.

Received 21*st October* 1985; *Paper* 5/1820