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ANACINE, A NEW BENZODIAZEPINE METABOLITE OF *PENICILLIUM AURANTIOGRISEUM* PRODUCED WITH OTHER ALKALOIDS IN SUBMERGED FERMENTATION

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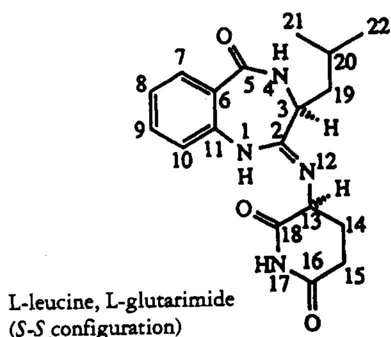
ABSTRACT.—A novel benzodiazepine metabolite of an isolate of *Penicillium aurantiogriseum* from Yugoslavia has been characterized from spectral and biosynthetic evidence. The compound, anacine [**1**], has been produced on the 60-liter fermenter scale after extensive sporulation induced by batched Ca^{++}, and its biosynthesis was preceded by that of the diketopiperazine fructigenine A [**7**], for which additional spectral and biosynthetic data have been obtained.

Benzodiazepines are natural products of several filamentous fungi and of streptomycetes (1). [^{14}C-carboxy] anthranilic acid is an effective biosynthetic probe for detecting benzodiazepine metabolites of fungi (2). Further application of this technique to penicillia isolated from Yugoslavia (Croatia) has revealed another novel compound which is a metabolite of an isolate of *Penicillium aurantiogriseum* Dierckx and which is given the trivial name anacine [**1**] because of the involvement of anthranilic acid and leucine in its biosynthesis. The dynamics of production in submerged fermentation have been explored, and other alkaloidal metabolites have been identified.

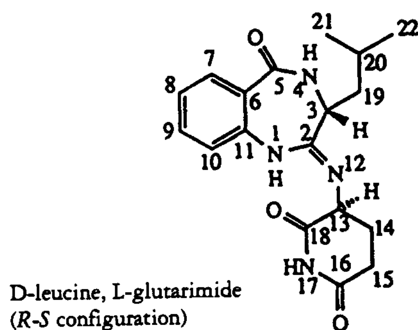
Autoradiography of metabolites extracted from the fermentation broth, resolved by tlc in a rather polar solvent system, showed radiolabel, presumably derived from the carboxy carbon of anthranilic acid, coinciding with a prominent compound at R_f 0.25. The compound, purified by hplc, had a molecular formula of $C_{18}H_{22}N_4O_3$ determined by accurate mass measurement of the molecular ion, $[M]^+$ 342, in the eims. Positive fabms confirmed the molecular ion assignment by the ion at *m/z* 343 and by the adducts with H^+ and Na^+. Prominent ions at *m/z* 326 and 298 were explained as extrusion of ammonia $[M+H-17]^+$ and successive loss of carbon monoxide $[M+H-17-28]^+$. Accurate mass measurement of important fragment ions in the eims showed losses of NH_3 (*m/z* 325), C_3H_7 (*m/z* 299), and C_4H_8 (*m/z* 286), the latter attributed to the loss of part of the isoprene component. The ion at *m/z* 130 is analogous to the indolic fragment of auranthine attributed to C_8H_4NO (2).

A ^{1}H-nmr spectrum (Table 1) showed four aromatic protons and three methine, three methylene, and two methyl groups. A broad doublet and two broad singlet signals corresponded to N-H protons. In both surface and submerged culture, experimental incorporation of ^{14}C from radiolabeled anthranilic acid, leucine, and glutamic acid implied that their carbon skeletons were constituents of anacine (Table 2). The expected cellular metabolic flux between glutamate and glutamine allowed experimental evidence from glutamate to apply equally to glutamine as discussed with respect to auranthine (3), so that equimolar condensations of glutamine with leucine and anthranilic acid accounted entirely for the molecular composition of anacine. Thus, in the ^{1}H-nmr spectrum the leucyl N-H proton was assigned by reference to the spectrum obtained for a model compound, cyclo-anthranilyl-leucine dipeptide [**5**] (Table 1). A COSY spectrum confirmed this assignment, since the leucyl N-H signal was coupled to the α-proton of leucine located at the 3 position of anacine at 4.39 ppm. Additional support for other proton assignments also came from the ^{1}H-nmr spectrum of L-pyroglutamide



L-leucine, L-glutarimide
(*S-S* configuration)

1



D-leucine, L-glutarimide
(*R-S* configuration)

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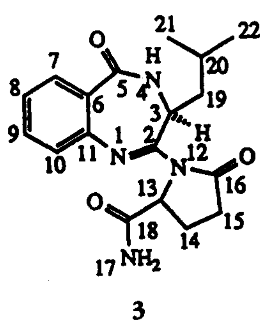

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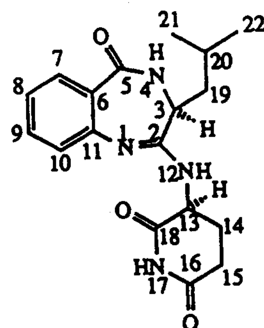

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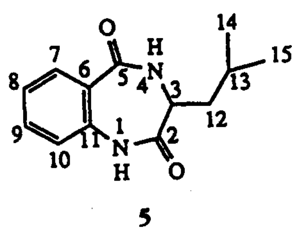

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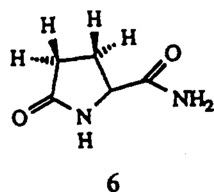

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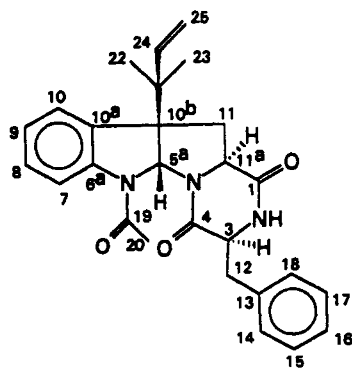

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TABLE 1. ^{1}H- and ^{13}C-nmr Data for Anacine [**1**], its Pyroglutaminyl Derivative [**2**], cyclo-Anthranilyl-leucine [**5**], and L-Pyroglutamide [**6**], in DMSO-d_6.

Position	Compound 1		Compound 2	Position	Compound 5	Compound 6
	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$		$\delta^{1}H$	$\delta^{1}H$
1(N)	6.74[a] s (broad)	—	6.72[a] s (broad)	1(N)	10.36 s	
2	—	152.04	—	2	—	
3	4.39 m	53.80[a]	4.72 dd	3	3.60 m	
4	8.87 d (broad)	—	8.53 d	4	8.43 d	
5	—	160.11[d]	—	5	—	
6	—	119.66	—	6	—	
7	8.13 dd	126.20	8.13 d	7	7.75 d	
8	7.52 dt	126.68	7.51 dt	8	7.21 t	
9	7.81 dt	126.71	7.81 dt	9	7.51 t	
10	7.63 dd	134.70	7.60 dd	10	7.09 d	
11	—	147.00	—	11	—	
12(N)	—	—	—			7.75
13	4.86 m	54.85[a]	5.09 t (broad)			3.92 dd
14	2.05 m 2H (1.96–2.14)	29.34	2.20 m 2H			1.88, 2.22 m 2H
15	2.33 m 2H (2.25–2.40)	32.16	2.15 m 2H (2.00–2.30)			2.1 m 2H (2.03–2.18)
16	—	166.59[b]	—			—
17(N)	7.31[a] s (broad)	—	7.24[a] s (broad)			7.08, 7.39 s
18	—	172.80[b]	—			—
19	1.74 m 2H (1.69–1.79)	47.18	1.65 m 2.28 m	12	1.55 m	
20	1.89 sept (broad) (1.84–1.93)	24.01	2.10 m	13	1.70m	
21	0.97 dd 3H	21.41[c]	0.97 dd 3H	14	0.85[a] d	
22	0.97 dd 3H	23.02[c]	0.97 dd 3H	15	0.77[a] d	

[a–c] May be interchanged.
[d] Assignment by analogy with auranthine (2).

[**6**] (Table 1). The ^{13}C-nmr spectrum of anacine showed 18 resonances (Table 1), and their assignment was assisted by reference to the data for auranthine (2), which also contains anthranilate and glutaminyl moieties.

Exposure of anacine to high temperature (180°) resulted in a doubling of all resonances in the ^{13}C-nmr spectrum, suggesting racemization at C-3. The ^{1}H-nmr spectrum correspondingly became much more complex. Hplc of the heated anacine showed an approximately 1:1 ratio of anacine to compound **2** of longer retention time (13 min) which co-chromatographed with a minor component in native fungal extract. The ^{1}H-nmr spectrum of **2** is shown in Table 1. Compound **2** also carried radiolabel from [^{14}C-carboxy] anthranilic acid. Thus, racemization of anacine at high temperature may only increase the amount of an apparently natural product arising by other means. An alternative structure **3** to account for the thermal effect has been rejected as theoretically contra-thermodynamic. Molecular mechanics calculations of relative heats of formation and optimized geometries of anacine and its putative rearrangment products enabled assessment of the relative stability of the amidine tautomer. The transoid amidine was less stable than the cisoid isomer. Racemization at the leucine α center (C-3) was exothermic (-2.4 Kcal·mol^{-1}), whereas conjugation of the amidine bond with the benzene ring of the anthranilyl moiety was endothermic ($+2.0$ Kcal·mol^{-1}).

Intramolecular hydrogen bonding in cyclic imides may decrease the carbonyl stretching frequency in the ir spectrum (4). The anacine carbonyl stretching frequency of the glutarimide moiety at 1688 cm^{-1}, as also the value (1695 cm^{-1}) for that in cycloheximide, is lower than that of glutarimide (1710 cm^{-1}) (5), suggesting that

TABLE 2. Incorporation of ^{14}C from Putative Biosynthetic Precursors by *Penicillium aurantiogriseum* into Anacine [1] and Fructigenine A [7] in Stationary and Submerged Cultures.

Precursor	Specific Radioactivity (mCi/mmol)	Given on days	Total ^{14}C	Harvested	^{14}C incorporation into purified	
					1[a]	**7**
Stationary culture						
^{14}C[Carboxy] anthranilic acid	10.5	5,8,	12 μCi	Day 11	0.15%	—
		6,8,10	50 μCi	Day 13	0.36%[b]	
L-[U-^{14}C] Leucine	342	4,6	10 μCi	Day 8	0.51%	—
		7,10,12	15 μCi	Day 17	0.07%	
L-[U-^{14}C] Glutamic acid	275	7,10,12	15 μCi	Day 17	0.01%	—
Submerged culture						
DL-[Methylene-^{14}C]tryptophan	59	1	10 μCi	Day 3	None	0.13%
L-[U-^{14}C] Leucine	342	2,3	12 μCi	Day 4	1.41%	None
L-[U-^{14}C] Glutamic acid	275	2,3	20 μCi	Day 4	0.04%	None

[a]Yield approximately 2 mg/flask.
[b]Additional 0.04% incorporation in the *RS* stereoisomer.

anacine and cycloheximide are hydrogen-bonded, as appears more likely in structure **1** than in structure **3**.

Assuming thermal racemization at C-3, the relative stability of the two chiral centers in anacine was addressed. Examination of the spectra of **1** and **2** showed the most significant differences to be in the shift of the leucine N-H proton and the shifts of the protons on C-19. In **2** the two protons on C-19 were at 1.65 and 2.28 ppm; in **1** they were together near 1.7 ppm. These two changes imply that the epimerization occurs at the leucine α center (C-3). Chemical shift changes in ^{13}C-nmr spectra were also greatest at C-19 (difference 7.85 ppm).

Exposure of anacine for 3 days to the trace amounts of H^+ in some spectroscopic $CDCl_3$ produced another compound which we assigned as structure **4**. The ^1H spectrum in $CDCl_3$ was complex and was not fully assigned. However, a new signal at 5.48 ppm was tentatively assigned to the new N-H proton at position 12 in structure **4**.

Autoradiography of chromatograms of fermentation extract following ^{14}C-tryptophan administration (Table 2) clearly revealed a second alkaloidal metabolite. However, the efficiency of radiolabeling was rather low because, as it was produced mainly during the first day of fermentation, its biosynthesis was competing with rapid growth processes for the administered tryptophan. Evidence from eims concerning fragment ions attributable to structural residues, together with the biosynthetic evidence of incorporation of ^{14}C-tryptophan, indicated a tryptophan-phenylalanine diketopiperazine with acetyl and prenyl substituents. ^1H- and ^{13}C-nmr spectroscopy revealed a structure which is identical to that described for fructigenine A [**7**], and much of the data (6) conforms to that which is published (7). However, since there is inconsistent assignment of the prominent eims fragment *m/z* 157 by Arai *et al.* (7), ms data, including metastable ion analysis using a link-scanning technique (summarized in Figure 1), and the ^1H- and ^{13}C-nmr data giving fuller assignment of prenyl and acetyl substituents through heteronuclear shift correlation, are included here (Table 3). Additional nOe experiments

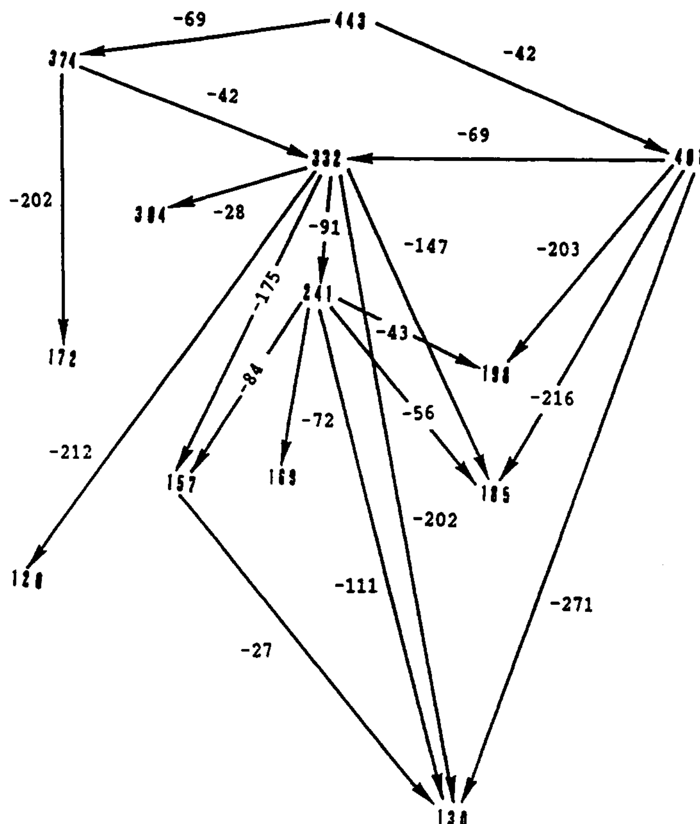

FIGURE 1. The origin of the important fragment ions in the eims of anacine [**1**], according to metastable ion analysis at a constant ratio of magnetic field strength to accelerating voltage (B/E).

(6) confirmed, for example, assignment of the C-7 proton because of the aromatic doublet response at H-7 after irradiating the *N*-acetyl protons. Further, the inverted prenyl and the proton at C-5a were cis to one another. The high field β-proton (1.60 ppm) of the tryptophan moiety at C-11 was cis to the prenyl group. The low field β-proton (2.31 ppm) was cis to the α-proton of tryptophan (C-11a). H-10 was also determined as close in space to the low field β-proton of tryptophan.

Tlc of extract of a surface culture treated with [^{14}C-carboxy] anthranilic acid showed, by autoradiography, a radiolabeled compound which was less polar than the radiolabeled anacine, and which had a chromatographic mobility similar to that of penicillic acid [R_f 0.6 in $CHCl_3$-Me_2CO (1:1) and 0.2 in $CHCl_3$-Me_2CO (93:7)]. In the eims of the purified compound, the important ions *m/z* 310 $[M]^+$, 253 $[M-MeNCO]^+$ together with other signals at *m/z* 252, 224, 196, 168, 119 were analogous to those of cyclopenin (8) if a 16 mass unit difference is taken into account. It was concluded that the compound was cyclopenol, which has previously been found in other penicillia closely related to *P. aurantiogriseum* (9). Cyclopenol and cyclopenin were also metabolites of the fungus *Penicillium fructigena* Takeuchi, used by Arai *et al.* (7) for the isolation of fructigenines A and B. In producing one benzodiazepine and one diketopiperazine in common the two fungi are probably very similar. Indeed, Pitt (10) commented that *P. fructigena* is probably a synonym of *Penicillium expansum*, which is at least in the sub-genus *Penicillium* as is *P. aurantiogriseum*.

TABLE 3. ^{1}H- and ^{13}C-nmr Data for Frutigenine A [7] in DMSO-d_6 Assigned According to the Heteronuclear Correlation Spectrum.

Position[a]	$\delta^{13}C$	DEPT	δ^1H	J(Hz)
1	165.20[b]	C	—	—
2(N)	—	—	8.19 s	—
3	55.54	CH	4.43 bt	to H-12 4.3, 5.0[c] to H-11a 1.3
4	166.57[b]	C	—	—
5(N)	—	—	—	—
5a	78.61	CH	5.92 s	—
6(N)	—	—	—	—
6a	143.20	C	—	—
7	117.72	CH	7.81 bd	to H-8 ortho 8.0
8	128.44	CH	7.24 dt	[d]
9	124.13	CH	7.12 dt	to H-7 meta 1.1 to H-8 ortho 7.5 to H-10 ortho 7.7
10	124.91	CH	7.37 dd	to H-8 meta 0.86 to H-9 ortho 7.7
10a	132.47	C	—	—
10b	60.55	C	—	—
11	36.32[e]	CH_2	1.6 t	geminal 12.2 trans to H-11a 11.8
			2.31 dd	geminal 12.2 cis to-11a 5.4
11a	58.53	CH	3.55 ddd	to H-3 1.3 trans to H-11 11.8 cis to H-11 5.4
12	36.02[e]	CH_2	2.99 dd	geminal 14.0 to H-3 5.0
			3.11 dd	geminal 14.0 to H-3 4.3
13	136.58	C	—	—
14	129.97	CH	7.17–7.26 m	—
15	128.06	CH		
16	126.50	CH		
17	128.06	CH		
18	129.97	CH		
19	169.52	C	—	—
20	23.67	Me	2.54 s	—
21	39.94	C	—	—
22	22.09	Me	0.78 s	—
23	22.86	Me	0.93 s	—
24	143.39	CH	5.63 dd	trans to H-25 17.3 cis to H-25 10.6
25	114.06	CH_2	5.02 m	trans to H-24 17.3 cis to H-24 10.6

[a]Positions according to Arai *et al.* (7).
[b]May be reversed.
[c]Cis and trans couplings can not be differentiated in the phenylalanine moiety due to free rotation about the C_α-C_β bond.
[d]Coupled to proton in position 9 according to COSY, and nOe experiment on H-9.
[e]The data for positions 11 and 12 may be reversed.

In experiments to assess the response of the *P. aurantiogriseum* isolate to $CaCl_2$ in submerged culture, 0.01% $CaCl_2$ did not induce conidiophore differentiation but sporulation occurred with 0.1, 0.5, and 2.0% $CaCl_2$ so that, for example, 2.4×10^8

conidia/ml were produced by day 3 in 2% $CaCl_2$. It was also evident that anacine and fructigenine A were only significantly elaborated in submerged cultures in which sporulation was induced by calcium. Consequently this stimulus was necessarily employed in all submerged cultures of this organism and had the added advantage of avoiding the apparent high viscosity of the filamentous habit which is an important factor at the 60-liter fermenter scale. In an example (Figure 2), the fermentation inoculum was in the form of long, infrequently branched hyphae. The calcium-supplemented fermenter medium encouraged radial proliferation of short hyphal branches from the inoculum hyphae, so that by 6 h hyphal branch tips were swollen as a prelude to conidiophore differentiation. Penicillus formation was evident by 12 h, and conidia became differentiated on phialides by 18 h. Maximum biomass (Figure 2) was achieved within 42 h through smooth incremental dynamics in which the values during the first 30 h expressed on a logarithmic scale give a linear plot. The finely divided form was at least partly influenced by impeller shear forces. Later values were more erratic due to sampling constraints of foaming, deposition of mycelia on fermenter surfaces, and continued evaporation during sparged aeration. Up to and including 18 h, biomass related only to filamentous mycelium. Thereafter, as profuse sporulation made the culture light green at 24 h and nearly black by 36 h, spores (approximately 10^8/ml) became an increasingly significant component of the biomass in the 18–42 h period. Culture filtrate became progressively amber-brown throughout the fermentation. Biomass accumulation ceased as saccharide nutrients were exhausted. Approximately 20% of the batched sucrose was hydrolyzed during sterilization, but the rest was transformed, presumably by an extracellular invertase within the first 18 h. The resultant glucose and fructose were consumed in a loosely diauxic manner so that the biomass that accumulated between 30 and 42 h was derived from a fructose carbon source (Figure 3). Within the first half hour fermenter broth pH decreased rapidly, but thereafter it gradually increased to stabilize near neutrality. Anacine was first observed at 30 h, when

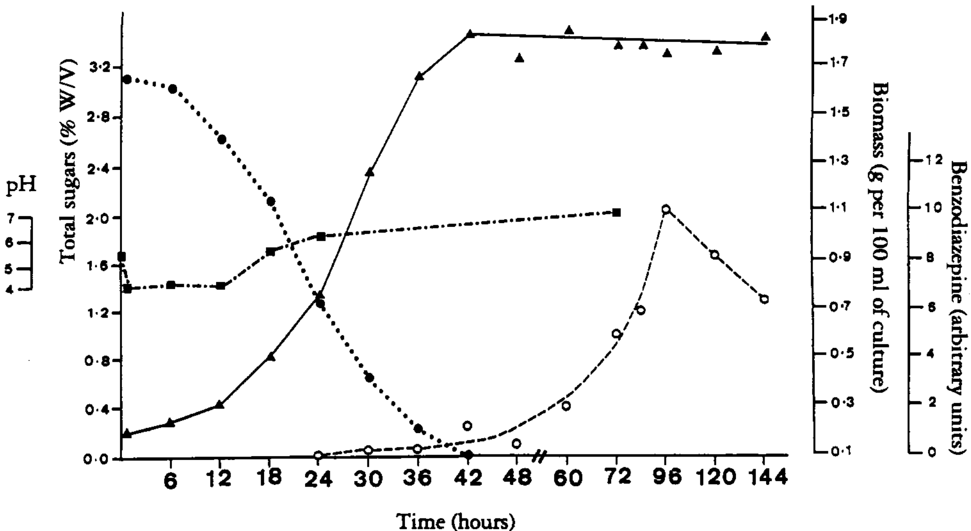

FIGURE 2. Progess of a 60-liter fermentation of *Penicillium aurantiogriseum* showing biomass accumulation (▲), sugar uptake (●) pH changes (■), and anacine [**1**] accumulation (o).

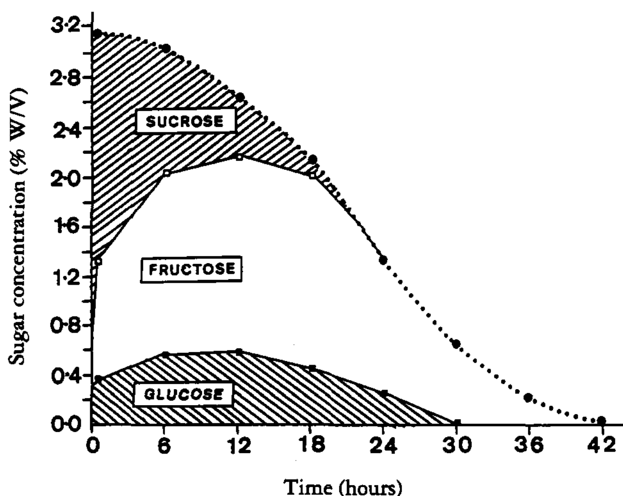

FIGURE 3. Changes in the concentration of glucose (■), fructose (□), and sucrose (●) in the broth in the first 2 days of a *Penicillium aurantiogriseum* fermentation.

glucose was no longer detected in the broth, and accumulated over 3 days. At harvest the fungus was in the form of small granular mycelial pellets and free spores, the latter making filtration difficult.

It is evident that the isolate of *P. aurantiogriseum* is particularly versatile in elaborating secondary metabolites derived from amino acids and exemplifies another way, complementary to that expressed in the biosynthesis of auranthine by other isolates of *P. aurantiogriseum* (11), in which glutamine can be incorporated with anthranilic acid into a benzodiazepine metabolite. Fructigenine A has also been isolated (12), and confirmed by eims, from a *P. aurantiogriseum* isolate M1 which did not produce benzodiazepines (11). The same isolate produced the polyketide verrucosidin (13), also fully characterized (14) as a metabolite of *P. aurantiogriseum* (IMI 180922) from which auranthine was first isolated (2). Verrucosidin, cyclopenol, and the substituted tryptophan-leucine diketopiperazine verrucofortine were all produced by a South African *Penicillium verrusosum* var. *cyclopium* [(syn *P. aurantiogriseum* (10)] (15), indicating similar biosynthetic potential in similar organisms of widely different origin. Verrucofortine was subsequently also described as fructigenine B (7), from which it could be assumed that the present *P. aurantiogriseum* might produce this leucyl analogue of fructigenine A. However, the concerted evidence of autoradiography of tlc of extracts of fermentations fed with ^{14}C-labeled tryptophan, leucine, and acetate did not support fructigenine B as a metabolite of this isolate of the fungus.

Anacine is also of interest in that glutamine forms a glutarimide moiety as occurs notably in cycloheximide (16), one of the few antibiotics effective against several yeasts. The glutarimide ring system is an important feature also of streptovitacin A, streptimidone, inactone, and actiphenol, which are cycloheximide-related antibiotics that inhibit peptide synthesis in eucaryotic organisms which have ribosomes of the 80S type (17).

Diversion of aromatic amino acids to so-called secondary metabolites is becoming a useful taxonomic character in the penicillia. A diverse range of alkaloids derived either from anthranilate or from tryptophan have been isolated from fungi identified directly as *P. aurantiogriseum* or assigned by implication (9). Tryptophan-derived compounds are mainly diketopiperazines but include other structural types such as indole-diterpenoid penitrems and ergoline-related alkaloids. The latter include the cyclopiazonic acids which are now regarded as metabolic indicators of the very closely related *Penicillium*

commune, recently re-elevated to species status (19). All the metabolites derived from anthranilate are at least biosynthesized via a benzodiazepine intermediate. Recent studies on *P. aurantiogriseum* recognize groupings arranged according to prominent secondary metabolites, among which the benzodiazepines and diketopiperazines have important roles (20), and the new benzodiazepine anacine fits into this current concept. Also, independent analysis (J.C Frisvad, personal communication) of metabolites of isolate M3 by spectrophotometry following hplc has recognized (in addition to the anacine, fructigenine A, and cyclopenol reported herein) the following compounds: penicillic acid, cyclopeptin, dehydrocyclopeptin, cyclopenin, viridicatol, 3-methoxy-viridicatin, rugulosuvine, verrucofortine, verrucosidin, and normethylverrucosidin. Further, isolates assigned to the broad concept of *P. aurantiogriseum* quite frequently produce anacine, including the fungus used by Hodge *et al.* (15).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker AM-500 spectrometer. Ir spectra were obtained with a Perkin-Elmer 881 spectrophotometer, and uv spectra were recorded on a Cary 210 uv spectrophotometer. High resolution mass spectra were obtained on a VG-Micromass 7070E spectrometer at 70 eV with a 100μA trap current, and low ionization energy spectra (12 eV) were also obtained.

ORGANISM.—The isolate of *P. aurantiogriseum* came from moldy maize in Kaniza, near Slavonski Brod, Yugoslavia (Croatia) in 1985, was designated M3, and was authenticated by Dr. J.I. Pitt (10). The fungus is deposited at the International Mycological Institute (IMI 357488).

CULTURE IN FLASKS.—Czapek Dox broth (Difco) supplemented with yeast extract (0.5%) (CDYE medium, 100 ml in 500-ml Erlenmeyer flasks) was used for stationary liquid culture and for a seed stage in baffled flasks for 24 h at 27° to provide inoculum (5 ml) for shaken production stage flasks of the same medium supplemented with $CaCl_2 \cdot 2H_2O$ (2%). Orbital shakers operated at 200 rpm and 10 cm eccentric throw. Media containing $CaCl_2$ concentrations of 0.01, 0.1, and 0.5% were also used.

CULTURE IN 60-LITER FERMENTERS.—Fermentation (60-liter) had the following batched composition: sucrose, 3%; yeast extract (Fould Springer), 0.5%; $NaNO_3$, 0.3%; K_2HPO_4, 0.1%, $MgSO_4 \cdot 7H_2O$, 0.05%; KCl, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001%; $CaCl_2$ (anhydrous), 2%; polyglycol P-2000, 6 ml; pH6.0. Primary inoculum was in the form of 4-day cultures on sterile moist bran at 27°. Stationary cultures were established by scattering bran inoculum on the surface of the medium. Seed stage submerged cultures in baffled flasks were each started with ca. 10 cm^3 of bran inoculum and incubated on a rotary shaker for 24 h at 27°. Second stage seed flasks (4 liters containing 1 liter medium) were inoculated with the contents (100 ml) of a baffled primary flask and incubated for a further 24 h. Two liters of this culture were used to start a 60-liter fermentation. The vessel characteristics were as previously described (11). Samples of decreasing volume (4 liters→300 ml) were taken at intervals throughout the fermentation. Biomass was measured in cells separated by filter paper on a glass sinter and then lyophilized to constant weight. Spore concentration was measured by hemocytometer after coarse filtration of fermenter culture through absorbent cotton wool. Culture mycelia were examined microscopically after staining with cotton blue in lactophenol.

Sugar composition (sucrose, glucose, and fructose) of culture broths was determined from Fehling's measurement of reducing sugars before and after hydrolysis and the direct measurement of glucose by glucose oxidase (18).

The course of anacine production in the fermenter was followed in combined Me_2CO extracts of lyophilized biomass and solvent extracts of broth. The least polar components were first removed on a small scale by dissolving solutes in $CHCl_3$ and applying to a silica Sep-pak cartridge (Waters Associates). $CHCl_3$-Me_2CO (5:1) (20 ml) followed, and then anacine was eluted with MeOH. Anacine was measured on an arbitrary scale by hplc in the Spherisorb ODS 2 system described below.

EXTRACTION OF ANACINE AND FRUCTIGENINE A FROM A 60-LITER FERMENTATION.—Cells and broth were separated by filtration, and the cells (spores and mycelium) were lyophilized (334 g). Dry cells were extracted with Me_2CO (2.5 liters) for 2 days and the extract evaporated to dryness. Culture broth was stirred vigorously with isobutyl methyl ketone (12.5 liters) and the organic solvent extract evaporated to a brown oil containing polyethylene glycol antifoam which was selectively removed by treatment with *n*-hexane. A flocculating precipitate containing benzodiazepine and diketopiperazine metabolites was separated by centrifugation, and the pellet washed twice with *n*-hexane (10 ml) and dried (200 mg). However, almost

certainly a considerable amount of the product remained in the *n*-hexane and antifoam phase. Combined extracts of cells and broth were processed by flash cc through a Si gel (Kieselgel 60, Merck) column (5 cm×8 cm). During elution with $CHCl_3$-Me_2CO (5:1), 17 fractions (50 ml) were collected. Seven more fractions were collected during elution with an equal mixture of solvents, and 20 fractions during MeOH elution. Fructigenine A eluted in fractions 4–8 and anacine in fractions 34–36.

PURIFICATION OF ANACINE.—Combined fractions 34–36 above were evaporated to dryness, dissolved in $CHCl_3$-MeOH (1:1) (1 ml), and loaded on preparative Si gel GF 254 layer plates and developed in $CHCl_3$-Me_2CO (1:3). Anacine (R_f 0.5) was eluted from the Si gel with $CHCl_3$-iPrOH (1:1) and purified finally by injecting aliquots of the solutes, dissolved in MeOH, into an hplc system using a Spherisorb ODS 2 (5 μm) column (5 cm×0.5 cm), a mobile phase of MeOH-H_2O (1:1) at 4 ml/min, and uv detection at 226 nm. Anacine (yield 22.5 mg) had a retention time of 7 min, and its diastereoisomer (compound **2**) 13 min. The low yield was constrained by the selective removal of antifoam by *n*-hexane earlier in this process (above).

PURIFICATION OF FRUCTIGENINE A.—Fractions 4–8 above were evaporated to dryness and dissolved in MeOH, and aliquots were processed by gradient hplc through a Separon SGX strong reversed-phase column. At a flow rate of 0.5 ml/min, linear gradients were programmed over 2-min intervals between the following isocratic steps: MeOH in H_2O, 65%, 0–6 min; 40%, 8–15 min; 80%, 17–25 min. Fructigenine A (yield 33 mg) had a retention time of 20–22 min.

BIOSYNTHESIS.—^{14}C-labeled putative precursors (Table 1) were added in sterile H_2O (600 μl) to cultures, injected beneath the surface mycelium of stationary liquid cultures, at intervals. At harvest the mycelia were lyophilized (0.96–1.1 g per flask) and extracted with Me_2CO (150 ml). Extracts were resolved by tlc (Polygram SIL G/UV_{254} 0.25mm, Camlab) in $CHCl_3$-Me_2CO (1:3), in which anacine moved to R_f 0.5. In $CHCl_3$-Me_2CO (1:1) the R_f value was 0.2. Chromatograms were autoradiographed (Fuji NIF-RX film) at −70°. Extracts were also resolved by hplc (above), and radioactivity in appropriate fractions was measured by scintillation counting.

Anacine [**1**].—An amorphous white solid, sparingly soluble in H_2O but soluble in organic solvents: uv λ max (EtOH) 226 (ε 16900), 270 (ε 4024); ir ($CHCl_3$) 1688 (ν max) 1217 cm^{-1}; ^1H and ^{13}C nmr (DMSO-d_6) see Table 1; ms *m/z* (rel. int.) $[M]^+$ 342 (100), 325 (30), 299 (20), 286 (72), 282 (42), 269 (79), 254 (35), 241 (25), 227 (63), 214 (48), 198 (20), 186 (18), 130 (23); hrms *m/z* $[M]^+$ 342.1683 (calcd for $C_{18}H_{22}N_4O_3$, 342.1692), $[M-NH_3]^+$ 325.1419 (calcd for $C_{18}H_{19}N_3O_3$, 325.1426), $[M-C_3H_7]^+$ 299.1145 (calcd for $C_{15}H_{15}N_4O_3$, 299.1144), $[M-C_4H_8]^+$ 286.1067 (calcd for $C_{14}H_{14}N_4O_3$, 286.1066), $[M-C_6H_3NO]^+$ 227.0691 (calcd for $C_{12}H_9N_3O_2$, 227.0695).

Fructigenine A [**7**].—Uv λ max (EtOH) 244; ms *m/z* (rel. int.) $[M]^+$ 443 (54); 401 (32), 374 (18) 332 (100), 304 (10), 241 (7), 198 (3), 185 (8), 172 (5), 157 (54), 130 (58), 120 (13), 91 (<1); hrms *m/z* $[M]^+$ 443.2209 (calcd for $C_{27}H_{29}N_3O_3$, 443.2209), $[M-C_7H_{11}O]^+$ 332.1403 (calcd for $C_{20}H_{18}N_3O_2$, 332.1399), $[M-C_{14}H_{18}O]^+$ 241.0849 (calcd for $C_{13}H_{11}N_3O_2$, 241.0851), $[M-C_{17}H_{20}NO_3]^+$ 157.0766 (calcd for $C_{10}H_9N_2$, 157,0766), $[M-C_{19}N_2O_3]^+$ 120.0814 (calcd for $C_8H_{10}N$, 120.0813, $[M-C_{20}H_{22}N_3O_3]^+$ 91.0547 (calcd for C_7H_7, 91.0548).

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