Flavipucine [3′-Isovaleryl-6-methylpyridine-3-spiro-2′-oxiran-2(1*H*),-4(3*H*)-dione], an Antibiotic from *Aspergillus flavipes*

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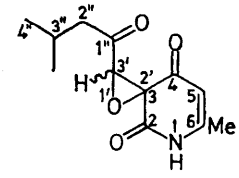
The structures of three metabolites produced by a strain of *Aspergillus flavipes* are discussed including a previously described antibiotic, which is shown to be 3′-isovaleryl-6-methylpyridine-3-spiro-2′-oxiran-2(1*H*),4(3*H*)-dione (1).

CASINOVI and his co-workers [1,2] have reported the isolation and biological activity of a new antibiotic substance, $C_{12}H_{15}NO_4$, produced by a strain of *Aspergillus flavipes*. On the basis of chemical and spectroscopic information, the structure 3-acetyl-4-isovaleryl-6-hydroxy-2(1*H*)-pyridone was proposed and the antibiotic was named ' glutamicine ' by these authors.

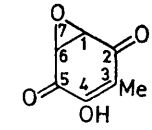
By large scale fermentation quantities of the order of 1 g of the antibiotic factor have been obtained for detailed study. It is now evident that the proposed structure is incompatible with spectroscopic data and new chemical results, and we present evidence in support of the novel structure (1) for the antibiotic and suggest the more appropriate name ' flavipucine '.

Chromatography (silica gel) of the chloroform extracts of *Aspergillus flavipes* [1] afforded terreic acid [3] (2) and the antibiotic, which was identical in all respects with material (' glutamicine ') used in the prior studies.[1,2] The n.m.r. spectrum of this material clearly indicated the presence of a second compound (20—25%). Only after ten recrystallizations from methanol–water was flavipucine obtained in a homogeneous state. Recrystallization from a variety of other solvents failed to remove the second component. While this purification did not significantly alter the m.p. or the i.r. and u.v. spectra of the material, the specific rotation $[\alpha]_D^{21}$ was enhanced from the previously reported value of −78 to −88° (*c* 1% in 95% ethanol).

Two features of the ' glutamicine ' structure [1,2] are an acetyl group and an isolated vinylic proton. By n.m.r. and double resonance techniques, it has now been ascertained that the signal [δ (C_5D_5N) 2·02 and ($CDCl_3$) 2·14 p.p.m.] formerly ascribed to the acetyl group is in fact coupled with the vinylic proton signal with a temperature-independent coupling constant (*J* 0·8 Hz). Similar coupling is also observed for a derivative discussed later (see Tables 1 and 2). In addition neither the antibiotic nor the derivative gave a positive iodoform test. These findings are clearly incompatible with an acetyl group, but are in accord with the structural feature ·CMe:CH· as in (1).



(1)



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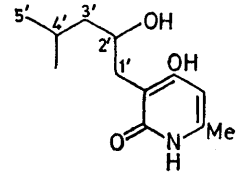

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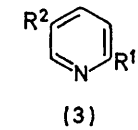

(3)

a; R^1=Me, R^2=CH_2·CH(OH)·CH_2·$CHMe_2$

b; R^1=CH_2·CH(OH)·CH_2·$CHMe_2$, R^2=Me

Spectroscopic evidence [1,2] attests to the presence

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[1] C. G. Casinovi, G. Grandolini, R. Mercantini, N. Oddo, R. Olivieri, and A. Tonolo, *Tetrahedron Letters*, 1968, 3175.

[2] C. G. Casinovi, G. Grandolini, R. Mercantini, N. Oddo, R. Olivieri, and A. Tonolo, *Ann. Ist. Super. Sanita* (*Rome*), 1969, **5**, 514.

[3] J. C. Sheehan, W. B. Lawson, and R. J. Gaul, *J. Amer. Chem. Soc.*, 1958, **80**, 5536.

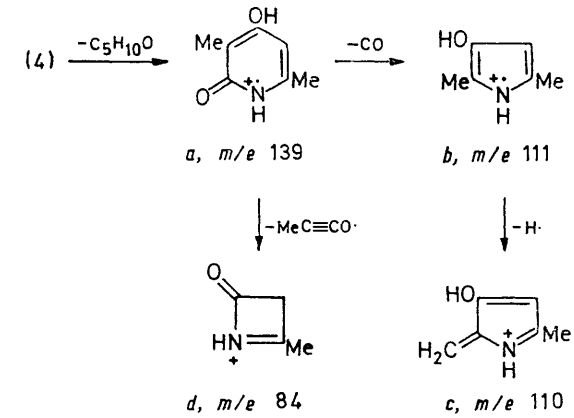
of a methyloxobutyl side-chain in the antibiotic. This accounts for all but two hydrogen atoms. One of these is readily exchanged for deuterium on equilibration with D_2O while the other is non-exchangeable and absorbs as a sharp singlet, δ ($CDCl_3$) 3·80 p.p.m., in the n.m.r. spectrum.

Reduction of flavipucine with lithium aluminium hydride leads to a complex mixture of neutral and basic products from which we have now isolated 2-methyl-5-(2-hydroxy-4-methylpentyl)pyridine (3a), albeit in very low yield. The 2,5-substitution pattern was readily established by comparison (n.m.r. and u.v.) with known 2,5-disubstituted pyridines.[4] The n.m.r. spectrum of (3a) showed that the 2′-proton (·C*H*·OH constituted the X part of an ABMNX system and this finding was corroborated by double resonance experiments. In the n.m.r. spectrum of 5-ethyl-2-methylpyridine the 2-methyl signal appears at δ 2·5 p.p.m. and the vinylic methylene at δ 2·7 p.p.m., the same positions as for the corresponding proton signals in (3). In addition, we find that the 2-methyl signals in 2,5-dimethyl-, 2,6-dimethyl-, and 2,4-dimethyl-pyridine are consistently near δ 2·5 p.p.m. while the 4- and 5-methyl signals are near δ 2·3 p.p.m. Thus the chemical shift data for the reduction product confirm structure (3a) but are inconsistent with the alternate formulation (3b).

Analytical and mass spectral data of the catalytic reduction product [1,2] show it to be a tetrahydrodeoxy-derivative, $C_{12}H_{19}NO_3$. By n.m.r. and double resonance we have shown that this compound possesses the same side-chain as the pyridine base (3a) and the ·CMe:CH· group as in flavipucine. The mass spectra of both reduction products display base peaks ($M - C_5H_{10}O$) corresponding to a retro-aldol reaction of the side-chain.

It is evident that the structure of tetrahydrodeoxyflavipucine can be arrived at by incorporating two oxygen atoms into the ring structure (3a), bearing in mind the coupling of the olefinic proton with the vinylic methyl group. A total of three exchangeable protons are present in the compound, and thus of the two remaining unassigned protons, one must be amidic. This is in accord with the i.r. spectrum, which shows strong absorptions at 1640 and 1600 cm^{-1} (conjugated amide) and multiple bands in the region 3250—3040 cm^{-1} (dimeric association of secondary amide [5]) as well as a strong band at 3380 cm^{-1} (OH). The u.v. spectrum shows a bathochromic shift from $\lambda_{max.}$ 287 to 269 nm upon acidification, as does that of 3-hexyl-4-hydroxy-6-methyl-2(1*H*)-pyridone [6] (from $\lambda_{max.}$ 288 to 269 nm). In basic solution, both compounds show slight hypsochromic shifts. Accordingly, we formulate tetrahydrodeoxyflavipucine as (4).

Mass spectral data provide corroboration for this conclusion. The mass spectrum of (4) is remarkably simple showing major fragments at *m/e* 139 (base peak), 111, 110, and 84. These ions are readily accounted for by the processes indicated in the Scheme. The decarbonylation of ion *a* to *b* is characteristic of 2-pyridones [7] and the subsequent loss of H· to give ions of type *c* is precedented by the spectra of 4-hydroxy-



SCHEME

6-methyl- and 6-methyl-2(1*H*)-pyridone. The former compound also fragments to an ion *m/e* 84, of the type *d*, postulated [7] to result from loss of the radical HC≡CO· from the molecular ion. The presence of metastable ions in the spectrum of the pyridone (4) at *m/e* 85·9 ($139^2/225$), 88·6 ($111^2/139$), and 50·8 ($84^2/139$) indicate the transitions (4) ⟶ *a*, *a* ⟶ *b*, and *a* ⟶ *d*, respectively, while accurate mass measurements confirmed the elemental composition of the molecular ion as well as ions *a* and *c*.

The presence of a lactam in flavipucine is supported by i.r. data [3240, 3160, and 3100 (NH) and 1630br cm^{-1} (conjugated amide)]. That the exchangeable proton of this molecule is amidic is in agreement with the presence of a low field n.m.r. signal [8] at δ ($CDCl_3$) 9·32 (8·25 in C_6H_6) p.p.m. which shifts on equilibration with D_2O. Since the signal for the remaining unassigned proton in flavipucine at δ 3·80 p.p.m. is non-exchangeable, the oxygen attached to the 4-position must be a carbonyl (or enol) oxygen since all formulations having hydrogen at C-4 are excluded by n.m.r. data. Thus all atoms of the optically active antibiotic are accounted for except for one oxygen atom and one proton. Accordingly there remain two sites to accommodate these atoms, namely, C-3 and C-1 of the pentyl substituent. An epoxide bridge located between these carbon atoms completely explains the observed spectral and chemical properties of the antibiotic. The 1- and 6-protons of terreic acid (2) give n.m.r. signals at δ ($CDCl_3$) 3·86 p.p.m., precisely the same field region as the signal arising from the similarly-shielded proton in flavi-

[4] Sadtler Standard Spectra, 1968, spectrum no. 571; C. T. Kyte, G. H. Jeffery, and A. I. Vogel, *J. Chem. Soc.*, 1960, 4454.

[5] R. M. Silverstein and G. C. Bassler, ' Spectrometric Identification of Organic Compounds,' 2nd edn., Wiley, New York, 1967, p. 94.

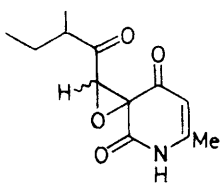
[6] Compound prepared according to the procedure described by K. Schreiber and G. Adam, *Chem. Ber.*, 1960, **93**, 1848.

[7] A. M. Duffield, C. Djerassi, G. Schroll, and S.-O. Lawesson, *Acta Chem. Scand.*, 1966, **20**, 361.

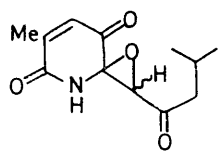
[8] L. M. Jackman and S. Sternhill, ' Applications of Nuclear Magnetic Resonance Spectroscopy to Organic Chemistry,' 2nd edn., Pergamon, New York, 1969, p. 216.

pucine. We thus arrive at structure (1) for the antibiotic in which only the stereochemistry of the side-chain remains to be clarified.

It was noted earlier that the purification of flavipucine is complicated by the presence and co-crystallization of a second compound (20—25%). While this minor component has not been isolated in pure form, spectral evidence suggests that it is probably the isomeric compound (5). The n.m.r. spectrum of the mixture clearly shows signals at δ ($CDCl_3$) 0·85 (t, J 7 Hz), 0·95 (d, J 7 Hz), 1·6 and 3·2 (both m), and 3·95 (s) p.p.m. with relative integrated intensities 3 : 3 : 2 : 1 : 1,



(5)



(6)

respectively, in agreement with the side-chain features in (5). It is reasonable to conclude that the signals for the remaining 5 protons are coincident with their counterparts in (1) and careful integration of the spectra of pure flavipucine and the mixture supports this speculation. In addition, the mass spectrum of the flavipucine prior to removal of the second component displays an ion (M − 29) corresponding to loss of an ethyl radical as would be expected from compound (5). Apart from the appearance of this fragment the mass spectrum of the mixture is virtually identical with that of pure flavipucine. Hence in addition to compounds (1) and (2), the flavipucine isomer (5) is probably also a metabolite of the *Aspergillus flavipes* strain under study.

The assignment of relative positions of the alkyl groups in the pyridine (3a) [and hence in (4), (1), and (5)] is based on comparison of n.m.r. data with that of appropriate 2,5-disubstituted pyridines and the alternate formulation (6) for the antibiotic has been excluded largely on these grounds. To confirm these findings synthetic studies are currently in progress.

EXPERIMENTAL

I.r. spectra were recorded on a Perkin-Elmer model 457 grating spectrophotometer and u.v. spectra with a Hilger and Watts Ultrascan H999 instrument. Mass spectra were determined with a Consolidated Electrodynamics Corporation R6-1 instrument. A Varian HA-100 spectrometer was employed for recording n.m.r. spectra. M.p.s were measured with a Kofler hot-stage apparatus and are uncorrected.

Flavipucine (1) *and Terreic Acid* (2).—The crude chloroform extract of the acidified fermentation broth of strain F2091/7 of *Aspergillus flavipes*,[1,2] was chromatographed on a silica gel column. Benzene eluted yellow crystalline terreic acid (2), m.p. 127—128° (from ether) (lit.,[3] 127—127·5°), $\nu_{max.}$ (KBr) 3300, 1690, 1655, 1630, 1390, 1380, 1355, 1310, 1250, 1220, 1200, 1135, 1035, 990, 865, 835, 790, 760, 715, 705, 625, 560, 520, 490, and 440 cm^{-1}, $\lambda_{max.}$ (95% ethanol) 214 (ε 10,500) and 316 nm (7250), δ ($CDCl_3$) 1·92 (3H, s), 3·86 (2H, s), and 7·02br p.p.m. (1H, s), *m/e* 154 (M^+) (Found: C, 54·55; H, 3·9. Calc. for $C_7H_6O_4$: C, 54·4; H, 3·95%).

The benzene–ether (3 : 1) eluates yielded flavipucine [3′-isovaleryl-6-methylpyridine-3-spiro-2′-oxiran-2(1*H*),-4(3*H*)-dione] (1), m.p. 130—131° (from benzene), δ ($CDCl_3$) 0·85 (3H, t, J 7 Hz), 0·95 (3H, d, J 7 Hz), 1·6 (2H, m), 3·2 (1H, complex m), and 3·95 p.p.m. (1H, s) in addition to signals assigned to flavipucine (see Tables 1 and 2) and of

TABLE 1

Chemical shift data [a]

(1)	5-H	3′-H			2″-H_a	2″-H_b	3″-H	4″-H_3	3″-Me	6-Me	*N*-H	Solvent
	5·85	3·80			2·64 *	2·72 *	2·18 *	0·95 *	0·98 *	2·14	9·32	$CDCl_3$
(4)	5-H	1′-H_a	1′-H_b	2′-H	3′-H_a	3′-H_b	4′-H	5′-H_3	4′-Me	6-Me	*N*-H + *O*-H_2	
	5·81	2·76 *	2·96 *	4·03 *	1·35 *	1·49 *	1·87 *	0·86	0·87	2·13	7—9	$CDCl_3$–C_5D_5N

[a] In δ/p.p.m. from Me_4Si. These spectra were recorded at 26°.

TABLE 2

Coupling constants, $J_{r,s}$/Hz

(1)	$J_{r,s}$	5-H,6-Me					
		0·8					
(4)	$J_{r,s}$	5-H,6-Me	1′-H_a,1′-H_b	1′-H_a,2′-H	1′-H_b,2′-H	2′-H,3′-H_a	2′-H-,3′-H_b
		0·8	−14·7 *	7·6 *	2·5 *	5·2 *	8·0 *
(1)	$J_{r,s}$	2″-H_a,2″-H_b	2″-H_a,3″-H	2″-H_b,3″-H	3″-H,4″-H_3	3″-H,3″-Me	ABMXY
		−16·7 *	7·2 *	6·6 *	6·7 *	6·7 *	
(4)	$J_{r,s}$	3′-H_a,3′-H_b	3′-H_a,4′-H	3′-H_b,4′-H	4′-H,5′-H_3	4′-H,4′-Me	AMBQXY
		−13·9 *	7·7 *	6·6 *	6·7	6·7	

* For these spectral parameters iterative calculations were performed using the LAOCN3 program of Bothner-By and Castellano.[9] Long-range couplings were assumed to be zero and the spectra were approximated by the simplified spin systems indicated in the final column. An IBM 7040 computer was employed for these calculations and the estimated accuracy is of the order of 0·1 Hz.

relative intensity (1 : 4—5). Pure flavipucine (1), m.p. 130—131°, was obtained by recrystallization of this mixture ten times from methanol–water, $\nu_{max.}$ (KBr) 3240, 3160, 3100, 2980, 2870, 1725, 1645, 1620, 1500, 1470, 1430, 1370, 1330, 1260, 1245, 1220, 1170, 1160, 1120, 1090,

[9] Computer Programs for Chemistry, ed. DeLos F. De Tar, Benjamin, 1968, p. 10.

1045, 930, 905, 850, 810, 750, 700, 600, 500, and 420 cm^{-1}, $\lambda_{max.}$ (95% ethanol, neutral or acidified) 330 nm (ε 5400) (addition of base causes irreversible time-, concentration-, and temperature-dependent shifts in $\lambda_{max.}$), *m/e* 237 (M^+), 152 (100%, $M - Me_2CH{\cdot}CH_2{\cdot}CHO$), 125, 84, and 57, $[\alpha]_D^{21} = -88°$ (*c* 1% in 95% ethanol).

Tetrahydrodeoxyflavipucine [4-*Hydroxy*-3-(2-*hydroxy*-4-*methylpentyl*)-6-*methyl*-2(1H)-*pyridone*] (4).—This compound, m.p. 189—190°, formerly called ' glutamicina idrogenata ' (hydrogenated glutamicine) was prepared as described,[2] $\nu_{max.}$ (KBr) 3380, 3250, 3120, 3040, 2960, 2930, 2870—2200, 1640, 1600, 1460, 1435, 1390, 1360, 1295, 1260, 1220, 1210, 1160, 1150, 1140, 1120, 1090, 1060, 1040, 1020, 1010, 960, 950, 900, 855, 845, 800, 785, 760, 630, 620, 555, 540, 532, 495, 480, 410, and 400 cm^{-1}, $\lambda_{max.}$ (95% ethanol) 287 nm (ε 7500), $\lambda_{max.}$ (95% ethanol + OH^-) 282 nm (ε 6500), $\lambda_{max.}$ (95% ethanol + H^+) 269 nm (ε 11,200), δ ($CDCl_3$) 2·13 (6-Me) and 5·81 p.p.m. (5-H), *m/e* 225 (M^+), 182 ($M - Me_2CH$), 168 ($M - Me_2CH{\cdot}CH_2$), 139 (100%, $M - Me_2CH{\cdot}CH_2{\cdot}CHO$), 138 [$M - Me_2CH{\cdot}CH_2{\cdot}CH(OH)$], 111, 110, and 84, [Found: *m/e* 225·136 (M^+). $C_{12}H_{19}NO_3$ requires 225·136; *m/e* 139·059. $C_7H_9NO_2$ requires 139·063; *m/e* 110·060. C_6H_8NO requires 110·061].

Reduction of Flavipucine (1) *with Lithium Aluminium Hydride.*—Flavipucine (2 g) in ether (150 ml) was added to a suspension of $LiAlH_4$ (6·73 g) in ether (400 ml) over 0·5 h. Stirring was continued at room temperature for 3 days. The excess of hydride was destroyed by addition of acetone and water and the mixture treated with 10% HCl (200 ml) and 10% H_2SO_4 (200 ml) and extracted with ether. The aqueous mixture was then neutralized with solid sodium carbonate, extracted ($CHCl_3$), dried (Na_2SO_4), and evaporated to dryness at 40° to yield a yellow oil (1·33 g). Chromatography [silica gel column; hexane–chloroform (3 : 2)] afforded 5-(2-hydroxy-4-methylpentyl)-2-methylpyridine (3a) (24 mg) as a pale yellow oil, $\nu_{max.}$ ($CHCl_3$) 3400, 1615, 1570, 1490, and 1460 cm^{-1}, $\lambda_{max.}$ (hexane) 264 (ε 3000), 269 (3080), and 274 nm (2420), *m/e* 193 (M^+), 178 (M — Me), 160 [$M - (H_2O + Me)$], 136 ($M - Me_2CH$), 107 (100%, $M - Me_2CH{\cdot}CH_2{\cdot}CHO$), 106 [$M - Me_2CH{\cdot}CH_2{\cdot}CH(OH)$], and 87 [$Me_2CH{\cdot}CH_2{\cdot}CH(OH)$].

The authors are grateful to Professor C. G. Casinovi for helpful discussions and assistance, and to Professor A. Tonolo for providing the crude extract from *Aspergillus flavipes*. Professor G. Grandolini (University of Perugia) kindly recorded some spectra and supplied a sample of ' glutamicina idrogenata ' and some other materials. We thank the Italian Ministry of Health for financial support.

[2/395 *Received*, 21*st February*, 1972]