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Isolation and identification of the fluorescent degradation product of some β-lactam antibiotics

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A fluorescent degradation product of cephalexin, recently employed in the determination of this antibiotic in aqueous solution and human plasma, has been isolated and identified as 2-hydroxy-3-phenyl-6-methylpyrazine. Spectroscopically and chromatographically the product is indistinguishable from that obtained by the same method from a series of other cephalosporins and penicillins bearing an α-amino group on the side-chain, and from the pyrazine structure produced by synthesis. Two reported methods for the fluorimetric determination of ampicillin have also been found to yield the same degradation product and not the suggested diketopiperazine structure.

A highly fluorescent product used in a sensitive fluorimetric assay for cephalexin (I, Scheme 1) in aqueous solution and plasma by Barbhaiya & Turner (1976a) can be obtained by different methods from β-lactam antibiotics having a side chain containing an α-amino group (Jusko, 1971; Durr & Schatzman, 1975; Miyazaki, Ogino & others, 1975; Barbhaiya & Turner, 1976b; 1977a, b, c; Barbhaiya, Turner & Shaw, 1977). Several published accounts refer to the fluorimetric determination of ampicillin (III, Scheme 1) (Jusko, 1971; Miyazaki, Ogino & Arita, 1974; Miyazaki & others, 1975). The structure of the degradation product was proposed tentatively to be the diketopiperazine (IV) derived from β-lactam opening followed by alternative recyclization (Jusko, 1971). Substituted diketopiperazine derivatives have been isolated and characterized from the benzene reflux treatment of cephalexin (Indelicato, Norvilas & others, 1974), and from room temperature aqueous sodium carbonate treatment of cephradine (Cohen, Funke & Puar, 1973). In neither case however, was the product reported to be fluorescent. However, some fluorescence was observed from the alkaline hydrolysis of cephalexin (I) and the structure of the fluorescent derivative was proposed to be a diketopiperazine (II) (Yamana, Tsuji & others, 1974; Aikawa, Nakano & Arita, 1976).

The methods described for fluorimetric determination of ampicillin (Jusko, 1971; Miyazaki & others, 1974, 1975), involved the same excitation and emission maxima as described by Barbhaiya & Turner (1976a). When the products from the procedures were compared chromatographically and spectroscopically, the fluorescent product of each was found to be the same substance. The emission and excitation maxima and the R_F values of the product did not vary whether it was derived from ampicillin, pivampicillin, hetacillin, cephalexin, cephaloglycin, cephradine or epicillin. Also the fluorescence profile of the ampicillin degradation product remained identical in aqueous solution and plasma (Barbhaiya & Turner, 1976a).

Previous work on the alkaline hydrolysis of cephalexin (I, Scheme 1) and cephradine provisionally identified the degradation product as the diketopiperazine (II) formed by rearrangement of the original compound (Cohen & others, 1973; Yamana & others, 1974) or a similar compound (IV) from acid hydrolysis of ampicillin (III) (Jusko, 1971). However, the compound isolated from these various degradation procedures had spectroscopic and other properties inconsistent with a diketopiperazine. The isolation, identification and independent synthesis of the fluorescent derivative was therefore undertaken.

After degradation of solutions of a series of cephalosporins and penicillins bearing an α-amino group on the side chain by the method previously used in the fluorescent assay of these materials (Barbhaiya & Turner, 1976a), the major degradation product from all (see Experimental) proved to have identical thin layer chromatographic behaviour (Table 1). A crystalline sample of this compound was obtained from ethyl acetate and purified. Examination of the mass spectrum showed that the

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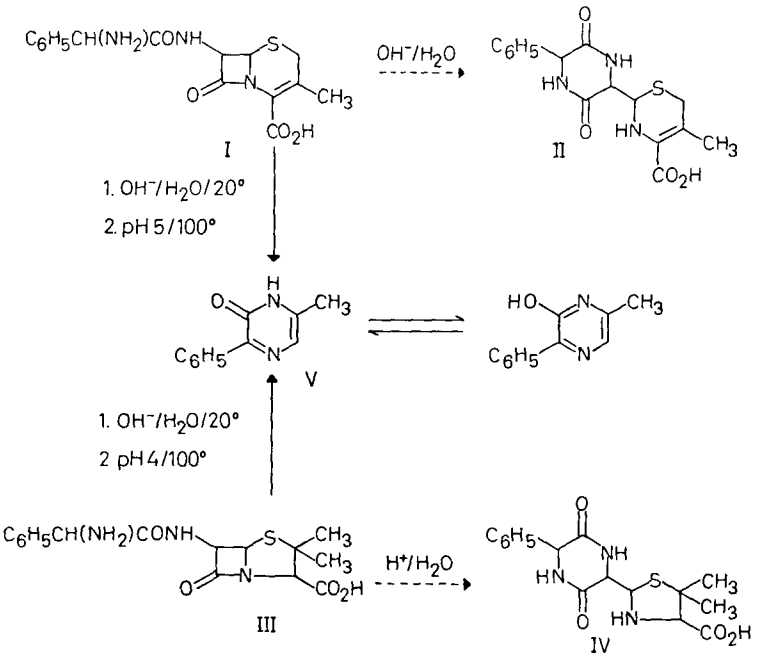

Scheme 1

molecular ion had an *m/e* value of 186 whilst mass measurement confirmed a molecular formula of $C_{11}H_{10}N_2O$. Diketopiperazine structures II and IV previously suggested were thus excluded.

In the proton magnetic resonance spectrum a broadened methyl singlet (δ 2·36 ppm) was suggestive of unresolved coupling whilst aromatic protons were distributed in two separate multiplets one at δ 8·35 (2H, *ortho* protons) and δ 7·42 (4H, *meta* and *para* protons plus one other). Irradiation at δ 2·36 caused partial collapse of the aromatic multiplet at δ 7·42 with concurrent appearance of a sharp superimposed singlet at δ 7·14; irradiation at δ 7·14 led to a sharpening of the methyl signal at δ 2·36 accompanied by a small (≃15%) nuclear Overhauser enhancement. These experiments suggested coupling between the methyl and a ring proton in the '*ortho*' position. With the appearance of an amide carbonyl frequency at 1650 cm^{-1}, a monohydroxypyrazine containing phenyl and methyl substituents seemed indicated. Two possibilities were consistent with these spectral requirements viz 2-hydroxy-3-phenyl-6-methylpyrazine and the corresponding 3-phenyl-5-methyl derivative. Of

Table 1. *Comparative thin layer chromatography of the hydrolysis products of some pencillins and cephalosporins.*

Solvent system	R_F values of fluorescent derivatives							
	Ampicillin*							
	1	2	3	4	Pivampicillin	Hetacillin	Cephalexin	Cephaloglycin
I	0·51	0·50	0·51	0·51	0·51	0·49	0·50	0·50
II	0·39	0·40	0·41	0·40	0·40	0·40	0·40	0·41
III	0·26	0·26	0·25	0·24	0·25	0·24	0·23	0·24
IV	0·63	0·64	0·63	0·62	0·62	0·61	0·62	0·62
V	0·24	0·25	0·25	0·25	0·25	0·23	0·23	0·23

Ampicillin 1. Fluorescent product from aqueous solution, by the present method.
2. Fluorescent product from aqueous plasma by the method of Barbhaiya & Turner (1977b).
3. Fluorescent product from aqueous acid by the method of Jusko (1971).
4. Fluorescent product from aqueous alkali by the method of Miyazaki (1974).

these the former had a m.p. in agreement with that of the antibiotic degradation product (Karmas & Spoerri, 1956).

Attempts to synthesize 2-hydroxy-3-phenyl-6-methylpyrazine via the intermediate 2-hydroxy-6-methylpyrazine by the method of Karmas & Spoerri (1952) were unsuccessful and an alternative approach utilizing the condensation of α-phenylglycine amide with methylglyoxal was used (cf. Cheeseman & Werstiuk, 1972). On admixture of the synthetic sample with that obtained from the antibiotics, no depression of m.p. was observed; ultraviolet, infrared, pmr, and mass spectral characteristics were identical as was the thin layer chromatographic behaviour. Therefore, the fluorescent product of the present study is assigned structure V, 2-hydroxy-3-phenyl-6-methylpyrazine, both through its coincidence of melting point with Karmas & Spoerri's product (crystallized from the same solvent), and the synthesized pyrazine.

The mechanism for the formation of the fluorescent product is not fully understood. However it is possible that the originally proposed diketopiperazines (II, IV) represent an intermediate stage in its formation. An apparent exception to the above is the observation of Roets, Vlietinck & others (1973) which links fluorescence with the isolation and spectroscopy of a diketopiperazine from the degradation of 6-epi-ampicillin (room temperature, pyridineacetic acid solution, five days).

Since aldehydes are more reactive than ketones towards nucleophiles (Reeves, 1966) and amines are far more nucleophilic than amides it was expected that the required compound would be the predominant condensation product of α-phenylglycine amide and methyl glyoxal. This hypothesis has been confirmed.

EXPERIMENTAL CHEMISTRY

Materials

Ampicillin trihydrate (Beecham), epicillin (Squibb), pivampicillin hydrochloride (Leo), hetacillin (Bristol), cephalexin monohydrate (Lilly), cephaloglycin (Lilly) and cephradine (Squibb) of reference standard quality were used. Aqueous methyl glyoxal (40% w/v, Sigma Chemicals) and DL-α-phenylglycine (95%, Aldrich) were used as received. All the other chemicals used were of analytical grade.

Conversion of β-lactam antibiotics to a fluorescent degradation product

The antibiotic (500 mg) in distilled water (50 ml) was treated (10 min; 20°) with sodium hydroxide solution (25 ml, M). Hydrochloric acid (25 ml, M) was then added followed by Sorensen's citrate buffer (150 ml) containing formaldehyde (1·0% v/v). The pH of the buffer was adjusted to 5·0 for cephalexin and cephaloglycin, and to pH 4·0 for ampicillin, pivampicillin and hetacillin. The mixture was then heated at 100° for 30 min, cooled and extracted repeatedly with ethyl acetate. The combined organic layers were then evaporated and the crude product thus obtained was crystallized from ethyl acetate.

The product had uncorrected m.p. 206–208° (Karmas & Spoerri (1956) cite m.p. 206°): found C, 70·4; H, 5·4; N, 14·4: calc. for $C_{11}H_{10}N_2O$ C, 70·95; H, 5·41; N, 15·05. Mixed melting points of the products of hydrolyses of cephalexin and ampicillin showed no depression.

The product had the following characteristics: λ_{max} (water) 250 nm (ϵ 5580), 346 nm (ϵ 10088); ν_{max} (KBr) 2800 cm^{-1} (OH), 1650 cm^{-1} (amide), 1617, 1295, 750 and 690 cm^{-1}; δ ($CDCl_3$ TMS) 2·36 (3H S), 7·42 (4H m), 8·35 (2H m) 13·45 (1H S); *m/e* 187, 186 ($M^{+\cdot}$); 185, 159, 158, 157, 130, 104, 103, 89. Mass measurement of the molecular ion gave 186·0793, $C_{11}H_{10}N_2O$ requires 186·0793). The ion *m/e* 158 formed from $M^{+\cdot}$ by the expulsion of 28 amu (CO) was also confirmed by mass measurement—158·0840: $C_{10}H_{10}N_2$ requires 158·0844.

Comparative thin layer chromatography of hydrolysis product

The purified fluorescent degradation products so obtained, and those for ampicillin obtained by the methods of Jusko (1971), Miyazaki & others (1974), and of Barbhaiya & Turner (1977b) from plasma, were subjected to thin-layer chromatography analysis on Merck silica gel F254 plates with solvent systems (v/v); (I) chloroform–acetone (1 : 1), (II) acetone–light petroleum (b.p. 40–60°) (1 : 1), (III) cyclohexane–ethanol (9 : 1), (IV) toluene–acetic acid (3 : 1) and (V) tetrahydrofuran–light petroleum (b.p. 40–60°) (1 : 1). The R_F values were identical (Table 1).

Synthesis of 2-hydroxy-3-phenyl-6-methylpyrazine (V)

A suspension of phenylglycine amide (3·5 g, 0·023 mol) (Neilson & Ewing, 1966) in methanol (27 ml) was stirred at −40° while aqueous sodium hydroxide (0·24 g in 2·5 ml) was added dropwise, followed by aqueous methylglyoxal (5 g of 40% w/v). The temperature rose to −30°. Aqueous sodium hydroxide (0·4 g in 2·5 ml) was added dropwise over 1 h during which time the temperature was not allowed to rise above −5°. After a further 2 h with stirring

at 20° the mixture was acidified to pH 2 with HCl and the precipitated solid filtered off. The filtrate was evaporated to dryness and the residue dissolved in chloroform. This solution was washed with water, dried ($MgSO_4$), and then evaporated to dryness leaving a red oil. Trituration with ether gave a solid which was crystallized from ethyl acetate as pale yellow needles, 0·65 g (15%), m.p. 206° (Karmas & Spoerri (1956) cite m.p. 206°). Found: C, 70·7; H, 5·6; N, 14·7: calc. for: $C_{11}H_{10}N_2O$ C, 70·95; H, 5·41; N, 15·05%. The mixed melting point of the synthesized and degraded products showed no depression.

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REFERENCES

AIKAWA, R., NAKANO, M. & ARITA, T. (1976). *Chem. Pharm. Bull.*, **24**, 2350–2355.

BARBHAIYA, R. H. & TURNER, P. (1976a). *J. Pharm. Pharmac.*, **28**, 791–792.

BARBHAIYA, R. H. & TURNER, P. (1976b). *Br. J. Pharmac.*, **58**, 473.

BARBHAIYA, R. H. & TURNER, P. (1977a). *Br. J. clin. Pharmac.*, **4**, 401–405.

BARBHAIYA, R. H. & TURNER, P. (1977b). *J. Antimicrob. Chemother.*, **3**, 423.

BARBHAIYA, R. H. & TURNER, P. (1977c). *Br. J. clin. Pharmac.*, **4**, 734.

BARBHAIYA, R. H., TURNER, P. & SHAW, E. (1977). *Clinica chim. Acta*, **77**, 373–377.

CHEESEMAN, G. W. H. & WERSTIUK, E. S. G. (1972). *Advances in Heterocyclic Chemistry*, Vol. 14, p. 99. London: Academic Press.

COHEN, A., FUNKE, P. & PUAR, M. (1973). *J. pharm. Sci.*, **62**, 1559–1561.

DURR, A. & SCHATZMAN, H. G. (1975). *Experientia*, **31**, 503–504.

INDELICATO, J. M., NORVILAS, T. T., PFEIFFER, R. R., WHEELER, W. W. & WITHAM, W. L. (1974). *J. mednl Chem.*, **17**, 523–527.

JUSKO, W. J. (1971). *J. pharm. Sci.*, **60**, 728–732.

KARMAS, G. & SPOERRI, P. E. (1952). *J. Am. chem. Soc.*, **74**, 1580–1584.

KARMAS, G. & SPOERRI, P. E. (1956). *Ibid.*, **78**, 4071–4078.

MIYAZAKI, K., OGINO, O. & ARITA, T. (1974). *Chem. Pharm. Bull.*, **22**, 1910–1916.

MIYAZAKI, K., OGINO, O., NAKANO, M. & ARITA, T. (1975). *Ibid.*, **23**, 178–183.

NEILSON, D. G. & EWING, D. (1966). *J. chem. Soc.*, 393.

REEVES, R. L. (1966). In: *Chemistry of the Carbonyl Group*, p. 567. Editor: Patai, S. London: Interscience.

ROETS, E. E., VLIETINCK, A. J., JANSSEN, G. A. & VANDERHAEFH, H. (1973). *Chem. Commun.*, 484–485.

YAMANA, T., TSUJI, A. J., KANAYAMA, K. & NAKANO, O. (1974). *J. Antibiotics*, **28**, 1000–1002.