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2-Hydroxy-3-aryl-6-methylpyrazines—fluorophores formed from the reaction of formaldehyde with cyclic enamine degradation products common to β-lactam antibiotics with aryl glycine side chains

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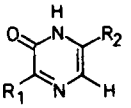
Sensitive fluorimetric assay procedures for β-lactam antibiotics containing the phenyl glycine side chain have been utilized since 1971 (Barbhaiya et al 1978; Lebelle et al 1979). Barbhaiya et al reported the isolation and identification of a fluorescent product, 2-hydroxy-3-phenyl-6-methylpyrazine, Ia, that formed when formaldehyde was used in the manipulation of degradation products from ampicillin, cephalexin, cephaloglycin, or cephradine. Recently Lebelle demonstrated that 2-hydroxy-3-phenylpyrazine, Id, is the fluorescent product formed directly from ampicillin degradation



I

a) R_1 = C_6H_5–; R_2 = Me

b) R_1 =  ; R_2 = Me

c) R_1 = HO-C_6H_4–; R_2 = Me

d) R_1 = C_6H_5–; R_2 = H

and that Ia is produced only in the presence of formaldehyde. A mechanism for the formaldehyde reaction was not proposed.

We now report the formation of Ia from both cephalexin and ampicillin via the method of Barbhaiya et al employing labelled formaldehyde [90% ^{13}C purchased from Stoher Isotope Chemicals (Rutherford, N.J.)]. Knowledge of the structural position of the formaldehyde carbon in Ia should suggest a mechanism for its formation from a degradation product obviously common to both ampicillin and cephalexin and probably common to other penicillins and cephalosporins containing aryl glycine side chains.

The crystalline products obtained from both ampicillin and cephalexin were identical spectroscopically when [^{13}C]formaldehyde was employed in the method of Barbhaiya et al. The 2-hydroxy-3-phenyl-6-methylpyrazines had the following characteristics: (i) unlabelled Ia, m.p. 206–208 °C; electron impact M^+ 186; δ(DMSO/d_6 at 360 MHz TMS) 2·21 (3H, s), 7·33 (1H, s), 7·40 (3H, m), 8·29 (2H, m), 12·52 (1H, s); (ii) ^{13}C enriched Ia, m.p. 208–210 °C; electron impact M^+ 187; δ(DMSO/d_6 at 360 MHz TMS) 2·21 (s) 2·21 (d, $^1J_{C\text{-}H}$ = 126 Hz), 7·33 (s), 7·40 (m), 8·29 (m), 12·52 (s). The only apparent differences between the product obtained from ^{13}C-enriched formaldehyde and the product obtained from formaldehyde were an increase in mass M^+ of 1 and a splitting of the methyl singlet at δ 2·21 into a doublet due to a ^{13}C–H coupling. The exclusive incorporation of the formaldehyde carbon in the methyl group of Ia is dramatically demonstrated by the ^{13}C n.m.r. spectra in Fig. 1. The pyrazines have been

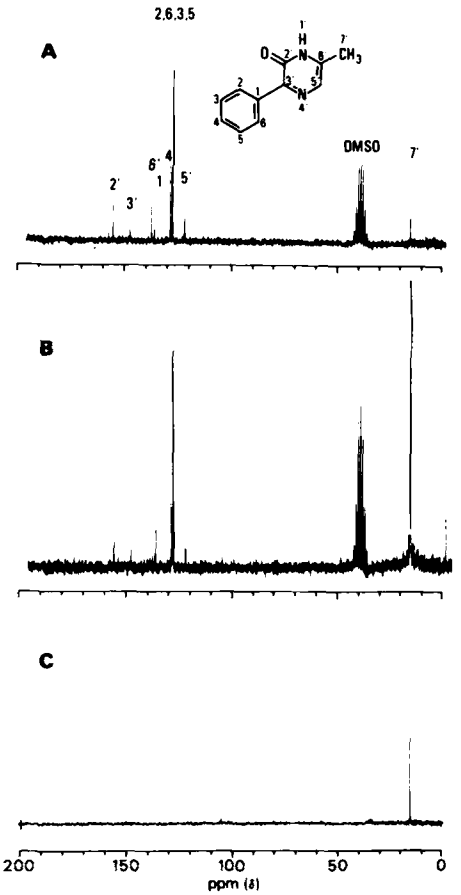
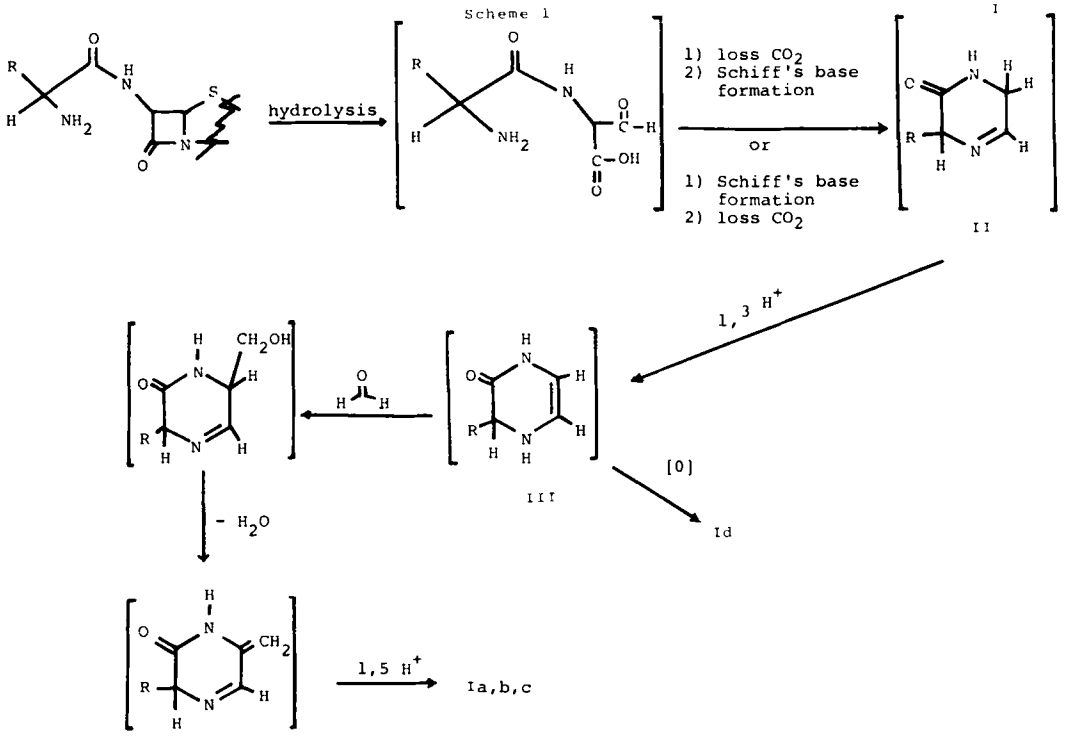


FIG. 1. ^{13}C n.m.r. spectra (DMSO/d_6). Key: A, compound Ia (natural abundance ^{13}C), 4500 transients; B, compound Ia (^{13}C enriched), 6300 transients; C, compound Ia (^{13}C enriched), 50 transients.

* Correspondence.



represented as the amide tautomer because no hydroxyl stretching frequency is observed in their i.r. spectra (KBr and $CHCl_3$).

These products (and other discussed herein) gave ^1H and ^{13}C n.m.r., i.r., and mass spectra fully consistent with structure Ia proposed by Barbhaiya et al, but definitive structure proof rests upon the independent synthesis by Karmas & Spoerri (1956).

Since the formaldehyde carbon becomes the 6-methyl carbon of Ia, we propose its formation by the mechanism shown in Scheme I. This scheme is essentially the scheme of Lebelle et al for the formation of Id from ampicillin but is now generalized to include cephalosporins and amoxicillin, and also to explain the formation of Ia. Extensive hydrolysis and decarboxylation of the penicillin or cephalosporin eventually lead to a cyclic Schiffs' base, II, which upon tautomerization to enamine, III, may serve as a common intermediate to both the Barbhaiya product, Ia, upon condensation with formaldehyde or to the Lebelle product, Id, upon oxidation in the absence of formaldehyde.

The product we obtained from cephradine by the Barbhaiya procedure was not Ia as suggested by Barbhaiya et al but Ib. 2-Hydroxy-3-(1,4-cyclohexadien-1-yl-6-methylpyrazine, Ib, had the following characteristics: mp 207–211 C; found C, 70·4; H, 6·3; N, 14·6; O, 8·6; calc. for $C_{11}H_{12}N_2O$; C, 70·2; H, 6·43; N, 14·88; O, 8·50; electron impact M^+ 188 (base peak 187); λ_{max} (CH_3OH) 245, nm (ϵ4,493), 342 nm (ϵ10,697); δ($CDCL_3$ at 360 MHz TMS) 2·38 (3H,s), 3·03 (2H,m), 3·17 (2H,m), 5·17 (1H,m), 5·87 (1H,m), 7·27 (1H,s), 7·69 (1H,broad s), 12·51 (1H,s). The n.m.r. spectrum suggested contamination with $\approx$ 5% of compound Ia.

The product we obtained from amoxicillin and cefadroxil by the Barbhaiya procedure was Ic. 2-Hydroxy-3-(4-hydroxyphenyl)-6-methylpyrazine, Ic, had the following characteristics: m.p. 328 °C dec.; found C, 65·2; H, 5·1; N, 13·7; O, 16·1: calc. for $C_{11}H_{10}N_2O_2$ C, 65·3; H, 5·0; N, 13·9; O, 15·8; electron impact M^+ 202; λ_{max} (CH_3OH) 245 nm (ϵ8,484), 357 nm (ϵ16,726); δ(DMSO/d_6 at 360 MHz TMS) 2·17 (3H,s), 7·23 (1H,s), 6·78 and 8·18 (4H aromatic AA' BB' pattern), 9·68 (1H,s), 12·34 (1H,s).

Scheme 1 presents a generalized degradation pathway for the hydrolysis of ampicillin and amoxicillin to fluorescent products and an alternative degradation pathway for aryl glycine cephalosporins to degradation by intramolecular nucleophilic attack of the α-amino function upon the β-lactam (Indelicato et al 1972, 1974; Yamana et al 1974).

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Diffusion coefficient determination using a filter-paper diaphragm cell technique

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While many diffusion coefficients have been measured and collected (Tuwiner 1962), the permutations of solute and solvent are almost limitless and it is therefore desirable to have a technique which is able, quickly and reproducibly, to determine the required value. The method reported in this paper uses a filter-paper diaphragm cell similar to that described by Kreevoy & Wewerka (1967).

It is a steady-state method based on the equation of Fick's 1st Law of Diffusion (Robinson & Stokes 1965). Diffusion cells described by previous workers (cf. Stokes 1950a) have a relatively thick glass sintered diaphragm and the measurement of a diffusion coefficient requires one to three days. The use of filter-paper diaphragms introduced by Gage (1948) allows the time-scale of the experiment to be considerably reduced.

The filter-paper diaphragm diffusion cell used in our studies is an adaptation of that described by Kreevoy & Wewerka (1967) (Figure 1). The diaphragm of the cell is a 0·45 μm pore size Millipore filter about 150 μm thick and cut from a larger (47 mm) disc, held firmly in position when the lower threaded cup is screwed into the upper part of the cell. Different Millipore discs are used in each run. The solutions in the two compartments are stirred magnetically: the 'flea' in the lower compartment is quite large (relative to the size of the compartment itself) while the smaller flea in the upper compartment sits directly on the filter. This arrangement is designed to preclude the possibility of stagnant diffusion layers of unknown thickness forming on either side of the filter.

The lower compartment containing a stirrer bar is filled with a previously thermostatted (25 °C) diffusant solution of known concentration until the surface tension holds the meniscus slightly above the level of the inner compartment: no air bubbles should adhere to the cell walls or to the stirrer bar. The filter is then positioned gently on the solution avoiding air bubbles; excess solution around the edges of the filter is removed with a clean tissue and the two halves of the cell are screwed firmly together and a smaller stirrer bar is placed on the filter. An appropriate volume of thermostatted (25 °C) pure solvent is added to the upper chamber, the cell is then thermostatted at 25 ± 0·02 °C and stirred.

After 30 min, complete thermal equilibration is achieved and a steady state concentration profile is produced in the diaphragm (with a much thicker glass sinter as the diaphragm, between 2 and 3 h are typically required). The upper compartment solution is then sampled, a stop-clock being started when approximately half the volume has been removed (time = 0). A further sample is withdrawn after another 25–60 minutes (time = t) and the concentration of solute in the two samples determined. Using the volumes of the two compartments (V_L and V_U), we may determine the concentration of solute in the lower and upper compartments at zero time and t, respectively c_1 and c_2, c_3 and c_4.

The results are analysed using the conventional diaphragm cell procedure (Robinson & Stokes 1965), the diffusion coefficient D being calculated from equation (1)

$$D = \frac{1}{\beta t} \ln \left\{ \frac{c_1 - c_2}{c_3 - c_4} \right\} \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

The calibration constant β is defined by equation (2)

$$\beta = \frac{A}{d}(V_L^{-1} + V_U^{-1}) \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where A is the total effective cross-sectional area of the diaphragm pores, the effective average length of which along the diffusion path is d.

The diaphragm cell has been used to measure solute diffusion coefficients in water and isopropyl myristate (IPM), the latter chosen because of its frequent use in drug absorption and distribution studies as a model for various membrane lipids (e.g. Poulsen et al 1968; Albery & Hadgraft 1979; Armstrong et al 1979). Although equation (2) predicts that the calibration constant of the cell is independent of the system studied, we have calibrated the cell for both water and IPM as

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