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Isolation and identification of a fluorophore from ampicillin degradation

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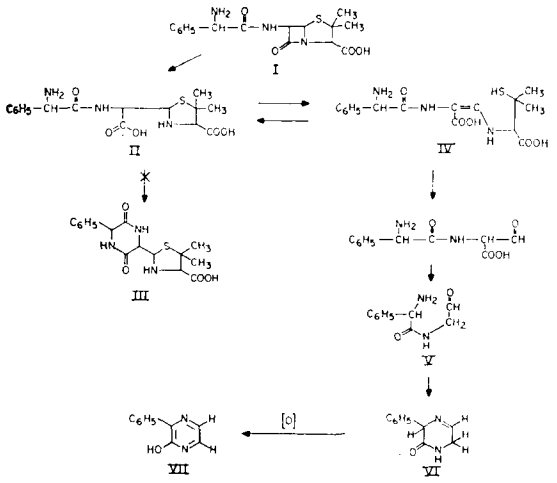
A fluorescent impurity in ampicillin has been isolated and identified as 2-hydroxy-3-phenylpyrazine. The product is formed under acidic conditions similar to those employed in some fluorometric assay procedures. The mechanism of the reaction is proposed to involve cyclization by condensation of the penilloaldehyde of ampicillin. The structure was confirmed by independent synthesis.

Fluorometric assay procedures based on the formation of fluorescent degradation products for the analysis of β-lactam antibiotics containing ampicillin-like side chains have been reported. Several of these (Barbhaiya & Turner, 1976a, b; 1977a, b) made use of a method originally developed for ampicillin (I, Scheme 1) by Jusko (1971) which employed acid hydrolysis of the easily formed alkaline degradation product, the penicilloic acid (II). The addition of



formaldehyde to the reaction solution resulted in enhanced fluorescence and a more reproducible assay. However, Barbhaiya et al (1977) reported a fluorometric assay for amoxicillin in which no formaldehyde was used. Other methods (Miyazaki et al 1974, 1975; Barbhaiya & Turner 1977c) required mild acid hydrolysis of the alkaline degradation product in the presence of mercuric chloride. All of these reports mentioned the formation of an intense fluorophore with excitation and emission maxima at about 345 nm and 425 nm respectively. Jusko (1971) originally proposed that the fluorescent species was the 2,5-diketopiperazine (III). Although diketopiperazines have been prepared from cephalosporins (Cohen et al 1973; Yamana et al 1974; Indelicato et al 1977; Dinner 1977), their synthesis from penicillins was not successful presumably because of steric hindrance (Indelicato et al 1972; Roets et al 1973).

That the fluorescent species formed from ampicillin degradation may be a 2-hydroxy-3-phenylpyrazine has been reported (LeBelle et al 1975; Hughes et al 1976) and recently Barbhaiya et al (1978) isolated a product formed under Jusko's conditions (using formaldehyde) and identified it as 2-hydroxy-6-methyl-3-phenylpyrazine.

While making stability studies on ampicillin we observed the presence of a fluorescent compound the ultra-violet absorption spectrum of which was similar to that of the fluorescent species reported in the previously mentioned fluorometric assay procedures (γ_{max} about 345 nm). Whether this product was identical to the fluorophore isolated by Barbhaiya et al (1978) was not known. Therefore the isolation and structure determination of this product was undertaken.

MATERIALS AND METHODS

Phenylglycinonitrile hydrochloride (Aldrich Chemical Co.) and 40% w/v aqueous glyoxal (Aldrich Chemical Co.) were used as received. All solvents were 'distilled in glass' grade.

Spectroscopy

Nuclear magnetic resonance spectra in chloroform-d solution with tetramethylsilane as internal standard were obtained on a Varian A-60A. Electron impact

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mass spectra were obtained on Varian Mat 311 and Hewlett-Packard Model 5985 instruments. Infrared spectra were recorded as potassium bromide disks on a Perkin Elmer 621. Ultraviolet spectra were recorded as aqueous solutions on a Beckman Acta CIII spectrophotometer.

Degradation of ampicillin to 2-hydroxy-3-phenylpyrazine (VII)

Sodium ampicillin (10 g) was dissolved in distilled water (200 ml) and the solution maintained at pH 11–12 by addition of sodium hydroxide solution (M). After 2 h the pH of the solution was then adjusted to 2 by the addition of hydrochloric acid (M). The solution was heated at 50°C for 5 h, cooled and extracted repeatedly with 25% methanol in chloroform. Evaporation of the extracts gave a yellow solid which upon preparative thin-layer chromatography (10% methanol in chloroform as eluant) yielded the pale yellow 2-hydroxy-3-phenylpyrazine (VII), m.p. 180–181°C: found C, 69·54; H, 4·80; N, 16·43: calc. for $C_{10}H_8N_2O$; C, 69·76; H, 4·68; N, 16·27.

The product had the following characteristics: λ_{max} (water) 243 nm (ϵ 6648), 336 nm (ϵ 11 252); ν_{max} (KBr) 2850, 1640, 1280, 1220, 810, 800, 740 and 685 cm^{-1}; $\delta(CDCl_3, TMS)$ 7·18 (1H, d), 7·45 (3H, m), 7·61 (1H, d), 8·33 (2H, m), 12·8 (1H, broad); *m/e* 172 ($M^{+\cdot}$), 144, 117, 104, 90, 89, 77, 63, 51. Mass measurement of the molecular ion gave 172·0634 ± 0·0004, $C_{10}H_8N_2O$ requires 172·0637. Direct analysis of daughter ions indicates 172 → 144 → 117. The fragments corresponding to 144 and 117 were confirmed by mass measurement to be $C_9H_8N_2$ (loss of CO) and C_8H_7N.

Synthesis of 2-hydroxy-3-phenylpyrazine (VII)

To a solution of phenylglycine amide (1·06 g) (Sandler & Karo 1968) in methanol (10 ml) at −5°C was added 40% w/v aqueous glyoxal (1·02 g) in methanol (5 ml) followed by sodium hydroxide solution (1·50 ml, 6 M). The solution was stored at −20°C for 2 h, allowed to warm to room temperature (20°C), acidified to pH 3 and extracted with chloroform. The chloroform extracts yielded 850 mg (70%) yellow solid which was recrystallized from chloroform–hexane to yield the 2-hydroxy-3-phenylpyrazine, m.p. 180–182°C (uncorrected). Jones (1949) reports m.p. 172–173°C.

High performance liquid chromatographic determination of 2-hydroxy-3-phenylpyrazine in ampicillin capsules

H.p.l.c. analysis was performed on a Waters ALC-100 chromatograph equipped with a Waters septumless injector (U6K) and a Schoeffel fluorescence detector (FS970). The excitation wavelength was 345 nm and emission filter was 389 nm. An RP-2 column (Brownlee Labs) and mobile phase of 25% methanol in ammonium acetate (0·1 M) were used. The flow rate was 1 ml min^{-1}. Integration was carried out using a Spectra Physics chromatography data system (SP 4000). The contents of an ampicillin capsule were dissolved to give a concentration of 10 mg ml^{-1} in methanol. 10 μl of the supernatant were injected into the chromatograph and the peak corresponding to the 2-hydroxy-3-phenylpyrazine integrated. The amount of (VII) present was calculated by injection of known amounts of pure VII. A mass spectrum of a sample of the fluorescent impurity collected from h.p.l.c. was identical with that of authentic 2-hydroxy-3-phenylpyrazine.

Thin layer chromatography

Thin layer chromatography was on Merck Silica gel F-254 plates using solvent systems v/v: (a) 15% methanol in chloroform; (b) 1:1 chloroform–acetone. The R_F values of the 2-hydroxy-3-phenylpyrazine and 2-hydroxy-6-methyl-3-phenylpyrazine were in solvent system (a) 0·52 and 0·58; (b) 0·36 and 0·39. The product isolated from ampicillin capsules by preparative h.p.l.c. had R_F values of 0·52 and 0·36 in solvent systems (a) and (b).

RESULTS AND DISCUSSION

The acidic degradation of a solution of ampicillin that previously had been hydrolysed in sodium hydroxide yielded upon workup a crystalline product, m.p. 180–181°C. The mass spectrum indicated a peak at *m/e* 172 (molecular ion). Mass measurement (172·0634 ± 0·0004) was consistent with a molecular formula of $C_{10}H_8N_2O$ (172·0637). The proton magnetic resonance (p.m.r.) spectrum contained 2 doublets ($J = 4$Hz) at δ7·18 (1H) and δ7·61 (1H), a three proton multiplet at δ7·45, a two proton multiplet at δ8·33 and a broad resonance at δ12·8 which disappeared upon exchange with deuterium oxide. These spectral features are similar to those reported for 2-hydroxy-6-methyl-3-phenylpyrazine (Barbhaiya et al 1978) except for lack of the methyl resonance. Structure (VII) was consistent with these p.m.r. and m.s. data. The multiplet at δ8·33 was assigned to the two phenyl *ortho* protons and that at δ7·45 to the remaining phenyl protons. The doublets at δ7·18 and δ7·61 were due to the protons of the pyrazine ring.

The structure of (VII) was confirmed by an alternative unambiguous synthesis of 2-hydroxy-3-phenylpyrazine (Jones 1949). No melting point depression occurred on admixture of the two compounds. In addition u.v., p.m.r. and m.s. data as well as t.l.c. and h.p.l.c. characteristics were identical.

Thus, unlike the fluorometric assay procedures which employ formaldehyde to yield the 2-hydroxy-6-methyl-3-phenylpyrazine, similar reaction conditions in the absence of formaldehyde gave (VII). Therefore, formaldehyde is not a catalyst as originally proposed (Jusko 1971; Barbhaiya & Turner 1976a; 1977a, b) but is actually incorporated into the reaction product. Only traces of (VII) were detected in the reaction with formaldehyde by thin-layer chromatography. The mechanism of the formaldehyde reaction has not been speculated upon.

Since fluorescent products are also formed under conditions known to involve the formation of the penamaldic acid (IV) derivative of ampicillin (Miyazaki et al 1974; 1975; Barbhaiya et al 1977c; Hou & Poole 1969; Hughes et al 1976) the reaction may proceed by degradation of the penamaldic acid through the penaldic acid to the penilloaldehyde (V) (Schwartz 1969). Cyclization could occur by formation of the Schiff's base between the side chain amino and the aldehyde group of penilloaldehyde (Thiel et al 1964) to give (VI). Subsequent oxidation would lead to structure (VII). The formation of pyrazines from dihydropyrazines has been reported (Cheeseman & Werstiuk 1972) to proceed in the presence of atmospheric oxygen.

Both t.l.c. and h.p.l.c. have indicated the presence of trace amounts of (VII) in some ampicillin trihydrate formulations. These were unexpired products commercially available on the Canadian market from various manufacturers and had been stored at ambient temperature for about one year. Of the products tested a maximum of 120 ppm of (VII) was found.

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