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Structure re-assignment of a metabolite of ampicillin and amoxycillin and epimerization of their penicilloic acids

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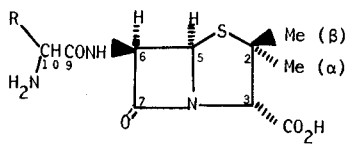
Products formed in-vitro from ampicillin and amoxycillin penicilloates have been examined by high-performance liquid chromatography, ultraviolet and nuclear magnetic resonance spectroscopy and thiol group determination and were found to be the 5*S* epimers of the penicilloic acids. This is in contrast to a published claim that the corresponding penamaldic acids were formed by the treatment used.

Masada et al (1980) reported the observation by high performance liquid chromatography of a new metabolite of ampicillin (I, R=C_6H_5) and an analogous metabolite of amoxycillin (I, R=4-OH.C_6H_4) which were also formed in-vitro from the corresponding penicilloic acids (II, R=C_6H_5 and 4-OH.C_6H_4) dissolved in aqueous mercuric chloride or sodium dihydrogen phosphate. They identified these compounds as the corresponding penamaldic acids (III, R=C_6H_5 and 4-OH.C_6H_4) and in subsequent pharmacokinetic studies (Uno et al 1981b; Haginaka et al 1981) refer to the assay of these metabolites. This identification is surprising because penamaldic acids, including that from ampicillin, have been reported (Woodward et al 1949; Schneider & de Weck 1966; Schwartz & Delduce 1969; Longridge & Timms 1971; Bundgaard & Larsen 1979) to be very unstable. The present paper shows that the interpretation by Masada et al (1980) is incorrect and that the new compounds observed by them are the penicilloic acids epimerized at C-5 to the 5*S* configuration. A penicilloic acid, when formed by hydrolysis of a penicillin, has the same 3*S*, 5*R*, 6*R* configuration as the parent penicillin. However, epimerization of penicilloic acids at C-5 on storage in aqueous solution has been reported (Carroll et al 1977; Busson et al 1976; Degelaen et al 1979) and our unpublished observations have suggested that it occurs readily with many penicilloic acids when dissolved in alkaline solution. Consequently the product from alkaline treatment of ampicillin penicilloic acid has been compared with that generated under the mercuric chloride and phosphate conditions of Masada et al (1980).



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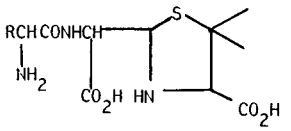

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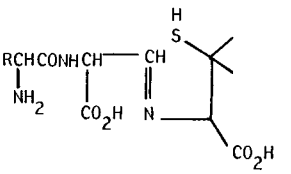

III

* Correspondence.

MATERIALS AND METHODS

Materials

Ampicillin and amoxycillin were Beecham commercial samples. Ampicillin and amoxycillin monosodium penicilloates were prepared by the method of Munro et al (1978). Sodium dideuterophosphate was prepared by freeze-drying a solution of sodium dihydrogen phosphate dihydrate (1·0 g) in deuterium oxide (10 ml). Deuterium oxide (99·8%)

and sodium deuteroxide (30% in D_2O) were obtained from Fluorochem Ltd. Acetonitrile was h.p.l.c. grade and other chemicals were reagent grade.

Nuclear magnetic resonance (n.m.r.) spectra

^1H Spectra were obtained at 60 MHz on a Perkin-Elmer R12A spectrometer or at 90 MHz on a Perkin-Elmer R32 spectrometer, both at a probe temperature of about 33 °C. A solution of tetramethylsilane in $CDCl_3$ sealed in a coaxial tube was used to provide a lock and external reference signal. Chemical shifts are reported downfield with respect to internal DSS and were obtained by subtraction of 0·14 from the measured shifts. This correction factor was determined from the spectra of a sample run with internal DSS and with the external TMS reference.

^{13}C Spectra were obtained on a Brüker WM 250 spectrometer at a probe temperature of about 21 °C. Broad band noise decoupling was used to decouple the proton resonances and the spectra were acquired into 16K data points with a spectral width of 16129 Hz. Chemical shifts are given relative to external dioxan at 67·4 p.p.m.

High-performance liquid chromatography (h.p.l.c.) and ultraviolet (u.v.) spectra

Reversed phase h.p.l.c. was carried out using a Waters model 6000 or an Altex model 110 pump, a stainless steel column (25 cm × 0·4 cm i.d.) prepacked with Zorbax C8 (du Pont Ltd) and fitted with a 20 µl fixed volume loop injector and a variable wavelength u.v. detector (Varian Vari-Chrom or Perkin-Elmer LC75) set at 230 nm 0·16 aufs or 260 nm 0·10 aufs. The mobile phase consisted of pH 5·5 0·1 M ammonium acetate (3 ml acetic acid in 450 ml water adjusted to pH 5·5 with 0·880 ammonia and diluted to 500 ml with water) containing 5% v/v acetonitrile for ampicillin penicilloate or 1% v/v acetonitrile for amoxycillin penicilloate. The flow rate was 1·5 ml min^{-1}. U.v. spectra were obtained from h.p.l.c. peaks by stopping the flow of mobile phase and using the LC75 in the scanning mode. Background spectra were obtained by stopping the flow and scanning just before elution of each peak. Other u.v. spectra were obtained with a Perkin-Elmer model 554 spectrophotometer.

Epimerization of penicilloic acids

Solutions for determination of u.v. spectra from h.p.l.c. peaks were prepared as follows: Solution A. 22 mg monosodium ampicillin penicilloate was dissolved in 20 ml 1 M NaH_2PO_4 and kept at 30 °C for 3·5 h. Solution B. 10·5 mg monosodium ampicillin penicilloate was dissolved in 20 ml of a 0·00125% aqueous solution of mercuric chloride and used within 10 min. Solution C. 19 mg monosodium ampicillin penicilloate was dissolved in 1 ml 1 M NaOH, kept at 30 °C for 30 min, 1 ml 1 M HCl added and diluted to 20 ml with 0·1 M pH 7 sodium phosphate.

Samples for ^{13}C and ^1H n.m.r. and for thiol group determination were prepared as follows: ca 400 mg monosodium ampicillin (Sample D) or amoxycillin (Sample E) penicilloate and ca 195 mg sodium dihydrogen phosphate dihydrate were dissolved in 10 ml deuterium oxide, kept at 36 °C for 24 h and then freeze dried.

Thiol group determination

Thiol content was determined colorimetrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman 1959).

RESULTS AND DISCUSSION

H.p.l.c., u.v. spectra and thiol content

Penamaldic acids give an absorption maximum at about 280 nm (Woodward et al 1949; Schneider & de Weck 1966; Schwartz & Delduce 1969; Longridge & Timms 1971) with a molar absorbance of about 9000 (Schwartz & Delduce 1969). Solutions A and B were prepared from ampicillin penicilloate by the methods given by Masada et al (1980) for its conversion to the penamaldic acid. Solution C was prepared under conditions shown by n.m.r. spectroscopy to result in partial conversion of the penicilloate to another compound. H.p.l.c. at 230 nm showed two peaks at retention times of about 4 and 6 min which were identical for the peaks from all three solutions. A fresh solution of ampicillin penicilloate in water gave only the peak at 6 min so the 4 min peak must be that ascribed to the penamaldic acid by Masada et al (1980). The u.v. spectrum between 230 and 320 nm of each peak from all three solutions was scanned by the stopped flow technique. All spectra showed a strong end absorption, peaking below 230 nm, and a broad ill-defined maximum at about 260 nm which can be assigned to the aromatic ring absorptions. Above 275 nm the spectra were identical to the background spectra and showed no sign of the presence of an absorption maximum around 280 nm.

Samples D and E were larger scale preparations by the phosphate method using ampicillin and amoxy-

cillin penicilloate respectively. Deuterium oxide was used to avoid a large HDO signal in the n.m.r. spectrum and to check for deuterium incorporation in the product. H.p.l.c. at 260 nm using 2 mg ml^{-1} for Sample D and 0·3 mg ml^{-1} for Sample E gave two peaks for each sample with an area ratio of about 4:1 for product to starting material. The retention times for Sample D were the same as those for Solutions A to C and for Sample E were about 4 and 5 min for product and starting material respectively. N.m.r. spectra of Samples D and E also showed about 80% conversion of penicilloate to the supposed penamaldic acid product. U.v. spectra of these samples, 0·003% in water, were obtained. Sample D showed a strong end absorption below 210 nm, an ill-defined maximum at about 260 nm and no significant absorption above 270 nm. Sample E showed a strong end absorption below 210 nm and maxima at 227 nm (ε 8500) and 270 nm (ε 1020), with a shoulder at 276 nm. This spectrum is very similar to that of amoxycillin in 0·1 M HCl (Bhattacharyya & Cort 1978) and is indicative of the phenolic chromophore. Neither spectrum showed the strong absorption peak at about 280 nm expected for a penamaldic acid.

Samples D and E gave a small response in the thiol group determination, equivalent to only 0·3% conversion of penicilloic acid to penamaldic acid in each sample.

Bundgaard & Larsen (1979) state that the penamaldate formed by addition of mercuric chloride to ampicillin penicilloate solution had disappeared, as judged by absorbance at ca 280 nm, 20 min after the mercuric chloride addition. This observation has been confirmed under the conditions given by Masada et al (1980) for reaction of the penicilloic acid with mercuric chloride. The spectrum of a solution of ampicillin penicilloate (0·24 mg ml^{-1}) in 0·00125% $HgCl_2$ was scanned at 10 min intervals, using an identical concentration of the penicilloate in water in the reference cell. Immediately after addition of $HgCl_2$ an absorption maximum at 276 nm was present but this was greatly reduced after 10 min and virtually absent after 20 min.

The absence of absorption at ca 280 nm, the very low thiol content and the demonstrated instability of ampicillin penamaldate show that the major new compound formed in the penicilloic acid solutions cannot be the penamaldic acid. This is further confirmed by the n.m.r. data below. Masada et al (1980) obtained a qualitative indication of the presence of thiol in their phosphate degraded sample both by a colorimetric method and from the Raman spectrum. The colorimetric test was done, like that on Samples D and E, on material prepared by freeze-drying a phosphate solution. Subsequently Uno et al (1981a) showed that ampicillin penicilloate in phosphate solution can given several minor degradation products including penicillamine. The presence of penicillamine is a plausible explanation for the observation of some thiol by the colorimetric method. However, the sample used for the Raman spectrum by Masada et al (1980) was obtained by preparative h.p.l.c. and so should have been free from penicillamine. Unfortunately the nature of the mobile phase and the procedure used to isolate the sample from it were not detailed and no evidence was given for the purity of the product. In this circumstance speculation about the origin of the apparent SH band in the Raman spectrum is pointless.

N.m.r. spectra

Preliminary 60 MHz ^1H spectra were obtained of ampicillin, amoxycillin and their monosodium penicilloates dissolved in D_2O adjusted to pD12 with NaOD. The chemical shifts of the penicilloic acid formed in-situ by hydrolysis of the penicillins were identical to those observed from a fresh solution of the monosodium penicilloates, thus confirming that these isolated salts have the 3*S*, 5*R*, 6*R* configuration of the parent penicillins. Repeat spectra on the aged solutions showed a gradual decrease in the initial penicilloate signals and progressive build up of a new set of signals. This behaviour is similar to that observed by Carroll et al (1977) in the alkaline hydrolysis of 6-aminopenicillanic acid and shown by them to be due to epimerization at C5. As the solutions of ampicillin penicilloate at pD12 aged beyond about 4 h, further signals appeared indicating formation of other minor components. This was not investigated further but could be due to epimerization at C-6 as well as C-5 and/or formation of degradation products such as pyrazines which have been observed in aged solutions of ampicillin penicilloate (Uno et al 1981a).

Subsequent spectra were obtained at 90 MHz and chemical shifts are given in Table 1 together with data from Masada et al (1980) for comparison. Repeat spectra on aged solutions of monosodium ampicillin penicilloate in D_2O at the natural pD of 5·9 and in NaD_2PO_4 showed similar behaviour to that in alkali, although the rate of change was slower than in alkali. The shifts vary with the pD of the solution but appropriate comparisons (e.g. Sample D adjusted to pD12 with the monosodium penicilloate isomerized at pD12) show that the same

product is formed in alkali, phosphate or D_2O alone. The n.m.r. data of Masada et al (1980) were obtained from solutions containing sodium phosphate but neither the phosphate concentration nor the pD of the solutions was stated. Shifts from a solution containing 2·4 M NaD_2PO_4, pD 3·9, are in good agreement with those of Masada et al, confirming that the product we have observed is that which they assigned as the penamaldic acid. The more limited results for amoxycillin penicilloate in Table 1 show that this behaves in a similar way to ampicillin penicilloate. In Table 1 shifts are not assigned separately to the α- and β- methyls or to 5-H and 6-H because such assignments cannot be made reliably on the basis of chemical shift data alone. It is noteworthy that Busson et al (1976) show inversion of chemical shift for 5-H and 6-H between the 5*R*6*R* and 5*S*6*R* isomers of dimethylbenzylpenicilloate in $CDCl_3$, where the protons can be unequivocally identified by coupling to the amide NH.

Table 1. ^1H Chemical shifts from 90 MHz spectra of solutions containing ca 40 mg penicilloate ml^{-1}.

Compound and solvent [pD]		Chemical shift, δ ppm[a] C-5[b]	C-2 methyls	3-H	5-H and 6-H[c]	10-H	Aromatics
II, R=C_6H_5, Na salt							
D_2O	*R*		1·10s, 1·16s	3·04s	4·25, 5·05q	5·17s	7·50s
[5·9]	*S*		0·51s, 1·43s	3·26s	4·83, 5·00q	5·23s	7·50s
II, R=C_6H_5, Na salt							
D_2O + NaOD	*R*		1·18s, 1·33s	3·26s	4·25, 5·11q	[d]	7·46s
[12]	*S*		0·84s, 1·55s	3·35s	4·83, 5·07q	[d]	7·46s
II, R=C_6H_5, Na salt							
D_2O, 2·4 M NaD_2PO_4	*R*		1·31s, 1·36s	3·51s	4·71, 5·35q	5·34s	7·63s
[3·9]	*S*		0·69s, 1·54s	3·56s	4·95, 5·14q	5·38s	7·63s
II, R=4-OH.C_6H_4, Na Salt							
D_2O	*R*		1·12s, 1·18s	3·08s	4·26, 5·06q	5·12s	6·95, 7·41dd
[6·2]	*S*		0·53s, 1·46s	3·29s	4·83, 5·01q	5·19s	6·95, 7·41dd
II, R=4-OH.C_6H_4, Sample E							
D_2O[e]	*R*		1·14s, 1·18s	3·12s	4·29, 5·10q	5·13s	6·94, 7·41dd
[5·9]	*S*		0·51s, 1·45s	3·28s	4·83, 5·00q	5·19s	6·94, 7·41dd
II, R=4-OH.C_6H_4[f]							
D_2O + NaH_2PO_4	*R*[f]		1·13s, 1·17s	[f]	4·31, 5 10q	[f]	[f]
	S[f]		0·54s, 1·53s	[f]	4·86, 5·02q	[f]	[f]
II, R=C_6H_5, Sample D							
D_2O[e]	*R*		1·13s, 1·18s	3·08s	4·31, 5·07q	5·21s	7·50s
[5·6]	*S*		0·50s, 1·44s	3·29s	4·84, 5·00q	5·27s	7·50s
II, R=C_6H_5, Sample D							
D_2O + NaOD[e]	*R*		1·18s, 1·31s	3·26s	4·24, 5·09q	[d]	7·46s
[12]	*S*		0·83s, 1·53s	3·35s	4·79, 5·05q	[d]	7·46s
II, R=C_6H_5[f]							
D_2O + NaH_2PO_4	*R*[f]		1·31s, 1·31s	[f]	4·65, 5·35q	[f]	[f]
	S[f]		0·69s, 1·55s	[f]	4·95, 5·12q	[f]	[f]

[a] The ratio of signal intensities was correct for the number of protons assigned in all spectra. Where both *R* and *S* isomers contributed to a single spectrum the intensity ratios were correct for each component.
[b] Configuration. Shifts for *R* isomer from spectra of fresh solutions and for *S* isomer from additional lines in spectra of mixtures formed in aged solutions, except for Samples D and E which were ca 4:1 mixtures of *S* to *R* isomer in fresh solution.
[c] $J_{5,6}$ 5 Hz for *R* and 3 Hz for *S* isomer in all spectra.
[d] Signal obscured by HDO peak.
[e] Solution contains 0·12 M NaD_2PO_4 from phosphate present in Samples D and E.
[f] Data from Masada et al (1980); sample and phosphate concentration and pD of solution unknown; shifts for *S* isomer are those assigned by Masada et al to the penamaldic acid; no shifts were given for 3-H, 10-H or aromatics.

Table 2 gives ^{13}C n.m.r. shifts and assignments which were based on off-resonance spectra to identify the quaternary carbon C-2 and on comparison with the shifts for the 5*R* and 5*S* isomers of the alkaline hydrolysis product of 6-aminopenicillanic acid (Carroll et al 1977).

The ^1H and ^{13}C n.m.r. data for the product from ampicillin and amoxycillin penicilloates is not consistent with the penamaldic acid structure. There is no one proton signal in the ^1H spectra at low field where the –CH=N– proton should be (Degelaen et al 1979, give 7·78 ppm for this proton in benzyl penamaldic acid) and the ^{13}C spectra show only the three carbonyl signals in the 168–175 ppm region where a –C=N– resonance would occur (Levy et al 1980), instead of the four signals which a penamaldic acid would give. By contrast the spectra are consistent with the products being a penicilloate isomer. The ^1H spectra showed no proton exchange accompanying product formation in D_2O, so the reaction cannot involve cleavage of a C–H bond. Cleavage and re-formation of C–C or C–N bonds necessary to isomerize C-3 or C-6 without C–H cleavage is impossible under the mild experimental conditions so the product must be the 5*S* penicilloate isomer formed by cleavage and re-formation of the C-5 bond via the penamaldic acid as a transient intermediate. Assignment of the product as the 5*S* epimer is supported by comparison of the ^1H n.m.r. data with that for isomeric dimethylbenzylpenicilloates (Busson et al 1976). The smaller shift difference and coupling constant between 5-H and 6-H for the 5*S*6*R* than the 5*R*6*R* isomer, the downfield shift of 3-H and the large upfield shift of one methyl and downfield shift of the other on conversion from 5*R*6*R* to 5*S*6*R* for the dimethylbenzylpenicilloates are all mirrored in the data in Table 1. Masada et al (1980) ascribe the high field signal of one methyl in their supposed penamaldic acids to the benzene ring current effect and state that the ring can be located above one methyl only when the C–S bond of the thiazolidine ring is cleaved. Examination of a molecular model of ampicillin penicilloic acid revealed no difficulty in locating the benzene ring close to one methyl, either in the 5*R* or 5*S* configuration, although it is obvious from the observed shifts that the preferred conformation for the 5*R* isomer does not have the ring

Table 2. ^{13}C Chemical shifts and assignments of II R=C_6H_5, II R=4-OHC_6H_4.

		Chemical shift, δ ppm[a]							
C-5	Methyls	C-2	C-3	C-5	C-6[b]	C-9	C-10[b]	Aromatics	Carboxy carbonyls
II, R=C_6H_5									
R[c]	26·6, 26·8	59·1	76·1	66·3	60·1	169·5	57·7	128·8(2C) 130·5(2C), 131·1, 133·2	175·7, 176·1
S[d]	27·9 28·8	58·8	76·2	67·3	56·3	170·1	57·7	128·8(2C) 130·9(2C), 131·2, 132·8	174·7, 174·8
II, R=4-OH.C_6H_4									
R[c]	26·4, 26·8	59·2	76·1	66·4	60·2	169·7	57·1	117·3(2C), 124·9, 130·7(2C), 157·9	175·8, 176·2
S[e]	27·6, 28·7	58·9	76·2	67·2	56·0	170·4	57·2	117(2C), 124·5, 130·6(2C), 157·9	174·7, 174·9

[a] All shifts are for a single carbon except where indicated otherwise.
[b] Tentative assignment—see text.
[c] From the spectrum of the monosodium salt.
[d] From the major signals in the spectrum of Sample D.
[e] From the major signals in the spectrum of Sample E.

positioned in this way. The downfield shift of both methyl carbons in the ^{13}C spectra (Table 2) on conversion from the 5*R* to 5*S* isomer provides a surprising contrast with the behaviour of the methyl signals in the proton spectra. It is not possible to assign C-6 and C-10 unambiguously but the results show a significant upfield shift for one of these carbons on conversion from the 5*R* to 5*S* isomer. This seems more likely to be C-6 than C-10 because of its proximity to the inverted centre, so tentative assignments have been given in Table 2 on that basis. Apart from this the differences between the ^{13}C shifts of the isomers are fairly minor.

The work described here refers only to ampicillin and amoxycillin penicilloates but the conclusion from it is clearly applicable also to the data in Masada et al (1980) assigned to the penamaldic acids of cyclacillin and 6-aminopenicillanic acid. ^{1}H Shifts for the 6-aminopenicillanic acid derivatives given by Masada et al (1980) are different from those in Carroll et al (1977), both for the initial hydrolysis product and for the compound described as the penamaldic acid by Masada et al (1980) or as 5-epipenicic acid by Carroll et al. These differences can be ascribed to the significant difference in salt content and pD of the solutions used to obtain the spectra.

Carroll et al (1977) comment that it is likely that all penicilloic acids epimerize at C-5 in alkali. We agree with this but would extend it to a wider pH range because the work reported here shows C-5 epimerization between pH 4 and 12 and Busson et al (1976) found that benzylpenicilloic acid epimerized at C-5 and C-6 on prolonged storage at pH 7. The ease of separation of diastereoisomers by h.p.l.c. and the procedure used here of following the change of n.m.r. spectrum with age of solution provide two valuable tools for investigating such reactions.

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