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Review

Application of ^{1}H nuclear magnetic resonance spectroscopy to the analysis of β-lactam antibiotics and their common degradation products

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Abstract: The ^{1}H NMR characteristics of the majority of penicillin and cephalosporin β-lactam antibiotics in world-wide clinical use are presented. Some of the data are novel and include several high resolution (220, 400 MHz) spectra. The influence of solvent and ionisation state upon spectral parameters is discussed and a scheme of analysis proposed for identifying an unknown β-lactam sample. Spectral features of common degradation products of benzylpenicillin and other penicillins are provided and the use of ^{1}H NMR spectroscopy in monitoring the breakdown of penicillin antibiotics described. Other aspects discussed are NMR studies of the stereochemistry, association and protein binding of β-lactam antibiotics.

Keywords: *^{1}H NMR spectroscopy; β-lactam antibiotics; penicillins; cephalosporins; degradation products.*

Introduction

Following the introduction of benzylpenicillin into clinical practice shortly after the end of the Second World War, a steady stream of novel β-lactam antibiotics of both the penicillin and cephalosporin class have found use in the treatment of bacterial infections. The present abundance of such agents is clear from inspection of the latest edition of Martindale's Extra Pharmacopoeia which lists 43 penicillins and 18 cephalosporins in clinical use together with several in the supplement of very recent introduction. This proliferation of agents of closely related structure and use presents a major challenge to pharmaceutical analysts both from the viewpoints of specific identification and quantification, and detection of impurities and products of degradation and isomerisation. It is the purpose of this review to demonstrate how ^{1}H nuclear magnetic resonance (NMR) spectroscopy may aid this analytical problem.

The value of NMR spectroscopy in differentiating groups of closely related compounds is well known, and numerous reports of the ^{1}H (and later ^{13}C) NMR features of β-

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lactam antibiotics have been made dating back to the early 1960s. These data, obtained in different laboratories throughout the world and recorded under a variety of conditions are collated in this review in juxtaposition with analyses of 60 and 100 MHz spectra obtained in our own laboratories under standard conditions. Some of our data are novel and include several high resolution (220 and 400 MHz) studies made to clarify features of lower resolution spectra and to provide information on isomeric nature where appropriate. Emphasis is upon application of the ^{1}H NMR data to the identification of specific β-lactam antibiotics in the single drug substance form rather than in formulations. Information on common degradation products of benzylpenicillin is also provided, and the potential of NMR in monitoring stability is discussed.

Because of the relative insensitivity of NMR spectroscopy as usually carried out employing continuous wave instruments, impurities are only likely to be detected if present in excess of a few percent. However, prospects for quantitative NMR procedures of greater sensitivity are better now as a result of the introduction of pulse (Fourier Transform, FT) spectrometers which require small sample sizes for analysis and have enhanced sensitivities over those of continuous wave instruments. Other applications of NMR, e.g. to association, protein-binding and stereochemical studies of the antibiotics, will also be described.

Material and Methods

60 and 100 MHz Spectra were obtained on JEOL PMX 60 and PS 100 spectrometers, respectively operating at normal ambient temperatures. 220 MHz spectra were provided by the Physico–chemical Measurement Unit, Harwell, UK, and 400 MHz spectra by the SERC WH-400 NMR spectroscopic service of the Department of Chemistry, University of Warwick. Samples were prepared in 5 mm o.d. tubes as *ca* 10% w/v solutions in D_2O, DMSO-d_6 and other solvents; weaker solutions (5–10 mg in 0.5 ml) were used for the 400 MHz work. Tetramethylsilane (TMS), sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and t-butanol were used as chemical shift standards. t-Butanol (chemical shift 1.28 ppm relative to DSS in D_2O) has the advantage over DSS of providing a single resonance line. Alkali metal salts were freely soluble in D_2O while the solution of free acids was promoted by minimal quantities of alkali (usually $NaHCO_3$), or acid (TFA or DCl) in the cases of β-lactams with amino substituents such as ampicillin. One neutral ester (penamecillin) was examined in $CDCl_3$.

Samples provided by the various pharmaceutical companies listed in the acknowledgements were generally of a purity suitable for pharmaceutical formulations and in some cases were described as analytical standards. Disodium salts of 5R,6R- and 5S,6R-benzylpenicilloic acids were prepared by treating benzylpenicillin sodium in water with aqueous NaOH at 0°C for 15 min (5R,6R product), or 25°C for 30 min (5S,6R product) [16]. Benzylpenilloic acid was obtained by treating the intact antibiotic with 0.1 M HCl for 1.5 h at the reflux temperature [47]. Benzylpenillic acid was separated after 24 h from a solution of benzylpenicillin sodium in water adjusted to pH 2.5 with 1 M HCl and stored at room temperature [35].

Results and Discussion

^{1}H NMR spectral characteristics of 25 penicillin and 15 cephalosporin derivatives are given in Tables 1 and 2, respectively, while Table 3 presents features of degradation

Table 1
^{1}H NMR characteristics of penicillin antibiotics*

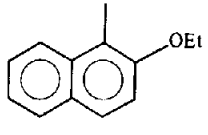
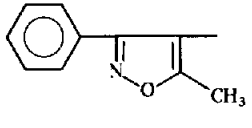
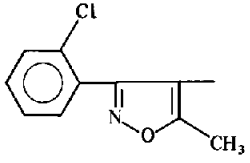
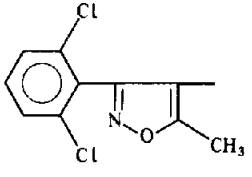
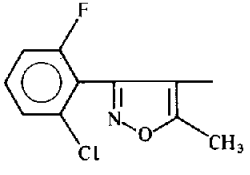
Compound and form†	Solvent and operating frequency (MHz)	2-methyl (α, β)	3-H	5-H, 6-H‡	Ar of R	Miscellaneous	Refs
benzylpenicillin Na R=$PhCH_2$	D_2O 100	1.50 s 1.57 s	4.23 s	5.40, 5.46 (~4)	7.29 s	$PhC\underline{H}_2$ 3.6 s	[1–4, 6, 14, 15]
	DMSO-d_6 100	1.47 s 1.57 s	3.88 s (free acid 4.2, ref. 2)	5.33 m, s§	7.17 s	PhCH 3.5 s NH 8.57 br d§	
phenoxymethylpenicillin K R=$PhOCH_2$	D_2O 100	1.52 s (α + β)	4.28 s	5.53, 5.59 (4.2)	6.8–7.6 m	$PhOC\underline{H}_2$ 4.52 s	[1, 2, 7]
phenethicillin K isomeric mixture R=PhOCH(Me)	D_2O 100	1.53 s (α + β)	4.25 s	α: 5.47, 5.63 (4.5) β: 5.50 s	6.8–7.5 m	PhOCHMe 1.44, 1.62¶ PhOCH 4.82‖	[2, 8]
	D_2O 200	within complex of s 1.60, 1.57, 1.60, 1.54, 1.52, 1.43	4.29 s	α: 5.48, 5.63 (3.92) β: 5.55	apparent t 6.95, 7.26	PhOCHMe within 1.4–1.6 complex PhOCH overlapping q centre 4.81 (6.4)	
methicillin Na R=2, 6diOMe.C_6H_3	D_2O 100	1.53 s 1.62 s	4.17 s	5.54, 5.61 (4.1)	6.71 d (8.6) 7.43 t (8.6)	$(OMe)_2$ 3.77 s	[1, 2]
nafcillin Na	D_2O 100	1.55 br s (α + β)	4.26 s	5.67, 5.87 (~4)	6.6–8.0 m	$OCH_2\underline{Me}$ 1.24 t $OCH_2\underline{Me}$ 3.87 q	[2]
R = 							
oxacillin Na	D_2O 60	1.43 s 1.47 s	4.13 s	5.50, 5.57 (4.0)	7.43 s (Ph)	Ar–Me 2.57 s	[1, 2]
R = 							

Table 1
(continued)

Compound and form†	Solvent and operating frequency (MHz)	2-methyl (α, β)	3-H	5-H, 6-H‡	Ar of R	Miscellaneous	Refs
cloxacillin Na	D_2O 100	1.45 s 1.49 s	4.12 s	5.50, 5.64 (4.5)	7.43–7.54 4 line m (C_6H_4)	Ar–Me 2.69 s	[2, 9]
R = 							
dicloxacillin Na	D_2O 60	1.42 s (α + β)	4.08 s	5.44, 5.60 (4.4)	7.47 s (C_6H_3)	Ar–Me 2.70 s	[2]
R = 							
flucloxacillin Na	D_2O 100	1.49 s (α + β)	4.15 s	5.51, 5.64 (4.1)	7.19–7.70 m (C_6H_3)	Ar–Me 2.72 s	
R = 							

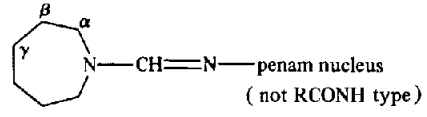
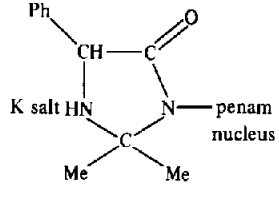
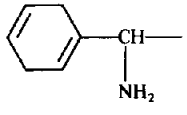
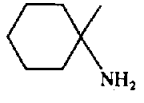
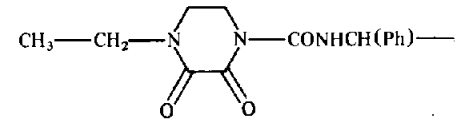
mecillinam HCl.2H_2O R =  (not RCONH type)	D_2O 100	1.61 s 1.78 s	4.61 s	5.54, 5.65 (4.5)	—	αCH_2 3.62, 3.79 br t $\beta/\gamma CH_2$ 1.43–1.88 m (overlaps 2-Me signal) CH=N 8.06 s	[11]** **
pivmecillinam HCl as above 3-$CO_2CH_2OCOCMe_3$	D_2O 100	1.51 1.70	4.71	5.55, 5.96 (~4)	—	αCH_2 3.59, 3.75 br t $\beta/\gamma CH_2$ 1.38–1.98 m** (overlaps 2-Me signal) CH=N 8.07 s CMe_3 1.20 s OCH_2O 5.82, 5.97 d (5–6)	** **
ampicillin 3H_2O	D_2O–TFA 60	1.39 s 1.44 s	4.49 s	5.59 s	7.63 s	PhCH 5.31 s	[1–3, 9]
R=PhCH(NH_2)	DMSO-d_6 60	1.42 s 1.53 s	4.12 s	5.37–5.57 br m; AB signal after D_2O centre 5.38 (4)	7.63–7.67 m	PhCH 4.95 s (resolved after D_2O) NH 8.57–9.57 br s	
pivampicillin HCl R=PhCH(NH_2) 3-$CO_2CH_2OCOCMe_3$	D_2O	1.43 br s ($\alpha + \beta$)	4.62 s	5.58 s	7.6 br s	PhCH 5.4 s CMe_3 1.19 s OCH_2O 5.76, 5.93 d (5–6)	
hetacillin††  K salt	DMSO-d_6 60	1.33 s 1.46 s	4.33 s	5.12, 5.50	7.2–7.7 m	CMe_2 1.48, 1.59 s (acetone-derived) Ar CH 4.67	[2]
	D_2O 60	1.44 s 1.49 s	4.28 s	5.05, 5.54	7.46 s	CMe_2 1.53, 1.63 s (acetone-derived) ArCH 4.73	[2]
free acid§§§	DMSO-d_6 270	1.30 s 1.42 s	4.24 s	5.10, 5.43 (3.8)	7.2–7.5 m	CMe_2 1.45, 1.56 ArCH 4.62	

Table 1
(continued)

Compound and form†	Solvent and operating frequency (MHz)	2-methyl (α, β)	3-H	5-H, 6-H‡	Ar of R	Miscellaneous	Refs
epicillin	D_2O–TFA 100	1.55 s 1.64 s	4.58 s	5.57, 5.65 (4.5)	—§§	vinylic protons of R 5.78, 6.20 br s CH_2 protons of R 2.80 br signal RC<u>H</u> 4.73 s	
R = 	DMSO-d_6, TFA 60	1.50 s 1.62 s	4.33 s	5.62 s	—	vinyl protons of R 5.78, 6.03 br s CH_2 protons of R 2.73 br s RC<u>H</u> 4.53 br s N<u>H</u> 8.6, 9.35 br s	
	DMSO-d_6, D_2O 400	1.39 s 1.49 s	4.10 s	5.37, 5.33††††	—	vinylic protons of R 5.65, 5.88 br s CH_2 protons of R 2.67 m RC<u>H</u> 4.30 s	
amoxycillin $3H_2O$ R=4OH.$C_6H_4CH(NH_2)$	D_2O–TFA 100	1.43 s 1.46 s	4.43 s	5.48 s	6.92, 7.32 AB d (9.0)	ArC<u>H</u> 5.17 s	[12]
ciclacillin R = 	D_2O–$NaHCO_3$ 100	1.52 s 1.62 s	4.26 s	5.41, 5.58 (4.5)	—	C_6H_{11} 1–2, m (beneath 2–Me signal)	—
piperacillin Na R = 	D_2O 100	1.41 s 1.47 s	4.16 s	only 2 lines of AB system resolved, 5.45 (overlaps PhC<u>H</u>) and 5.50	7.41 s (br at base)	$(CH_2)_2$ of piperazine dione 3.6 (overlaps CH_2Me q), 3.9 br m NC<u>H</u>$_2$Me 1.17 t (7.5) NCH$_2$<u>M</u>e 3.48 q (7.5) PhC<u>H</u>I 5.45 s	[13(amide, 3-$CONH_2$)]

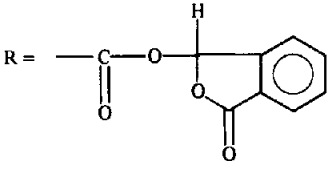
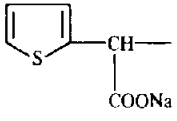
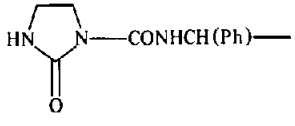
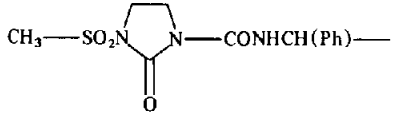
bacampicillin HCl isomeric mixture, A major, B minor component R=PhCH(NH_3^+) 3-CO_2CH(Me)O$CO_2$$CH_2$Me	CD_3OD¶¶ 100	4 line group near 1.5 (includes d due to Me of 3-side chain)	4.38 s (lower intensity s resolved to low field)	5.58 centre of m, higher field d resolved 5.49 (4.5)	7.52 s (br at base)	PhCH 5.23 br s *3-side chain* OCH(Me) d within 1.5 signal OCH(Me) 6.77 q (4.5) CH_2Me 1.28 t (7) CH_2Me 4.22 q (7)	—
	CD_3OD 400 A major, B minor resonances (ratio ~2:1)	A: 1.32, 1.34 s B: 1.28, 1.33 s	A: 4.49 s B: 4.50 s	A: 5.51, 5.48 (3.9) B: 5.44, 5.52 (3.9)	7.45 centre of complex m	PhCH A: 5.16 s B: 5.16 s *3-side chain* OCH(Me) A + B: 1.50 d (5.4) OCH(Me) A: 6.74 q (5.4) B: 6.74 q (5.4) CH_2Me A: 1.21 t (7.2) B: 1.22 t (7.2) CH_2Me A + B: 4.18 centre of overlapping q	
talampicillin HCl isomeric mixture (ratio ~1:1 from 2-Me signal intensities)	CD_3OD 100	1.46, 1.47, 1.49, 1.52 closely placed s	4.53 s 4.55 s	5.55 centre of 5-line m	7.53 s br at base	PhCH 5.16 s Ar protons of 3-substituent 7.82 m	—
R=PhCH(NH_3^+)	CD_3OD 220	1.42, 1.48 s 1.46, 1.51 s	4.54 s 4.56 s	5.49, 5.53 (4) 5.62 centre of 4 closely placed lines (2) of unusual AB form	7.55*** centre of narrow m	PhCH 5.18 s Ar protons of 3-substituent 7.7–8.0 m***	
R = 							
carbenicillin 2 Na isomeric mixture (ratio ~1:1)	D_2O 100	3 lines 1.54, 1.57, 1.64	4.30 s 4.33 s	5.58 centre of m	7.76 s	PhCH not resolved (4.56, 4.61 s at 60)	—
R=PhCH(CO_2Na)	D_2O 220	4 lines 1.51, 1.54, 1.55, 1.62	4.32 s 4.36 s	5.5–5.7 m 2 overlapping AB systems (4)	7.53 s	PhCH 4.58, 4.64 s	

Table 1
(continued)

Compound and form†	Solvent and operating frequency (MHz)	2-methyl (α, β)	3-H	5-H, 6-H‡	Ar of R	Miscellaneous	Refs
ticarcillin 2 Na isomeric mixture (ratio ~1:1)	D_2O 100	3 lines 1.52, 1.55, 1.62	4.28 s 4.31 s	5.6 centre of m	2 m, centres 7.18, 7.46	PhCH 4.67 (isomeric signal within HDO band)	—
R = 	D_2O 220	3 lines 1.53, 1.55, 1.62	4.31 s 4.34 s	5.5–5.7 m 2 AB systems of unusual form (4)	3 m, centres near 7.2, 7.45 and 7.55	PhCH 4.69, 4.74 s	
azlocillin Na	D_2O 100	1.43 s 1.48 s	4.22 s	5.46 s	7.32–7.47 m	PhCH coincides with 5–H, 6–H signal, $(CH_2)_2$ of R 3.35–3.51 3.69–3.93 m	
R = 							
mezlocillin Na	D_2O 100	1.43 s 1.47 s	4.17 s	5.46 centre of ill-defined AB signal	7.28–7.46 m	PhCH 5.42 s, overlaps 5-H, 6-H signal, $(CH_2)_2$ of R 3.6–4.0 m, SO_2Me 3.35 s	
R = 							

penamecillin R=CH_2Ph 3-CO_2CH_2OCOMe	$CDCl_3$ 100	1.47 α + β	4.40 s	within 5.4–5.9 9 line m	7.29 s br at base	$PhCH_2$ 3.63 s NH 6.44 br d (5–6) *3-side chain* COMe 2.1 s CH_2O 5.76, 5.78 central lines of AB signal
	$CDCl_3$ 400	2 lines just resolved, centre 1.45	4.38 s	5.48 d (4) 5.65 dd (4, 8.8) H-6 is lower field	7.2–7.39 m	$PhCH_2$ 3.64 s NH 6.08 d (7–8) *3-side chain* COMe 2.13 s CH_2O 5.74, 5.79 d (5.6)

*Chemical shifts in ppm (mostly to nearest 0.01 ppm with FT spectrometer values taken from print-outs, δ scale) relative to TMS or DSS. Resonance descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Multiplet separations (first-order *J* values) in parentheses (Hz). Data corresponds closely with those of previous reports with allowance for differences in ionisation state, solvent and concentration.

†Structures shown beneath name refer to R in **1** and C-3 substituents other than underivatised CO_2H.

‡4-Line AB system, chemical shifts calculated or first-order values; specific spectral assignments (higher field d to 6-H) are by analogy with benzylpenicillin unless NH coupling evidence is available.

§Absent or appearance after D_2O.

¶Outer lines of α, β doublets centre lines overlap α, β 2-Me signal to give a distorted net triplet.

‖Central lines of α, β quartets obscured by HDO signal; resolution is better at 60 MHz, 4.77, 4.78 ppm.

**Of the hexahydroazepine ring.

††From data of ref. 2.

§§Low intensity signal near 7.5 ppm is indicative of ampicillin impurity.

¶¶All features are apparent at 60 MHz in D_2O–acetone-d_6 except that the 5-H, 6-H resonance forms a broad singlet.

‖‖Signals resolved.

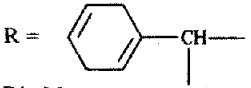
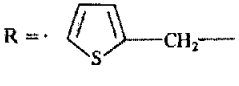
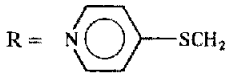
***Methine signal of C-3 side chain falls within aryl resonance.

†††Broader of the two doublets, even more so in absence of D_2O hence assigned to 6-H.

‡‡‡Spectrum run at 220 MHz provides no further information.

§§§Our data: spectrum displayed signals due to ampicillin (1.36, 1.47, 4.13, 5.0 s, 5.38 d, all ppm) present as impurity in the sample or formed on dissolution of hetacillin.

Table 2
^{1}H NMR characteristics of cephalosporin antibiotics*

Compound and form†	Solvent and operating frequency (MHz)	2-CH_2‡	6-H, 7-H‡	Ar of R	Miscellaneous	Refs
cephalexin§§ R=PhCH(NH_2) R′=Me	D_2O–TFA 100	3.19, 3.45 d (18)	4.99, 5.65 d (4.5)	7.42 s	3-Me 2.09 s PhC<u>H</u> 5.26 s	[19, 22]
cefadroxil R=4-OH.C_6H_4CH(NH_2) R′=Me	D_2O–TFA 100	3.19, 3.45 d (18)	4.96, 5.66 d (4.5)	7.01, 7.46 AB d (9)	3-Me 2.09 s ArC<u>H</u> 5.25 s	[19]
cephradine R =  R′=Me	D_2O–TFA 100	3.39, 3.61 d (18)	5.14, 5.70 d (4.5)	§	3-Me 2.15 s vinylic protons of R¶ 5.78, 6.20 br s CH_2 protons of R¶ 2.81 br s RC<u>H</u> 4.7 s	[19, 20]
cefaclor R=PhCH(NH_2) R′=Cl	D_2O–$NaHCO_3$ 60	3.39, 3.53 d (18)	5.15, 5.72 d (4.6)	7.43 s	PhC<u>H</u> 5.24 s	[19, 21]
cephaloglycin‖ R=PhCH(NH_2) R′=CH_2OCOMe	D_2O–Na_2CO_3 60	3.25** (18)	4.90, 5.68 (4.5)	7.40 s	PhC<u>H</u>†† COMe 2.08 s C<u>H</u>$_2$O††	[19]
cephalothin Na R =  R′=CH_2OCOMe	D_2O 100	3.35, 3.59 d (18)	5.08, 5.69 d (4.5)	C_4H_3S 7.06 d, 7.37 t	ArCH_2 3.89 s OCOMe 2.12 s CH_2O 4.76, 4.89 d‡ (12)	
cefapirin‖ R =  R′=CH_2OCOMe	D_2O–Na_2CO_3 60	3.45** (18)	4.97, 5.56 d (5)	7.33, 8.35 d (7)	ArSCH_2 3.97 s OCOMe 2.11 s CH_2O 4.72** (13)	[19]

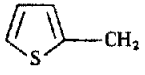
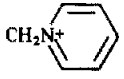
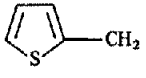
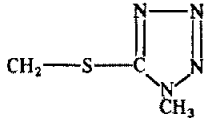
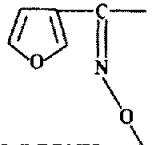
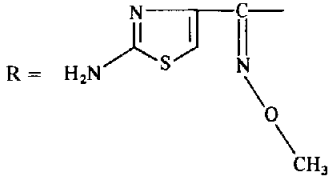
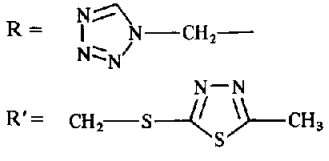
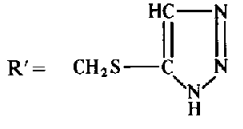
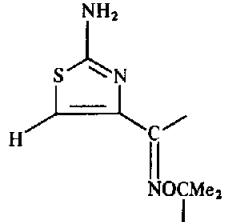
cephaloridine R =  R′= 	D_2O 100	3.09, 3.57 d (16.5)	5.10, 5.66 d (4.5)	6.96, 7.26 m	$ArCH_2$ 3.83 s C_5H_5N 8.07, 8.55 m 8.96 d (4) CH_2N 5.27, 5.58 d (15)	[1, 2, 19, 39]
cefoxitin Na R =  R′=CH_2OCONH_2 7-OMe	D_2O 100	3.22, 3.56 d (18)	6-H 5.10 s 7-OMe 3.51 s	7.05, 7.35 m	$ArCH_2$ 3.93 s CH_2O 4.58, 4.89 outer lines of AB signal, inner lines obscured by HDO band (12.5)‡‡	[19, 24]
cephamandole R=PhCH(OH) R′= 	D_2O 100	3.38, 3.68 d (18)	5.05, 5.61 d (4.5)	7.46 s br at base	CH_2S 4.05, 4.27 d (12) NMe 3.99 s PhC<u>H</u> 5.27 s	[19, 23]
cephamandole nafate‖ R=PhCH(OCHO) R′ as for cephamandole	D_2O–Na_2CO_3 60	3.57** (18)	4.91, 5.55 d (5)	7.15–7.55 m	CH_2 4.06** (13) NMe 3.85 s PhC<u>H</u> 6.20 s PhCH(OC<u>H</u>O) 8.25 s	[19]
cefuroxime Na R =  R′=CH_2OCONH_2	D_2O 100	3.44, 3.69 d (18)	5.23, 5.84 d (4.9)	C_4H_3O 6.65 m, 6.91 d (3.4) 7.70 br s	CH_2O 4.72, 4.89 d (12) OMe 4.03 s	[19]

Table 2
(continued)

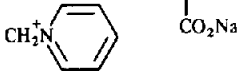
Compound and form†	Solvent and operating frequency (MHz)	2-CH_2‡	6-H, 7-H‡	Ar of R	Miscellaneous	Refs
cefotaxime Na  R′=CH_2OCOMe	D_2O 60	3.46, 3.73 d (18)	5.27, 5.88 d (4.8)	Ar–H 7.04 s	COMe 2.14 s CH_2O 4.81, 4.98 d (12.8) OMe 4.06	[19, 25]
cephazolin Na 	D_2O 100	3.44, 3.81 d (18)	5.10, 5.70 d (4.5)	Ar–H 9.27 s	NCH_2 5.57 s Ar Me 2.75 s	[19, 26]
cefatrizine R=4–OH.$C_6H_4CH(NH_2)$ 	D_2O–Na_2CO_3 60	3.30** (18)	4.96, 5.56 d (5)	6.82, 7.32 d (9)	ArCH 4.58 s CH_2S 3.75** (13) Ar–H(R′)7.50 s	[19]

moxalactam structure¶¶	D_2O 400	4.32, 4.47 d (17.4)	6-H 5.05 s 7-OMe 3.46 s	6.81, 7.18 d (8)	ArCH 4.44 s CH_2S 3.93, 4.13 (13.5) NMe 3.93 s
ceftazidime Na (Fortum)	DMSO-d_6 400	3.09, 3.52 d (17.7)	6-H 5.08 d (5) 7-H 5.72 dd (5, 8.2)	7.3 br s (overlaps NH signal)	CH_2N 5.16, 5.66 d (13.4) C_5H_5N 8.15 t, 8.58 t, 9.45 d (overlaps NH signal) CMe_2 1.39 d (just resolved)

R =



R′ =



*Footnote * of Table 1 applies.

†Structures shown beneath name refer to R and R′ substituents in **2**.

‡4-Line AB system, chemical shifts calculated or first-order values.

§Low intensity signal 7.6 ppm indicative of cephalexin impurity, cf. ref. [51].

¶cf. Similar features of epicillin (Table 1).

‖Data from ref. [19].

**Centre of AB signal.

††Obscured by HDO signal near 4.65 ppm.

‡‡From 60 MHz spectrum.

§§Features of the precursor 7-aminodesacetoxycephalosporanic acid (7-ADCA), Na salt in D_2O: 2-CH_2 3.21, 3.56; 6-H 4.67; 7-H 4.99; 3-Me 1.91 ppm (Ref. [52]).

¶¶

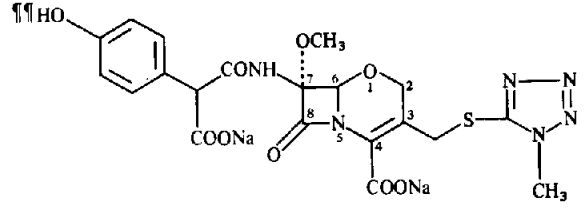


Table 3
^{1}H NMR characteristics of penicillin precursors, transformation and degradation products*

Compound		Solvent and operating frequency	2-methyl (α, β)	3-H	$PhCH_2$ (ArCH)	5-H, 6-H‡	Ph(Ar)	Refs
6-aminopenicillanic acid (6-APA)	Na salt	D_2O 60	1.57, 1.67 s	4.24 s	—	4.64, 5.54 d (4)	—	[1]
(3)	free acid	CD_3OD–TFA 100	1.63, 1.76 s	4.53 s	—	5.00, 5.64 d (4.5)		
		D_2O–TFA 60	1.57, 1.70 s	4.60 s	—	5.05, 5.67 d (4.4)		
penicic acid¶ (4)		D_2O, pH 8.5 60	1.33, 1.75 s	3.62 s	—	3.8, 5.06 d (7.0)	—	[17]
5-epipenicic acid¶		D_2O, pH 8.5 60	1.26, 1.60 s	3.45 s	—	3.58, 4.93 d	—	[17]
penicillamine		D_2O 60	1.97, 2.07 s	4.17 s	—	—	—	—
benzylpenicilloic acid 2Na 5R,6R isomer (5)		D_2O 60	1.23, 1.48 s (1.01, 1.55 s)†	3.37 s	3.63 s (3.72 s)†	4.22, 5.03 d (5.4)	7.3 s	[14, 16, 31, 29]
5S,6R isomer 2Na		D_2O 60	1.02, 1.53 s (1.20, 1.47 s)†	3.37 s	3.72 s (3.63)†	4.68, 5.00 d (3.0)	7.3 s	
Aldrich free acid (chiefly 5R,6R isomer)		DMSO-d_6 100	1.19, 1.47, 1.56 (lowest field line of minor intensity)	3.57 s	3.68 s	6H: 4.41 dd (6,9)§ 5H: 5.01 d (6)	7.29 s	[14]
penicilloic acid from ampicillin§§	5R,6R	D_2O, pD 5.9 90	1.10, 1.16 s	3.04 s	5.17 s (ArCH)	4.25, 5.05 d (5)	7.5 s	[18, 31]
	5S,6R		0.51, 1.43 s	3.26 s	5.27 s	4.83, 5.00 d (3)		

penicilloic acid from amoxycillin§§	5R,6R	D_2O,pD 5.9 90	1.14, 1.18 s	3.12 s	5.13 s (ArCH)	4.29, 5.10 d (5)	7.41 dd	[18, 31]
	5S,6R		0.51, 1.45 s	3.28 s	5.19 s	4.83, 5.00 d (3)	7.41 dd	
benzylpenilloic acid isomeric mixture (6)		D_2O,acetone-d_6 60	1.14, 1.20, 1.55 lowest field line of highest intensity (0.98 impurity)	3.47 (major) 3.57 s	3.66 (major) 3.81 s	6-H_2, 5-H unresolved band 3.4–3.9 ppm beneath 3-H and $PhC\underline{H}_2$ signals	7.35, 7.41 (minor) s	[15, 29, 31]
		D_2O, $NaHCO_3$ 400 fresh solution‖	1.21, 1.56 (1.18, 1.58) s	3.48 (3.40) s	3.60 (3.64) s	5-H 4.77 t (7.16) (4.67 t, 4.9 Hz) 6-H_2 (8-line double AB signal) centre 3.33 (13.6, 7.2) (centre ~3.58, 14.5, 5.3)	7.4 m	
benzylpenillic acid (7)		D_2O,$NaHCO_3$ 100	1.47, 1.49 s	4.16 s	absent due to exchange with D_2O**	4.62‡‡, 5.76 d (4.5)	7.37 m	[15(ph 2.5)]
benzylpenamaldic acid*** (8)		D_2O–DCl pD 2.5 270	1.45, 1.62 s	3.9 s	3.73 s	5–H(vinylic) 7.78 s	not recorded	[15]
6-S mecillinam HCl†††		CD_3OD 60	1.53, 1.62 s (1.66, 1.73)‖‖	4.54 s (4.28)	C<u>H</u>-N,8.23 s (8.10)	5.07, 5.43 d (1.6) [5.37, 5.56 d (4)]	††	[11]
6-R-formamidopenicillanic acid Na salt††† (11)		D_2O 60	1.58, 1.67 s	4.32 s	OC<u>H</u> 8.23 s	5.67 m		[11]

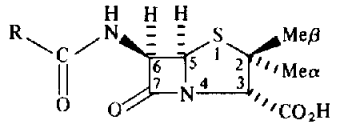
Table 3
(continued)

Compound	Solvent and operating frequency	2-methyl (α, β)	3-H	$PhCH_2$ (ArCH)	5-H, 6-H‡	Ph(Ar)	Refs
6S-formamidopenicillanic acid K salt†††	60	1.52, 1.60	4.33 s	OCH 8.18 s	4.93, 5.34 d (1.8)		[11]
5-epibenzylpenicillin Na‡‡‡	D_2O 60	1.44, 1.57 s	3.63 s¶¶	3.63 s¶¶	4.82, 5.10 d (2)	7.31 s	[45]
sulphoxide§§§ of phenoxymethylpenicillin methyl ester¶¶¶	$CDCl_3$ 100	1.23, 1.73 s (1.49, 1.60)	4.69 s (4.47)	4.54 s (4.56)	5.03, 6.10 d (5.58, 5.74)	not recorded	[27]

*Footnote * of Table 1 applies.
†Minor signals.
‡4-Line AB system.
§CONH 8.39 ppm d (9).
‖Minor signals (in parentheses) reached similar intensities to major signals when solution examined 48 h after preparation. Major signals of the fresh solution probably relate to the 5-R diastereoisomer.
¶Data from ref. [17].
**Observed at 3.63 ppm in spectrum of the dimethyl ester in $CDCl_3$ (^{13}C resonance 35.48 ppm).
††CH_2 3.5–4.0 m (3.5–3.9)‖‖, β/γ CH_2 1.5–2.1 m (1.5–2.1)‖‖.
‡‡Higher field line of doublet obscured by HDO band at 60 MHz.
§§Data from ref. [18].
‖‖Data on 6-R mecillinam given in parentheses: cf. data of Table 1 in D_2O.
¶¶Coincident signals; note high field 3-H resonance compared with 3-H of benzylpenicillin (4.23 ppm).
***Data from ref. [15].
†††Data from ref. [11].
‡‡‡Data from ref. [45].
§§§Data for methyl ester of phenoxymethylpenicillin in parentheses.
¶¶¶Data from ref. [27].

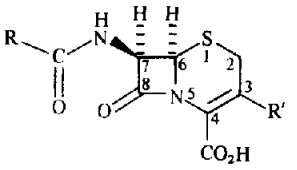
products of benzylpenicillin and its precursor 6-aminopenicillanic acid. General structures, ring numbering and absolute stereochemistries of free acid forms of the antibiotics are given in formulae **1** and **2**; details of substituents and generic names are included in the Tables for easy reference.

1



Penam nucleus: 7-oxo-1-thia-4-azabicyclo [3.2.0] heptane
Absolute configuration: 3-S, 5-R, 6-R

2



Cepham nucleus: 8-oxo-1-thia-5-azabicyclo [4.2.0] octane
Absolute configuration: 6-R, 7-R

Assignments of penicillin spectra are mostly trivial although there are uncertainties in some cases. Evidence for the specific assignment of 5-H and 6-H resonances, and of α- and β-2-methyl signals is restricted to benzylpenicillin and phenoxymethylpenicillin, respectively, and signals of other derivatives are assigned by analogy unless other facts are available.

Signals common to all *penicillin* spectra are the 2α- and 2β-methyl, the 3-H and the 5-H, 6-H resonances. The *2α/2β Me resonance* falls in the chemical shift range 1.39–1.78 ppm when D_2O is the solvent with α/β signals usually resolved (Δδ 0.03–0.17 ppm). Coincident signals are found for phenoxymethylpenicillin, phenethicillin, dicloxacillin and flucloxacillin at 100 MHz, while three lines or more appear in spectra of bacampicillin, talampicillin, carbenicillin and ticarcillin indicative of isomeric mixtures. The spectrum of hetacillin displays four lines in the 1.3–1.6 ppm range but in this case the additional two are derived from the acetone molecule which is condensed with the two NH groups of the C-6 side chain of ampicillin to give hetacillin. In the case of the methyl ester of phenoxymethylpenicillin, there is evidence from nuclear Overhauser effect experiments (NOE) that the β-methyl signal has the lower field chemical shift [27]. The *3-H resonance* occurs as a singlet in the range 4.08–4.33 ppm in spectra of alkali metal salts in D_2O but has a lower field position (4.38–4.61 ppm) when the 3-carboxylic acid function is unionised (a result of the greater deshielding influence of CO_2H over CO_2^- upon α-protons). From relative 3-H chemical shifts, the carboxylate functions of ampicillin and epicillin appear to be extensively ionised in D_2O and DMSO-d_6 (i.e. zwitterions are the chief species) but not so when TFA is present.

The *5-H,6-H resonance* most usually takes the form of an AB quartet in the 5.4–5.65 ppm range with doublet separations (^3J) of 4–4.5 Hz characteristic of *cis* protons in lactams [28]. Chemical shifts coincide in a few cases, e.g. ampicillin, amoxycillin, azlocillin, mezlocillin and one isomer of phenethicillin. In the case of benzylpenicillin, evidence of the spectrum of biosynthetic material produced in D_2O (in which 6-H but not

5-H is replaced by deuterium) allows assignment of the higher field doublet to H-6 [6], and this is assumed to be generally true for data of the Tables. In some instances operation of N-H, 6-H coupling is clear enough to allow a specific assignment; thus the 6-H signal of penamecillin forms a doublet of doublets to a *low* field of the 5-H doublet (400 MHz data in $CDCl_3$).

Only spectra of mecillinam, epicillin and ciclacillin lack *aromatic* features in the range 6.71–7.76 ppm; multiplet variations of the signal, e.g. apparent singlet for benzylpenicillin and ampicillin, near doublet/triplet for methicillin, provide valuable clues to identity as will be discussed. Many spectra display a one proton singlet due to ArC<u>H</u> in the range 4.56–5.46 ppm (above 5 ppm for most α-amino/amido derivatives) which may be close to or overlap the 5-H/6-H resonance. This feature is absent in spectra of derivatives of the type 6-ArCONH such as methicillin, nafcillin and the isoxazole derivatives, and is replaced by a 2-proton singlet in spectra of benzylpenicillin (3.6 ppm), phenoxymethylpenicillin (4.52 ppm) and a few others due to $ArC\underline{H}_2$.

Additional features are observed in many spectra. Some fall within or close to chemical shift ranges already made reference to, e.g. the OCH<u>Me</u> doublets of phenethicillin (1.44–1.62 ppm), the OMe singlets of methicillin (3.77 ppm) and the vinylic resonances of epicillin (5.78–6.20 ppm), but in general these extra signals are resolved and have diagnostic value. The analysis of overlapping signals in 60 and 100 MHz spectra has been facilitated by resorting to higher frequency data, e.g. the cases of bacampicillin and penamecillin (Figs 1 and 2), and phenethicillin. The $OC\underline{H}_2O$ protons of penamecillin provide an interesting case of magnetic non-equivalence since they give rise to a 4-line AB signal in spite of being three bonds removed from the nearest chiral centre. The same feature is seen in spectra of pivampicillin and pivmecillinam (2,2-

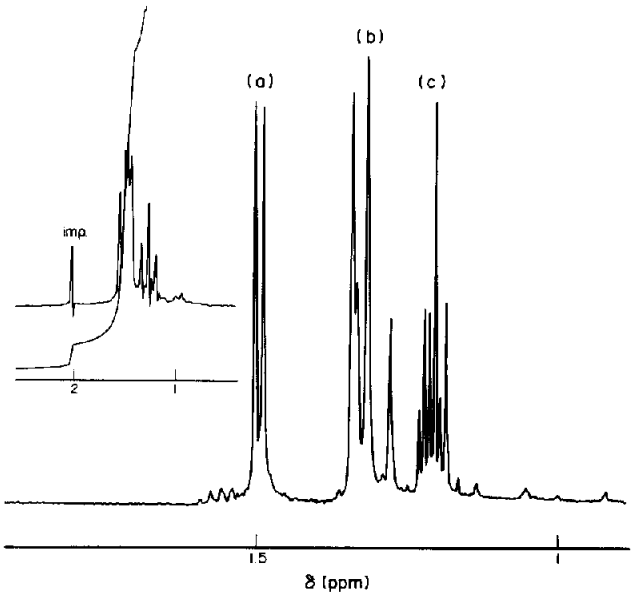


Figure 1
1.0–1.5 ppm region of the 400 MHz ^1H NMR spectrum of bacampicillin HCl in CD_3OD. **a** single Me doublet; **b** major and minor 2- α,β Me singlets; **c** major and minor $CH_2\underline{Me}$ triplets. Insert shows the same spectral region recorded at 60 MHz (imp. = impurity).

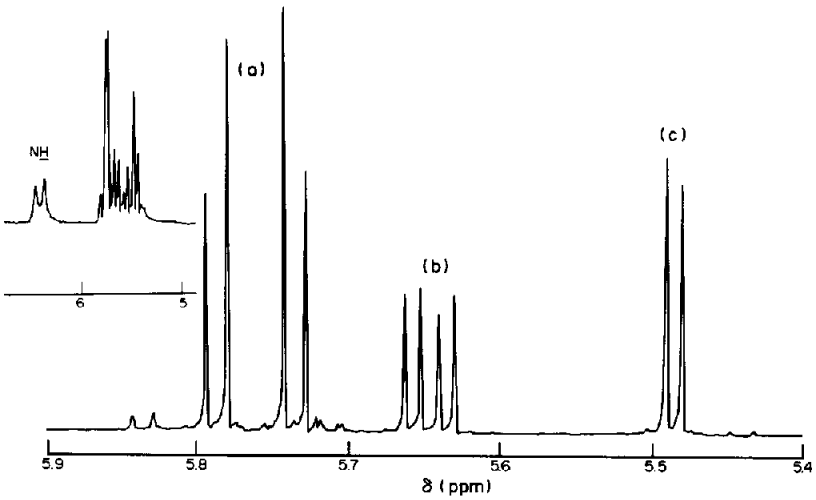


Figure 2
5.4–5.9 ppm region of the 400 MHz ^{1}H NMR spectrum of penamecillin in $CDCl_3$. a OCH_2O 4-line AB signal; b 6-H (coupled to 5-H and N-H); c 5-H doublet. Insert shows the same region recorded at 100 MHz and includes the NH resonance.

dimethyl-l-oxopropoxymethyl esters of the free acids) which are characterised in addition by nine-proton singlets due to the CMe_3 feature. Spectra of freshly prepared and overnight stored solutions did not differ significantly, a result which indicates that these pro-drug forms are stable in D_2O at room temperature (they rapidly degrade in the presence of human or mouse serum) [50].

In some cases spectral complexity is the result of samples being employed as isomeric mixtures rather than single components as in phenethicillin, bacampicillin, talampicillin, carbenicillin and ticarcillin; relative areas of corresponding signals when resolved provide approximate measures of the isomeric content (near 1:1 except for phenethicillin). Isomers of bacampicillin and talampicillin differ in C-3 substituent, and the others in C-6 substituent chirality.

Benzathine penicillin and procaine penicillin are sparingly soluble salts of benzylpenicillin with the organic bases *N,N'*-dibenzylethylamine and procaine, respectively. The spectrum of the former salt in DMSO-d_6 has a more complex and intense aromatic proton signal near 7.2 ppm than that of the parent and displays additional singlets near 3.0 and 4.0 ppm due to the two types of methylene proton present in the diamine. Procaine penicillin's spectrum in the same solvent is distinguished by a high field triplet (~1 ppm) due to the $NCH_2\underline{Me}$ protons and aromatic AB doublets (~6.6. and 7.75 ppm) which flank the 7.2 ppm signal of the antibiotic [2].

Common features of *cephalosporin* spectra (Table 2) are the 2-CH_2, and 6-H,7-H resonances which both form AB doublet pairs. The former is distinguished by its higher chemical shifts (range 3.09–3.81 ppm) and large separations (^2J, 18 Hz). The 2-CH_2 resonances are lower field in 1-oxo analogues such as moxalactam (4.32, 4.47 ppm, ^2J 17.4 Hz) due to the greater deshielding influence of oxygen compared with that of sulphur. The 6-H,7-H resonance (4.96–5.88 ppm, ^3J, 4.5–5 Hz) overlaps the range of the corresponding 5-H,6-H signals of penicillins but there are no instances of chemical shift coincidence and $\Delta\delta_{6,7}$ values are generally greater than $\Delta\delta_{5,6}$ values of the penicillin

spectra. ArCH (or CH_2) singlets are common features of all spectra except those of compounds like cefuroxime and cefotaxime (7-ArC=NOMe.NH derivatives), and these have chemical shift ranges similar to those of corresponding resonances of penicillins. Low field signals due to aromatic protons occur in all spectra except that of cephradine which displays a vinylic resonance akin to that of the related penicillin, epicillin. The aromatic signals fall mostly in the range 6.65–7.7 ppm but are lower field in spectra of cephaloridine and cefapyrin (due to pyridyl moieties) and cefazolin (s, 9.27 ppm, 1 proton). Details of these low field resonances have diagnostic value. Except for cefaclor, resonances due to the C-3 substituent add further complexities to cephalosporin spectra. This is least for 3-methyl examples (s, 2.09–2.15 ppm). Most other derivatives are of the type 3-CH_2X which add an additional 4-line AB signal. In such cases care is required to distinguish the three AB systems especially as outer lines are often of low intensity and tend to be overlooked. The extra signal falls between the 6-H,7-H and 2-CH_2 resonances (overlap may occur) and is further identified by separations (^2J, 12–15 Hz) of magnitudes intermediate between those of the other two systems (Fig. 3 shows a typical example). Certain 3-proton singlets due to methyl attached to nitrogen or oxygen in the range 3.5–4.1 ppm are valuable aids to identification, e.g. 7-OMe (3.51 ppm) of cefoxitin (replacing the usual 7-H signal while the 6-H resonance at 5.1 ppm is a singlet in consequence), N-Me (3.99 ppm) of cefamandole and OMe (near 4.05 ppm) of cefuroxime and cefotaxime. The spectrum of the non-clinical example cephalosporin C is distinguished by the absence of resonances downfield of 5.65 ppm and a 4-proton multiplet (1.55–1.95 ppm) due to methylene protons of the α-aminoadipoyl side chain [19].

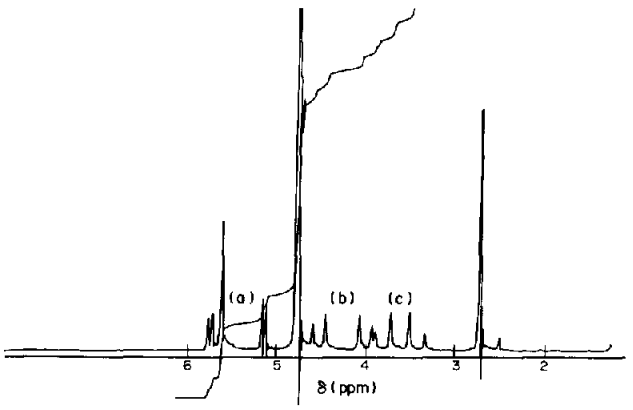


Figure 3
Part of the 60 MHz ^{1}H NMR spectrum of cephazolin Na in D_2O. Approximate centres of 4-line AB signals: **a** 6-H, 7-H; **b** 3-CH_2Ar; **c** 2-CH_2.

Analytical potential of ^{1}H NMR *data for* β*-lactam antibiotics*

The data of Tables 1 and 2 clearly demonstrate the highly specific nature of most individual spectra, and a variety of schemes may be devised for the rapid identification of a β-lactam antibiotic based upon ^{1}H NMR information. In the analytical protocol presented here, the first step is to establish the solubility properties of the unknown. Substances readily soluble in water are probably alkali-metal salts; those less freely

soluble may be hydrochloride salts (an indication of β-lactams with α-amino substituents), a fact readily confirmed by the usual tests for chloride. Samples insoluble or sparingly soluble in water are probably free acids — if dilute acid promotes solution they are most likely to be amino derivatives. If dilute alkali fails to bring about dissolution, the material must be an ester and will require $CDCl_3$ or DMSO-d_6 for examination by NMR.

Having recorded the ^1H NMR spectrum of the sample in the appropriate solvent, examination of the 1.4–1.8 ppm region allows differentiation of penicillins from cephalosporins: penicillin spectra are characterised by the presence of a 6-proton signal in the form of two equally intense singlets of variable (but usually small) separation (an apparent 6-proton singlet may be seen). If signals are absent in this region, a 4-line AB signal in the range 3.1–3.8 ppm with separations near 18 Hz shows the sample to be a cephalosporin derivative.

Diagnostic features of β-lactam spectra

(1) 6-Proton signal 1.4–1.8 ppm as above.

(2) 3-H one proton signal: a singlet of chemical shift near 4.3 ppm dependent upon the ionisation state of the 3-carboxylate substituent (ionised 4.08–4.33, unionised 4.4–4.6 ppm).

(3) 5-H,6-H two proton signal: a 4-line AB quartet in the region 5.4–5.65 ppm of separations 4–5 Hz (sometimes the signals coincide to yield a single line). Cephalosporin spectra display signals of this kind in the same region due to the 6-H,7-H protons, but chemical shift separations ($\Delta\delta$ 0.5–0.6 ppm) between the doublets are usually greater than those of penicillin spectra.

Once features 1–3 have been identified in a β-lactam spectrum, it may be assumed to be that of a penicillin derivative and specific evidence of identification among this group may now be sought. In the first instance this may be carried out by examining the nature of the aromatic proton signal which usually falls between 7 and 8 ppm. The approach is summarised in Table 4. In all cases an identification must be confirmed by ensuring that all other resonance features are present, and that the solubility characteristics are in accord.

A similar approach may be made if the unknown β-lactam has been identified as a cephalosporin. There are five commonly met amino derivatives (detected by their solubility characteristics). Spectra of cefadroxil and cefatrizine are quickly identified by their typical AB aromatic resonances (6.8–7.5 ppm, 2 doublets); the former displays a 3-Me resonance (2.09 ppm) and the latter a $\underline{CH_2}S$ signal centred at 3.75 ppm. Cephradine's spectrum shows diagnostic broad signals at 5.58 and 6.2 ppm due to olefinic protons of the cyclohexadiene grouping. Spectra of cephalexin and cefaclor both display a 5-proton aromatic singlet near 7.4 ppm and may be distinguished by the absence of a 3-Me signal near 2 ppm in the spectrum of cefaclor. The spectrum of the less common agent cephaloglycin may be confused with that of cephalexin; spectra in which the CH_2O signal of the former is resolved (it overlaps the HDO band at 60 MHz in D_2O, Na salt) should allow a differentiation.

Spectra of cephalothin, cephaloridine and cefoxitin are well distinguished by their thienyl proton resonances (3-line signal near 7.3 ppm, 2-line signal near 7.0 ppm). Of these, that of cephaloridine is identified by its additional low field resonance (8–10 ppm) due to pyridyl protons, while the singlet 6-H (5.1 ppm) and C_7-OMe (3.51 ppm) resonances of the cefoxitin spectrum are likewise diagnostic.

Table 4
Procedure for identifying a penicillin antibiotic from features of the 6.6–8.1 ppm region of its ^{1}NMR spectrum

Signal in the 6.6–8.1 ppm region	*Penicillin indicated*	*Additional diagnostic features**
No signal	ciclacillin	1–2 16-proton signal composed of 2 s (1.52, 1.62) emerging from a br band†
	epicillin	5.78, 6.2 br s (vinylic protons)
	mecillinam‡‡	8.06 s (NC<u>H</u>=N)
Singlet or near-singlet (3-proton intensity for dicloxacillin, 5-proton for the rest)	*ampicillin‡§§*	*5.31 s* (ArC<u>H</u>)§
	azlocillin	3.35–3.51, 3.69–3.93 m (2 × CH_2 of R substituent)¶
	bacampicillin	5.2 s (ArC<u>H</u>) and complex signal near 1.5 ppm
	benzylpenicillin	3.6 s (PhC<u>H</u>$_2$)
	carbenicillin	100 MHz: 3 lines 1.54, 1.57 (highest intensity), 1.64
	dicloxacillin	2.70 s (Ar–Me)‖
	hetacillin‡	4 lines in 1.47–1.7 ppm region due to methyls of C-2 and acetone adduct
	mezlocillin	3.35 s (O_2SMe)
	oxacillin	2.57 s (ArMe)‖
	penamecillin	3.63 s (PhC<u>H</u>$_2$) in $CDCl_3$**
	piperacillin	1.41, 1.47 s (α, β 2-Me) 1.17 t (NCH$_2$<u>Me</u>)

Of the remaining four common examples, cefotaxime and cefazolin are readily identified by the simplicity of the 6–10 ppm region of their ^{1}H NMR spectra; that of cefotaxime displays a one proton singlet at 7.1 ppm and that of cefazolin, the same feature at 9.3 ppm. Distinguishing features of spectra of cefamandole and cefuroxime are a broad 5-proton singlet (7.5 ppm) and low field complex (7.7 and 6.9 ppm narrow doublets, 6.65 ppm doublet), respectively. As usual, initial evidence of structure must be confirmed by a complete spectral analysis.

Precursors and common degradation products of penicillins

The data of Table 3 refer principally to compounds related to benzylpenicillin; information of this kind, however, is clearly of general value to stability studies of β-lactam antibiotics. 6-Aminopenicillanic acid (**3**, 6-APA) in either sodium salt or

Table 4
(continued)

Signal in the 6.6–8.1 ppm region	Penicillin indicated	Additional diagnostic features*
Complex signal within 7–8 ppm	cloxacillin	2.69 s (ArMe)††
	flucloxacillin	2.72 s (ArMe)††
	talampicillin	100 MHz: 4 lines 1.46, 1.47, 1.49, 1.52 of similar intensities
	ticarcillin	100 MHz: 3 lines 1.52, 1.55 (highest intensity) 1.62
Complex signal extending beyond 7–8 ppm (range 6.6–8.1 ppm)	amoxycillin	6.92, 7.32 d (9) (Ar signal, unique among the set)
	methicillin	3.77 s [6-proton $Ar(OMe)_2$ signal]
	nafcillin	1.27 t ($COCH_2\underline{Me}$) 3.87 q ($COC\underline{H}_2Me$)
	phenethicillin	1.44, 1.53, 1.62, distorted t (α, β 2-Me plus $PhOCH\underline{Me}$)
	phenoxymethylpenicillin	4.52 s ($ArO\underline{CH}_2$)

* Allowance should be made for chemical shift differences from listed values (taken from Table 1) as a result of variations in solvent and ionisation state; chemical shifts are in ppm and abbreviations as in Table 1.

† The 1–2 ppm signal of mecillinam is similar in appearance but the two singlets (1.61, 1.78) are of greater separation.

‡ In free acid forms examined in DMSO-d_6, ampicillin and hetacillin display broad aromatic signals.

§ In all other members of this group with $Ar\underline{CH}$ features except bacampicillin, this signal overlaps with the 5-H, 6-H resonance; spectra of ampicillin and bacampicillin are readily distinguished by differences in signals near 1.5 ppm.

¶ The spectrum of mezlocillin displays a broad multiplet in the same region (3.6–4.0 ppm) but is distinguished by a singlet at 3.35 ppm (SO_2Me).

‖ Spectra of oxacillin and dicloxacillin are distinguished by differences between the 2-Me_2 signals.

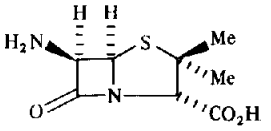
** Penamecillin is defined by its solubility in $CDCl_3$ and insolubility in $NaOH–H_2O$.

†† Spectra of cloxacillin and flucloxacillin are distinguished by fine points of difference between their 2-Me_2 and Ar signals (Table 1).

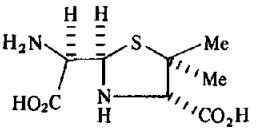
‡‡ The spectrum of pivmecillinam is distinguished by a 9 proton singlet at 1.2 ppm and a 2 proton 4-line signal centred at 5.9 ppm.

§§ The spectrum of pivampicillin is distinguished by a 9 proton singlet at 1.2 ppm and a 2 proton 4-line signal centred at 5.84 ppm.

protonated amine form gives the anticipated simple spectra; doublets of the 5-H,6-H resonances show a chemical shift difference greater than that of the penicillins. 6-APA is rapidly transformed when the pH is raised since its spectrum in $D_2O–NaHCO_3$ showed overlapping doublet pairs between 5 and 6 ppm; these are due, presumably, to 5-H,6-H protons but their chemical shifts correspond to neither those of the sodium salt [1] nor the isomeric penicic acids (Table 3) and probably arise from the 8-oxo analogue of benzylpenillic acid that is formed by degradation of 6-APA in the presence of carbon dioxide [40, 41, 57]. Treatment of 6-APA with β-lactamase or brief exposure to alkali at 0° opens the β-lactam ring to give penicic acid **4** which isomerises to the more stable 5-epipenicic acid on prolonged storage at pH 9–12 [17]. ^1H NMR features of the epimers are given in Table 3; note that the $^3J_{5,6}$ magnitude is greater for penicic acid in which the coupled protons are *cis*.



3 (6–APA)



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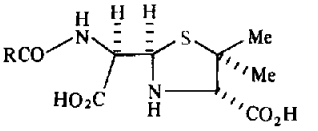
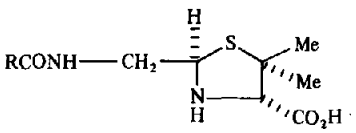


5 (5R, 6R) (R=$PhCH_2$)

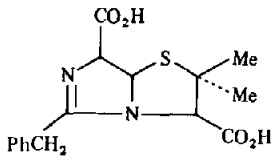
Isomerically pure samples of *benzylpenicilloic acid* **5** could not be obtained but spectra of samples in which the 5R,6R and 5S,6R diastereoisomers preponderated respectively allowed documentation of all the resonances. Spectral features are similar to the precursor antibiotic but differ sufficiently to allow differentiation of all three compounds. Separation of α/β 2-methyl signals of the 5R,6R isomer is enhanced over that of the parent (0.07 → 0.2 ppm) and even more so for the 5S,6R isomer (Δδ 0.52 ppm), and the higher field 2-Me singlets of the penicilloic acids are well resolved from the 2-Me resonances of the intact antibiotic. Chemical shift differences between 5-H,6-H doublets are in the order benzylpenicillin <5S,6R <5R,6R penicilloic acids while ^3J magnitudes of the latter (5R,6R 5.4 Hz, 5S,6R 3.0 Hz) confirm *cis* and *trans* stereochemistry of the 5,6 protons, respectively. The H-3 resonance of salts moves upfield (4.23 → 3.37 ppm) when the β-lactam ring is opened. Data of Ghebre–Sellassie *et al.* [16] are close to those of Table 3.

Spectral differences of the same kind were noted for penicilloic acids derived from ampicillin and amoxycillin (details in Table 3) [18]. Earlier Munro *et al.* [31] reported 60 MHz data on penicilloic acids derived from 11 penicillins isolated after storage at pH 12 for 1.5 h. No indication of isomeric nature is apparent from the chemical shifts (isomerisation is not discussed) but products from ampicillin and amoxycillin appear to be the 5R,6R isomer from comparison with the 1983 data [18]. The spectrum of a sample of free acid supplied by Aldrich (chiefly the 5R,6R isomer) in DMSO-d_6 allowed assignment of the 6-H resonance through observation of coupling to the NH proton, and overall features were similar to those of a reference spectrum [14]. Except for the 2-methyl resonances, the free acid data compared reasonably with chemical shifts reported at pH 2.5 in D_2O at 37°C (270 MHz study) [15]. Details of the ^1H NMR features of the four possible 3-S benzylpencillinoates (as dimethyl esters) have been reported [42]; the 5S,6S (γ) and 5R,6S (β) isomers were obtained by adaptation of the Sheehan total synthesis. There is much interest in benzylpenicilloates and penilloates as reagents for detecting penicillin allergy, and in a recent study of the stability of such mixtures 5R,6R penicilloic acid was shown to isomerise to both the 5S,6R isomer (major) and 5S,6S form [43]. The diastereoisomeric structures were established by reference to the HPLC behaviour of authentic samples characterised by elemental analyses and ^1H NMR. In this work the 5R,6S isomer was obtained by treating 6-epipenicillin [44] with alkali.

Spectra of *benzylpenilloic acids* **6** are more complex than those of the penicilloic acids as a result of the poorer isomeric purity attainable and the non-equivalence of the methylene protons produced at C-6 after decarboxylation. Full analysis was possible, however, from a 400 MHz spectrum which revealed four 2-methyl resonances and duplicate 3-H, $PhCH_2$, 5-H and 6-H_2 signals, all evidence of the isomeric nature of the sample. The 6-H_2 signals were both composed of 8 lines as required by the ABX system (CH_2CH) (Fig. 4). Signals which increased in intensity on storage of the solution are assumed to arise from the 5-S diastereoisomer. In 60 MHz spectra of the free acid, only



6 (5R)
(R=$PhCH_2$)



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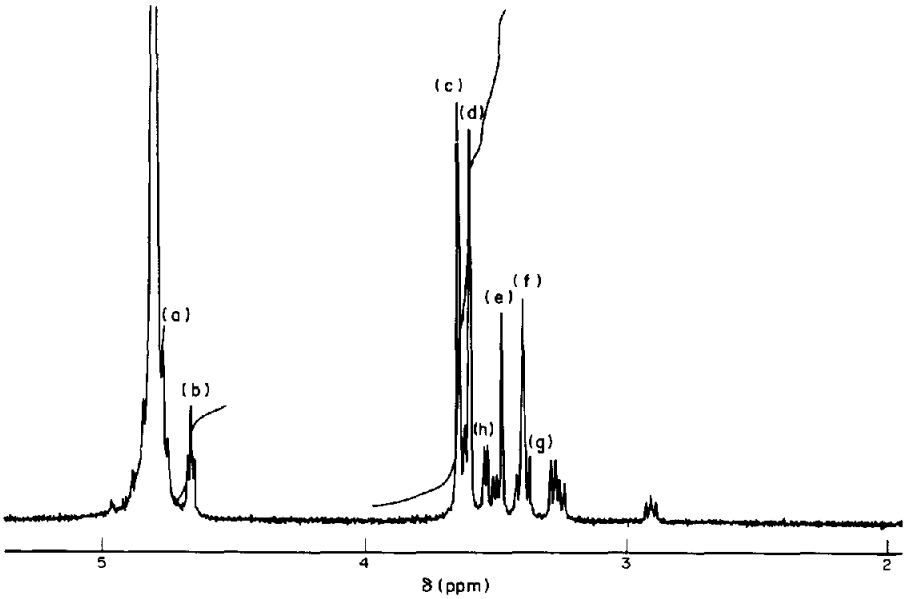
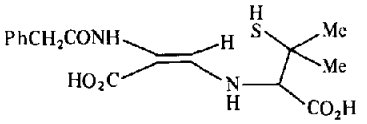


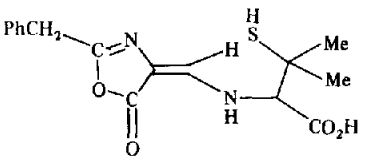
Figure 4
3–5 ppm region of the 400 MHz ^1H NMR spectrum of benzylpenilloic acid in D_2O–$NaHCO_3$ recorded 48 h after preparation: **a**, **b** major and minor 5-H triplets; **c**, **d** minor and major $PhCH_2$ singlets; **e**, **f** major and minor 3-H singlets; **g** approximate centre of major 6-CH_2 signal (8 lines, one obscured by **f**); **h** approximate centre of minor 6-CH_2 signal (several lines obscured).

three 2-methyl signals were resolved while the 6-H_2, 5-H multiplet formed an unresolved band beneath the 3-H and $PhCH_2$ signals. Munro *et al.* [31] report data on six penilloic acids; those for benzylpenilloic acid approximate to the present report.

Benzylpenillic acid **7** prepared by a reported procedure gave a relatively simple spectrum (Na salt in D_2O) indicative of isomeric purity (its configuration is unknown). The α- and β-methyl signals were just resolved at 60 MHz but no signal due to $PhCH_2$ was observed due, presumably, to exchange with D_2O as occurs in other 2-benzyl-imidazoline derivatives [32]. The $PhCH_2$ signal was apparent in ^1H and ^{13}C NMR spectra of the corresponding dimethyl ester run in $CDCl_3$. The spectrum of the free acid in DMSO-d_6 was complex and gave evidence of formation of isomeric/transformation products, e.g. four signals appeared in the 2-Me resonance region. Chemical shifts of *benzylpenamaldic acid* **8** were reported as part of a 270 MHz study of the degradation of benzylpenicillin at pH 2.5 [15]. Data on this unstable material was obtained from studies of the acid degradation of *benzylpenicillenic acid* **9** which has been shown by Longridge *et al.* [34] to form penamaldic acid in the pH range 2.0–2.9.



8 (enamine form)



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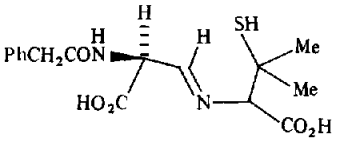
Several workers have monitored degradations of benzylpenicillin by ^{1}H NMR spectroscopy. Mitsumori *et al.* [33] recorded spectral changes of benzylpenicillin, 10 mg ml^{-1} in deuterated phosphate buffer (0.3 M, pH 7), at 100 MHz using a correlation technique (32–128 accumulations were made over 10 min). An overall degradation rate ($k = 0.8 \times 10^{-2}$ h^{-1}) consistent with a pseudo-first order reaction was calculated from intensity decreases of 2-Me, $PhCH_2$ and 3-H resonances. A peak at 1.27 ppm (which appeared after 2.5 h) was identified as due to penicilloic acid by comparison with a spectrum of penicillin degraded by penicillinase. Another peak seen after 8 h was attributed to penilloic acid but its chemical shift (1.07 ppm) shows it due to 5S,6R penicilloic acid (see Table 3). Hem and colleagues [16] calculated the epimerisation rates of disodium penicilloates in D_2O from integral data upon 2-methyl resonances (5R,6R 1.07 ppm, 5S,6R 0.87 ppm); at equilibrium the 5R:5S ratio was 0.2 in agreement with an HPLC analysis, i.e. the initially formed acid is the least thermodynamically stable form. Amongst other evidence of an imine **10** rather than enamine **8** form of penamaldic acid intermediate, was the fact that interconversions of the epimers in D_2O–NaOD did not lead to deuteration at C-6 as must occur if the enamine species is involved.



10 (imine form of **8**)

A more ambitious study of the degradation of benzylpenicillin was carried out by Feeney and others [15] by FT–NMR who achieved high resolution by working at 270 MHz. Changes in the spectra of solutions (5×10^{-3}–5×10^{-2} M) in DCl–D_2O adjusted to pH 2.5 and held at 37° were monitored and signal assignments made by comparisons with spectra of standards recorded under the same conditions. In the early stages signals due to penillic, penicilloic and penamaldic acids appeared almost simultaneously, diagnostic resonances being 5.9 ppm (5-H), 1.26, 1.39 ppm (2-Me) and 7.78 ppm (5-H), respectively. Most of the penicillin had degraded after 100 min as judged by intensities of the 5.45 and 5.53 ppm resonances (5-H,6-H) and an apparent 1st-order rate constant of 0.44 min^{-1} was calculated from integral data. After day 1, lines due to penilloic acid appeared which steadily increased with concomitant fall in the intensities of signals due to the initially formed acids. After 30 days the spectrum was essentially that of penilloic acid (Fig. 5). Since chemical shifts of the degradation products were often close, the successful extraction of kinetic data from the spectral results was only possible under the conditions of very high resolution employed, especially as epimers also contributed to

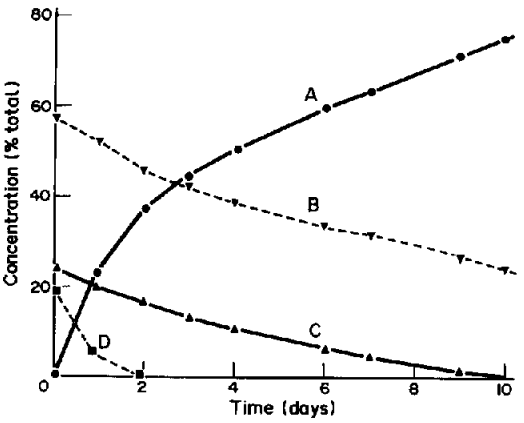
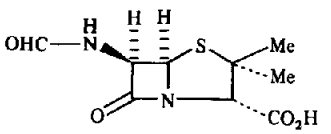


Figure 5
Plot of the concentration of the degradation products of benzylpenicillin at pH 2.5 and 37°C versus time: A, penilloic acid; B, penillic acid; C, penamaldic acid; D, penicilloic acid.

the spectral complexity. If the short-lived benzylpenicillenic acid is a common precursor, as proposed by Blaha *et al.* [35, 56] then all products derived from it should carry a deuterium atom at C-6. However, examination of the 5-H and 6-H resonances of penillic and penicilloic acids formed in the degradation showed only 30–35% deuterated material to be present, evidence that these acids must also result from other pathways. No penicillamine or penilloaldehyde were detected. HPLC evidence has been advanced for the original scheme [36, 37] and the mechanism and routes of acid-catalysed degradation of benzylpenicillin remain controversial.

^{1}H NMR also aided study of the breakdown of mecillinam [11]. The key degradation product was the 6R-formamidopenicillanic acid **11** which, together with mecillinam itself, underwent reversible epimerisation at C-6 in basic solutions (Table 3). Products with formamido or amidine substituents at C-6 (including penicilloic and penilloic acids) gave rise to low field singlets and could be monitored by examination of the 7.5–8.5 ppm spectral region. Epimerisation of an intact penicillin at C-6 is rare and only previously reported for hetacillin [38] and other derivatives chemically modified at the amido-nitrogen atom [30, 53–55]. The 6-epi products showed the characteristic decrease in the ^3J values of 5-H and 6-H protons (4–1.6 Hz for the mecillinams). Under acid conditions no compounds of the penillic or penicillenic acid type were detected.

Although 5-epibenzylpenicillin is unlikely to be encountered in general analytical practice, this stereoisomer has been obtained by treating benzyl 6-phthalimidopenicillanate with chlorine/$SnCl_2$ and conversion of the 5S product to the corresponding benzylpenicillin by standard methods [45]. Its ^{1}H NMR features are included in Table 3.



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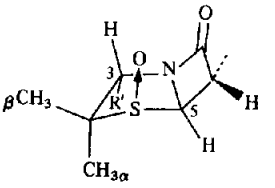
Sulphoxides (and sulphones) are well known as degradation products of therapeutic agents containing cyclic sulphide moieties such as phenothiazine tranquillizers, but do not appear to have been sought amongst degraded penicillins. Sulphoxides have, however, been employed in stereochemical studies [27, 46] and ^{1}H NMR data on the sulphoxide of phenoxymethylpenicillin reported for the methyl ester (Table 3). 2-α-Methyl and 5-H protons are shielded, and 2-β-methyl and 3-H protons deshielded in the sulphoxide relative to corresponding chemical shifts of the sulphide. These results support proposals for the stereochemistry of the sulphoxide (**12**) (shielding effects of S–O were assumed similar to those of C≡C), which was subsequently confirmed by X-ray crystallography.



R′ = CO_2Me

12

Miscellaneous applications

Chemical shifts of β-lactam proton resonances are concentration dependent as is evident from a study of potassium benzylpenicillin in D_2O [4]. All proton resonances except H-3 displayed significant changes (mostly upfield shifts) as the concentration was raised from 0.01 to 1.0 M. Upfield shifts were most pronounced for the benzylic unit (Ph: 7.82 → 7.56 ppm; $PhC\underline{H}_2$: 4.14 → 3.90 ppm) and attributed to self-association of the antibiotic molecules. In the aggregates that result, overlapping or stacking of benzyl side chains in the non-polar core leads to mutual shielding effects. Behaviour of one of the β-lactam protons (assigned to H-5 but probably due to H-6) was unusual in showing a chemical shift minimum. Plots of chemical shift versus 1/M gave two linear regions which intersected at values of M close to the critical micelle concentration (CMC) determined by cryoscopic and other methods. Concentration effects should always be taken into account when comparing β-lactam spectra, especially when polar solvents are employed. Minimal differences are expected, however, over the range 1–10%, i.e. between extremes of concentration usually employed for pulse and continuous wave experiments, respectively.

So far this review has dealt exclusively with chemical shift and coupling constant NMR parameters. Much useful information, however, may be derived by study of the relaxation of excited spin populations characterised by the time constants T_1 (spin–lattice, longitudinal) and T_2 (spin–spin, transverse). Present day instrumentation allows the ready acquisition of such data but its application to β-lactam antibiotics appears to be limited to a 20-year old study by Fischer and Jardetzky [5].* These authors investigated the binding of benzylpenicillin to bovine serum albumin by measuring the

*Two further reports have been traced since completion of this review [58, 59].

broadening of proton resonances induced by the presence of protein, and calculated T_2 from the expression

$$1/T_2\ (s^{-1}) = \pi\Delta\nu_{1/2},$$

where $\Delta\nu_{1/2}$ is the line width at one-half the maximum height (the method gives only the apparent T_2 value since magnetic field inhomogeneities and other factors also contribute to the line width — more accurate methods are now available). When a small molecule is bound to a macromolecule its rates of molecular motion, particularly rotational motion, are generally greatly diminished, and restrictions of this kind will be revealed by increases in the relaxation rates (1/T) of protons in the bound molecules. It is probable that the rotational freedom of the various parts of a bound molecule will not be affected to the same extent, the more tightly attached units being more restricted ("frozen") than features not so directly involved at the site of binding. In such cases a *selective* change in correlation times will occur and be detected by selective broadening of the NMR signals of the bound molecule [48].

In the case of the penicillin study the line widths of all resonances increased progressively as the concentration of albumin was raised; selective broadening was greatest for the Ph signal followed by that of the $\underline{C}\underline{H}_2$Ph peak (Fig. 6). Control experiments, such as the effects of antibiotic concentration and viscosity, were necessary to establish the extent to which T_2 changes could be attributed to binding factors. Evidently the benzyl group is intimately involved in the binding process while other portions of the molecule retain freedom of motion to a much greater extent.

Effects of albumin upon chemical shifts were small, and the fact that the ^{1}H NMR signals (which are largely due to free species) display significant broadening means that the relaxation times of the free and bound forms must differ by much larger factors than those of the chemical shifts of the two species. Mathematical treatment of the data allowed assessment of the fraction of total penicillin bound.

Phenoxymethylpenicillin, another antibiotic which is known to bind to albumin, also exhibited line broadening of its ^{1}H NMR signals in the presence of protein. The

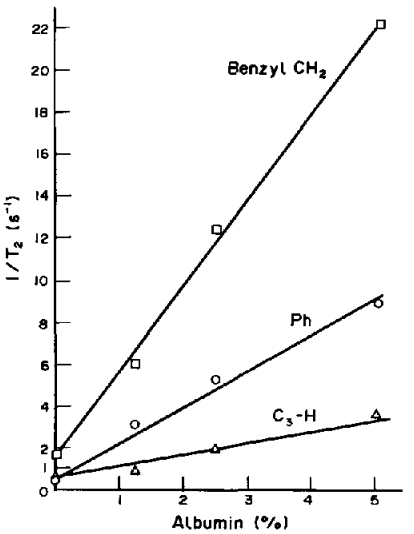


Figure 6
Effect of albumin on the $1/T_2$ relaxation values of three ^{1}H NMR signals of benzylpenicillin [5].

methylene signals of the two penicillins were resolved in mixtures — a spectrum of a solution plus albumin showed slightly less broadening of the benzyl CH_2 resonance compared with controls, indicative of competition for binding sites on the albumin molecule. The spectrum of the phenoxymethylpenicillin–albumin mixture was not affected by 6-aminopenicillanic acid or by its acetyl derivative; in contrast, the line width of the methylene signal was reduced in the presence of phenoxyacetic acid or phenoxyacetamide.

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