Sweeney and B. T. Poon of the Walter Reed Army Institute of Research are gratefully acknowledged.

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Substituent Effects upon the Base Hydrolysis of Penicillins and Cephalosporins. Competitive Intramolecular Nucleophilic Amino Attack in Cephalosporins

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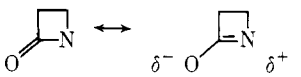
The chemical reactivity of a series of β-lactam antibiotics was found to be sensitive to steric strain and to inductive effects. Steric strain as reflected by the β-lactam ir carbonyl frequency correlates with the log of the rate constant for base hydrolysis. Insignificant changes in β-lactam reactivity resulted from C-6- and -7-acylamido side-chain modification in penicillins and cephalosporins. Cephalosporins that have α-amino-containing acylamido side chains may undergo intramolecular nucleophilic attack at the β-lactam, while ampicillin cannot because of steric hindrance. In contrast, substituent effects as a result of substitution at the C-3 methylene of cephalosporins were significant and correlate with σ_I values and calculated electron densities at the β-lactam carbonyl.

Two classes of extremely valuable clinical antibiotics, the penicillins and cephalosporins, are β-lactam-containing compounds that interfere with the three-dimensional crosslinking of peptidoglycan strands by an enzyme, transpeptidase,[1] during the final stage of cell wall biosynthesis.[2] This enzyme cleaves the C-terminal D-alanine residue from a peptide chain which terminates with D-alanylalanine on one peptidoglycan strand and replaces it with a free amino group connected to a neighboring peptidoglycan strand. Strominger has proposed that the penicillins and cephalosporins resemble the D-alanylalanine peptide fragment and that transpeptidase mistakes the β-lactam-containing molecule as its substrate. When the β-lactam opens, the transpeptidase becomes irreversibly acylated and inactive.[3]

Because acylation of transpeptidase is necessary for antibacterial activity, the chemical reactivity of the β-lactam moiety of a penicillin or cephalosporin may reflect its antibiotic activity. A number of such correlations have been explored.

Woodward has suggested that isolated β-lactams, due to resonance stabilization, are considerably less reactive than the strained penicillin β-lactam.[4] Simple amides and isolated β-lactams **1** may exist in a planar resonance stabilized configuration **2**. However, in penicillins and cephalosporins the nitrogen bond to the carbonyl function cannot become planar because of ring strain. The infrared carbonyl frequencies of isolated β-lactams (1730 cm^{-1}) and penicillins (1775 cm^{-1}) reflect these differences in strain and resonance stabilization. Morin, *et al.*, have shown a qualitative correlation between the infrared β-lactam carbonyl frequency (a proposed measure of acylation ability) of a series of penicillins and cephalosporins and their antibacterial activity.[5] (A qualitative correlation is all that should be expected because antibacterial activity depends upon factors such as enzyme recognition, cell permeability, and β-lactamase resistance in addition to acylating ability.[6]) Hermann's CNDO/2 calculations of the inductive effect of 3-methylene substituents upon cephalosporin β-lactam reactivity also correlate with gram-negative antibacterial activity.[7] Sweet and Dahl, using X-ray structure analysis, have correlated the carbonyl and carbon–nitrogen bond lengths of penicillins and cephalosporins with their antibacterial activity.[8] These investigators have also suggested (from qualitative literature data) a correlation between the ease of base hydrolysis of the β-lactam moiety and antibacterial activity. The effect of 6-acylamido substitution in penicillins upon the reactivity of the β-lactam ring has been examined by Kinget and Schwartz.[9]



1 **2**

The effect of substituents on the reactivity of isolated β-lactams was first reported by Holley and Holley[10] and more recently by Washkuhn and Robinson.[11] The effect of ring size on ring-fused β-lactam reactivity has been studied by Earle,[12] *et al.*, and Moll.[13] To our knowledge a quantitative comparison of the relative reactivities of a series of penicillins and similarly substituted cephalosporins has not been previously reported.

Our purposes for this study were threefold: (1) to further examine Morin's prediction that the infrared carbonyl frequency of a series of β-lactam antibiotics correlates with the β-lactam reactivity; (2) to correlate linear free energy substituent constant values with the relative rate constants of base hydrolysis of 3-methylene-substituted cephalosporins; and (3) to examine the effect of various acylamido side chain moieties upon the observed hydrolysis rate constants of penicillins and cephalosporins.

Results and Discussion

β-Lactam Infrared Frequency, a Measure of Chemical Reactivity. Morin (using methyl esters) assumed that

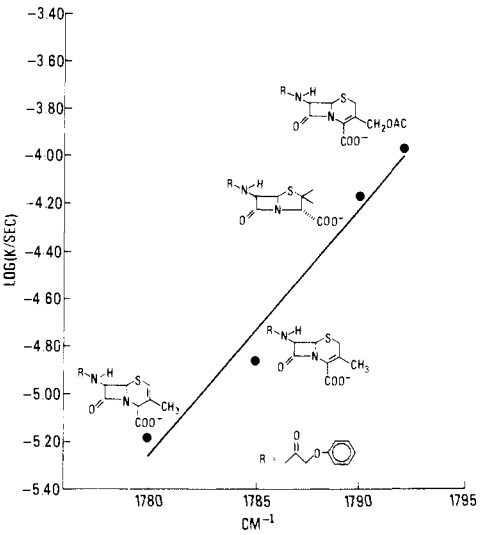


Figure 1. Correlation of observed pseudo-first-order rates of penicillin and cephalosporin β-lactam ring opening at pH 10, 35°, and ir (CCl_4) frequency of β-lactam carbonyl.

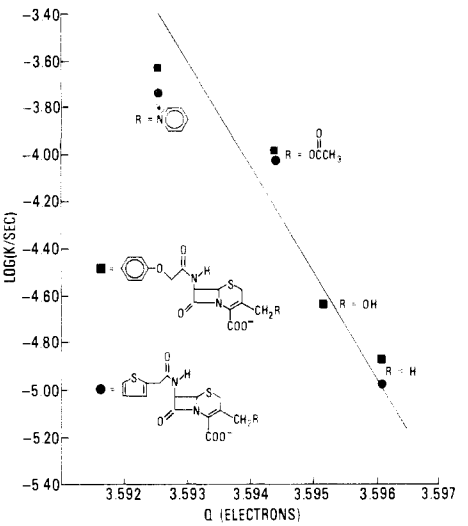
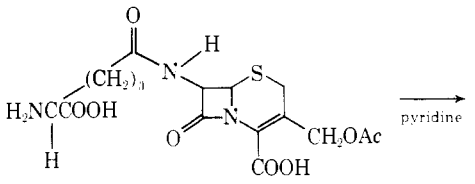


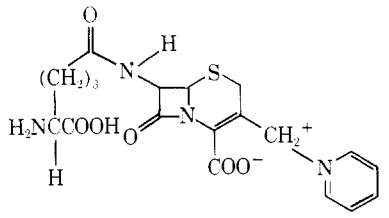
Figure 2. Correlation of observed pseudo-first-order rates of cephalosporin β-lactam ring opening at pH 10, 35°, and calculated electron density (CNDO/2) on β-lactam carbonyl.

the infrared carbonyl frequency of a β-lactam was an indicator of the acylating power of the β-lactam.[5] Our data for the limited series of penicillins and cephalosporins reported here (Figure 1) support this assumption, assuming that there is a direct relationship between ease of hydrolysis and acylating power.

Effect of 3-Methylene Substituents upon Cephalosporin β-Lactam Reactivity. During early work on the purification of naturally occurring cephalosporin C, 3, the first biologically active chemical derivative, **4**, was isolated from pyridine–acetate buffer.[14] Since then, displacement of the acetoxy group at the 3-methylene position of cephalosporins by other nucleophiles was found to be a facile reaction and many derivatives have been prepared.[15]

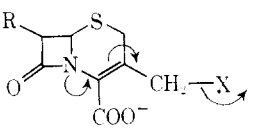


3



4

Although 3-methylene substituents are four atoms removed from the β-lactam carbonyl, these substituents may inductively exert considerable influence upon the chemical reactivity of the β-lactam. This long-range inductive effect may be conjugationally transmitted as suggested in **5**. Hermann has recently calculated (by CNDO/2 methods) the inductive effect of 3-methylene substituents upon the β-lactam and correlated these data with antibacterial activity against gram-negative bacteria.[7] We have observed (Figure 2) that the rates of β-lactam hydrolysis correlate with Hermann's calculated electron density at the β-lactam carbonyl as the 3-methylene substituent is varied. In addition, we have observed (Figure 3) that these same rate constants (plus the rate constant for a 3-methylmercaptomethyl derivative) correlate with aliphatic σ_I values.[16]



5

Effect of C-6- and -7-Acylamido Modification upon Penicillin and Cephalosporin Reactivity. Intramolecular Amino Attack on β-Lactams. Studies by Behrens, *et al.*, in 1948 showed that antibacterial properties of penicillin depend to a large extent upon the nature of the acylamido group.[17] Vast numbers of penicillins and cephalosporins with modified C-6- and -7-acylamido groups, respectively, have been prepared and new side-chain moieties continue to be reported in the literature. We chose to study the effect upon β-lactam reactivity of a few of the common acylamido side chains found on clinically used penicillins and cephalosporins.

The effect of side-chain alteration on the rate of hydrolysis at pH 10.0 of the β-lactam moiety of 7-aminocephalosporanic acid (7-ACA), 6-aminopenicillanic acid (6-APA), and 7-aminodeacetoxycephalosporanic acid (7-ADCA) is illustrated in Figure 4. The results suggest that while acylation of these nuclei generally increases the reactivity of the β-lactam, there is little difference in reactivity among the variously acylated compounds. Exceptions are the somewhat greater reactivity of D-α-aminophenylacetyl-7-ACA (D-cephaloglycin) and, particularly, the substantially greater reactivities of the L-α-aminophenylacetyl derivatives of both 7-ACA (L-cephaloglycin) and 7-ADCA (L-cephalexin). Both corresponding D- and L-α-aminophenylacetyl isomers of 6-APA (D- and L-ampicillin), however, appear to fit the general rule and are not noticeably different in their reactivity toward hydrolysis at pH 10.0.

We believe that this unusual increase in reactivity of cephalosporins having α-aminophenylacetyl side chains is due to an additional reaction which competes with hydroxide attack on the β-lactam at pH 10 and that there is no substantial difference in the intrinsic reactivity of the β-lactam. Intermolecular or intramolecular nucleophilic attack by the α-amino group on the β-lactam is obviously implicated. We discount the intermolecular reaction be-

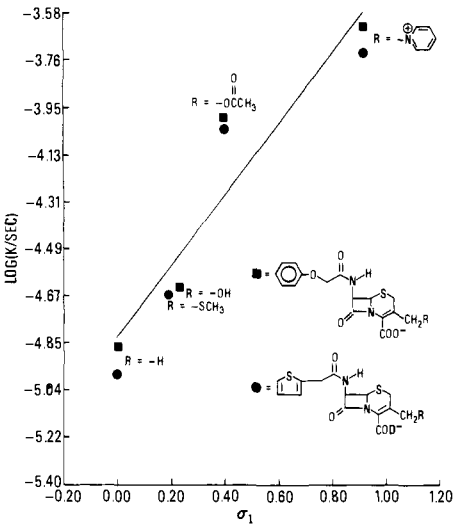


Figure 3. Correlation of observed pseudo-first-order rates of cephalosporin β-lactam ring opening at pH 10, 35°, and aliphatic σ_1 values of the 3-methylene substituent.

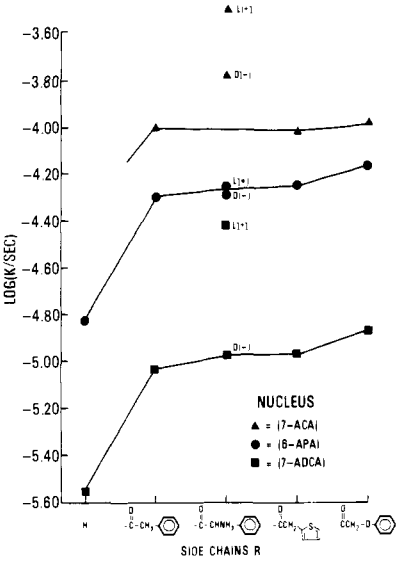


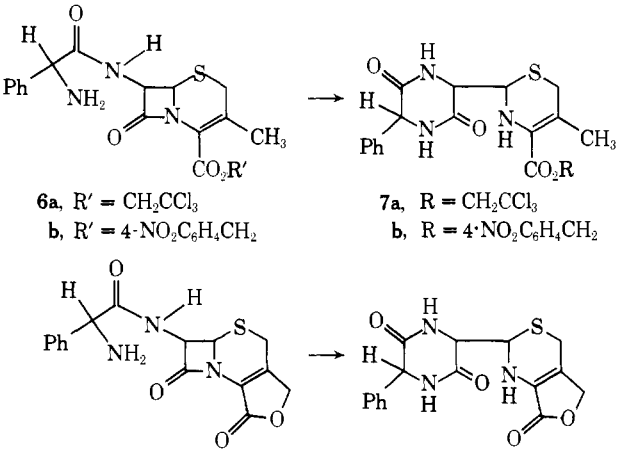
Figure 4. Substituent effect of acylamido side-chain modification upon observed pseudo-first-order rates of penicillin and cephalosporin β-lactam ring opening at pH 10, 35°.

cause we found the hydrolysis rate constants to be independent of β-lactam concentration at constant pH.

Intramolecular nucleophilic attack of the α-amino group has been suggested as a decomposition mechanism for cephaloglycin by Hoover and Stedman[18] and for ampicillin by Jusko[19] but such mechanisms have not been demonstrated. We therefore sought products that would confirm the occurrence of this mechanism.

Intramolecular α-amino attack in α-aminophenylacetyl cephalosporins should result in piperazine-2,5-dione products. Although the hydrolysis of cephalosporins results in a number of unstable products,[20] we were unsuccessful in attempts to isolate a piperazine-2,5-dione product from aqueous hydrolyses of cephalosporins.† However, we obtained the respective piperazine-2,5-diones **7a,b** and **9** by heating cephalexin trichloroethyl **6a** and *p*-nitrobenzyl **6b** esters and cephaloglycin lactone **8** in benzene under reflux overnight. (For a preliminary communication of this work, see ref 22. See also Supplementary Material Available paragraph.) In contrast, ampicillin trichloroethyl ester was recovered unchanged under these reaction conditions. These product studies in refluxing benzene along with the aqueous rate data suggest that intramolecular nucleophilic α-amino side-chain attack may occur in aqueous solution with cephalosporins but not with ampicillin.

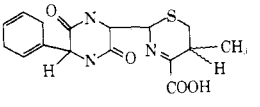
In view of the structural similarity of cephalosporins and penicillins, this difference in reactivity was surprising. Inspection of molecular models reveals that intramolecular attack on the β-lactam of these compounds must occur from the β face, *i.e.*, cis to the amide side chain. The models also show that steric hindrance by the *gem*-dimethyl group and/or the 3-hydrogen might prevent "cis" attack in the penicillin, while the cephalosporins are not sterically hindered to "cis" attack.‡ (However, the molecular models did not show why the L side-chain configuration should be more favorable than the D configuration for intramolecular attack in cephalosporins.)



Analysis of the X-ray structures of ampicillin and cephaloglycin confirms the steric conclusions drawn from molecular models.§ In the crystalline state, the 3-proton of ampicillin is at an interatomic distance of 2.79 Å from the carbonyl carbon of the β-lactam. Inasmuch as the interatomic distance of 2.79 Å approaches the van der Waal's carbon–hydrogen distance of 2.70 Å,[24] the 3-proton of ampicillin may block nucleophilic attack at the β face to the extent that a similar conformation exists in solution. In contrast, the nearest proton of the β-methyl group in the ampicillin crystal is at an interatomic distance of greater than 5.28 Å from the carbonyl and is not a steric obstacle as suggested by molecular models. The X-ray data for cephaloglycin confirm that the β-lactam carbonyl is unhindered as suggested by molecular models.

We have provided data to support the assumption that the chemical reactivity or acylation ability of a β-lactam antibiotic is sensitive to both steric strain and inductive effects of substituents. Steric strain as reflected by the β-lactam ir carbonyl frequency was found to correlate with the log of the relative rate constant for base hydrolysis. Substituent effects due to C-6- and -7-acylamido side-chain modification result in insignificant changes in reactivity. In contrast, substituent effects upon β-lactam reac-

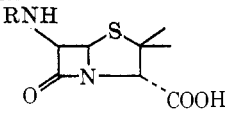
†During the preparation of this manuscript the following piperazine-2,5-dione was isolated from an aqueous sodium carbonate solution of cephradine.[21]

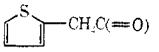


‡Intramolecular nucleophilic attack has been observed recently in 6-epiampicillin, where α-face attack is possible.[23]

§Private communication from M. O. Chaney (Eli Lilly and Co.). Calculated from coordinates reported in the unpublished work of M. James (University of Alberta) and ref 8.

Table I



Side chain (R)	k_{obsd} × 10^5 sec^{-1}	Compd ref
H	1.50 ± 0.08	*a*
$PhOCH_2C(=O)$	6.90 ± 0.48	*b*
$PhCH_2C(=O)$	5.08 ± 0.15	*c*
	5.65 ± 0.28	*d*
$PhCH(NH_2)C(=O)$ (D)	5.25 ± 0.18	*e*
$PhCH(NH_2)C(=O)$ (L)	5.60 ± 0.18	*e*

[a]F. R. Batchelor, F. P. Doyle, J. H. C. Nayler, and G. N. Rolinson, *Nature (London)*, **183**, 257 (1959). [b]O. K. Behrens, J. Corse, J. P. Edwards, L. Garrison, R. G. Jones, Q. F. Soper, F. R. Van Abeele, and C. W. Whitehead, *J. Biol. Chem.*, **175**, 793 (1948). [c]Penicillin G. [d]R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. A. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. Van Heyningen, *J. Amer. Chem. Soc.*, **84**, 3401 (1962). [e]F. P. Doyle, G. R. Foster, J. H. C. Nayler, and H. Smith, *J. Chem. Soc.*, 1440 (1962).

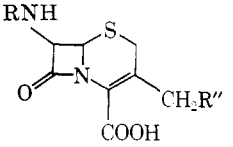
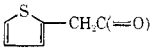
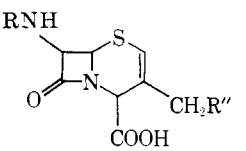
tivity as a result of substitution at the C-3 methylene in cephalosporins are significant and correlate with σ_I values and calculated electron densities at the β-lactam carbonyl. Cephalosporins that have α-amino-containing acylamido side chains may undergo intramolecular nucleophilic attack at the β-lactam—a reaction pathway which could compete with transpepidase acylation at pH's where the amino group is unprotonated.

Experimental Section

β-Lactams. The penicillins and cephalosporins used in this study were synthesized by the authors or by colleagues at Lilly Research Laboratories, by procedures referenced in Tables I and II. Ampicillin was purchased from several commercial sources.

Kinetic Methods. The hydrolysis rates of the penicillins and cephalosporins, with the exception of the 3-acetoxymethyl, 3-pyridiniummethyl, and 3-methylmercaptomethyl cephalosporins were followed by constant pH titration (Schwartz method).[9] The penicillinoic and cephalosporinoic acids produced in the hydrolysis at pH 10.0 were titrated automatically with a Radiometer TTT2 pH-stat, fitted with an A. H. Thomas (4094-15) combination electrode and calibrated with Fisher pH 10.00 certified buffer. Rate determinations were conducted at 35.0 ± 0.1° and constant ionic strength (0.102 *N* ± 0.003) was established by prior addition of potassium chloride. β-Lactam concentrations varied between 0.001 and 0.005 *M*. Carbon dioxide was excluded from the system with argon. From the recorded volume of standard sodium hydroxide solution consumed as a function of time, plots of log [β-lactam] *vs.* time were constructed. From these plots the pseudo-first-order rate constants were calculated. The faster reactions were followed for 2 half-lives, while the very slow reactions were followed to 25% completion. In all cases straight line plots

Table II

Side chain (R′)	3 (R″) substituent	k_{obsd} × 10^5 sec^{-1}	Compd ref
			
H	H	0.27 ± 0.04	*a*
$(NH_3)^+CH(COO^-)(CH_2)_3C(=O)$	OAc	10.4 ± 0.11	*b*
$PhOCH_2C(=O)$	OAc	10.5 ± 0.4	*c*
		23.8 (one run)	*d*
	H	1.37 ± 0.03	*e*
	OH	2.33 ± 0.17	*c*
$PhCH_2C(=O)$	OAc	10.0 ± 1.0	*c*
	H	0.93 ± 0.03	*f*
	OAc	9.70 ± 0.65	*c*
	H	1.07 ± 0.01	*f*
		18.75 ± 0.6	*g*
	SMe	2.20 ± 0.2	*h*
$PhCH(NH_2)C(=O)$ (D)	OAc	16.7 ± 0.6	*i*
	H	1.05 ± 0.01	*j*
$PhCH(NH_2)C(=O)$ (L)	OAc	31.62 ± 0.45	*i*
	H	3.8 ± 0.1	*k*
			
$PhOCH_2C(=O)$	H	0.65 ± 0.04	*l*

[a]E. P. Abraham and G. G. F. Newton, *Biochem. J.*, **62**, 658 (1956). [b]G. G. F. Newton and E. P. Abraham, *ibid.*, **62**, 651 (1956). [c]R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. A. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. Van Heyningen, *J. Amer. Chem. Soc.*, **84**, 3401 (1962). [d]J. L. Spencer, F. Y. Siu, E. H. Flynn, B. G. Jackson, M. V. Sigal, H. M. Higgens, R. R. Chauvette, S. L. Andrews, and D. E. Bloch, *Antimicrob. Ag. Chemother.*, 273 (1966). [e]R. B. Morin, B. G. Jackson, R. L. Mueller, E. R. Lavagnino, W. B. Scanlon, and S. L. Andrews, *J. Amer. Chem. Soc.*, **85**, 1896 (1963). [f]R. S. Stedman, K. Swered, and J. R. E. Hoover, *J. Med. Chem.*, **7**, 117 (1964). [g]J. L. Spencer, F. Siu, B. G. Jackson, H. M. Higgins, and E. H. Flynn, *J. Org. Chem.*, **32**, 500 (1967). [h]J. C. Clark, J. Kennedy, and A. G. Long, U. S. Patent 3,668,203 (1972). [i]J. L. Spencer, E. H. Flynn, R. W. Roeske, F. Y. Siu, and R. R. Chauvette, *J. Med. Chem.*, **9**, 746 (1966). [j]C. W. Ryan, R. L. Simon, and E. Van Heyningen, *ibid.*, **12**, 310 (1969). [k]T. Takahashi, Y. Yamazaki, K. Kato, and M. Isomo, *J. Amer. Chem. Soc.*, **94**, 4035 (1972). [l]Reference 5.

were obtained when one assumed that 1 equiv of acid was produced for each mole of β-lactam hydrolyzed.

The rates of hydrolysis of 3-acetoxymethyl, 3-pyridiniummethyl, and 3-methylmercaptomethyl cephalosporins were measured spectrophotometrically. Loss of absorbance at 260 mμ is characteristic of β-lactam opening either chemically or enzymatically.[25] The previously described procedure was used to maintain the reactions at constant pH, temperature, and ionic strength and to exclude carbon dioxide. Samples were removed at appropriate time intervals and quenched with pH 6 phosphate buffer. The absorbance was immediately read from a Cary 15 spectrophotometer. The data were treated by the Guggenheim method for determining rates of first-order reactions.[26]

Rate constants determined by the above methods are sensitive to experimental conditions, such as stirring rate, rate of addition of titrant, response time of various brands of glass electrodes, and the condition of the electrode. The electrodes, probably due to poisoning by decomposition products, were found to slow in response time after several experimental runs necessitating frequent replacement. Slight variations in the standard buffers used to calibrate the system also affect the observed rates. However, by carefully standardizing the procedure and frequently cross-checking the various rates using different electrodes and buffers, the precision noted in Tables I and II was obtained.

Because the observed rate constant values are sensitive to experimental conditions, conversion to the absolute second-order rate constants was not justified. For our discussions, only the relative rates of hydrolysis are of importance.

Decomposition of β,β,β-Trichloroethyl 7-[D(−)-α-Aminophenylacetamido]-3-methyl-Δ³-cephem-4-carboxylate (6a). A C_6H_6 solution (500 ml) of **6a**[27] (1.5 g, 3.14 mmol) was stirred under reflux for 24 hr. The mixture was allowed to cool to room temperature and the C_6H_6 was concentrated *in vacuo* to yield 0.650 g (43%) of the piperazine-2,5-dione **7a** as a tan crystalline solid: mp 179° dec. *Anal.* ($C_{18}H_{18}Cl_3N_3O_4S$) C, H, N. Further concentration of the C_6H_6 solution yielded an additional 0.088 g of **7a**.

Decomposition of p-Nitrobenzyl 7-[D(−)-α-Aminophenylacetamido]-3-methyl-Δ³-cephem-4-carboxylate (6b). A C_6H_6 solution (3 l.) of **6b**[28] (5 g, 10.4 mmol) was stirred under reflux for 48 hr. The C_6H_6 solution was allowed to cool and was concentrated *in vacuo* until the piperazine-2,5-dione **7b** crystallized: 3.35 g (66%); mp 165° dec. *Anal.* ($C_{23}H_{23}N_4O_4S$) C, H, N.

Decomposition of 7-[D(−)-α-Aminophenylacetamido]-3-hydroxymethyl-Δ³-cephem-4-carboxylic Acid Lactone (8). A suspension of 8[29] (2.2 g, 6.3 mmol) was refluxed in C_6H_6 (3.5 l.) and filtered. The resulting saturated solution of **8** was stirred under reflux for 48 hr. Cooling and concentration *in vacuo* yielded piperazine-2,5-dione **9**: 0.2 g (10%); mp 145° dec. A satisfactory analysis was not obtained because of occluded benzene.

β,β,β-Trichloroethyl 6-[D(−)-α-Aminophenylacetamido]penicillinate (10). To a stirred suspension of sodium *N*-[1-methyl-2-carbomethoxyvinyl]-D(−)-α-aminophenylacetate[30] (5.85 g, 21.6 mmol) in EtOAc (600 ml) was added β,β,β-trichloroethyl 6-aminopenicillinate hydrochloride[=] (8.25 g, 21.6 mmol). Stirring was continued and an EtOAc solution of dicyclohexylcarbodiimide (4.44 g, 21.6 mmol) was added dropwise. The mixture was stirred for 18 hr.

A mixture of dicyclohexylurea and NaCl was collected by filtration (4.9 g, 6.06 g, theory). The filtrate was concentrated *in vacuo* and the residue was dissolved in 150 ml of dioxane–H_2O (2:1). Hydrochloric acid was added to keep the mixture at pH 2.5 (10 ml × 1 *N* required). The dioxane–H_2O was removed *in vacuo* and the residue was dissolved in water and extracted with EtOAc. The pH of the aqueous layer was adjusted to 8.0 with 10% aqueous $NaHCO_3$ and extracted with EtOAc (2 × 50 ml). The combined extracts were dried (Na_2SO_4) and concentrated *in vacuo* to a foam. This material was dissolved in C_6H_6, filtered, and concentrated at 0° *in vacuo* to yield **10** as an amorphous solid: 1.3 g (10%); nmr ($CDCl_3$) δ 1.65 and 1.70 (2 s, 6, *gem*-dimethyl), 1.95 (br s, 2, $-NH_2$), 4.65 (s, 2, ester methylene), 4.7 (2, s, α-H and C-3 H), 5.6 (2, m, β-lactam collapsed to AB quartet in $CDCl_3$–D_2O), 7.3 (5, s, aromatic), and 8.0 (d, 1, amide NH).

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Supplementary Material Available. The 100-Hz proton chemical shifts and coupling constants for compounds **7a,b** and **9** and for 3-phenyl-2,5-piperazinedione in DMSO-d_6 will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JMED-74-523.

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