solution. A mixture of 0.1 ml of this homogenate, 0.1 ml of 0.2 *M* phosphate buffer, pH 6.4 (containing the test compound where indicated), and 0.1 ml of a mixture of 1.5 ml of labeled tyrosine, 0.6 ml of water, and 0.9 ml of tyrosine (26.6 μg/ml) was incubated. Final compound concentrations were 10^{-4} *M* at which concentration DL-phenylalanine, DL-*p*-fluorophenylalanine, and DL-α-methyl-*m*-tyrosine decreased the conversion to about 25% of the control. Only **32** showed some activity (24% decrease of conversion) but less than α-methyldopa.

Acknowledgments.—We are grateful to Dean Windsor Cutting and Dr. E. Furusawa of the Department of Pharmacology, University of Hawaii, for the antiviral tests, and to Dr. Edith G. McGeer, Kinsmen Laboratory of Neurological Research, The University of British Columbia, who carried out the determinations with tyrosine hydroxylase.

Chemistry of Cephalosporin Antibiotics. V.[1] Amides and Esters of Cephalothin[2]

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Received March 21, 1966

Chemical alteration of cephalothin (I) which might lead to orally active derivatives was investigated by preparing a number of C-4 carboxyl modifications.[3] Isomerization of the double bond in the thiazine ring was encountered under many conditions of amidation and esterification, giving rise to Δ^2-cephalosporins which were completely devoid of antimicrobial activity.

7-(Thiophene-2-acetamido)cephalosporanic acid (I) is an outstanding member of a series of cephalosporins prepared some time ago in these laboratories.[4] There are many reports of its desirable antibacterial activities against gram-positive and gram-negative organisms and penicillin-resistant staphylococci in the laboratory and in clinical practice.[5] As cephalothin and many of its analogs lack oral efficacy, an investigation of the effect of modification at the C-4 carboxyl group on oral absorption and biological activity was undertaken.

Both amides and esters were considered. An interesting speculation was the possibility that amides derived from a cephalosporanic acid and an amino acid might cross the intestinal wall and be cleaved in the body. Although simple esters, like the methyl ester, are known to possess diminished antibiotic activity compared to the free acids,[4] the possibility exists that more easily hydrolyzable esters (by enzymatic or chemical means) might exhibit significant *in vivo* activity. A therapeutic advantage might be anticipated from derived compounds if the structural environment of the carboxyl group is a bar to absorption through the gastric or intestinal walls. Activity could be inherent in the derivative or be produced as a result of enzymatic cleavage to the parent compound after absorption has occurred. Gastric acidity, often a negative influence in oral absorbability of penicillins, would seem to be an unlikely factor in cephalosporin absorption because of the relatively good acid stability of this class of antibiotics. Objectives similar to these are not uncommon in the literature of penicillin chemistry.[6] A second motivation for this work was provided by a recurring need for an easily cleaved blocking group for the carboxylic acid in cephalosporin synthetic chemistry.

This paper reports the chemistry involved in amidations and esterifications of 7-(thiophene-2-acetamido)-cephalosporanic acid (I).

To form peptides from a cephalosporin required that the carboxyl at C-4 be appropriately activated for acylation of a protected amino acid. Nefkens, *et al.*,[7] have demonstrated that N-hydroxyphthalimide condenses with carboxylic acids, in the presence of a carbodiimide, to give oxyphthalimide esters that are suitable intermediates in peptide synthesis. Using their conditions, I was treated with N-hydroxyphthalimide to yield the expected cephalosporanoyloxyphthalimide (III) in respectable yield. The isolable products from reaction of III with a number of amines, however, were not the anticipated Δ^3-cephalosporinamides. With ethyl glycinate, for example, III gave a good yield of IV in which the thiazine ring double bond had completely isomerized to the Δ^2 position.

Ready isomerization to Δ^2-cephalosporins accompanied many of the reactions included in this study. Identification of these Δ^2 isomers was possible from the characteristics which follow. (1) The ultraviolet absorption near 260 mμ, which is correlated with β-lactam double bond conjugation in the normal Δ^3-cephalosporin ring system, is lacking. (2) In the nmr spectra, lone protons at C-2 and C-4, visible as single peaks near τ 3.6 and 5.0, respectively, replace the methylene protons adjacent to the sulfur, evident as doublets centered near τ 6.4 and 6.7 in the normal Δ^3-cephalosporin series. Further, the centers of the single-proton quartet and the single-proton doublet

(1) Paper IV: E. Van Heyningen and C. N. Brown, *J. Med. Chem.*, **8**, 174 (1965).

(2) Cephalothin is the generic name for 7-(thiophene-2-acetamido)cephalosporanic acid; cephalothin sodium salt, Keflin®.

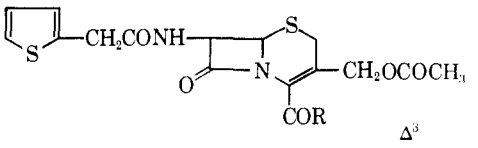
(3) For naming and numbering of the cephalosporins, see R. B. Morin, B. G. Jackson, E. H. Flynn, and R. W. Roeske, *J. Am. Chem. Soc.*, **84**, 3400 (1962).

(4) R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. B. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. Van Heyningen, "Antimicrobial Agents and Chemotherapy," American Society for Microbiology, Detroit, Mich., 1962, p 687.

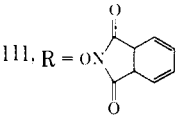
(5) W. S. Boniece, W. E. Wick, D. H. Holmes, and C. E. Redman, *J. Bacteriol.*, **84**, 1292 (1962); T. Chang and L. Weinstein, *ibid.*, **85**, 1022 (1963); R. S. Griffith and H. R. Black, *J. Am. Med. Assoc.*, **189**, 823 (1964).

(6) A. B. A. Jansen and T. J. Russell, *J. Chem. Soc.*, 2127 (1965); D. A. Johnson, *J. Am. Chem. Soc.*, **75**, 3636 (1953); H. F. J. McDuffie and D. E. Cooper, U. S. Patent 2,650,218 (1959).

(7) G. H. L. Nefkens, G. I. Tesser, and R. J. F. Nivard, *Rec. Trav. Chim.*, **81**, 683 (1962).

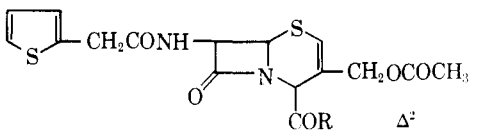


I, R = OH, Δ^3
II, R = O^-Na^+, Δ^3

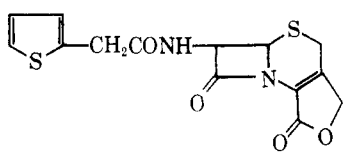


III, R = ON , Δ^3

IV, R = $NHCH_2COOC_2H_5$, Δ^3
V, R = $NHCHCH_3COOC(CH_3)_3$, Δ^3
VI, R = $NHCHCH_3COOH$, Δ^3
VII, R = $OCH_2CHOHCH_2OH$, Δ^3 and Δ^2
VIII, R = $OCH_2CH(CH_3)_2$, Δ^2
IX, R = $OCOOCH_2CH(CH_3)_2$, Δ^3



X, R = $OCOOC_2H_5$, Δ^3
XI, R = OCH_2OCH_3, Δ^3
XII, R = OCH_2COCH_3, Δ^3 and Δ^2
XIII, R = OCH_2OCOCH_3, Δ^3 and Δ^2
XIV, R = $(OCH_2)_2O$, Δ^3 and Δ^2
XV, R = $OCH_2COOC_2H_5$, Δ^3
XVI, R = p-$OCH_2C_6H_4OCH_3$, Δ^3
XVII, R = $NC_6H_{11}CONHC_6H_{11}$, Δ^3 and Δ^2
XVIII, R = p-$OC_6H_4OCH_3$, Δ^3



XIX

representing β-lactam hydrogens at C-7 and C-6, respectively, are generally τ −0.4 unit apart in the Δ^2 isomers and over τ −0.8 unit apart in the Δ^3-esters.[8] (3) Antimicrobial activity against organisms normally sensitive to the Δ^3-cephalosporins is absent.

In contrast to the amide reaction mentioned above, when the carboxyl group was activated directly by N,N′-dicyclohexylcarbodiimide in the formation of V from *t*-butyl alaninate, isomerization did not occur. The alanine ester (V) was hydrolyzed successfully to furnish the cephalosporanoylalanine (VI). The carboxyl group of this derivative was a weaker acid than that of previous known cephalosporins. Both of the alanine derivatives V and VI showed a very low order of antibacterial activity.

Because the absorption and hydrolysis of esters need not be identical with that of amides, easily hydrolyzable esters were also investigated. Attempts to synthesize a glyceryl ester using N,N′-dicyclohexylcarbodiimide in pyridine, a procedure described by Kochetkov, *et al.*,[9] for condensing amino acids with monosaccharides, yielded no isolable product other than the urea amide XVII. In a mixed anhydride reaction with isobutyl chloroformate, I reacted poorly with anhydrous glycerol. The neutral product was a mixture which required chromatography over silica to effect separation. The glyceryl ester VII was isolated, however, as a mixture of Δ^3- and Δ^2-cephalosporins in an approximate ratio of 1:1. Repeated recrystallizations did not appear to alter this ratio. A second product was isolated and characterized as the isobutyl ester VIII (presumed to result from loss of carbon dioxide by the mixed isobutylcarbonate anhydride intermediate under the conditions of the reaction). It was predominantly the Δ^2 isomer. Repeated recrystallizations of this ester only served to concentrate the less desirable isomer to give pure isobutyl Δ^2-cephalosporanate. The Δ^3-ester was not recovered in pure form.

Although the outcome of the mixed anhydride reaction just described was disappointing as a means for synthesizing a series of esters, formation of the mixed monoalkylcarbonate anhydride was indicated. This intermediate was interesting in itself so that isolation was attempted. The sodium salt of cephalothin (II) was treated with a number of chloroformates under conditions modified to protect the product from loss of carbon dioxide. Although the crude neutral products in some cases showed little ultraviolet absorption near 260 mμ, it was feasible to prepare and isolate pure samples of the cephalosporanic acid isobutyl (IX) and ethyl (X) carbonate anhydrides.

Preparation of ester derivatives by the interaction of "activated" alkyl halides with the sodium salt of cephalothin (II) met with varied success. Generally, the crude neutral products could be shown to contain both Δ^3 and Δ^2 unsaturation by nmr. While purification of the methoxymethyl ester XI (from reaction with chloromethyl methyl ether) and the acetonyl ester XII (from chloroacetone) was uncomplicated, we were not able to obtain a pure cephalosporin from the coupling with chloromethyl acetate. The acetoxymethyl ester XIII was of special interest since it incorporates a terminal group that could facilitate hydrolysis. Dichloromethyl ether, which was a contaminant of the chloromethyl acetate used (evident from the nmr), gave rise to the dimeric biscephalosporanate ester XIV as a coproduct.

Reaction of I with ethyl diazoacetate occurred as expected to give the derivative XV. Diazomethane was reported earlier to give the methyl ester of cephalothin.[4]

In addition to seeking easily hydrolyzable esters for oral absorption studies, we wished to devise suitable blocking groups for the carboxyl, which might be removed later without disruption of the β-lactam ring. The acid lability of *p*-methoxybenzyl esters which have been investigated by Weygand, *et al.*,[10] for use in peptide synthesis, was of interest. When I was allowed to react with anisyl alcohol in the presence of N,N′-dicyclohexylcarbodiimide, a mixture of neutral products was obtained. Fractional crystallization separated the *p*-methoxybenzyl ester XVI as a pure Δ^2-cephalosporin, from an amide which proved to be the N,N′-dicyclohexylurea amide (XVII), found predominantly as the Δ^3 isomer.

In like manner, the *p*-methoxyphenyl ester XVIII was prepared. Although nmr of the crude neutral product indicated the material to be largely Δ^2, a pure sample of the desired Δ^3-cephalosporanate ester was successfully isolated by crystallization. Treatment of

(8) G. F. H. Green, J. E. Page, and S. E. Staniforth, *J. Chem. Soc.*, 1955 (1965).

(9) N. K. Kochetkov, V. A. Derevitskaya, and L. M. Likhosherstov, *Chem. Ind. (London)*, 1532 (1960).

(10) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

XVIII with cold, anhydrous trifluoroacetic acid for 5 min caused cleavage of the *p*-methoxyphenyl grouping. A bioautograph of the product displayed a single biologically active component duplicating the mobility of cephalothin.

An improved preparation of the lactone of cephalothin (I), heretofore made from 7-(thiophene-2-acetamido)cephalosporadesic acid,[11] was developed. When either cephalothin or its sodium salt was dissolved in a 1 *N* HCl–aqueous dioxane solution, the water-insoluble lactone XIX precipitated in excellent yield. One recrystallization from either hot acetonitrile or dimethylformamide–ether gave an analytically pure product.

No satisfactory general method of synthesizing cephalosporin amides and esters, akin to those for the penicillins, was found. It was evident that the thiazine double bond in the cephalosporin ring system was particularly labile in carboxyl-substituted derivatives. The equilibrium existing between the Δ^3- and Δ^2-unsaturated products discussed here did not consistently favor one form or the other. Though both isomeric forms might have been formed in all the reactions cited, it was often possible or practical to isolate one. The biological testing of these materials was unrewarding. While all normal cephalosporins prepared could be shown to have antibiotic properties, there was little evidence of conversion of the derivatives to cephalothin *in vivo*, as measured by antibiotic blood levels in mice. The compounds were administered at a dosage of 20 and 40 mg/kg orally for these determinations.

Experimental Section[12]

N-[7-(Thiophene-2-acetamido)cephalosporanoyloxy]phthalimide (III).—Following the general procedure of Nefkens, *et al.*,[7] a mixture of I (4 g, 10 mmoles) and phthaloxime (2 g, 12.2 mmoles) in 30 ml of dry tetrahydrofuran (THF) was cooled in an ice bath and stirred during the addition of N,N'-dicyclohexylcarbodiimide (2.1 g, 10 mmoles) in 10 ml of ethyl acetate. The reaction mixture was stored in the cold for 3 days. The N,N'-dicyclohexylurea which had precipitated was collected by filtration and air dried. Its weight represented 98% of the theoretical amount. The solvents were evaporated and replaced by ethyl acetate. An extraction with 5% $NaHCO_3$ solution removed unreacted phthaloxime. The ethyl acetate solution was further washed with water, dried ($MgSO_4$), and evaporated. The residue was a light yellow amorphous solid which crystallized from ethyl formate. A recrystallization from hot ethyl formate–diisopropyl ether afforded pure ester in over 50% yield, mp 146–150° dec; λ_{max} 219 and 268 mμ (ε 41,500 and 7000, respectively); λ_{max} 2.94, 5.58, 5.71, 5.91, 6.58, and 8.0 μ; nmr was consistent with a Δ^3-cephalosporanate ester characterized by the methylene doublets centered at τ 6.49 for the hydrogen at C-2. A signal at τ 2.23, representing four aromatic protons, was assigned to the phthalimide. The bioautograph showed a major fast-moving activity spot near the solvent front, streaking lightly to form a minor spot corresponding to I. This activity streak was attributed to gradual hydrolysis of the derivative during development of the chromatogram with the aqueous solvent system used. For analytical purposes, a small sample was recrystallized from ethyl formate–2-propanol.

Anal. Calcd for $C_{24}H_{19}N_3O_8S_2$: C, 53.22; H, 3.53; N, 7.76. Found: C, 53.00; H, 3.83; N, 7.80.

N-(Carbethoxymethyl)-3-acetoxymethyl-7-(thiophene-2-acetamido)-2-cephem-4-carboxylic Acid Amide (IV).—Compound III (1.1 g, 2 mmoles) and ethyl glycinate hydrochloride (280 mg, 2 mmoles) reacted following the procedure of Nefkens, *et al.*[7] The product was a light yellow amorphous solid weighing 1 g. It was crystallized from warm ethyl formate–petroleum ether. One recrystallization from the same solvents gave 700 mg (73%) of pure product: mp 174°; λ_{max} 233 mμ (ε 12,900) and no 260 mμ absorption; λ_{max} 2.94, 5.63, 5.72, 5.91, and 7.92 μ; nmr was consistent with a Δ^2-cephalosporin, characterized by single-proton peaks at τ 3.52 for the vinyl hydrogen at C-2, at τ 4.88 and 5.42 for the α and β forms of the allyl hydrogen at C-4. A three-proton triplet centered at τ 8.76, and a two-proton quartet centered at τ 5.77 identified the ethyl ester. A two-proton methylene signal near τ 6 completed the characterization of the carbethoxymethyl moiety.

Anal. Calcd for $C_{20}H_{23}N_3O_7S_2$: C, 49.88; H, 4.81; N, 8.73. Found: C, 50.19; H, 5.04; N, 8.62.

***t*-Butyl α-Aminopropionate Hydrochloride.**—A method described by Roeske[13] was used. Liquid isobutylene (300 ml) was added to a precooled solution of L-alanine (30 g, 336 mmoles) in 300 ml of dry dioxane and 30 ml of concentrated H_2SO_4 in a pressure vessel. The mixture was shaken at room temperature for 20 hr. The pressure bottle was cooled in an ice bath, opened, and immediately poured into a cold, stirred mixture of 2.4 l. of ether and 1.5 l. of 1 *N* NaOH. The ether was separated, combined with another cold ether wash of the aqueous layer, dried ($MgSO_4$), concentrated to a smaller volume, and then treated with dry HCl. The precipitated salt was separated by filtration and recrystallized from methanol–ether. The yield was 10.9 g (18%); mp 170° dec; λ_{max} 5.73 μ for the ester carbonyl; electrometric titration in 66% aqueous dimethylformamide showed a pK_a = 8.0 and an apparent molecular weight of 183 (calcd 182).

Anal. Calcd for $C_7H_{16}ClNO_2$: C, 46.29; H, 8.88; N, 7.72. Found: C, 46.27; H, 8.99; N, 7.82.

N-[1-(Carbo-*t*-butoxy)ethyl]-7-(thiophene-2-acetamido)cephalosporanic Acid Amide (V).—*t*-Butyl α-aminopropionate hydrochloride was converted to free amino ester by neutralizing a cold aqueous solution of the salt to pH 9 with K_2CO_3 and subsequently extracting into ether which was dried ($MgSO_4$) and evaporated for immediate use. A solution of I (15.8 g, 40 mmoles), *t*-butyl α-aminopropionate (40 mmoles), and N,N'-dicyclohexylcarbodiimide (8.25 g, 40 mmoles) in 300 ml of dry CH_2Cl_2 was stored at room temperature for 4 hr. The precipitation of N,N'-dicyclohexylurea began immediately. Its weight when the reaction was terminated indicated about a 75% reaction. The CH_2Cl_2 solution was cooled, washed successively with cold 1 *N* HCl, 1 *N* $NaHCO_3$ solution, and water, and evaporated. The residual oil was crystallized and recrystallized from warm acetone–petroleum ether (bp 30–60°), yielding 9 g of pure product (additional crops furnished 6.2 g of slightly lower melting material which did not depress the melting point of first crop material): mp 190–191°; λ_{max} 235 and 265 mμ (ε 15,760 and 8150, respectively); λ_{max} 2.94, 5.58, 5.74, 5.92, 6.59, and 8.0 μ; nmr was consistent in every detail with a Δ^3-cephalosporin. In addition, the spectrum showed a methyl group attached to carbon as a doublet at τ 8.64 and lying under the nine-proton signal of the *t*-butyl at τ 8.53. A single-proton quartet representing the hydrogen at the α carbon was centered near τ 5.41.

Anal. Calcd for $C_{23}H_{29}N_3O_7S_2$: C, 52.75; H, 5.58; N, 8.02. Found: C, 53.01; H, 5.68; N, 7.83.

N-(1-Carboxyethyl)-7-(thiophene-2-acetamido)cephalosporanic Acid Amide (VI).—Compound V (7.4 g, 14 mmoles) was dissolved in 180 ml of formic acid (98%) and then diluted carefully with 210 ml of water to keep the ester in solution. The mixture was heated for 4 hr in a water bath at 40°. The solvents were evaporated. The residue was redissolved in cold ethyl acetate for extraction with cold 5% $NaHCO_3$ solution. The bicarbonate extract was acidified to pH 2.5 with 1 *N* HCl in the presence of ethyl acetate. The organic layer was separated, dried ($MgSO_4$), and evaporated. The crude residue crystallized from acetone–petroleum ether to give 3.4 g (52%) of an acid, mp 178–180° dec. Recrystallization from hot methanol

(11) R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. B. Morin, R. A. Mueller, R. P. Ploch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. Van Heyningen, *J. Am. Chem. Soc.*, **84**, 3401 (1962).

(12) All melting points were taken on a Mel-Temp melting point apparatus and are uncorrected. All evaporations were performed at temperatures below 50°, in a rotary vacuum evaporator. Starting materials I and II were predried in a vacuum oven at 60° for 2 hr. Ultraviolet spectra were obtained in ethanol. Infrared spectra were obtained in $CHCl_3$. Nmr spectra were taken on a Varian Associates Model HR-60 spectrometer, in $CDCl_3$ with tetramethylsilane as an internal standard. Bioautographs (against *Bacillus subtilis* seeded agar plates) of paper chromatograms, developed in either methyl ethyl ketone saturated with water or in 70% aqueous 1-propanol, showed single biologically active spots for all Δ^3-cephalosporins prepared and no spots for those products reported as pure Δ^2-cephalosporins.

(13) R. W. Roeske, *J. Org. Chem.*, **28**, 1251 (1963).

raised the melting point to 190–192° dec; λ_{max} 235 and 260 mμ (ε 15,500 and 8200, respectively); λ_{max} 5.65, 5.73, 6.01, 6.15, 6.51, and 7.94 μ; electrometric titration in 66% aqueous dimethylformamide showed a pK_a = 5.85 and an apparent molecular weight of 460 (calcd 468); nmr showed the methylene adjacent to the sulfur as doublets centered at τ 6.41, a methyl attached to carbon as a three-proton doublet centered at τ 8.35, and the α-hydrogen of the alanine moiety as a single-proton quartet centered at τ 5.14.

Anal. Calcd for $C_{19}H_{21}N_3O_7S_2$: C, 48.81; H, 4.52; N, 8.99. Found: C, 48.61; H, 4.70; N, 8.97.

2,3-Dihydroxypropyl 7-(Thiophene-2-acetamido)cephalosporanate (VII).—Compound **I** (4 g, 10 mmoles) was dissolved in 15 ml of dry dioxane and 10 ml of analytical grade acetone containing triethylamine (1.1 g, 11 mmoles). The solution was cooled in an ice-alcohol bath and stirred during dropwise addition of isobutyl chloroformate (1.37 g, 10 mmoles) in 10 ml of dry dioxane. About 10 min was allowed for the mixed anhydride to form and then anhydrous glycerol (0.92 g, 10 mmoles) in 10 ml of dry dioxane–acetone was added. The reaction mixture was stirred in the cold for 2 hr and then at room temperature overnight. The solvents were evaporated, and the residual oil was taken up in chloroform for successive washes with 5% HCl, 5% $NaHCO_3$ solution, and water. The $CHCl_3$ solution was dried ($MgSO_4$) and evaporated. The residue weighed about 2 g and showed three components in thin layer chromatography using silica gel and a chloroform–ether (1:2) system for development and an iodine chamber to visualize the spots. A 12.5 × 3.1 cm column of silica (45 g, activated at 120° for 2 hr) was prepared in an ethyl acetate–ether (1:3) system. The neutral product (1.5 g), dissolved in 7 ml of ethyl acetate, was added; when the solution had completely entered the adsorbent, the column was eluted successively with 300 ml of ethyl acetate–ether (1:3) [300 ml (1:1)], 150 ml of chloroform, and 150 ml of chloroform–ethanol (1:1). The effluent was collected in 150-ml portions. Fraction 6, on evaporation, gave 600 mg of a yellow oil which crystallized from ethylene chloride–ether to yield a glyceryl ester: mp 111–112°; λ_{max} 235 and 260 mμ (ε 14,600 and 3900, respectively); λ_{max} (Nujol mull) 2.92, 5.68, 5.71, 6.01, 6.55, and 8.01 μ; nmr was consistent with a nearly equal mixture of Δ^2- and Δ^3-cephalosporanate esters, supported by the low ultraviolet absorption at 260 mμ. Signals at τ 3.62 and 5.01 identified single protons at C-2 and C-4, respectively. Slightly less intense signals, as doublets, centered near τ 6.42 pointed to the presence of some CH_2 protons adjacent to the sulfur.

Anal. Calcd for $C_{19}H_{27}N_3O_9S_2$: C, 48.50; H, 4.71; N, 5.95. Found: C, 48.44; H, 4.93; N, 5.78.

Fraction 1, on evaporation, gave 400 mg of a yellow oil which crystallized from THF-ether-petroleum ether; mp 106–107° (the melting point was depressed by mixing with material obtained from fraction 6); λ_{max} 235 and 260 mμ (ε 12,800 and 3480, respectively). Further recrystallizations from ethylene chloride–petroleum ether yielded pure **isobutyl 3-acetoxymethyl-7-(thiophene-2-acetamido)-2-cephem-4-carboxylate (VIII),** mp 110–111°, λ_{max} 235 (ε 14,600) and no 260 mμ absorption; nmr was typical of a Δ^2-cephalosporanate ester. Single protons at C-2 and C-4 were responsible for signals at τ 3.62 and 5.01, respectively. The presence of the isobutyl ester was indicated by a two-proton CH_2 quartet centered at τ 6.03, by two C-methyl peaks at 9.02 and τ 9.13, and one-proton multiplet centered near τ 8.0.

Anal. Calcd for $C_{20}H_{24}N_2O_6S_2$: C, 53.08; H, 5.34; N, 6.19. Found: C, 53.13; H, 5.50; N, 6.11.

Fraction 4, on evaporation, gave 200 mg of an oil which could not be crystallized or identified.

7-(Thiophene-2-acetamido)cephalosporanic Acid Monoisobutylcarbonate Anhydride (IX).—A solution of **I** (4 g, 10 mmoles) in 15 ml of dry dioxane and 20 ml of analytical grade acetone containing triethylamine (1 g, 10 mmoles) was cooled in an ice–alcohol bath. To this was added dropwise, a solution of isobutyl chloroformate (1.37 g, 10 mmoles) in 10 ml of acetone. The reaction mixture was kept near 0° for several hours, then poured into a stirred mixture of ice-cold water and chloroform. The organic layer was separated, washed successively with cold 5% HCl, 5% $NaHCO_3$ solution, and water, dried ($MgSO_4$), and evaporated. The neutral residue was an oil weighing about 1.6 g. When dissolved in THF and diluted first with ether and then petroleum ether, the oil gave 800 mg of crystalline product: mp 114–116°; λ_{max} 234 and 266 mμ (ε 12,100 and 6800, respectively); λ_{max} 2.93, broad 5.45–5.59, 5.72, and 5.91 μ; nmr corresponded in every respect to a Δ^3-cephalosporin. The presence of isobutyl was indicated by a two-proton methylene quartet centered at τ 5.94, by two C-methyl peaks at τ 9.00 and 9.11 and a single-proton multiplet centered near τ 8.0. The bioautograph showed an activity streak comparable for the one discussed for III.

Anal. Calcd for $C_{21}H_{24}N_2O_8S_2$: C, 50.79; H, 4.87; N, 5.64; CO_2, 8.86. Found: C, 50.67; H, 5.17; N, 5.62; CO_2, 8.60.[14]

7-(Thiophene-2-acetamido)cephalosporanic Acid Monoethylcarbonate Anhydride (X).—A solution of **I** (16 g, 40 mmoles) in 310 ml of dry THF containing triethylamine (4 g, 40 mmoles) was cooled in an ice–alcohol bath. Ethyl chloroformate (4.3 g, 40 mmoles) in 40 ml of the same solvent was added dropwise. The reaction mixture was stirred and kept cold for 1 hr. The solvent was evaporated and the residue was dissolved in chloroform. The chloroform solution was washed successively with cold 5% HCl, 5% $NaHCO_3$ solution, and water, then dried ($MgSO_4$), and evaporated. The residue was a light yellow oil weighing about 10 g. An ultraviolet spectrum of the neutral product showed little absorption in the region of 260–270 mμ. Two recrystallizations from CCl_4 gave 3.5 g of a product with constant melting point and ultraviolet absorption: mp 97–98°; λ_{max} 234 and 266 mμ (ε 10,800 and 6150, respectively); λ_{max} 2.93, broad 5.51–5.58, 5.70, 5.91, and 7.98 μ. The bioautograph showed an activity streak like the one described for III.

Anal. Calcd for $C_{19}H_{20}N_2O_8S_2$: C, 48.71; H, 4.30; N, 5.98. Found: C, 49.41; H, 4.22; N, 5.95.

Methoxymethyl 7-(Thiophene-2-acetamido)cephalosporanate (XI).—A suspension of **II** (12.6 g, 30 mmoles) in 50 ml of N,N-dimethylacetamide was stirred and cooled at ice–alcohol temperature during the dropwise addition of chloromethyl methyl ether (2.4 g, 30 mmoles) in 10 ml of the same solvent. The reaction mixture was stored at 0° overnight, then poured into 500 ml of cold phosphate buffer (pH 6.6) causing an oil to separate. The oil crystallized during attempted extraction with ether. The white crystalline material weighing 10 g was recrystallized from hot 2-propanol yielding 8.8 g (66%, in two crops) of pure ester: mp 154–155°; λ_{max} 236 and 261 mμ (ε 11,700 and 7500, respectively); λ_{max} 2.94, 5.59, 5.71, 5.91, and near 8 μ.

Anal. Calcd for $C_{14}H_{16}N_2O_7S_2$: C, 49.08; H, 4.57; N, 6.36. Found: C, 49.20; H, 4.64; N, 6.15.

2-Ketopropyl 7-(thiophene-2-acetamido)cephalosporanate (XII) was prepared in a manner identical with that of XI, using 1 equiv of chloroacetone. One recrystallization of the product from acetone–petroleum ether afforded analytically pure ester: mp 147–148°; λ_{max} 236 and 260 mμ (ε 12,450 and 7160, respectively); λ_{max} 2.94, 5.59, 5.74, and 5.91 μ. In the nmr spectrum, in addition to the CH_2 (C-2) doublets centered at τ 6.49, normal for Δ^3-cephalosporanate esters, a small peak at τ 3.52 (associated with the vinyl proton at C-2 in the case of Δ^2-cephalosporins) was present. This represented less than 15% of the Δ^2 isomer and corresponded proportionally to the lower extinction coefficient of the 260 mμ absorption in the ultraviolet. The acetonyl ester portion was responsible for a three-proton singlet at τ 7.79 for the terminal C-methyl and for two-protons in the region of τ 4.95 for the methylene.

Anal. Calcd for $C_{15}H_{16}N_2O_7S_2$: C, 50.43; H, 4.46; N, 6.19. Found: C, 50.57; H, 4.57; N, 6.28.

Acetoxymethyl 7-(Thiophene-2-acetamido)cephalosporanate (XIII).—To a suspension of **II** (12.6 g, 30 mmoles) in 75 ml of N,N-dimethylacetamide was added a solution of chloromethyl acetate (prepared after Descudé,[15] still containing dichloromethyl ether to the extent of 20–25% after repeated distillations) (3.5 g, 33 mmoles) in 25 ml of the same solvent. The mixture was stirred at room temperature for 5 hr, filtered, and concentrated *in vacuo* to remove the solvent. The residue was taken up in ethylene chloride, washed with phosphate buffer (0.1 M, pH 7), dried ($MgSO_4$), and evaporated to dryness. The amorphous residue weighed nearly 5 g. A thin layer chromatogram developed with ethylene chloride–ether (1:2) (using a silica gel plate and stained with iodine) of this crude material indicated three components. The bioautogram also indicated three components. A 2.4-g sample of the crude product was chromatographed over silica (150 g) using ethylene chloride–ether (1:3)

(14) Obtained from a measure of CO_2 evolution from a solution of the compound in 0.01 *N* HCl–aqueous acetone at room temperature.

(15) M. Descudé, *Bull. Soc. Chim. France*, [3] **27**, 867 (1902); L. H. Ulich and R. Adams, *J. Am. Chem. Soc.*, **43**, 662 (1921).

as eluent. The progress of the separation was followed by thin layer chromatography on the residues from evaporation of periodic fractions. Those fractions, containing a material corresponding to the leading spot in the thin layer chromatogram of the mixture, were combined to give 500 mg of a white powder which after repeated recrystallizations from methylene chloride–diisopropyl ether was characterized as the acetoxymethyl ester of cephalothin: mp 91–93°; λ_{max} 235 and 270 mμ (ε 13,300 and 3030, respectively); nmr showed the presence of nonequivalent CH_2 doublets centered at τ 6.54, assigned to the hydrogens at C-2, and single-proton signals at 5.00 and 3.56 assigned to the hydrogens at C-4 and C-2, respectively, indicating a mixture of Δ^3- and Δ^2-type cephalosporins. Signals at τ 7.95 and 7.88 represent two different types of acetoxy methyls. Also, signals at τ 4.23 and 4.14 were assigned to nonequivalent methylenes in the two different kinds of acetoxymethyl esters in the mixture.

Anal. Calcd for $C_{19}H_{20}N_2O_8S_2$: C, 48.71; H, 4.30; N, 5.98. Found: C, 49.11; H, 4.86; N, 5.98.

Intermediate fractions, representing 100 mg of material, gave two spots in thin layer chromatograms and were discarded. Compound XIV, **oxydimethyl bis-7-(thiophene-2-acetamido)cephalosporanate,** was removed from the column by changing the eluent to ethylene chloride–ether (1:2). The following fractions, giving a material corresponding to the second spot in the thin layer chromatogram of the crude product, were combined and evaporated to furnish 300 mg of an amorphous powder. This was twice recrystallized from ethylene chloride–ether–petroleum ether and identified as the oxydimethyl bis-ester of cephalothin: mp 155–156° dec; λ_{max} 235 and 270 mμ (ε 22,400 and 4725, respectively); nmr was somewhat indistinct but absorption peaks for both Δ^2- and Δ^3-cephalosporins could be distinguished.

Anal. Calcd for $C_{34}H_{34}N_4O_{13}S_4$: C, 48.91; H, 4.11; N, 6.71. Found: C, 49.19; H, 4.48; N, 6.54.

The remaining fractions, obtained after altering the eluent to ethylene chloride–ether (1:1.5), furnished 330 mg of material which no longer possessed biological activity and which appeared to be a complex hydrocarbon in the nmr spectrum. This material, related to the third or slow-moving spot in the thin layer chromatogram of the mixture, resulted from decomposition during extended contact with silica.

Carbethoxymethyl 7-(Thiophene-2-acetamido)cephalosporanate (XV).—A cooled solution of **I** (8 g, 20 mmoles) in 100 ml of dry dioxane was treated with ethyl diazoacetate (2.5 g, 22 mmoles) under dry nitrogen. As no reaction was apparent in the cold or at room temperature, the mixture was placed in an oil bath at 65–70° for 4 hr. Coloration and gas evolution resulted. The solvent and excess reagent were removed by evaporation. The residue was redissolved in chloroform for successive washes with 5% HCl, 5% $NaHCO_3$ solution, and water. The $CHCl_3$ solution was dried ($MgSO_4$) and evaporated to dryness leaving 5.8 g of crystalline residue. This was recrystallized from warm ethylene chloride–petroleum ether to give 5.0 g (86%) of pure ester: mp 133–135°; λ_{max} 236 and 260 mμ (ε 12,600 and 7270, respectively); nmr was consistent with a Δ^3-cephalosporanate ester as evidenced by the two-proton CH_2 doublets centered at τ 6.46 for the hydrogens at C-2. The carbethoxymethyl ester was represented by a three-proton triplet centered at τ 8.70, a two-proton quartet centered at τ 5.67 for the ethyl group, and a two-proton CH_2 signal (not resolved if a doublet) at τ 4.9 fused to the doublet for the β-lactam hydrogen at C-6.

Anal. Calcd for $C_{24}H_{22}N_2O_8S_2$: C, 49.78; H, 4.59; N, 5.81. Found: C, 50.00; H, 4.76; N, 5.57.

***p*-Methoxybenzyl 3-Acetoxymethyl-7-(thiophene-2-acetamido)-2-cephem-4-carboxylate (XVI).**—A solution of I (4.0 g, 10 mmoles) and anisyl alcohol (1.4 g, 10 mmoles) in 100 ml of dry THF was treated with N,N′-dicyclohexylcarbodiimide (2.1 g, 10 mmoles) and stirred at room temperature overnight. The precipitated N,N′-dicyclohexylurea was removed by filtration, the solvent was evaporated and replaced by chloroform for successive washes with 5% HCl, 5% $NaHCO_3$ solution, and water. The $CHCl_3$ was removed by evaporation. The residue (2.5 g) crystallized from warm 2-propanol. Fractional recrystallization from ethanol gave two compounds, A and B. Compound A was the *p*-methoxybenzyl ester, 900 mg, mp 143–144°; the ultraviolet spectrum had no 260-mμ absorption; λ_{max} 2.95, 5.60, 5.72, 5.92, 6.20, 6.61, 8.10, 8.52, 9.70, and 12.08 μ; nmr was consistent with a Δ^2-cephalosporanate ester with single-proton peaks at τ 3.62 and 5.00 assigned to hydrogens at C-2 and C-4, respectively. In addition, a signal at τ 6.20, representing three protons, was assigned to the *p*-methoxy grouping. Nonequivalent two-proton CH_2 signals near τ 4.8 and 5.4 identified the acetoxymethyl at C-3 and the benzyl ester. Seven aromatic protons were accounted for in the region of τ 2.7–3.2.

Anal. Calcd for $C_{24}H_{24}N_2O_7S_2$: C, 55.80; H, 4.68; N, 5.42. Found: C, 56.08; H, 4.59; N, 5.39.

Compound B was **1,3-dicyclohexyl-1-[7-(thiophene-2-acetamido)cephalosporanoyl]urea (XVII),** 500 mg, mp 150–152°; λ_{max} 234 and 266 mμ (ε 14,650 and 6500, respectively); nmr showed the two-proton doublets centered at τ 6.67 characteristic of the CH_2 adjacent to the sulfur as well as small peaks at τ 3.93 and 5.08 associated with the single protons at C-2 and C-4, respectively. The intensity of the signals was indicative of a mixture of cephalosporins composed of approximately four parts of Δ^3 and one part of Δ^2. Twenty-two alicyclic protons were accounted for in the region τ 8.2–8.9 for the hydrogens of the dicyclohexylurea amide.

Anal. Calcd for $C_{29}H_{38}N_4O_6S_2$: C, 57.78; H, 6.35; N, 9.29. Found: C, 57.38; H, 6.64; N, 9.12.

***p*-Methoxyphenyl 7-(thiophene-2-acetamido)cephalosporanate (XVIII)** was prepared in the manner described for XVI. The product was crystallized from ethyl acetate–ether–petroleum ether; mp 171–172°; λ_{max} 2.23, 2.37, and 2.68 mμ (ε 17,950, 13,700, and 9950, respectively); nmr was consistent with a Δ^3-cephalosporanate ester. The CH_2 adjacent to the sulfur appeared as doublets centered at τ 6.49, the protons of the *p*-methoxy grouping appeared at τ 6.19 and seven aromatic protons were accounted for by signals in the τ 2.7–3.2 region. The bioautograph showed no activity at 5-μg application.

Anal. Calcd for $C_{23}H_{22}N_2O_7S_2$: C, 54.96; H, 4.41; N, 5.57. Found: C, 55.13; H, 4.58; N, 5.32.

7-(Thiophene-2-acetamido)cephalosporanolactone (XIX).—Compound I (7.9 g, 20 mmoles) was dissolved in 50 ml of dioxane and diluted with 50 ml of 2 *N* HCl. The solution was stirred overnight at room temperature. The precipitated lactone was filtered off, washed with cold aqueous dioxane, and dried in a vacuum dessicator. The yield of crude lactone was 5.5 g (82%). Recrystallization from dimethylformamide-ether gave an 80% recovery and afforded analytically pure lactone: mp 230–232°; λ_{max} 236 and shoulder at 260 mμ (ε 12,350 and 6800, respectively); nmr (in DMF) was consistent in every respect with a Δ^3-cephalosporin. In addition, the spectra showed at the expected two-proton peak at τ 4.90 for the lactone methylene at C-3.

Anal. Calcd for $C_{14}H_{12}N_2O_4S_2$: C, 49.98; H, 3.59; N, 8.33. Found: C, 49.99; H, 3.90; N, 8.62.

Acknowledgment.—The authors gratefully acknowledge contributions made by the following groups to the experimental data: Mr. W. L. Brown, Mr. H. L. Hunter, and Mr. D. L. Cline for microanalyses; Mr. L. G. Howard, Mr. D. O. Woolf, Dr. H. Boaz, and associates for spectrometry; Mr. W. E. Wick for microbiological testing; and Mr. C. T. Pugh for bioautography.