β-LACTAM ANTIBIOTICS DERIVED FROM NITROGEN HETEROCYCLIC ACETIC ACIDS

2. CEPHALOSPORIN DERIVATIVES†

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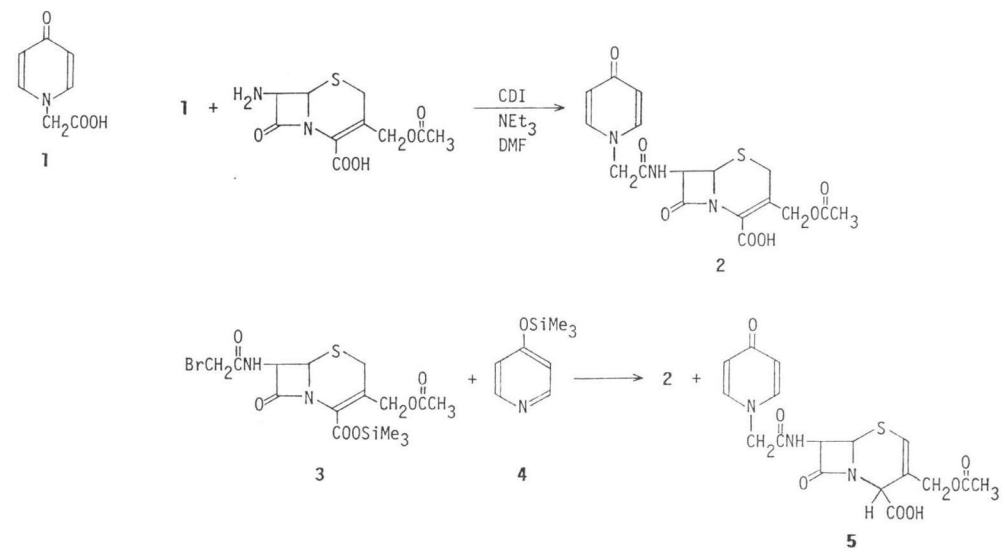
Three cephalosporin derivatives were prepared from 1,4-dihydro-4-oxypyridine-1-acetic acid. These were the 7-aminocephalosporanic acid (7-ACA) derivative and the compounds with 5-methyl-1,3,4-thiadiazol-2-thiol and 1-methyl-1,2,3,4-tetrazole-5-thiol at C-3 of the cephalosporin nucleus. The antibacterial activity of the 7-ACA derivative was comparable to cephalothin, and that of the other two derivatives was comparable to cefazolin. The 7-ACA derivative, compared to cephalothin, was significantly less metabolized, was less protein bound, and had a longer half life.

In the previous paper of this series† we reported the antibacterial activity of penicillins derived from a variety of heterocyclic acetic acids. The penicillin derived from 1,4-dihydro-4-oxopyridine-1-acetic acid (**1**) was the most active of those tested against a variety of Gram-negative bacteria. This paper reports the synthesis of the cephalosporin derivatives of **1** and their antibacterial activity. Other workers have reported data on similar compounds.[1)]



† Paper 1. EDWARDS, M. L.; R. E. BAMBURY & H. W. RITTER: β-Lactam antibiotics derived from nitrogen heterocyclic acetic acids. 1. Penicillin derivatives. J. Med. Chem. 20: 560~563, 1977

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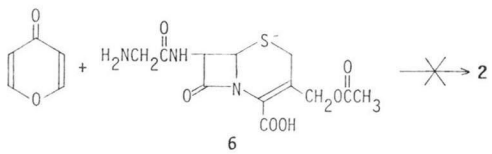
The zwitterionic nature of **1** necessitated the use of a polar solvent (DMF) in coupling reactions with 7-aminocephalosporanic acid derivatives. We found the reagent of choice to be carbonyldiimidazole (CDI). Alternatively, the trimethylsilyl ester **3** was alkylated with the ether **4** to give, after hydrolysis of the TMS ester with methanol, the desired product **2** contaminated with the Δ^2 isomer **5**. The formation of **5** is due to isomerization of the TMS ester of the product by excess **4**.[2] Compound **2** (Na salt) was crystallized from a mixture of the sodium salts of **2** and **5** dissolved in methanol by addition of isopropyl alcohol. We also attempted the synthesis of **2** by reaction of **6** with γ-pyrone in aq.-$NaHCO_3$. The reaction of γ-pyrones with a primary amine to give 4-pyridones is known.[3] Decomposition products which may be due to an intramolecular attack of the β-lactam by the amino group at C-7 precluded isolation of **2**. Reaction of **2** with the appropriate thiol gave **7a, b**.[4] Careful monitoring of the reaction by HPLC (see Experimental) was necessary. Attempts to prepare **7a, b** from **1** and **8** with CDI gave mixtures of **7**, **8** as well as **1** due to lack of appreciable solubility of the triethylammonium salt of **8** in DMF.



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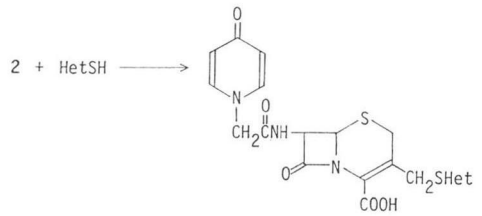

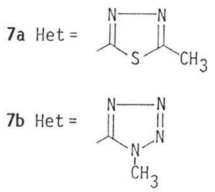

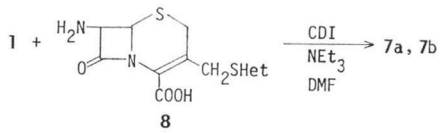

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Materials and Methods

1. Antibiotics

Cephalothin was obtained from Eli Lilly and Company and Cefazolin from Smith Kline and French Laboratories.

2. Antibiotic Spectrum

Standard strains, some of clinical origin, were used in determining minimal inhibitory concentrations (MICs) by serial twofold dilutions of compound in MUELLER-HINTON agar and inoculation of the agar surface using the multiple inoculator of STEERS *et al.*[5]. To obtain inocula for MIC determinations, all cultures except *Streptococcus pneumoniae* and *Streptococcus pyogenes* were grown 18~24 hours in trypticase soy broth (Difco) and diluted in MUELLER-HINTON broth (Difco) 10-fold for *Staphylococcus aureus* and 100-fold for the others. *Streptococcus pneumoniae* and *Streptococcus pyogenes* were grown for 24 hours in brain heart infusion broth (Difco) and diluted 10-fold in the same broth. The incubation temperature for the growth of all cultures was 37°C. A volume of approximately 0.003 ml of inoculum was applied to the agar surface, and after incubation for 17 hours at 37°C the lowest concentration causing complete or virtually complete inhibition of visible growth was considered to be the MIC.

3. Binding to Serum Proteins

The extent to which cephalothin and compound **2** were bound to mouse serum was determined by an ultrafiltration method. Fifteen 20 ($\pm$1) g albino CD-1 male mice for each cephalosporin were dosed at 40 mg/kg subcutaneously and bled from the orbital sinuses at a time when there would be between 10 and 20 μg/ml in the blood. The blood from each group was pooled and the sera were col-

lected. Additionally, the cephalosporins were added to mouse sera to provide concentrations of 15~20 μg/ml. These were allowed to incubate at 37°C for 1 hour to allow complete binding to occur. To obtain protein free filtrates, a portion of each sample was transferred to an Amicon Centriflo Ultrafiltration Membrane Cone (2100 CF 50) and centrifuged at 500×*g* for 20 minutes at 4°C.

4. Antibiotic Concentrations in Mouse Blood

Cephalothin and **2** were administered subcutaneously as a single 40 mg/kg dose to 20 (±1) g albino CD-1 male mice. Four mice were bled from the orbital sinuses for each sampling time and then sacrificed. The blood was pooled and the serum collected.

5. Bioassays

Serum was assayed by a standard agar diffusion procedure using *Bacillus subtilis* ATCC 6633 as the assay organism seeded in antibiotic medium #1 (Difco). Standard curves were prepared from serum samples to which known concentrations of antibiotic had been added and which had been diluted with 0.1 M pH 6.0 potassium phosphate buffer in the same proportion as were the samples to be assayed. Protein free filtrates containing antibiotic were diluted in the pH 6.0 phosphate buffer and assayed against a standard curve obtained from dose responses to known concentrations of antibiotic dissolved in the buffer. Protein free filtrates had previously been shown to have no effect on responses to the antibiotics tested.

6. Metabolism of Cephalosporins

Five 20 (±1) g albino CD-1 male mice were administered a single 80 mg/kg dose of either cephalothin or **2**. After 1 hour, urine was collected from the bladders of each group, pooled, and frozen at −20°C until chromatographed. Urine from mice dosed with cephalothin were chromatographed by descending chromatography on Whatman #1 paper using acetone - water (9: 1 v/v); urine from mice dosed with **2** were chromatographed on Eastman 13254 cellulose thin-layer sheets by ascending chromatography using acetone - water (3: 1, v/v). Chromatograms were bioautographed on antibiotic medium #1 seeded with *Bacillus subtilis* ATCC 6633. Concentrations in urine were determined by chromatography and bioautography of known amounts of reference compounds. Deacetylated derivatives of cephalothin and **2** were prepared by the method of JEFFERY *et al.*[6].

7. Treatment of Experimental Infections in Mice

Albino CD-1 male mice weighing 20 (±1) g were infected by intraperitoneal injection of a bacterial suspension containing a sufficient number of organisms to produce uniformly lethal infections. The suspensions were in brain heart infusion broth and were 0.1 to 1.0 ml in volume, depending upon the organism. Suspensions of *S. aureus* M238 and *Escherichia coli* Es 59 contained 2.5% mucin. Groups of ten mice each were treated subcutaneously with appropriate concentrations of antibiotic at 1 and 4 hours after infection. The number of mice in each group surviving the challenge for 4 days was recorded and the ED_{50} (the dose in mg/kg required to protect 50% of the infected mice) determined by the method of REED and MUENCH[7].

Results and Discussion

The microbiological comparisons of **2** with cephalothin are noteworthy. While the *in vitro* potency of the two compounds was the same or favored cephalothin, the *in vivo* potency often favored **2**. For example, **2** and cephalothin were equipotent *in vitro* against *Escherichia coli,* and cephalothin possibly was the more potent of the two against *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Table 1), yet **2** was the more potent against mouse infections caused by these organisms (Table 2). Cephalothin was significantly more potent than **2** *in vitro* against a benzylpenicillin-sensitive strain of *Staphylococcus aureus* and possibly more potent against a resistant strain. Again, the two compounds were equipotent against the sensitive strain and **2** was significantly more potent against the resistant strain *in vivo*. Finally, the superiority shown by cephalothin against *Salmonella schottmuelleri in vitro* was not reflected by the results obtained *in vivo* against this organism.

Table 1. Activity *in vitro* of **2**, **7a**, **7b**, cephalothin and cefazolin.

Organism	MIC (μg/ml)				
	2	**7a**	**7b**	Cephalothin	Cefazolin
Staphylococcus aureus[a]	0.8	0.4	0.4	0.2	0.2
Staphylococcus aureus[b]	1.6	1.6	1.6	0.8	1.6
Streptococcus pneumoniae	0.2	0.1	0.2	0.1	0.05
Streptococcus pyogenes	0.2	0.2	0.2	0.1	0.2
Escherichia coli	6.2	3.1	1.6	6.2	1.6
Klebsiella pneumoniae	6.2	3.1	1.6	3.1	1.6
Proteus mirabilis	12.5	12.5	6.2	6.2	6.2
Salmonella schottmuelleri	12.5	3.1	1.6	3.1	1.6
Enterobacter aerogenes	>100	>100	25	100	50
Enterobacter cloacae	>100	>100	12.5	>100	50

[a] Benzylpenicillin-sensitive.
[b] Benzylpenicillin-resistant.

Table 2. Activity *in vivo* of **2**, **7a**, **7b**, cephalothin and cefazolin.

Organism	Challenge (CFU)[b]	ED_{50} (mg/kg/dose)[a]				
		2	**7a**	**7b**	Cephalothin	Cefazolin
Staphylococcus aureus[c]	3.5×10^8	0.28	0.76	0.71	0.2	1.1
Staphylococcus aureus[d]	6×10^7	5.0	4.5	4.7	14.8	11.9
Streptococcus pneumoniae	1.5×10^8	2.9	0.5	0.65	29	0.76
Streptococcus pyogenes	2×10^4	0.61	0.68	0.49	2.7	0.83
Escherichia coli	1×10^8	15.2	2.6	1.5	34.5	2.1
Klebsiella pneumoniae	4.5×10^8	102	13.8	21.2	93	19.0
Proteus mirabilis	1×10^8	140	35.9	54.3	163	52
Salmonella schottmuelleri	1×10^7	33.5	4.9	2.1	27.5	2.9

[a] Results are averages of two or more experiments. Mice were treated 1 and 4 hours after infection.
[b] CFU, colony forming units.
[c] Benzylpenicillin-sensitive.
[d] Benzylpenicillin-resistant.

Table 3. Comparative *in vivo* deacetylation of **2** and cephalothin.

Compound administered	Chromatographic system[a]	Rf	Bioactive component in urine; percent of total based on bioautography	
2	A	0.65	**2**;	85±5
		0.48	Desacetyl **2**;	15±5
Cephalothin	B	0.66	Cephalothin;	15±5
		0.53	Desacetylcephalothin;	85±5

[a] A, urine chromatographed on Eastman 13254 cellulose thin-layer using acetone-water (3:1 v/v); B, urine chromatographed by descending chromatography on Whatman #1 paper using acetone-water (9:1 v/v).

These findings could be rationalized by the results of subsequent investigations. First, as compared to cephalothin, **2** was significantly less metabolized to the less active desacetyl metabolite (Table 3).

Table 4. Mouse serum binding of cephalothin and **2**.

Compound	Serum sample[a]	μg/ml[b]	% bound
2	Unfiltered from dosed mice	15.9	17
	Filtered from dosed mice	13.2	
	Unfiltered from spiked mouse serum	19.5	20
	Filtered from spiked mouse serum	15.6	
Cephalothin	Unfiltered from dosed mice	13.5	68.5
	Filtered from dosed mice	4.25	
	Unfiltered from spiked mouse serum	17.5	68.6
	Filtered from spiked mouse serum	5.5	

[a] Filtered samples were obtained by ultrafiltration using Amicon Cones and are considered to be protein free filtrates.
[b] Obtained by standard bioassay using *Bacillus subtilis* ATCC 6633 and antibiotic medium #1.

Table 5. Serum levels of mice dosed with **2** and cephalothin.

Compound	Serum level (μg/ml)[a]							
	5 min.	10 min.	15 min.	20 min.	30 min.	40 min.	60 min.	90 min.
2	67.5	75.0	60.0	60.0	49.5	21.6	10.5	3.75
Cephalothin	30.6	39.0	24.0	28.8	9.1	3.4	N.M.[b]	N.M.

[a] Obtained by standard bioassay using *Bacillus subtilis* ATCC 6633 and antibiotic medium #1.
[b] N.M., not measurable.

Extensive deacetylation of cephalothin by the mouse has been previously reported.[8,9] Second, **2** was 17~20% bound by mouse serum as compared to cephalothin which was *ca* 69% bound (Table 4). Finally, a significantly higher and a more prolonged blood level of antibiotic activity was obtained with **2** than with cephalothin after subcutaneous administration (Table 5). These results were similar to those found for **2** in human subjects.[10]

The synthesis of analogous compounds with heterocyclic thiomethylene groups in the three position of the cephalosporin nucleus produced two compounds **7a** and **7b** with potent biological activity. The *in vitro* spectrum of activity and potency is quite comparable to that of cefazolin (Table 1). Preliminary data showed **7b** to be more active than cefazolin against *Enterobacter* sp.; **7a** was inactive. As shown in Table 2, activity *in vivo* of the two analogs was comparable to that of cefazolin, with one important exception; both **7a** and **7b** were more potent against the benzylpenicillin-resistant *S. aureus*.

Experimental

IR spectra were determined in pressed KBr disks. NMR spectra in D_2O were obtained with DSS as an internal standard. In addition to spectral data, purity of the compounds was determined by HPLC assay, which was carried out as follows:

The HPLC assay of compounds **2**, **7a**, and **7b** was conducted using a Bondapak C_{18}/corasil column 1 m×2 mm i.d. The eluent used was a 0.1 M phosphate buffer pH 3.2 containing 3% v/v acetonitrile for compounds **2** and **7b** and containing 7% v/v acetonitrile for compound **7a**. The work was performed on a Varian Model 4200 liquid chromatograph equipped with an injection valve fitted with a 20 μl sample loop. The analysis was carried out by making successive injections of solutions of a reference sample of the compound and of the sample to be analyzed. The peak height/unit weight response for

that peak on each chromatogram associated with the desired compound was determined; the assay result was calculated by comparing these two values.

The solvolysis reaction forming **7a** from **2** was followed by taking successive aliquots from the reaction mixture, diluting them, and injecting the dilution as above. The height of the **7a** peak was measured on each chromatogram; **2**, the thiol, and **7a** were well separated by this chromatographic system.

3-Acetoxymethyl-7-[2-(4-oxo-1(4H)-pyridinyl)acetyl] amino-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid, sodium salt (**2**)

Method A

A mixture of **1** (6.2 g, 0.04 mole), CDI (7.8 g, 0.048 mole) and 75 ml of dry DMF was stirred for 1 hour under a dry nitrogen atmosphere. At the end of this period the reaction mixture consisted of a thick white suspension of the imidazolide. This suspension was added to a solution of 10.8 g (0.04 mole) of 7-aminocephalosporanic acid, chloroform (280 ml, hydrocarbon stabilized) and triethylamine (16 g, 0.16 mole) which had been stirred with Linde 4A molecular sieves for 20 minutes. The resulting mixture was stirred under a dry nitrogen atmosphere for 4.5 hours. Nuchar activated carbon (3.0 g) was added to the reaction mixture and stirring was continued for 30 minutes. The reaction mixture was filtered through celite and the filtrate was cooled in an ice-acetone bath. The stirred filtrate was treated with 40 ml of 2 N sodium 2-ethylhexanoate in *n*-butyl alcohol.

After stirring for 20 minutes, the mixture was filtered and the precipitate was washed with $CHCl_3$, acetone and dry ether. The crude product was dried overnight over P_2O_5 at 0.1 mm. This material was dissolved in 325 ml of methanol and the soln. was decolorized with 3 g of Nuchar for 20 minutes and filtered through celite. The filtrate was treated with 250 ml of isopropyl alcohol and allowed to stand 20 minutes to crystallize. An additional 750 ml of isopropyl alcohol was then added to the stirred mixture, followed by cooling in an ice bath. The mixture was filtered and the solid was washed with isopropyl alcohol and dry ether. A second recrystallization resulted in a 45% yield of product of 94% purity by HPLC assay. IR (KBr) 1760, 1700, 1650, 1620, 1550, 1410, 1380, 1230, 1195, 1020 and 850 cm^{-1}; NMR (D_2O) δ 2.11 (s, 3H), 3.55 (dd, J=9 Hz, J=3 Hz, 2H), 4.82 (d, J=6 Hz, 2H), 5.0 (s, 2H), 5.45 (dd, J=6 Hz, J=2 Hz, 1H), 5.75 (d, J=4 Hz, 1H), 6.65 (d, J=8 Hz, 2H), 7.87 (d, J=8 Hz, 2H).

Anal. Calcd. for $C_{17}H_{16}N_3O_7SNa \cdot H_2O$: C, 45.63; H, 4.06; N, 9.39; S, 7.17; H_2O, 4.03.
Found: C, 45.56; H, 3.72; N, 9.38; S, 7.27; H_2O, 4.00.

Method B

Bis (trimethylsilyl)acetamide (125 ml) was added to a suspension of **3** (105 g, 0.267 mole) in 625 ml of $CHCl_3$ (hydrocarbon stabilized) and the mixture was stirred under a nitrogen atmosphere until solution occurred. Compound **4** (50 ml) was added and the mixture was stirred 18 hours at room temperature, then poured into methyl alcohol (400 ml). The resulting mixture was filtered through a plug of Nuchar sandwiched between two layers of celite. The filter cake was washed with 400 ml of a chloroform-methyl alcohol (3: 2, v/v) mixture. Ether (2.5 liters) was added to the filtrate and the mixture was stirred until the supernatant liquid was clear. The solvent was decanted and the residue was dissolved in methyl alcohol (1.6 liters) and 325 ml of a 2 N solution of sodium 2-ethylhexanoate in *n*-butyl alcohol was added. Isopropyl alcohol (1.5 liters) was added over 15 minutes. The mixture was filtered and the precipitate was washed with isopropyl alcohol and vacuum dried over P_2O_5 to give the product (65 g, 57% yield) which assayed 98% purity by HPLC.

3-[(5-Methyl-1,3,4-thiadiazol-2-yl) thio] methyl-8-oxo-7-[(4-oxo-1 (4H)-pyridinyl) -acetyl] amino- 5 -thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, sodium salt (**7a**)

A mixture of 2-methyl-1,3,4-thiadiazole-5-yl thiol (3.25 g, 0.025 mole), sodium bicarbonate (2.1 g, 0.025 mole) and water (10 ml) was stirred at 60°C until solution occurred. Potassium thiocyanate (50 g) was added and the mixture was heated at 75~80°C until all solids had dissolved. To this solution **2** (5.0 g, 0.017 mole) was added and when the solution was clear the pH was adjusted to 6.8 with 40% phosphoric acid. The reaction mixture was heated at 80°C for 1 hour, cooled to room temperature and filtered to remove unreacted thiol.

The filtrate was pipetted onto a preconditioned Amberlite XAD-4 resin column (4 cm×27 cm)

and the column was eluted with 400 ml of 12.5% acetonitrile-triethylammonium formate buffer solution (pH 3.2) followed by 700 ml of a 25% solution of the buffer. The fractions which contained the product were concentrated to 75 ml, chilled and filtered to give 2.3 g of product (40% yield), HPLC assay 96%. IR (KBr) 1760, 1680, 1640, 1600, 1460, 1380, 1190 and 845 cm^{-1}; NMR (D_2O) δ 2.8 (s, 3H), 3.73 (dd, J=6.5, 2.5 Hz, 2H), 4.25 (dd, J=12, 8 Hz, 2H), 5.02 (s, 2H), 5.18 (d, J=2 Hz, 1H), 5.72 (d, J=2 Hz, 1H), 6.72 (d, J=4 Hz, 2H), and 7.85 (d, J=4 Hz, 2H).

Anal. Calcd. for $C_{18}H_{16}N_5NaO_5S_3 \cdot 2.5H_2O$: C, 39.55; H, 3.50; N, 12.81; S, 17.60; H_2O, 8.0.
Found: C, 39.35; H, 3.21; N, 13.02; S, 18.45; H_2O, 7.9.

In a similar manner, **7b** was prepared (50% yield, HPLC assay 83%). IR (KBr) 1765, 1690, 1645, 1620, 1560, 1400, 1360, 1200, and 850 cm^{-1}; NMR (D_2O) δ 3.65 (dd, J=9.5, 3.2 Hz, 2H), 4.06 (s, 3H), 4.21 (q, J=9.5, 3.2 Hz, 2H), 4.89 (s, 2H), 5.12 (d, J=3 Hz, 1H), 5.67 (d, J=3 Hz, 1H), 6.63 (d, J= 6 Hz, 2H) and 7.85 (d, J=6 Hz, 2H).

Anal. Calcd. for $C_{17}H_{16}N_7NaO_5S_2 \cdot 4H_2O$: C, 36.62; H, 4.34; N, 17.58; S, 11.50; H_2O, 12.91.
Found: C, 36.62; H, 4.41; N, 15.60; S, 11.78; H_2O, 13.9.

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