DIASTEREOMERIC 7-α-UREIDOACETYL CEPHALOSPORINS

V. ANTIMICROBIAL ACTIVITY, β-LACTAMASE STABILITY AND PHARMACOKINETICS OF 7-(α-UREIDO-2-AMINO-4-THIAZOLYLACETYL)-CEPHALOSPORINS

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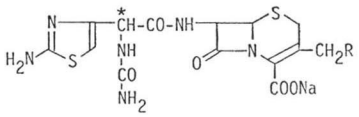
Diastereomeric 7-(α-ureido-2-amino-4-thiazolylacetyl)cephalosporins are broad-spectrum antimicrobial agents with activity mainly against Gram-negative bacteria. The L-isomers were more potent than the D-isomers. All compounds showed some degree of stability to β-lactamases. The compounds reached high serum and tissue fluid levels and were excreted unchanged mainly in urine.

In a previous report[1] BREUER *et al.* described the synthesis, chemical properties and *in vitro* antimicrobial activity of a series of new cephalosporin derivatives. These compounds, with a ureidoaminothiazolyl moiety in the 7 side chain, differ from each other in stereochemistry and by substitution at position 3. The present paper describes the influence of the 3-substituent and of stereochemistry on the antibacterial potency, stability towards β-lactamases, and pharmacokinetic behaviour of 7-(α-ureido-2-amino-4-thiazolylacetyl)-cephalosporins.



Compound No.	R	*
1	STz	D, L
2	STz	L
3	STz	D
4	OAc	D, L
5	OAc	L
6	OAc	D

Material and Methods

Test compounds were prepared in the Organic Chemistry Department as sodium salts. Reference cephalospornis were commercial preparations.

Minimum inhibitory concentrations (MIC's) were determined by the agar plate dilution method (MUELLER-HINTON agar, BBL) using 10^4 and 10^6 colony forming units (CFU) as the final inoculum concentration.

β-Lactamase stability was determined spectrophotometrically[2]. Crude enzyme preparations were obtained from *Enterobacter cloacae* SC 10,435 (Type Ia), *Escherichia coli* SC 10,404 (Type IIIa), *E. coli* SC 11,101 (Type IIIb) and *Klebsiella aerogenes* (Type IVc)[2], by sonicating the bacterial suspension followed by centrifugation and dialysis. In some cases the enzyme solution was purified by chromatography using Sephadex G75.

Efficacy studies were performed in male or female CF_1 mice (obtained from Hagemann, Bösingfeld, Germany), 18 ~ 20 g, infected with *E. coli* SC 8294 (100 × median lethal doses (LD_{50})), and treated with the test cephalosporin subcutaneously (s.c.). The treatments were administered in divided doses at 1 and 5 hours post infection. Mice were observed for one week after treatment and all deaths were recorded. Median effective doses (ED_{50} values) were calculated according to LITCHFIELD and WILCOXON[3].

Urinary excretion studies were carried out in male CF_1 mice (25~30 g) and male Sprague-Dawley rats (180~200 g) purchased from Wiga, Sulzfeld, Germany. The animals were fed the diet Ssniff R (from Ssniff Versuchsdiäten, Soest, Germany) and had free access to water. Before the experiment they were water-loaded and treated with test compounds s.c. (25 mg/kg). Mice were placed in wide-mouth porcelain ointment jars fitted with a wire screen, as described by WHEELER *et al.*[4] The vessels were standing in ice. Rats were seated in steel metabolic cages and urine was collected over a 6-hour period in calibrated glass cylinders.

Biliary excretion was determined in male urethan-anaesthesized Sprague-Dawley rats (260~320 g) with cannulated biliary duct. Test compounds were administered intravenously (i.v.), 2.5 mg/kg (volume: 10ml/kg), and bile was collected over a 4~5-hour period.

Drug serum levels were measured in male or female mice (28~32 g) treated i.v. or s.c. (25 mg/kg volume: 10 ml/kg). The animals were distributed at random in several groups, 5 animals per group, and sacrificed at intervals ranging from 3 to 60 minutes post injection by decapitation. Serum half-lives were obtained graphically from concentration curves, using a semi-log graph paper.

Serum and tissue fluid levels were also determined in rats. Six male Sprague-Dawley rats (180~200 g) were provided with subcutaneous multiperforated plastic capsules[5] that became filled with fluid. Approximately 3 weeks after implantation of capsules, rats were administered with a cephalosporin derivative (25 mg/kg intramuscularly (i. m.)). At intervals up to 3 hours the sublingual vein was punctured to get a blood sample, and a sample of the capsule fluid was obtained by percutaneous aspiration. For use in animal experiments the test compounds were dissolved in water.

Serum protein binding was estimated using the ultrafiltration method[6]. Test compounds were dissolved in 4% human serum albumin and diluted to obtain solutions of 10^{-4} to 10^{-8} M. Solutions were placed in Amicon Centriflo membrane cones CF 25 and centrifuged at 140 g for 30 minutes.

All antibiotic concentrations were measured by microbiological assay using agar plates seeded with *Micrococcus luteus* ATCC 9341. Samples of body fluids (20 μl) were applied to 6-mm paper discs (Oxoid) and transferred onto the plates. The inhibition zones were read after 18-hour incubation period at 37°C with a Fisher-Lilly zone reader and antibiotic concentration estimated from standard curves.

Urine and bile were examined by thin-layer chromatography (Merck silica gel plates 60 F 254) developed in a mixture of methyl-ethylketone - water - acetonitril - acetic acid (30: 10: 5: 5) or *n*-butanol - acetic acid - water (3: 1: 1). After developing and drying, the chromatograms were laid on agar plates seeded with *M. luteus*. After 10 minutes, the chromatograms were removed and the plates were incubated overnight.

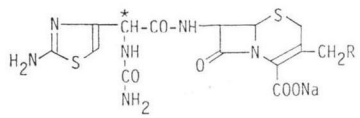
Results

1. Experiments *In Vitro*

As demonstrated in the previous report[1], the test compounds are broad-spectrum antibiotics, the effect of which is comparable with cefotaxime (CTX). Table 1 shows more MIC data. It is evident that the inoculum effect is low and that L-isomers or racemates were often more active than the corresponding D-isomers.

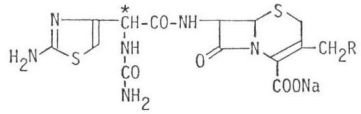
This observation was further confirmed in experiments using recent clinical isolates. As an example, the antibacterial activity against 20 strains of *E. coli* is shown in Fig. 1. The figure demonstrates that 70~80% of the strains are inhibited by compound **2** at 8 ng/ml, compound **1** at 16 ng/ml, and compounds **3**, **4**, **5**, and CTX at 60 ng/ml, whereas **6** is clearly inferior. The superiority of L-isomers over DL-isomers, D-isomers and/or CTX was confirmed in similar experiments using clinical isolates of *Enterobacter* sp., *Klebsiella pneumoniae*, *Serratia marcescens*, and *Salmonella*; CTX was superior to L-, DL- and D-isomers (in this order) in experiments with indole-positive *Proteus* sp. and *Staphylococcus aureus*. None of the compounds was significantly active against *Pseudomonas aeruginosa*. The S-

Table 1. *In vitro* minimum inhibitory concentrations.



Compound No. R *	1 STz D, L		2 STz L		3 STz D		4 OAc D, L		5 OAc D		6 OAc L		Cefotaxime	
CFU	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
S. aureus 2400	3.1	6.3	3.1	6.3	6.3	12.5	6.3	6.3	6.3	6.3	6.3	25	1.6	1.6
S. aureus 2399	3.1	3.1	3.1	6.3	3.1	3.1	6.3	6.3	6.3	6.3	6.3	6.3	0.8	1.6
E. coli TEM+ 10404	<0.2	<0.2	<0.2	<0.2	<0.2	0.8	<0.2	<0.2	<0.2	<0.2	0.4	1.6	<0.2	<0.2
E. coli TEM− 10439	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2
E. coli RGN 238 10854	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2	<0.2	<0.2	0.4	0.8	<0.2	0.8
Citr. freundii 10204	<0.2	1.6	<0.2	<0.2	<0.2	3.1	<0.2	25	<0.2	3.1	0.8	50	<0.2	6.3
Shig. sonnei 10944	<0.2	0.8	<0.2	0.4	<0.2	3.1	0.4	6.3	<0.2	3.1	3.1	25	0.8	6.3
Sal. typhimurium 10943	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2
E. cloacae P99+ 10435	50	>50	25	50	>50	>50	>50	>50	>50	>50	>50	>50	50	>50
E. cloacae P99− 10441	<0.2	<0.2	<0.2	0.4	<0.2	0.4	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2
E. cloacae 8415	<0.2	6.3	<0.2	0.8	<0.2	6.3	<0.2	1.6	<0.2	0.4	0.8	25	<0.2	3.1
K. aerogenes K1+ 10436	0.4	>50	0.8	>50	6.3	>50	0.8	50	0.8	50	12.5	>50	0.8	>50
K. aerogenes K1− 10440	<0.2	<0.2	<0.2	<0.2	<0.2	0.8	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2
K. pneumoniae 8340	<0.2	<0.2	<0.2	<0.2	<0.2	1.6	<0.2	<0.2	<0.2	<0.2	<0.2	0.4	<0.2	<0.2
K. pneumoniae 11066	<0.2	1.6	<0.2	<0.2	<0.2	3.1	<0.2	0.4	<0.2	0.2	0.4	3.1	<0.2	0.8
Prot. rettgeri 8217	<0.2	0.4	<0.2	<0.2	<0.2	1.6	<0.2	<0.2	<0.2	<0.2	<0.2	1.6	<0.2	1.6
Prot. vulgaris 10950	<0.2	>50	<0.2	>50	<0.2	>50	<0.2	>50	<0.2	>50	1.6	>50	<0.2	>50
Prot. vulgaris 10951	<0.2	>50	<0.2	>50	0.4	>50	<0.2	>50	<0.2	>50	1.6	>50	<0.2	>50
Pr. mirabilis 9574	<0.2	<0.2	<0.2	<0.2	<0.2	0.8	<0.2	<0.2	<0.2	<0.2	0.4	0.8	<0.2	<0.2
Ps. aeruginosa 9545	1.6	25	3.1	6.3	25	25	50	>50	25	>50	>50	>50	0.8	6.3
Ps. aeruginosa 8329	>50	>50	50	>50	50	>50	>50	>50	50	>50	>50	>50	12.5	50
Ser. marcescens 9782	<0.2	1.6	<0.2	1.6	<0.2	>50	0.4	25	<0.2	3.1	6.3	50	<0.2	1.6

Table 2. β-Lactamase stability.

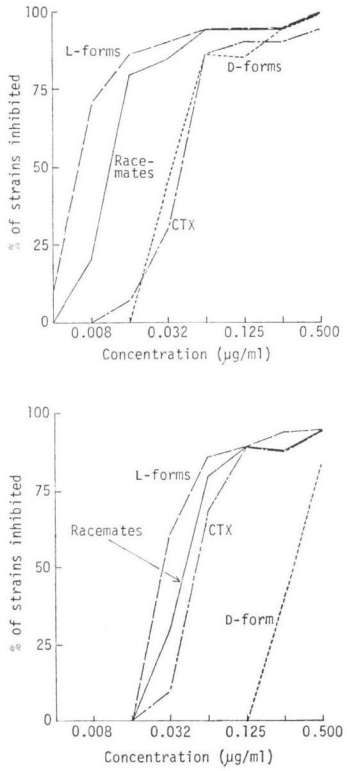


Compound No.	R	*	Relative stability against enzyme type			
			Ia	IIIa	IVc	TEM 2
Cephaloridine			100	100	100	
1	STz	D,L	2.7	43	48	
2	STz	L	0.5	4.1	4.8	
3	STz	D	2.1	73	55	
4	OAc	D,L	0.7	24	34	
5	OAc	L	0.2	1.0	2.8	
6	OAc	D	2.1	46	61	
Cefuroxime			0	0.7	25	
Cefotaxime					100	100
2	STz	L			117	1580
5	OAc	L			78	967
Cefuroxime					555	278

Fig. 1. Antibacterial activity against 20 clinical isolates of *Escherichia coli*.

Top: S-tetrazole derivatives and CTX.

Bottom: O-acetyl derivatives and CTX.



tetrazoles were in all cases more effective antibiotics than the corresponding acetoxy compounds.

All six products were more stable to three different β-lactamase preparations than cephaloridine (Table 1), and both L-isomers were markedly more stable than the other derivatives. To judge the stability more precisely, in a further experiment the K 1 enzyme was used at a higher concentration and the TEM 1 enzyme was replaced by TEM 2 from another strain of *E. coli*. Under these conditions both L-isomers and CTX were about equally stable against inactivation by the *Klebsiella* enzyme, whereas cefuroxime was clearly inferior, but less stable against the TEM 2 enzyme than CTX and cefuroxime.

Protein binding was generally low. It was 29 and 23% for compounds **1** and **4**, respectively at the concentrations used.

2. Experiments *In Vivo*

The therapeutic efficacy of aminothiazolylureido cephalosporins and CTX was compared in mice experimentally infected with *E. coli* SC 8294. The results are summarized in Table 2 and indicate once more that the L-isomers are superior to the racemate, the D-form, and also to CTX.

Mice infected systemically with a pathogenic β-lactamase-producing strain of *E. coli* SC 20,132, which is resistant against a number of penicillins and cephalosporins, responded to compounds **2** and **4** more effectively than to CTX.

On parenteral administration to mice the test compounds reached high blood levels (Fig. 2). Extrapolated serum concentrations at time 0 were approximately 100 μg/ml. Serum half-life was 17~19 minutes (CTX only 10 minutes).

Fig. 2. Serum level in mice after i.v. (top panel) or s.c. administration (25 mg/kg).

Experiments were performed with racemates. Six mice per group.

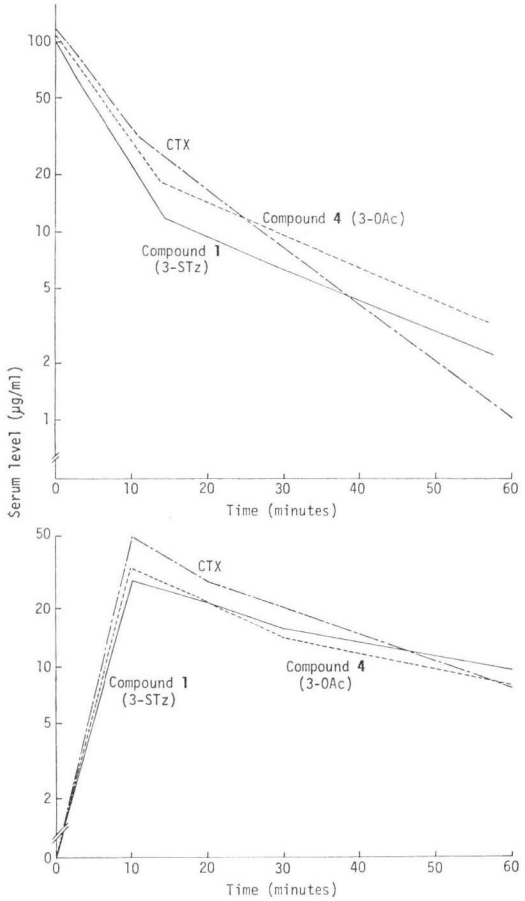
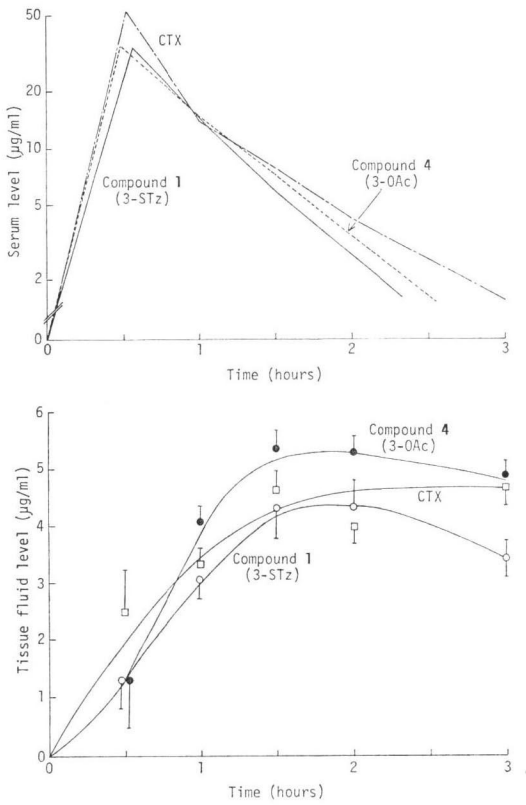


Fig. 3. Serum (top panel) and capsule fluid kinetics in rats (25 mg/kg i.m.).

The bars in the bottom panel indicate standard error of the mean. Six rats per group.



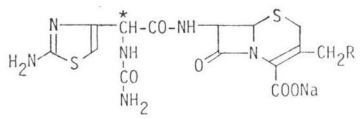
Compounds **1**, **4**, and CTX were also administered i.m. to rats bearing subcutaneous tissue chambers. Results of the serum analyses are shown in Fig. 3 (top). Antibiotic activity reached a maximum 30 minutes after dosing, with no detectable antibiotic at 3 hours. In contrast, tissue fluid levels peaked at 2 hours and showed longer duration (Fig. 3, bottom).

The predominant part of the administered amount is excreted unchanged in urine of mice and rats (Table 3). S-Tetrazolyl derivatives were generally recovered in urine at a higher amount than the acetoxy compounds. Also, the overall recovery was higher for a representative of the former group. Thin-layer chromatography and bioautography failed to detect any active metabolic product except the original compound.

Discussion

The experiments performed *in vitro* and *in vivo* confirmed that aminothiazolyl ureido cephalosporins are a group of potent antimicrobial agents, with broad-spectrum activity mainly against Gram-negative bacteria. In the majority of tests the L-isomers were clearly more potent than the D-isomers while the

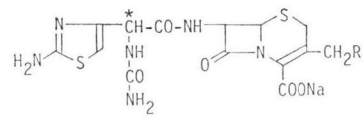
Table 3. Therapeutic effectiveness in mice.



Compound No.	R	*	ED_{50}, mg/kg s.c.	
			E. coli SC 8294	*E. coli* SC 20,132
1	STz	D, L	0.18	0.14
2	STz	L	0.064	NT[a]
3	STz	D	0.41	NT
4	OAc	D, L	0.20	0.80
5	OAc	L	0.08	NT
6	OAc	D	1.70	NT
Cefotaxime			0.21	3.40

[a]) not tested

Table 4. Urinary and biliary excretion.



Compound No.	R	*	Urinary excretion, % of s.c. dose		Biliary excretion, % of i.v. dose rat
			Mouse	Rat	
1	STz	D,L	60	70	13
2	STz	L	72	81	24
3	STz	D	75	73	17
4	OAc	D,L	50	57	2
5	OAc	L	NT[a]	52	NT
6	OAc	D	NT	65	NT
Cefotaxime			53	99	1

[a]) not tested

racemates were of intermediate activity.

On the other hand, the pharmacokinetic behaviour of the compounds were not significantly different from each other. On parenteral administration the compounds reached high serum levels. Measurements of the tissue fluid concentration indicate that the compounds penetrate well through the vessel wall in tissue fluid. This tissue distribution may explain the relatively short serum half-life.

Although all compounds were found at high concentration in the 6-hour urine, part of the S-tetrazolyl derivatives also occurred in bile. The amounts found in urine and bile account fairly well for the administered dose, so accumulation in the body does not seem probable. On the other hand, the total recovery of the acetoxy derivative **4** in rat urine and bile was about 60%. It can be assumed that these compounds are metabolized to a certain extent to inactive products which are not detectable by the analytical methods we used.

References

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