DIASTEREOMERIC 7-UREIDOACETYL CEPHALOSPORINS. III

CONTRIBUTION OF D- AND L-ISOMERS TO THE GROWTH INHIBITING ACTIVITIES OF 7α-H AND 7α-OCH_3 DERIVATIVES FOR GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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A series of 7β-ureidoacetyl, 7α-H and 7α-OCH_3 cephalosporin antibiotics have shown broad-spectrum antibacterial activity *in vitro*. In the 7α-H but not in the 7α-OCH_3 series, contrary to experience in the antibiotic field, the L-isomers were substantially more active than the D-isomers both *in vitro* and *in vivo* particularly, but not exclusively, against *Enterobacteriaceae* that produce potent chromosomal cephalosporinases. Enhanced resistance to and inhibition of β-lactamase (s) appeared to be responsible for this effect. Studies *in vitro* specifically with 7β-thienylureidoacetyl derivatives showed that D-isomers interacted with L-isomers in the 7α-OCH_3 series in a synergistic manner against "cephalosporinase-type" enzyme producers while isomers in the 7α-H series did not. Examples were presented in which this favorable event resulted in improved efficacy of the racemic mixture over the pure D- or L-isomer alone in appropriate experimental infections.

An earlier paper has described the synthesis of 7β-(ureidoacetyl), 7β-H cephalosporins carrying a methyl tetrazolylthiomethyl substituent in the 3-position[1]. In this series it was noted that the L-diastereomer of compounds in which the terminal nitrogen of the ureido group was unsubstituted were substantially more active against certain Gram-negative bacteria *in vitro* than the corresponding D-side chain isomers. The introduction of a 7α-OCH_3 group into the two most active isomeric pairs reversed this relationship[2].

It is the purpose of the present study to expand these original observations by comparing the extent of and mechanism(s) of increased antibacterial activity and interaction of the D- and L-isomers in this 7α-OCH_3 series with the aid of suitable *in vitro* and *in vivo* experiments.

Materials and Methods

Antibiotics

The new semisynthetic cephalosporins reported herein were synthesized in the chemical laboratories of the Squibb Institute in Princeton, N. J., U.S.A. and/or in the Squibb International Research Center, Regensburg, Germany. When required reverse phase high performance liquid chromatography as described by YOUNG[3] was used to separate and quantitate the diastereomeric purity of the compounds. Clavulanic acid and cephaloridine were gifts of Beecham Pharmaceuticals, Betchworth, England and Glaxo Research Limited, Greenford, Middlesex, England, respectively.

Animals

Female, white Swiss mice of the CFI strain (Carworth Farms, New City, N. Y., U. S. A.), average weight 18 ~ 20 g were used in all efficacy trials.

Bacterial Cultures

With but few exceptions all test organisms were obtained over the last five years from a number of metropolitan hospitals in the U. S. and are considered representative with respect to antibiotic resistance

of cultures isolated through 1976[4]. A few special organisms were received as follows: *Escherichia coli* W-3110 ^{R}TEM[5], *E. coli* RGN 238[5], and *Klebsiella aerogenes* (*pneumoniae*) 1082E[5] from M. H. RICHMOND (Department of Bacteriology, University of Bristol, England), *Neisseria gonorrhoeae* CDC 76–061782 (SC 10,735) from C. THORNSBERRY (Center for Disease Control, Atlanta, Ga., U. S. A.), *Bacteroides fragilis* subsp. *fragilis* SC 9844 from D. LAMBE (Emory University, Atlanta, Ga., U.S.A.), and all strains of ampicillin-resistant *Hemophilus influenzae*, type b from L. HARDING (Children's Hospital, Boston, Mass., U.S.A.). All isolates were maintained under liquid nitrogen or under mechanical refrigeration (Revco) at -90°C as part of the Squibb stock culture collection and regrown for use on appropriate media several days prior to study.

Several groups of organisms were screened for their ability to elaborate β-lactamase (s) as demonstrated by their ability to produce a color change from yellow to red when incubated in the presence of the cephalosporin substrate 87/312 described by O'CALLAGHAN and coworkers[6].

Antibacterial Activity

Minimum inhibitory concentrations (MIC) were determined by conventional twofold dilution methods with broth or agar media. Broth dilution susceptibility tests were carried out in antibiotic assay broth (BBL) as described earlier[7] containing an initial concentration of *ca.* 10^3 colony-forming units (CFU)/ml (low inoculum) and 10^6 CFU/ml (high inoculum). Incubation was at 37°C for 18 hours.

Agar dilution susceptibility tests were conducted in MUELLER-HINTON (MH) agar (BBL) that was supplemented with 2% rabbit blood for Group A streptococci and *Streptococcus pneumoniae*, with 5% "chocolatized" sheep blood that was supplemented with 1% dextrose, and 1% supplement C (DIFCO) for *Hemophilus* and *Neisseria*, or with 5% sheep blood for *Bacteroides*. All other organisms were cultured on unsupplemented MH medium. Stock solutions of the test substances were made in 0.05 M phosphate buffer (pH 7.0) to an initial concentration of 1 mg/ml. Further dilutions of each antibiotic were prepared in MH broth (pH 7.4); 1 ml amounts added to 90-mm Petri dishes, mixed with 9.0 ml of MH agar and allowed to harden. Test cultures grown in appropriate broth media for *ca.* 18~24 hours at 37°C were deposited on the solidified surface of the agar using an automated multiple-pronged replicator designed to deposit 0.03 ml of culture containing either 10^3, 10^4, 10^5, 10^6, or 10^7 CFU on the agar surface. All plates were incubated aerobically at 37°C except those containing blood which were incubated under reduced oxygen tension in a candle jar (*Hemophilus*; *Neisseria*) or Gas Pak® (BBL) (*Bacteroides*). The MIC was defined as the lowest concentration of antibiotic that suppressed visible growth.

Chemotherapeutic Studies

Experimental infections were produced by the intraperitoneal injection of suitably diluted cultures of *Enterobacter cloacae* SC 9965, *Proteus rettgeri* SC 8217, and *Serratia marcescens* SC 9782, each constituting a challenge dose of *ca.* 100 LD_{50}. Under these conditions, all untreated control animals died within 72 hours. Medication was given s. c. in divided doses at 1 and 5 hours after infection. At least three different doses of the test antibiotics were used; each dose group consisted of 10 mice. All animals were observed for a period of 6 days and the median effective dose (ED_{50}) from duplicate experiments was determined by the method of REED and MUENCH[8].

Interaction of Combinations of Diastereomers with Gram-negative Organisms *In Vitro*.

Combinations of D- and L-diastereomers were tested using MH medium and broth dilution susceptibility tests according to the "checkerboard pattern"[9] in order to determine the MIC of the isomers alone and in combination. A culture inoculum of each isolate (*E. cloacae* SC 9965; *P. rettgeri* SC 8217; *S.marcescens* SC 9782) representing *ca.* 1×10^4 CFU/ml was added to each tube. Incubation was at 37°C for 18 hours.

Preparation of β-Lactamases

Crude sonicates of *E. cloacae* SC 9965 (Id) were prepared by first growing the cultures in shake flasks containing Brain Heart Infusion (Difco) broth for 18 hours at 25°C and a shaker speed of 300 rpm. *E. cloacae* was induced with 500 μg/ml benzylpenicillin introduced *ca.* 2 hours after addition of the inoculum. The cells were harvested in a Sorvall RC2-B centrifuge (RCF=24,350; 30 minutes, 5°C), the culture supernatant was discarded and the cell cake resuspended in 0.05 M phosphate buffer (pH 7.0)

to a slurry with flow characteristics. Cells were disrupted batchwise with constant ice-bath cooling using a Biosonik III ultrasonic disintegrator (80% max. voltage, 3 minutes). The sonicates were cleared by ultracentrifugation in a Beckman centrifuge, Model L2-651B (RCF = 105,536, 2 hours, at 5°C).

Enzyme from *E. coli* W-3110 (SC 10,404)[5] and *S. aureus* SC 2400 was recovered by means of the preparative procedures described by RICHMOND[10].

Activity of β-lactamase was determined spectrophotometrically using the cephalosporin substrate 87/312 as described by O'CALLAGHAN[6]. Enzyme class and type was verified with the published literature[5] by determining the substrate profile for five antibiotics using the macro-iodometric method of PERRET[11].

Enzymatic Hydrolysis of Cephalosporins

Hydrolysis rates of 7α-ureidoacetyl cephalosporin antibiotics for the *Enterobacter* enzyme were determined with the spectrophotometric method of O'CALLAGHAN[6].

Inhibition of Cephalosporin 87/312 Hydrolysis

The chromogenic cephalosporin 87/312[6] is very sensitive to hydrolysis by all known β-lactamases. In order to determine the ability of certain 7α-ureidoacetyl cephalosporins to inhibit the hydrolysis of this substrate, all compounds to be tested were solubilized in 0.5 M phosphate buffer (pH 7.0), diluted appropriately and mixed with 0.2 ml of 87/312 (1,000 μg/ml). Immediately after equilibration at 30°C, or after 10 minutes pre-incubation, an amount of enzyme was added which in a control reaction had been shown to hydrolyze the substrate in 5 minutes. The O. D. was monitored at 482 nm in a Spectromic 20 (Bausch & Lomb) spectrophotometer. The I_{50} was defined as the concentration of inhibitor (μg/ml) required to cause a 50% reduction in β-lactamase activity.

Determination of Crypticity

Enterobacter cloacae SC 9965 was grown on BHI agar for 18 hours at 37°C. The culture was washed from the slants with antibiotic assay broth (BBL), sub-divided and one-half of the culture fluid subjected to ultrasonic (Heat-Systems Sonifier Model 350-W, 1/2 in. dia. horn, setting #7, 80% output) pulsation for 6 minutes under constant ice-bath cooling. Crypticity was determined for each substrate or substrate mixture by estimating the amount of substrate destroyed[7] (as a measure of β-lactamase activity available) by washed intact cells/sonicated broken cells.

Kinetic Measurements

The resistance of 7α-ureidoacetyl cephalosporins to degradation by the β-lactamase from *E. cloacae* SC 9965 was determined by monitoring the optical density at the wavelength associated with the maximum absorption of the compounds and varied within the range of 230~265 nm. Protein was determined by the method of LOWRY *et al.*[12] The maximal rate of reaction (V_{max}) and the dissociation constant (K_m) were derived from a LINEWEAVER-BURK plot[13].

Results

Antibacterial Activity *In Vitro*

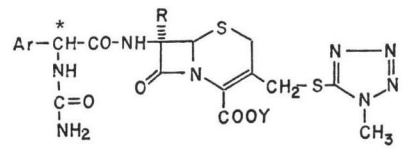
An in-depth evaluation of all compounds (Table 1) was conducted by comparing the susceptibility of *ca.* 20 individual recent clinical isolates from human sources, organisms that represent the most important genera of bacteria. Meaningful differences in geometric mean MIC favoring the L-isomer in the 7α-H and the D-isomer in the 7α-OCH_3 series were found only in selected genera belonging to the family Enterobacteriaceae (Table 2). This relationship was demonstrated by all (100%) of the *Proteus* and *Citrobacter*, but only 75~85% of the *Serratia* and *Enterobacter* strains studied. In the case of the latter two genera the difference is accounted for by frank resistance at 100 μg/ml for each diastereomeric pair of 15% of the *Serratia* and 25% of the *Enterobacter* strains tested.

Close scrutiny of the test data obtained from other Enterobacteriaceae (*Escherichia, Klebsiella, Salmonella, Shigella, Providencia, E. aerogenes, S. liquefaciens, Citrobacter* sp.) and an unrelated group

of Gram-negative rods (*Bacteroides*) showed an occasional strain from each group to yield data similar to that described above. All of these isolates were potent producers of β-lactamase (s); representing both chromosomally mediated and R-plasmid mediated enzymes. These observations were expanded by determining the MIC's of representative strains of bacteria from these groups and selected other organisms at two inoculum levels (Table 3). While the MIC's in certain instances were very high indicating low activity for the particular isomer against the test strain, in each instance the L-isomer in the 7α-H series was more potent than the D-isomer, most notable at the low inoculum level. The reverse was true in the 7α-OCH_3 series. Where comparisons were possible, the L-isomer, based on the high/low inoculum ratio, appeared to be more stable to the β-lactamase(s) produced by these organisms especially when the compound carried a 7α-OCH_3 group (compound **5b** and **6b**). Apparently, the class of enzyme produced by the organism had no bearing on the relationship demonstrated.

Susceptibility of similar numbers of isolates of staphylococci (benzylpenicillin-sensitive and -resistant), streptococci (Groups A and D; *S. pneumoniae*), *Neisseria gonorrhoeae*, and *Hemo-*

Table 1. 7-Ureidoacetyl, 7α-H and 7α-OCH_3 cephalosporanic acids



Compounds	Ar	R	Y	Config. at C*
1a	C_6H_5	H	Na	D
1b	C_6H_5	H	Na	L
2a	3-Thienyl	H	K	D
2b	3-Thienyl	H	K	L
3a	2-Thienyl	H	Na	D
3b	2-Thienyl	H	Na	L
4a	2-Furyl	H	Na	D
4b	2-Furyl	H	Na	L
5a	2-Thienyl	OCH_3	Na	D
5b	2-Thienyl	OCH_3	Na	L
5c	2-Thienyl	OCH_3	Na	D,L
6a	2-Furyl	OCH_3	Na	D
6b	2-Furyl	OCH_3	Na	L
6c	2-Furyl	OCH_3	Na	D,L

Table 2. Comparison of the susceptibility of selected clinical bacterial isolates to 7β-ureidoacetyl, 7α-H and 7α-OCH_3 cephalosporins

Compounds	Geometric mean MIC (μg/ml)[a]			
	Enterobacter cloacae	*Proteus* sp. (indole-positive)	*Serratia marcescens*[b]	*Citrobacter freundii*
1a	>100	12.5	>100	3.9
1b	11.2	4.1	10.5	1.2
2a	100	9.8	93	2.8
2b	1.6	3.2	8.6	1.0
3a	82	7.8	>100	1.6
3b	12.5	3.4	22.5	0.5
4a	64	8.7	>100	1.4
4b	0.7	1.3	2.9	0.3
5a	6.7	2.4	1.2	0.3
5b	11.5	4.6	3.1	0.8
6a	19.1	5.1	1.4	0.9
6b	29.5	17.8	11.6	3.2

a) A total of 21 clinical isolates of each species was tested in an agar-dilution procedure; an inoculum of *ca.* 10^5 CFU was placed on the agar surface with a multipoint inoculator. All strains were known to produce β-lactamase (s) based on tests conducted with the chromogenic cephalosporin 87/312.

b) Includes both pigmented and non-pigmented strains.

Table 3. Antibacterial activity of diastereomeric 7-ureidoacetyl

Compounds	MIC (μg/ml)											
	I[b]											
	E. cloa.		*Pr. mor.*		*C. freu.*		*S. enter.*		*Ser. lique.*		*Pr. inc.*	
	10^{6c}	10^3	10^6	10^3	10^6	10^3	10^6	10^3	10^6	10^3	10^6	10^3
3a	>100	>100	100	3.1	>100	6.3	100	25	>100	>100	>100	25
3b	100	1.6	25	1.6	25	0.2	25	1.6	>100	50	50	6.3
4a	>100	100	100	25	>100	>100	50	12.5	>100	>100	100	12.5
4b	50	6.3	50	1.6	50	6.3	3.1	0.2	50	6.3	50	3.1
5a	50	3.1	12.5	0.8	12.5	0.8	1.6	0.8	6.3	1.6	50	6.3
5b	50	12.5	12.5	6.3	50	6.3	12.5	3.1	25	6.3	>100	25
6a	50	12.5	12.5	1.6	25	3.2	3.2	0.8	12.5	3.2	12.5	3.2
6b	>100	50	12.5	6.3	100	12.5	25	3.1	100	12.5	25	12.5

[a] *E. cloa.*: *Enterobacter cloacae* SC 8415; *Pr. mor.*: *Proteus morganii* SC 9774; *C. freu.*: *Citrobacter freundii* SC 10,204; *S. enter.*: *Salmonella enteritidis* SC 9686; *Ser. lique.*: *Serratia liquefaciens* SC 9068; *Pr. inc.*: *Proteus inconstans* SC 10,187 (formerly *Providencia*); *Sh. son.*: *Shigella sonnei* SC 10,944; *B. frag.*: *Bacteroides fragilis* SC 9844; *E. coli* (T): *Escherichia coli* SC 10,404 (W3110) R6K-R$^+$TEM; *S. typhi*: *Salmonella typhi* SC 9201; *K. aer.*: *Klebsiella aerogenes* SC 10,436 (Richmond 1082E); *E. coli* (R)

Table 4. Antibacterial activity of diastereomeric 7-ureidoacetyl cephalosporins to ampicillin-resistant strains of *S. aureus*, *H. influenzae* and *N. gonorrhoeae*

Compounds	MIC (μg/ml)[a]					
	Staph. a.[b]		*H. infl.*[b]		*Neiss. gon.*[b]	
	10^6 [b]	10^3	10^6	10^3	10^6	10^3
3a	6.3	1.6	1.6	1.6	3.13	0.2
3b	12.5	12.5	6.3	6.3	4.7	0.8
4a	6.3	6.3	1.6	1.6	1.6	0.2
4b	12.5	12.5	1.6	1.6	1.6	0.4
5a	1.6	0.8	0.2	0.2	0.1	0.01
5b	6.3	6.3	0.8	0.4	0.6	0.2
6a	3.13	1.6	0.3	0.3	0.3	0.1
6b	6.3	6.3	0.8	0.6	0.6	0.2

[a] Agar-dilution test; an inoculum of *ca.* 10^6 or 10^3 was placed on the agar surface with a multi-point inoculator.

[b] *Staph. a.*: *Staphylococcus aureus* SC 2400 produces penicillinase; *H. influ.*: *Hemophilus influenzae* SC 10,556 produces a Class III, R-plasmid mediated β-lactamase; *Neiss. gon.*: *Neisseria gonorrhoeae* SC 10,735 produces Class III. R-plasmid mediated β-lactamase. All of these organisms are resistant to ampicillin at an inoculum level of 10^6 CFU/ml (MIC$\geq$200 μg/ml).

philus influenzae (ampicillin-sensitive and -resistant), data that are not shown, favored the D-isomer in both the 7α-H and 7α-OCH_3 series. A representative sample of such data for ampicillin-resistant *S. aureus*, *N. gonorrhoeae*, and *H. influenzae* is shown in Table 4.

Antibacterial Activity *In Vivo*

Experimental infections established with the aid of three Gram-negative organisms (*E. cloacae* SC 9965; *P. rettgeri* SC 8217; *S. marcescens* SC 9782) have responded to therapy with subcutaneous doses of most of the cephems tested. In almost all cases the MIC was a good predictor of chemotherapeutic

cephalosporins to selected Gram-negative bacteria

MIC (μg/ml)											
I		III						IV		V	
Sh. son.		*B. frag.*[e]		*E. coli* (T)		*S. typhi*		*K. aer.*		*E. coli* (R)	
10^6	10^3	10^6	10^3	10^6	10^3	10^6	10^3	10^6	10^3	10^6	10^3
50	25	50	25	>100	25	>100	100	>100	>100	12.5	3.13
12.5	6.3	25	25	>100	6.3	>100	50	>100	>100	6.3	3.13
25	6.3	25	25	50	25	>100	25	>100	>100	6.3	3.13
12.5	0.8	6.3	6.3	12.5	0.8	50	3.13	>100	50	0.8	0.8
6.3	3.13	0.4	0.4	3.13	1.6	0.4	0.4	0.4	0.2	3.13	1.6
12.5	3.13	6.3	3.13	12.5	12.5	3.13	3.13	6.3	1.6	12.5	6.3
6.3	3.13	NT[d]	NT	NT	NT	1.6	0.8	1.6	0.4	1.6	1.6
12.5	3.13	NT	NT	NT	NT	6.3	3.13	12.5	1.6	3.2	3.2

SC 10,854 (Richmond RGN238).
[b] Enzyme class
[c] CFU/ml.
[d] Not tested
[e] Agar dilution test, inoculum indicates CFU applied to plate.

Table 5. Comparative chemotherapeutic efficacy of D- and L-diastereomers of selected 7β-ureidoacetyl, 7α-H and 7α-OCH_3 cephalosporin antibiotics

Compounds	Animal pathogen[a]					
	Enterobacter cloacae SC 9965		*Proteus rettgeri* SC 8317		*Serratia marcescens* SC 9782	
	MIC[b] (μg/ml)	ED_{50}[c] (mg/kg)	MIC (μg/ml)	ED_{50} (mg/kg)	MIC (μg/ml)	ED_{50} (mg/kg)
1a	>100	NT[e]	12.5	>400	>100	NT
1b	3.1	34	3.1	200	12.5	179
2a	100	245	6.3	193	>100	NT
2b	1.6	18	1.6	84	50	202
3a	6.3	28	12.5	175	>100	>400
3b	1.6	4	1.6	69	25	220
3c[d]	25	>100	18.7	100	>100	184
4a	100	158	1.6	201	>100	NT
4b	0.8	11	0.8	63	25	148
5a	3.1	76	6.3	128	0.8	7.4
5b	12.5	86	12.5	>400	1.6	44
5c[d]	3.1	6.5	6.3	92	0.8	4.7

[a] Infecting dose of each pathogen was *ca.* 100 LD_{50}; all compounds were given s. c. in divided doses at 1 and 5 hours after infection.
[b] Method same as described in footnote to Table 2.
[c] Median effective dose.
[d] Ratio of D: L in these mixtures was *ca* 60: 40.
[e] Not tested.

efficacy, hence the superior activity shown by the L-isomers in the 7α-H series *in vitro* has been translated to meaningful activity *in vivo*. In the 7α-OCH_3 series, using the 7β-thienylureidoacetyl derivatives as an example, the D-isomer (compound **5a**) was naturally more efficacious than the L-isomer (compound **5b**)

in these animal experiments. Interestingly, administration of the 7α-OCH_3 DL-mixture (compound **5c**) demonstrated substantially improved efficacy over that of its pure D-isomer suggesting a beneficial interaction between the D- and L-isomers *in vivo*. No such improvement in efficacy was noted when the 7α-H mixture (compound **3c**) was given; in fact reduction in efficacy, lower than that of either isomer resulted in one case.

Interaction of Isomers *In Vitro*

Several experiments were conducted in order to define, characterize, and quantify the interaction of the D- and L-isomers using examples from both the 7α-H and 7α-OCH_3 series of 7β-ureidoacetyl cephalosporins.

Fig. 1. Isobolograms showing synergism between D (Compound **5a**) and L (Compound **5b**) diastereomers of 7β-thienylureidoacetyl, 7α-OCH_3 cephalosporin antibiotics *vs. E. cloacae, S. marcescens* and *P. rettgeri*

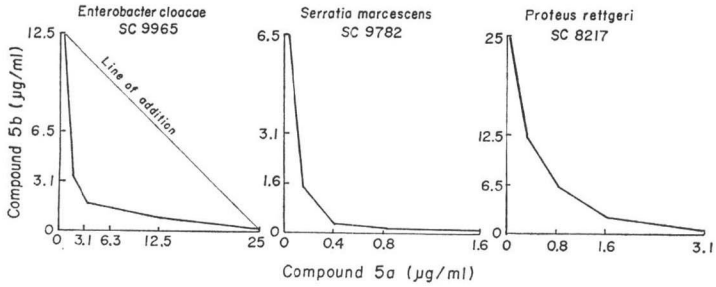
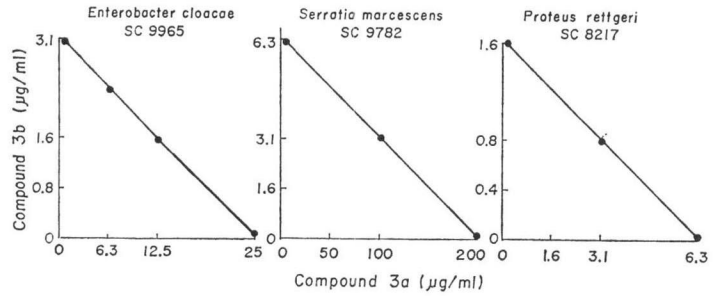


Fig. 2. Isobolograms showing indifference between D (Compound **3a**) and L (Compound **3b**) diastereomers of 7β-thienylureidoacetyl, 7α-H cephalosporin antibiotics *vs. E. cloacae, S. marcescens* and *P. rettgeri*



In the first test, the isomers were combined in test tubes in a "checkerboard" pattern as suggested by SABATH and co-workers[9] using the same strains of bacteria that were employed in the chemotherapeutic trials and with organisms listed in Table 3. The MIC results were plotted on an arithmetic scale according to the method of LOEWE[14]. When the D and L 7α-OCH_3 (compounds **5a** and **5b**) were combined in such a manner, marked synergism, as defined by WEINSTEIN *et al*,[15] occurred with each strain belonging to enzyme Class I, but not with organisms that elaborate "penicillinase-like" β-lactamases.

Table 6. Relative rates of hydrolysis and median inhibitory concentrations (I_{50}) of diastereomeric 7α-

Enzyme class[a]	Host cell	Specific activity (katal/ml)	Relative activity[b]							
			3a	**3b**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**
Id	*E. cloacae* SC 9965	6.3×10^{-3}	60	4	17	4	>1	>1	9	12
IIIa	*E. coli* (W3110) SC 10,404	1.75×10^{-3}	84	138	71	75	>1	>1	>1	>1
IVc	*K. aerogenes* (1082E) SC 10,436	3.38×10^{-2}	63	151	74	140	>1	>1	>1	>1
Pen	*S. aureus* SC 2400	7.8×10^{-4} (katal/g)	1.8	2.1	3.4	3.3	>1	>1	>1	>1

a) Classification scheme after RICHMOND and SYKES[5]
b) Relative to a rate of hydrolysis of 100% for benzylpenicillin.
c) I_{50} values are the concentrations of the β-lactam compound that reduced the rate of hydrolysis of cephalo-

Three typical examples are illustrated in Fig. 1. Indifference or no effect (Fig. 2) was noted when the corresponding D- and L-(7α-H) isomers were combined in an identical manner. These observations in part support the results of drug efficacy studies in mice.

Susceptibility to β-Lactamases

The relative susceptibility of selected 7β-thienyl- and 7-furylureidoacetyl cephalosporin derivatives to hydrolysis by various β-lactamases in cell-free sonicates was determined and related to that of benzylpenicillin. The results showed that 7α-OCH_3 derivatives on the whole (compounds **5a** and **b**, **6a** and **b**) were poor substrates for a representative member of enzyme class I, III, IV, and the staphylococcal β-lactamase (Table 6). Corresponding 7α-H derivatives, with the exception of the staphylococcal enzyme generally, and *Enterobacter* enzyme specifically, *vis-a-vis* the 7β-thienyl and 7-furyl derivatives were less resistant to the hydrolytic effects of the other Gram-negative β-lactamases.

Enzyme Inhibition of 7α-Ureidoacetyl Cephalosporins

The ability of certain 7α-ureidoacetyl cephalosporins to inhibit the hydrolysis of a very sensitive substrate (cephalosporin 87/312) by various β-lactamases was compared with that of clavulanic acid[16]. The new compounds selected for testing all were poor inhibitors of the *E. coli* W3110 and *K. aerogenes* 1082E enzymes (Table 6). The most potent inhibitors of *E. cloacae* SC 9965 enzyme were the L-diastereomers of the 7β-thienyl- and 7-furylureidoacetyl derivatives in both the 7α-H and 7α-OCH_3 series. The reverse was true for the staphylococcal β-lactamase in which the D-diastereomers were the most active. Clavulanic acid was a potent inhibitor of the *Escherichia, Klebsiella* and *Staphylococcus* enzyme, but a poor inhibitor of the *Enterobacter* enzyme.

Kinetics of Hydrolysis of 7β-Thienylureidoacetyl Cephalosporins by *Enterobacter cloacae* β-Lactamase

The resistance of 7β-thienylureidoacetyl cephalosporins to β-lactamase degradation was quantitated by determining the kinetics of the enzyme reaction using the subject cephems or mixtures as substrates. The L-isomer in the 7α-H series (compound **3b**) and both isomers in the 7α-OCH_3 series (compounds **5a** and **b**), in agreement with data presented in Table 6, were poor substrates for the *Enterobacter* β-lactamase (Table 7). On the other hand, the D-isomer in the 7α-H series (compound **3a**) was 40 times more labile to the degradative effects of the enzyme than the corresponding L-isomer. Both isomeric mixtures exhibited low rates of hydrolysis. No major differences in affinity of the enzyme for any of the

thienyl and furylureidoacetyl, 7α-H and 7α-OCH_3 cephalosporin derivatives

I_{50} (μg/ml)[c]						
3a	**3b**	**4a**	**4b**	**5a**	**5b**	Clavulanic acid
25	0.63	7.0	1.25	0.13	0.08	>125 (88)[d]
>125	>125	>125	>125	>125	>125	0.125 (0.03)
>125	>125	>125	>125	>125	>125	0.50 (0.125)
0.125 (0.08)	50 (20)	3.75 (3.75)	125 (50)	3.75 (0.5)	>125 (12.5)	60 (0.025)

sporin 87/312 to 50% of the value obtained without the inhibitor.

[d] Value obtained after preincubation for 10 min. at 37°C before assay in parenthesis.

Table 7. Kinetics of hydrolysis of diastereomeric 7β-thienylureidoacetyl, 7α-H and 7α-OCH_3 cephalosporin antibiotics by β-lactamase from *E. cloacae* SC 9965

Substrate compound or mixture	$Km(\mu M)\times10^{-2}$	$V_{max}(\times10^{-3})$[b]
3a	0.79	71
3b	0.52	1.76
3a+**3b**[a]	0.19	0.85
5a	0.24	0.70
5b	0.47	0.96
5a+**5b**[a]	0.43	0.07
Cephaloridine	1.43	426

[a] Equal mixtures of the two isomers

[b] Expressed as micromoles per minute per milligram of protein.

isomers or mixtures were noted.

Crypticity

The crypticity values for all of the compounds or mixtures listed in Table 7 against the *Enterobacter* β-lactamase were equal to unity, *i. e.* 1.

Discussion

Historically, β-lactam antibiotics synthesized as side-chain diastereomeric pairs have demonstrated vastly superior antimicrobial activity for the diastereomer whose absolute configuration is related to D-phenyl glycine[1,17]. The recent identification of a series of ureido cephems in which the L-isomers were substantially more active than the D-isomers *in vitro*[1] has been further explored herein. This relationship was found to be limited to all tested isolates of *Citrobacter freundii* and indole-positive *Proteus*, most strains of *Enterobacter cloacae* and *Serratia marcescens*, and occasional isolates of *Bacteroides* sp. and other Enterobacteriaceae. The common denominator among all of these pathogens seemed to be the production of relatively high levels of cell-bound β-lactamase(s), quantitatively most prominent among the chromosomal cephalosporinase producers, and the presence of a permeability barrier. Bacteria such as β-lactamase producing staphylococci, *H. influenzae*[18], and *N. gonorrhoeae*, in which one or both of these conditions are not met, were more susceptible to the D- than to the L-isomers. These observations were further supported by studies which demonstrated that L-isomers of the most active 7α-H compounds were poorer substrates and better inhibitors of a chromosomally-mediated *Enterobacter* β-lactamase, and thus showed a lower rate of hydrolysis, than did the D-isomers. Confirmatory evidence was provided in the case of the thienyl derivatives by kinetic experiments which showed the affinity of the enzyme for both isomers to be similar, but the rate of hydrolysis of the D-isomer was substantially greater than that of the L-isomer. Since the crypticity was equal to 1 in both cases, it was not surprising that the growth inhibiting properties of these isomers *in vitro* would be reflected in appropriate activity *in vivo*, *i. e.* the L-isomer was in each instance more efficacious than the D-isomer. In contrast to the findings made with the isomers in the 7α-H series, the D-isomers in the 7α-OCH_3 series were always more active than the L-isomers against both Gram-positive and Gram-negative pathogens. The most active compounds were extremely poor substrates for all of the enzymes tested and in the case of the 7β-thienyl derivatives were better inhibitors than their 7α-H counterparts of the *Enterobacter* enzyme, but poorer inhibitors of the staphylococcal enzyme. These observations were also reflected in the kinetic studies conducted with the *Enterobacter* enzyme. As previously noted for the 7α-H isomers, the MIC was also a good predictor of chemotherapeutic efficacy in experimental infections for the 7α-OCH_3 compounds.

A fortuitous observation which deserves special comment was made when 7β-thienylureidoacetyl, 7α-H and 7α-OCH_3 derivatives were compared with their racemates in two separate *in vivo* experiments with three chromosomal β-lactamase producers (*E. cloacae, P. rettgeri*, and *S. marcescens*). In all of these life-threatening infections in mice the 7α-OCH_3 DL-mixture in contrast to its 7α-H counterpart was more effective than the corresponding D- or L-isomers. This finding was later attributed to a synergistic interaction between the D- and L-isomers in appropriate *in vitro* experiments. Based on kinetic experiments with the *Enterobacter* enzyme it appears that the relative rate of hydrolysis of the DL-mixture is in part responsible for the observed effect.

The most active compound in these series, the 7β-thienylureidoacetyl-7α-OCH_3 derivative (D-isomer), SQ 14,359, is presently being evaluated extensively in comparison with other known 7α-OCH_3 cephalosporins, particularly, cefoxitin[19~21] and CS-1170[22].

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