Chem. Pharm. Bull. 30(9)3333—3339(1982)

Effect of 3-Substitution in Oxyiminocephalosporins on the Stability to and the Inhibition of Various β-Lactamases

Mikio Arisawa, Junko Ohshima, and Hiromi B. Maruyama*

Department of Microbiology and Chemotherapy, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247, Japan

(Received March 6, 1982)

Nine 2-aminothiazolyl methoxyiminoacetamidocephems (ATOICs) as well as several 4-furyl methoxyiminoacetamidocephems (FOICs) were compared for stability to and inhibition of various β-lactamases. Although ATOICs are generally less stable to cefuroxymases (CXases) from *Proteus vulgaris* or *Bacteroides fragilis*, the relative susceptibility among them was greatly affected by the substitution at the 3-position: the compound unsubstituted at C-3 was most stable, while thiomethyleno-2-methyl-6-hydroxytriazine-5-one substitution gave the most labile compound. A similar tendency was also seen with FOICs, which, however, were in general more susceptible to those CXases than were ATOICs.

The substitution at C-3 had lesser effects on the stability to some cephalosporinases (CSases), and also had little effect on the inhibitory activity of ATOICs and FOICs on various CSases, though the compound unsubstituted at C-3 (ceftizoxime) exhibited the least inhibition among ATOICs tested. The K_i values of typical ATOICs except ceftizoxime were 10^{-8}—10^{-9} M for enzymes from *Citrobacter freundii*, and *Enterobacter* sp. and 10^{-6}—10^{-7} M for those from *Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*.

Keywords——oxyiminocephalosporins; β-lactamase; ceftriaxone; *syn-anti* configuration; C-3 substitution

Introduction

It is well known that the introduction of an oxyimino acetamido group into the 7-position of cephalosporins results in a high resistance to various β-lactamases. In particular, aminothiazolyl methoxyiminoacetamido cephalosporins (ATOICs), which have extraordinarily potent and broad-spectrum antibacterial activity, are emerging as 3rd generation cephalosporins which differ from each other only in the substituent at the 3-position. It is therefore interesting to see how substitution at the 3-position affects the β-lactamase stability as well as the inhibitory action. We have carried out a comparative study among 9 ATOICs including ceftriaxone (Ro 13-9904), cefotaxime, ceftizoxime and cefmenoxime as well as several 4-furyl methoxyiminoacetamido cephalosporins (FOICs), with β-lactamases from 21 *Enterobacteriaceae* strains, 2 *Pseudomonas aeruginosa* strains and a strain of *Bacteroides fragilis*. This paper deals with the effect of 3-substitution.

Experimental

Bacterial Strains——Microorganisms used in this study were the same strains as described in reference 1, and were stored at −70°C in 12.5% glycerol until use.

Chemicals——Cephaloridine was the product of Shionogi Pharmaceuticals, and all other cephalosporins (Chart 1) were provided by F. Hoffmann-La Roche Co. Ltd., Basle, Switzerland, for this study.

Preparation of Crude Enzymes——Overnight cultures (12 to 14 h in Trypto-soy broth (EIKEN)) were diluted 40-fold with the same broth and the cultivation was continued at 37°C. After 2 h ($OD_{650} \fallingdotseq 0.5$—0.8) benzylpenicillin (200 μg/ml) was added to the culture to induce β-lactamase and incubation was continued for a further 2 h. Cells were then harvested, washed with 0.85% NaCl, suspended in 4—6 ml of 0.1 M phosphate buffer (pH 7.0) and disrupted by sonication. After removal of cell debris by centrifugation (Spinco L-2, 100000×*g* for 30 min at 4°C), the extract was treated with streptomycin (Meiji Seika) at a final concen-

tration of 2% to remove nucleic acid and then dialyzed against 0.01 M phosphate buffer (pH 7.0). The supernatant obtained by centrifugation of the dialyzed extract was used as the crude enzyme, which contained 5—20 mg protein per ml (Lowry method). Under these conditions most of the enzymes were stable for several months, except for those from *Bacteroides fragilis*, which lost their activity with a half-life of about 4 months at −20°C. Crude enzymes thus obtained were stored at −70°C or −20°C until use for the assay of β-lactamase.

β-Lactamase Assay——Ultraviolet (UV) spectrophotometric assay according to Ross and O'Callaghan[6] was adopted for the determination of β-lactamase activity. An aliquot of the enzyme solution (usually 10 μl) was added to a test tube containing 2 ml of the substrate (usually 100 μM) dissolved in 0.1 M phosphate buffer in a cuvette (light path; 2, 5 or 10 mm). The decrease of optical density at 37°C was recorded on a spectrophotometer (Hitachi 124 with a temperature controller (Sharp Thermo Electric model TE-12K)). The wavelength of the maximum absorption associated with the β-lactam ring and the change in optical density at the wavelength for each cephalosporin used were determined by measuring the difference in spectrum between intact and completely hydrolyzed cephalosporin.

The β-lactamase inhibiting activity of the cephalosporins was measured by the same method as mentioned above after preincubating the substrate with the inhibitor to be tested for 5 min at 37°C. The optical density of the enzyme solution itself was less than 0.1 under the assay conditions.

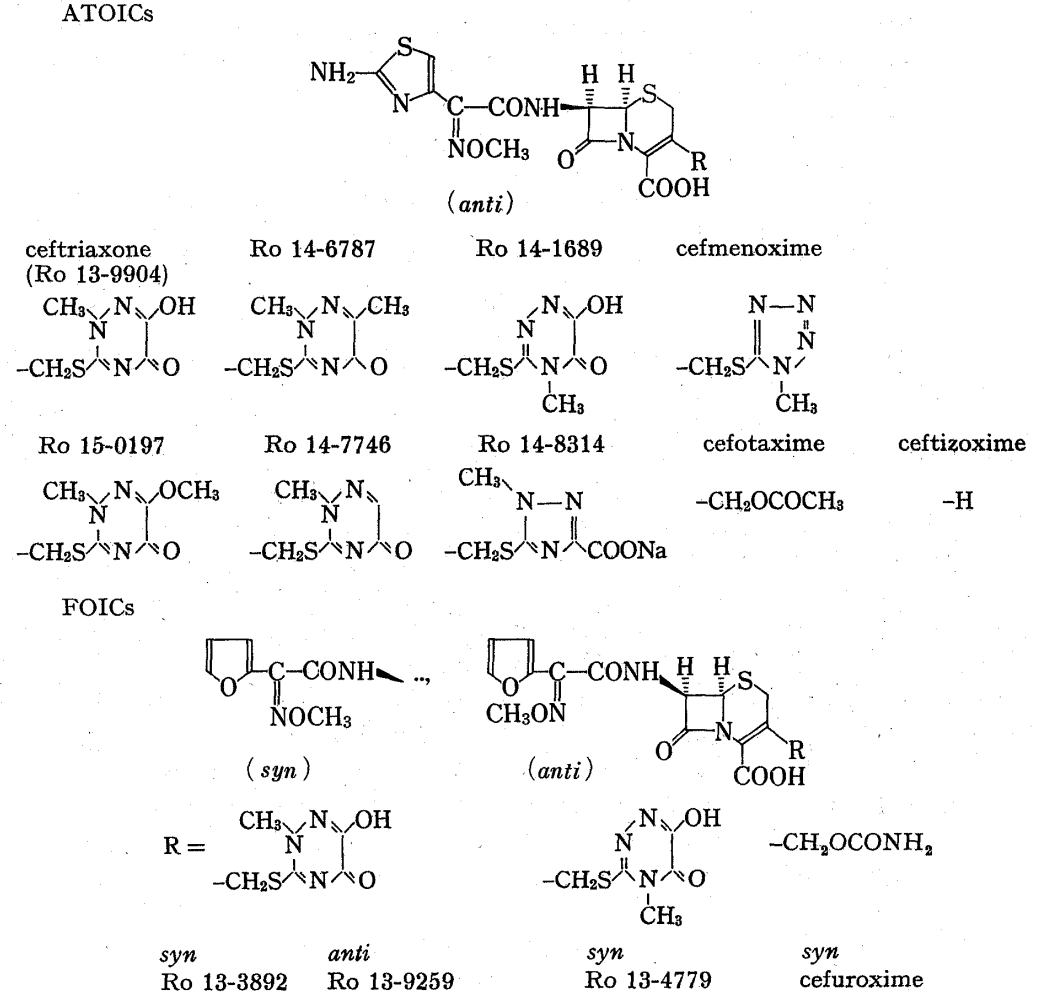

Chart 1. Structures of ATOICs and FOICs Studied

Results

Effect on 3-Substitution on the Stability to Various β-Lactamases

Confirming the previous reports,[1,5] Table I (left shows that all ATOICs having the 2-aminothiazolyl methoxyiminoacetamido moiety at the 7-position of cephalosporins were stable

Table I. Susceptibility of ATOICs and FOICs to Various β-Lactamases from G-Negative Bacteria

Enzyme source	Relative hydrolysis of standard β-lactams (PCG or CER =100			Type of β-lactamase[a]	Relative hydrolysis of ATOICs (CER=100)									Relative hydrolysis of FOICs (CER=100)			
	PCG	CER	CPZ		Ceftriaxone	Ro 15-0197	Ro 14-6787	Ro 14-7746	Ro 14-1689	Ro 14-8314	Cefmenoxime	Cefotaxime	Ceftizoxime	Ro 13-3892	Ro 13-9259	Ro 13-4779	Cefuroxime
E. coli																	
1V57	100	45	2	PCase	0	0	0	2	0	0	0	3	11	0	0	0	0
1U591	77	100	5	CSase	14	9	10	<6	<12	11	6	9	<2	29	15	15	13
K. pneumoniae																	
1X165	—	100	—	CSase	0	2	0	0	0	0	0	0	0	<10	4	5	0
C. freundii																	
1U589	4	100	2	CSase	0	0	0	0	0	0	0	0	33	0	0	0	0
1X142	0	100	40	CSase	0	0	0	1	0	0	0	0	1	1	25	1	0
1R524	0	100	37	CSase	0	0	0	0	1	0	0	4	9	1	17	0	0
P. vulgaris																	
5D63-1	17	100	16	CXase	82	40	36	25	21	38	38	40	4	132	55	57	146
5F96-1	16	100	13	CXase	88	35	30	19	14	32	34	34	3	122	38	38	126
1X113	100	19	14	PCase	0	0	1	1	0	0	1	0	0	1	39	3	1
P. morganii																	
1V39	—	100	—	CSase	0	0	0	0	0	0	0	0	2	0	0	0	0
P. rettgeri																	
1X120	—	100	—	CSase	0	0	0	0	0	2	1	0	1	0	0	0	2
S. marcescens																	
5A405-1	—	100	—	CSase	0	0	0	0	0	1	1	2	8	0	0	0	1
E. cloacae																	
6D63-2	—	100	—	CSase	0	0	0	0	0	0	0	0	1	0	0	0	0
1U599	—	100	—	CSase	11	13	11	12	13	15	1	9	1	25	38	19	14
E. aerogenes																	
5I497-0	—	100	—	CSase	0	0	0	0	0	0	0	0	1	5	37	<5	2
Ps. aeruginosa																	
5E81-1	100	72	3	PCase	0	0	0	0	0	0	0	1	0	0	0	0	0
B. fragilis																	
1X97	—	100	—	CXase	33	10	0	0	0	33	15	33	0	<18	<11	<12	<26

a) PCase, penicillinase-type; CSase, cephalosporinase-type; CXase, cefuroximase-type (see Ref. 4).

Table II. β-Lactamase Inhibitory[a] Activity of Oxyiminoacetamido Cephalosporins

β-Lactamase from	ATOICS (inhibition %)									FOICs (inhibition %)			
	Ceftriaxone	Ro 15-0197	Ro 14-6787	Ro 14-7746	Ro 14-1689	Ro 14-8314	Cefmenoxime	Cefotaxime	Ceftizoxime	Ro 13-3892	Ro 13-4779	Cefuroxime	Ro 13-9259
C. freundii													
5D60-1	100	100	100	100	100	100	100	100	94	100	100	100	100
1U589	98	98	98	98	98	100	100	100	89	100	98	100	100
1R523	52	46	43	41	43	44	44	50	46	56	44	43	51
P. vulgaris													
1X113	3	6	6	6	4	4	31	7	21	4	4	9	14
P. morganii													
1V39	97	93	94	93	97	96	98	100	93	97	97	100	100
P. rettgeri													
1X120	0	6	0	9	28	3	0	0	0	58	47	53	96
S. marcescens													
5A405-1	90	89	90	92	97	75	81	64	19	96	97	85	100
E. cloacae													
6D63-2	100	100	100	100	100	100	100	100	92	100	100	100	100
1U599	7	9	17	8	10	9	10	4	2	13	1	9	22
Ps. aeruginosa													
6E300-1	0	15	9	18	12	6	1	8	0	12	14	11	22

a) Substrate: cephaloridine 100 μM, inhibitor 10 μM.

to both penicillinase-type and cephalosporinase-type β-lactamases from various G-negative bacteria, except for those from some *Proteus vulgaris* strains and *Bacteroides fragilis*, which are classified, together with the *Pseudomonas cepacia* enzyme, as so-called cefuroximase-type.[4)]

Table I (left) also clearly demonstrates that the substituents at the 3-position greatly affect the susceptibility of ATOICs to cefuroximase (CXase) type enzymes from *Proteus vulgaris* and *Bacteroides fragilis*. Thus thiomethyleno-2-methyl-6-hydroxytriazine-5-one substituted cephem (ceftriaxone) at C-3 was most labile and was hydrolyzed almost as fast as cephaloridine, while the compound without 3-substitution (ceftizoxime) was least susceptible to the enzyme. The other 7 ATOICs tested seemed to have similar susceptibilities, with Ro 14-7746 and Ro 14-1689 being relatively less susceptible. It should be noted that the geometric isomers of 3-substituents, *i.e.* ceftriaxone and Ro 14-1689, differ considerably in CXase susceptibility.

Cephalosporinases (CSase) from certain strains of *Escherichia coli* (IU591) and *Enterobacter cloacae* (IU599) also hydrolyzed ATOICs at much lower rates, though in contrast to cefuroximases, the substitution at the 3-position seemed to have little effect on the relative susceptibility.

In order to confirm the effect of 3-substituents, the stability of available furylmethoxyiminoacetamido cephalosporins (FOICs) with similar substitution at the 3-position was examined with the same set of β-lactamases; the results are summarized in Table I (right). Enzymes that are able to hydrolyze ATOICs (Table I, left) apparently hydrolyzed FOICs more easily except that from *B. fragilis*, to which both groups showed similar susceptibility. Thus, Ro 13-3892 and Ro 13-4779 having the same substituent at the 3-position as ceftriaxone and Ro 14-1689, respectively, were hydrolyzed by *P. vulgaris* CXases 1.5—2 times more rapidly than were their ATOIC counterparts. However, the stability order and overall susceptibility profile were unchanged.

In comparing Ro 13-3892 with Ro 13-9259, which has the same 3-substituent, it is evident that the *syn-anti* configuration of 7-substituents had more profound effects on the stability and led to considerable changes in the susceptibility profile. Thus, the *anti*-isomer, Ro 13-9259, showed a higher stability than the *syn*-isomer, Ro 13-3982, to *Proteus* CXases, but was fairly labile to various other β-lactamases, *e.g.* CSase from *Citrobacter freundii*, and PCases from *P. vulgaris* 1X113 and *Enterobacter aerogenes* 5I479-0, that could hardly hydrolyze Ro 13-3892 at all.

Comparison of β-Lactamase Inhibitory Activities

The activity of ATOICs and FOICs in inhibiting cephaloridine hydrolysis by each β-lactamase is shown in Table II. All ATOICs exhibited the same inhibition profile and a similar activity, indicating that the C-3 group has little effect on the β-lactamase inhibitory activity. However, ceftizoxime having no substituent at C-3 showed relatively low inhibitory activities. The enzymes from *C. freundii*, IU589 and *E. cloacae* 6D63-2 were completely inhibited by 10 μM ATOICs other than ceftizoxime, which also showed a lower inhibitory activity against the *Serratia marcescens* enzyme. Although there seems to be no essential difference in β-lactamase inhibitory activity between ATOICs and FOICs, FOICs inhibited the *Proteus rettgeri* 1X120 enzyme twice as strongly as did ATOICs. From comparison of Ro 13-3892 with Ro 13-9259, it can be seen that the *anti*-form exhibited a slightly stronger inhibition, in contrast to its lower stability (Table I) as compared with the *syn*-form.

Since the C-3 substituent effect was small, the K_i values were calculated with ceftriaxone as a representative for each species (Table III). Ceftriaxone was found to be a highly effective inhibitor of the β-lactamases from *C. freundii*, *Enterobacter aerogenes* and *E. cloacae* with K_i values of 10^{-9}—10^{-8} M, compared with 10^{-7}—10^{-6} M for the enzymes from *E. coli*, *S. marcescens*, *Proteus morganii* and *Ps. aeruginosa*.

Table III. K_i Values of Ceftriaxone for Various Enzymes

β-Lactamases from	K_i (M)	Mode of inhibition
E. coli 1V 57	4.9×10^{-6}	Competitive
1X 131	3.1×10^{-7}	Competitive
C. freundii 5D 60-1	6.0×10^{-9}	Non-competitive
1U 589	2.3×10^{-8}	Competitive
1U 592	7.1×10^{-9}	Non-competitive
S. marcescens 5A 405-1	5.4×10^{-7}	Competitive
1T 1	8.9×10^{-7}	Competitive
1T 9	6.4×10^{-7}	Competitive
E. cloacae 6D 63-2	1.5×10^{-8}	Competitive
6I 212-1	8.9×10^{-8}	Competitive
1X 176	8.2×10^{-8}	Competitive
1T 405	1.7×10^{-8}	Competitive
1V 655	3.3×10^{-8}	Competitive
E. aerogenes 1X 183	3.6×10^{-8}	Competitive
1Y 243	7.0×10^{-8}	Competitive
1U 595	5.0×10^{-8}	Competitive
P. morganii 1V 36	4.6×10^{-7}	Competitive
1V 39	2.8×10^{-7}	Competitive
1X 117	2.1×10^{-7}	Competitive
1Y 226	2.2×10^{-7}	Competitive
1W 443	2.0×10^{-7}	Competitive
1R 554	2.4×10^{-7}	Competitive
1AB 669	1.7×10^{-7}	Competitive
P. inconstans 1X 360	5.0×10^{-7}	Competitive
Ps. aeruginosa 5E 81-1	2.9×10^{-7}	Competitive
6F 120-1	2.7×10^{-7}	Competitive
5E 267-1	3.0×10^{-7}	Competitive

Discussion

Two groups of β-lactamase resistant cephalosporins, *i.e.* ATOICs and FOICs, were examined in order to determine the effect of C-3 substituents on the interaction with β-lactamases. It is well known that cephalosporins that have a *syn*-methoxyimino group in the C-7 side chain become more stable to β-lactamase,[2] but these cephalosporins are hydrolyzed by the β-lactamase called cefuroximase.[1] In addition to confirming these points we found that the stability of *syn*-methoxyimino cephalosporins to cefuroximases from *P. vulgaris* and *B. fragilis* was considerably affected by their C-3 substituents. Among nine ATOICs tested, ceftriaxone having a 2-methyl-6-hydroxytriazine-5-one moiety at C-3 was the most labile, being hydrolyzed as fast as cephaloridine, while ceftizoxime without a C-3 substituent was the most stable, being hydrolyzed at less than one-thirtieth of the rate of cephaloridine. The stability order of other ATOICs with a triazine ring as the C-3 substituent was Ro 14-1689>Ro 14-7746>Ro 14-6787>Ro 15-0197≫ceftriaxone. From the results, the following relationships between β-lactamase stability and the structure of the triazine ring were deduced. (i) Methylation at N-2 (Ro 13-9904) makes ATOICs more labile than N-6 methylation (Ro 14-1689). The same effect was observed with FOICs when Ro 13-4779 and Ro 13-3892 were compared. (ii) In the 2-methyltriazin group, the substituent at the C-6 position of the triazine ring influenced the stability. Ro 14-7746, without a substituent at this position, was stable and the stability decreased with increase in the polarity of the substituents; namely, electron-withdrawing ability increases in the order of –H, $-CH_3$, $-OCH_3$ and –OH, which is in accord with the stability order of ATOICs having 2-methyl-triazine substituted with the corresponding group at the C-6 position of the triazine ring to the *P. vulgaris* enzyme.

The enzyme from *E. cloacae* IU599 hydrolyzed all ATOICs, except cefmenoxime and ceftizoxime, as well as FOICs at the same velocity, indicating that the C-3 group has little effect. This is a different type of enzyme from that of *P. vulgaris*.

Ro 13-9259, the anti-isomer of Ro 13-3892, was hydrolyzed more easily than was the *syn*-form by a typical cephalosporinase from *C. freundii*, penicillinases from *P. vulgaris* and *Ps. aeruginosa* and *E. aerogenes* 5I497-0 β-lactamase, confirming the previous report.[2] On the contrary, Ro 13-9259 became more stable to *P. vulgaris* cefuroximase. These results may indicate that *syn* configuration of the methoxy-imino group is necessary for making cephalosporins resistant to some β-lactamases (typical cephalosporinase from *C. freundii*, penicillinase from *P. vulgaris* and *Ps. aeruginosa* and cephalosporinase from *E. coli*, *etc.*), but *syn* configuration is not necessary for susceptibility to other enzymes (cephalosporinase from *C. freundii* 5D60-1 or others, TEM-type enzyme, β-lactamase from *S. marcescens* and *P. morganii*, *etc.*) and, surprisingly, the *syn*-form was the preferred substrate configuration for *P. vulgaris* cefuroximase.

On the other hand, as regards β-lactamase inhibitory activity, the substituent at 3-position had little effect, though significantly lower activity was seen with ceftizoxime. The *anti*-methoxyimino containing FOIC, Ro 13-9259, showed potent activity against the *P. rettgeri* enzyme.

References and Notes

1) M. Arisawa, J. Ohshima, E. Ohsawa, H.B. Maruyama, Y. Sekine, and S. Mitsuhashi, *Chem. Pharm. Bull.*, **30**, 2544 (1982).
2) Y.A. Charbbert and J.L. André, *Antimicrob. Agents Chemother.*, **14**, 749 (1978).
3) K. Hirai, S. Iyobe, M. Inoue, and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **17**, 355 (1980).
4) S. Mitsuhashi and M. Inoue, "β-Lactam Antibiotics," ed by M. Salton and G.D. Shockman, Academic Press, New York, 1981, pp. 361—375.
5) R. Reiner, U. Weiss, U. Brombacher, P. Lanz, M. Montavon, A. Furlenmeier, P. Angehrn, and P.J. Probst, *J. Antibiot.*, **33**, 783 (1980).
6) G.W. Rose and C.H. O'Callaghan, "Methods in Enzymology," Vol. 43, ed. by S.P. Colowick, and N.O. Kaplan, Academic Press, New York, 1975, pp. 69—85.