EFFECT OF SIDE-CHAIN SUBSTITUTION OF A CH_2 GROUP BY SULFUR ON THE ANTIMICROBIAL ACTIVITY OF NATURAL PENICILLINS AND CEPHALOSPORINS

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The *S*-carboxymethyl-D-cysteine side-chain analogs of cephalosporin C and deacetoxycephalosporin C were found to have markedly increased *in vitro* activity against Gram-positive and Gram-negative bacteria compared to the natural antibiotics. The *S*-carboxymethyl-L-cysteine analogs were less active than the *S*-carboxymethyl-D-cysteine analogs but against most organisms tested, still more active than the natural compounds. The effect of replacement of CH_2 with S was less dramatic in the case of penicillin N and isopenicillin N. The *S*-carboxymethyl-D-cysteine analog of deacetoxycephalosporin C was found to be orally available in rats.

Isopenicillin N synthetase ("cyclase")[1], catalyzes the ring cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N. Studies of the substrate specificity of this enzyme have led to the *in vitro* production of new penicillins and cephalosporins by the technique of enzymatic biosynthesis[2~8]. We have recently isolated the enzyme in pure form from both *Cephalosporium acremonium*[9] and *Streptomyces clavuligerus* (unpublished data). The cyclases from *C. acremonium*[2,4] and *S. clavuligerus*[3] yield penicillins and cephalosporins in which a CH_2 group in the side-chain is replaced by sulfur when the analog tripeptide δ-(*S*-carbonylmethyl-L-cysteine)-L-cysteinyl-D-valine is employed as substrate in place of ACV. Because of the lability of the isopenicillin N epimerase of *C. acremonium*, the fungal preparation stops at the stage of the penicillin analogs[2,4]. However, the *S. clavuligerus* epimerase is more stable[3], and cell-free experiments with this organism proceed to cephalosporin analogs. The present work is concerned with the *in vitro* activities of these antibiotics and reveals a marked increase in activity resulting from substitution of CH_2 by S in the side-chain.

Materials and Methods

The antibiotics used in this study were synthesized from the appropriate 6-amino or 7-amino precursor, as described by BOWERS, *et al.*[3]. Minimum inhibitory concentrations (μg/disc) of the antibiotics were determined by an agar diffusion assay using 6.5 mm paper discs to apply the antibiotic. Alternatively, minimum inhibitory concentrations (μg/ml) were also determined by broth dilution assay. The purity of each penicillin was determined by the chemical procedure of HOLM[10] using ampicillin as a standard. A modified assay was devised (C. LÜBBE and A. L. DEMAIN, in preparation) using *En-*

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Table 1. MIC (μg/disc) of β-lactam antibiotics and their sulfur analogs.

Test strain	Isopenicillin N	S Analog	Penicillin N	S Analog	Deacetoxycephalosporin C	S Analog	S Analog (L)**	Cephalosporin C	S Analog	S Analog (L)**
Bacillus brevis Nagano	0.69	0.41	0.19	0.03	0.57	0.13	5.5	0.15	0.08	0.55
B. subtilis BL151	3.7	1.6	0.57	1.5	10	0.53	1.4	0.70	0.16	0.22
Streptococcus pyogenes ATCC 10389	0.45	0.12	0.08	0.07	10	0.60	1.0	1.6	0.24	0.27
Micrococcus luteus ATCC 381	0.18	0.15	0.15	0.06	>20	1.6	10	0.64	0.19	0.20
Staphylococcus aureus ATCC 25923	1.3	1.1	0.62	0.66	>20	7.5	15	15	1.6	0.74
S. epidermidis ATCC 14990	4.2	4.4	0.46	6.0	>20	6.4	15	8.7	2.2	1.2
Escherichia coli Ess*	0.72	0.52	0.09	0.005	0.03	0.002	0.63	0.004	0.004	0.07
E. coli ATCC 25922	15	15	10	6.3	>20	10	>20	10	1.9	15
Salmonella typhimurium ATCC 13311	15	4.4	0.09	0.07	20	2.7	>20	2.0	0.23	3.3
Shigella sonnei ATCC 11060	>20	>20	>20	5.5	>20	6.4	>20	10	2.3	>20
Klebsiella pneumoniae ATCC 27736	>20	>20	>20	20	>20	6.3	>20	4.5	0.79	>20
Enterobacter cloacae ATCC 13047	>20	>20	15	>20	>20	>20	>20	>20	>20	>20
Serratia marcescens MIT B-43	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
Pseudomonas aeruginosa ATCC 27853	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20

* Super-sensitive to β-lactam antibiotics.

** Derived from *S*-carboxymethyl-L-cysteine.

Table 2. MIC (μg/ml) of selected cephalosporins.

Test strain	Cephalosporin C	Cephalexin	Deacetoxy-cephalosporin C S analog	Deacetoxy-cephalosporin C
Escherichia coli Ess*	0.1	1.0	0.1	0.25
E. coli B/5	25	10	>100	>100
Salmonella typhimurium ATCC 13311	10	2.5	20	>100
Comamonas terrigena ATCC 8461	10	10	2.5	1.0
Micrococcus luteus ATCC 9341	2.5	0.25	5	25
Staphylococcus aureus ATCC 25923	50	2.0	50	>100
S. aureus (penicillin resistant)	50	2.5	50	>100

* Super-sensitive to β-lactam antibiotics.

terobacter cloacae β-lactamase instead of *Bacillus* penicillinase to determine purity of cephalosporins. Cephalosporin C was used as the standard in this case. Oral availability studies were conducted using male Sprague Dawley rats (approx 300 g). Antibiotics were administered in aqueous solution at 50 mg/kg body weight. Blood was drawn into citrate containing syringes 1 hour after administration of the antibiotics. One hundred and fifty microliter amounts of plasma (four replicates) were bioassayed by agar diffusion assay on 12.7 mm paper discs using *Escherichia coli* Ess as the indicator organism.

Results

Activities of isopenicillin N, penicillin N, deacetoxycephalosporin C, cephalosporin C and their sulfur analogs are shown in Table 1. In general, it can be seen that substitution of S for CH_2 in the side-chain having the D-configuration results in a marked increase in biological activity against both Gram-positive and Gram-negative bacteria.

The effect of replacing S by CH_2 in the side-chain is seen (Table 1) most clearly with cephalosporin C. Higher activity is observed with the D-side-chain against all of the Gram-positive bacteria and four of the eight Gram-negative species. The L-side-chain has a smaller effect; activity is higher against five of the six Gram-positive bacteria, but is decreased or unchanged against all Gram-negative species.

Analogous results are obtained for the analogs of deacetoxycephalosporin C. With the *S*-carboxymethyl-D-cysteine side-chain, increased activity is found against all Gram-positive strains and five of eight Gram-negative strains. However, enhancement is minimal when the L-side-chain is present.

The effect of the carboxymethylcysteine side-chain is much less impressive in the penicillin series. Replacement of D-α-aminoadipyl (penicillin N) by *S*-carboxylmethyl-D-cysteine leads to increased activity against *Micrococcus luteus*, *Bacillus brevis*, *Salmonella typhimurium* and *Shigella sonnei*, but decreased activity against *Bacillus subtilis* and *Staphylococcus epidermidis*. Replacement of L-α-aminoadipyl (isopenicillin N) by L-carboxymethylcysteine has no effect apart from slightly increased activity against *S. typhimurium*.

The same trends are observed when minimum inhibitory concentrations of various cephalosporins are determined by broth dilution assay (Table 2). Replacement of the D-α-aminoadipyl side-chain of deacetoxycephalosporin C by *S*-carboxymethyl-D-cysteine (CMC-DAOC) increases activity for all strains except *E. coli* B/5 (unchanged) and *Comamonas terrigena*. The orally active compound cephalexin has been included here. Cephalexin exhibits higher activity than CMC-DAOC for five of

Table 3. Oral availability of deacetoxycephalosporin C (S analog) and cephalexin.

Antibiotic administered[a]	Zone of inhibition[b] (mm)	Plasma concentration[c] (μg/ml)
Cephalexin	20.6	17.7
	20.1	16.4
	20.4	17.0
Deacetoxy-	20.0	0.35
cephalosporin C	20.5	0.38
(S analog)	20.2	0.37

[a] Each antibiotic was administered to three rats.
[b] Average of quadruplicate samples, each 150 μl.
[c] Determined from standard curves using *Escherichia coli* Ess as indicator organism.

the seven strains tested.

The oral availability of CMC-DAOC in comparison to that of cephalexin was also investigated. Antibiotic solutions were administered to Sprague Dawley rats by oral gavage at 50 mg/kg body weight, and plasma from blood obtained 1 hour after administration of the antibiotics was tested by bioassay against *E. coli* Ess. Both antibiotics were absorbed and plasma samples gave similar zones of inhibition (Table 3). The greater sensitivity of the indicator organism to CMC-DAOC as compared to cephalexin, however, meant that the plasma concentration of cephalexin was actually much higher (average 17 μg/ml) than that of CMC-DAOC (average 0.37 μg/ml).

Discussion

The present study reveals a significant increase in activity when the D-α-aminoadipyl side-chain of cephalosporin C is replaced by its sulfur analog, *S*-carboxymethyl-D-cysteine. With the exception of the cephalosporin-resistant species (*E. cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa*), all Gram-positive and Gram-negative bacteria tested show 2 to 13-fold increases in susceptibility. Similar effects are found with deacetoxycephalosporin C. The effect of sulfur substitution is less impressive with penicillin N and isopenicillin N.

It is noteworthy that the sulfur analog of penicillin N, RIT 2214, was obtained some years ago[11] when a lysine auxotroph of *C. acremonium* was supplied with *S*-carboxymethyl-L-cysteine. Although the *in vitro* activity of RIT 2214 was not impressive, the compound was found to be more active *in vivo* than ampicillin, presumably due to higher plasma concentrations. In semi-synthetic cephamycins[12], the *S*-carboxymethyl-D-cysteine side-chain also confers high levels of *in vitro* activity and an even higher level of *in vivo* activity. The present study is consistent with the findings[11,12] that *in vitro* activity is not markedly altered by the analog side-chain in penicillins but is markedly increased in cephalosporins.

Although estimates of *in vivo* activity were not attempted in the present work, the oral availability of CMC-DAOC makes this compound deserving of continued investigation, since lack of oral availability is a major drawback of most current semi-synthetic cephalosporins. The ease of preparation of cephalosporins containing the *S*-carboxymethyl-D-cysteine side-chain may make enzymatic synthesis a viable alternative to chemical synthesis[7].

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References

1) KONOMI, T.; S. HERCHEN, J. E. BALDWIN, M. YOSHIDA, N. A. HUNT & A. L. DEMAIN: Cell-free conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine into an antibiotic with the properties of isopenicillin N in *Cephalosporium acremonium*. Biochem. J. 184: 427~430, 1979

2) SHIELDS, J. E.; C. S. CAMPBELL, S. W. QUEENER, D. C. DUCKWORTH & N. NEUSS: Synthesis of an *S*-(carboxymethyl)cysteine analog of (L-2-amino-6-adipyl)-L-cysteinyl-D-valine and its cell free biosynthetic conversion into 6-[2-((D-2-amino-2-carboxyethyl)thio)acetamido]penicillanic acid. Helv. Chim. Acta 67: 870~875, 1984
3) BOWERS, R. J.; S. E. JENSEN, L. LYUBECHANSKY, D. W. S. WESTLAKE & S. WOLFE: Enzymatic synthesis of the penicillin and cephalosporin nuclei from an acyclic peptide containing carboxymethylcysteine. Biochem. Biophys. Res. Commun. 120: 607~613, 1984
4) WOLFE, S.; I. J. HOLLANDER & A. L. DEMAIN: Enzymatic synthesis of a sulfur-analog of penicillin using the "cyclase" of *Cephalosporium acremonium*. Bio/Technology 2: 635~636, 1984
5) BAHADUR, G. A.; J. E. BALDWIN, J. J. USHER, E. P. ABRAHAM, G. S. JAYATILAKE & R. L. WHITE: Cell-free biosynthesis of penicillins. Conversion of peptides into new β-lactam antibiotics. J. Am. Chem. Soc. 103: 7650~7651, 1981
6) KUPKA, J.; Y.-Q. SHEN, S. WOLFE & A. L. DEMAIN: Studies on the ring-cyclization and ring-expansion enzymes of β-lactam biosynthesis in *Cephalosporium acremonium*. Can. J. Microbiol. 29: 488~496, 1983
7) WOLFE, S.; A. L. DEMAIN, S. E. JENSEN & D. W. S. WESTLAKE: Enzymatic approach to synthesis of unnatural beta-lactams. Science 226: 1386~1392, 1984
8) BALDWIN, J. E.; R. M. ADLINGTON, N. J. TURNER, B. P. DOMAYNE-HAYMAN, H.-H. TING, A. E. DEROME & J. A. MURPHY: Penicillin biosynthesis: Enzymatic synthesis of new cephams. J. Chem. Soc. Chem. Commun. 1984: 1167~1170, 1984
9) HOLLANDER, I. J.; Y.-Q. SHEN, J. HEIM, A. L. DEMAIN & S. WOLFE: A pure enzyme catalyzing penicillin biosynthesis. Science 224: 610~612, 1984
10) HOLM, K. N.: Automated colorimetric determination of penicillin in fermentation samples using a molybdoarsenic acid-mercuric acid reagent. Anal. Chem. 44: 795~799, 1972
11) TROONEN, H.; P. ROELANTS & B. BOON: RIT 2214, a new biosynthetic penicillin produced by a mutant of *Cephalosporium acremonium*. J. Antibiotics 29: 1258~1267, 1976
12) IWAMATSU, K.; S. INOUYE, T. TSURUOKA, K. MIZUTANI, S. OMOTO, H. OGINO, K. MIYAUCHI, T. WATANABE & T. NIIDA: Synthesis and biological activity of 7β-(2-amino-2-carboxy)ethylthioacetamido-7α-methoxy-cephalosporin derivatives. J. Antibiotics 36: 229~241, 1983