SIDE-CHAIN CONFIGURATION OF THE SULFUR-ANALOG OF PENICILLIN PRODUCED BY *CEPHALOSPORIUM ACREMONIUM*

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WOLFE *et al.*[1] recently demonstrated the production of δ-(carboxymethylcysteinyl)penicillin by exposure of δ-(L-carboxymethylcysteinyl)-L-cysteinyl-D-valine (LLD-CMC-CV) to a semi-purified preparation of isopenicillin N synthetase ("cyclase")[2,3] from *Cephalosporium acremonium* strain C-10 (*Acremonium chrysogenum* ATCC 48272). LLD-CMC-CV is an analog of the normal tripeptide substrate, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV), which is converted to isopenicillin N by *C. acremonium* cyclase. In LLD-CMC-CV, a sulfur atom exists in the position occupied by methylene in LLD-ACV. Since the L-stereochemistry of the δ-(L-α-aminoadipyl) moiety is unchanged in the conversion of LLD-ACV to isopenicillin N by *C. acremonium* isopenicillin N synthetase (demonstrated unequivocally by several independent methods, *e.g.* biological spectrum[2], specificity of L-amino acid oxidase[3], and derivatization and HPLC[4]), it was suggested that the corresponding δ-(L-α-carboxymethylcysteinyl) moiety was unchanged in the conversion of LLD-CMC-CV to the δ-(carboxymethylcysteinyl)penicillin. The preparation of isopenicillin N synthetase used to produce the δ-(carboxymethylcysteinyl)penicillin was free of isopenicillin N epimerase[2]. [This epimerase isomerizes the δ-(L-α-aminoadipyl) moiety in isopenicillin N to the corresponding D side chain present in penicillin N]. SHIELDS *et al.*[5] prepared bis[L-cysteine-(*S*-acetyl)-L-hemicysteinyl ($S^2 \rightarrow S^{2'}$)-D-valine] and following reduction of this disulfide dimer to LLD-CMC-CV, independently demonstrated cell-free conversion of LLD-CMC-CV to a penicillin by a partially purified preparation of *C. acremonium* isopenicillin N synthetase that was not characterized as to the presence or absence of isopenicillin N epimerase. The penicillin was isolated and identified on the basis of its NMR spectrum as a δ-(carboxymethylcysteinyl)penicillin*. The stereochemistry of the δ-(carboxymethylcysteinyl) side chain was assumed without supporting evidence to be the same as that in the δ-carboxymethylcysteinyl)penicillin isolated from a previous *in vivo* study[6]. In that earlier *in vivo* study, δ-(D-carboxymethylcysteinyl)penicillin** had been produced by feeding L-CMC to intact cells of a *C. acremonium* lysine auxotroph.

In order to clarify the nature of the reaction catalyzed by isopenicillin N synthetase, we have experimentally determined the stereochemistry of the δ-(carboxymethylcysteinyl)penicillin formed *in vitro* by the action of isopenicillin N synthetase on LLD-CMC-CV. Here we demonstrate that isopenicillin N synthetase from *C. acremonium* C-10, when free of isopenicillin N epimerase[2,7], catalyzes the conversion of LLD-CMC-CV to δ-(L-carboxymethylcysteinyl)penicillin.

Presumptive identification of the penicillin product was initially carried out by disk diffusion bioassays against four microorganisms: *Escherichia coli* ESS (a mutant super-sensitive to β-lactam antibiotics), *Micrococcus luteus* ATCC 381, *Streptococcus pyogenes* ATCC 10389 and *Salmonella typhimurium* ATCC 13311. In the case of the product produced from the analog tripeptide, the ratios of zone sizes were compared to those obtained with the synthetic sulfur analog of isopenicillin N and the synthetic sulfur analog of penicillin N. As a further control set,

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* In nomenclature used by SHIELDS *et al.*[5], this compound is (6-[2-((amino-2-carboxyethyl)thio)-acetamido]penicillanic acid.

** In nomenclature used by SHIELDS *et al.*[5], this compound is 6-[2-((D-2-amino-2-carboxyethyl)thio)-acetamido]penicillanic acid.

Table 1. Antibacterial activities of enzymatic products from LLD-ACV and LLD-CMC-CV.

Compound	Zone diameter in mm				Ratios	
	Ec[a]	*Ml*	*Sp*	*St*	*Ec*/*Ml*	*Sp*/*St*
Product from LLD-ACV[b]	14.0	27.4	20.7	<7.0	0.51	>2.9
Synthetic isopenicillin N	10.1	24.7	19.3	<7.0	0.40	>2.7
Synthetic penicillin N	27.4	32.6	28.5	11.7	0.84	2.4
Product from LLD-CMC-CV[b]	10.0	20.8	19.0	<7.0	0.48	>2.7
Synthetic S analog of isopenicillin N	13.1	27.6	24.0	<7.0	0.47	>3.4
Synthetic S analog of penicillin N	36 0	28.0	25.0	19.7	1.3	1.3

[a] Abbreviation: *Ec*; *Escherichia coli* ESS, *Ml*; *Micrococcus luteus*, *Sp*; *Streptococcus pyogenes*, *St*; *Salmonella typhimurium*.

[b] The products were prepared with a partially purified enzyme preparation of *Cephalosporium acremonium* C-10 as previously described[1].

product was also produced with the normal tripeptide LLD-ACV and its zone size ratios were compared to those of synthetic isopenicillin N and penicillin N. Synthesis of synthetic peptides and penicillins were described earlier[5]. Since the synthetic products were not pure, the actual zone sizes are not as important as the ratios of zone sizes comparing *E. coli* to *M. luteus* and *S. pyogenes* with *S. typhimurium*. Table 1 shows (as expected) that the product from LLD-ACV resembles isopenicillin N, not penicillin N. The product from LLD-CMC-CV resembles the synthetic sulfur analog of isopenicillin N and not the synthetic sulfur analog of penicillin N.

BOWERS *et al.*[8] found that HPLC could be used to separate the sulfur analogs of penicillin N and isopenicillin N. To further examine the identity of our product, HPLC was used under the following conditions: column, Waters C_{18} Bondapak; solvent, 95% 50 mM KH_2PO_4 (pH 4.0) and 5% CH_3OH; pressure, 141 kg/cm^2; detector, 214 nm; flow rate, 2 ml/minute; volume injected, 30 μl. The retention time of the synthetic sulfur analog of isopenicillin N was found to be 4.2 minutes and that of the sulfur analog of penicillin N was 3.7 minutes. A mixture of the two synthetic analogs showed retention times of 4.2 and 3.7 minutes. We next examined reaction mixtures which had been incubated with LLD-CMC-CV. A peak was found in reaction mixtures incubated for 60 minutes; it was absent in the unincubated control and its retention time was similar to that of isopenicillin N (4.1 minutes), thus showing that the penicillin had the L-CMC side-chain.

The finding that the product of the reaction is the CMC analog of isopenicillin N is consistent with the absence of isopenicillin N epimerase activity in the partially purified enzyme preparation used[1]. Due to the lability of isopenicillin N epimerase in *C. acremonium* extracts[2,7], even crude extracts of *C. acremonium* CW-19 (after freezing and thawing) would be expected to stop at the isopenicillin N analog stage. The D-configuration of the penicillin isolated by mutasynthesis[6] is consistent with the presence of isopenicillin N epimerase inside intact mycelia of *C. acremonium*. In contrast to the situation with *C. acremonium*, the stability of epimerase in crude extracts of *Streptomyces clavuligerus* allows LLD-CMC-CV to be converted all the way to the CMC analog of deacetoxycephalosporin C[8].

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