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

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Wolfe et al. recently demonstrated the production of \(\delta\)-(carboxymethylcysteinyl)penicillin by exposure of \(\delta\)-(L-carboxymethylcysteinyl)-L-cysteinyl-D-valine (LLD-CMC-CV) to a semi-purified preparation of isopenicillin N synthetase ("cyclase") from Cephalosporium acremonium strain C-10 (Acremonium chrysogenum ATCC 48272). LLD-CMC-CV is an analog of the normal tripeptide substrate, \(\delta\)-(L-(L-\alpha-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 \(\delta\)-stereochimistry of the \(\delta\)-(L-\alpha-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, specificity of L-amino acid oxidase, and derivatization and HPLC), it was suggested that the corresponding \(\delta\)-(L-carboxymethylcysteinyl) moiety was unchanged in the conversion of LLD-CMC-CV to the \(\delta\)-(carboxymethylcysteinyl)penicillin. The preparation of isopenicillin N synthetase used to produce the \(\delta\)-(carboxymethylcysteinyl)penicillin was free of isopenicillin N epimerase. [This epimerase isomerizes the \(\delta\)-(L-\alpha-aminoadipyl) moiety in isopenicillin N to the corresponding \(\delta\) side chain present in penicillin N]. Shields et al. prepared bis[L-cysteine-(S-acetyl)-L-hemicycysteinyl \((S^\gamma\rightarrow S^\delta)\)-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 \(\delta\)-(carboxymethylcysteinyl)penicillin. The stereochemistry of the \(\delta\)-(carboxymethylcysteinyl) side chain was assumed without supporting evidence to be the same as that in the \(\delta\)-(carboxymethylcysteinyl)penicillin isolated from a previous in vivo study. In that earlier in vivo study, \(\delta\)-(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 \(\delta\)-(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, catalyzes the conversion of LLD-CMC-CV to \(\delta\)-(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 \(\beta\)-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,
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 earlier8). 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 I 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 C18 Bondapak; solvent, 95% 50 mm KH₂PO₄ (pH 4.0) and 5% CH₃OH; pressure, 141 kg/cm²; 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 used9). Due to the lability of isopenicillin N epimerase in C. acremonium extracts6,7), even crude extracts of C. acremonium CW-19 (after freezing and thawing) would be expected to stop at isopenicillin N analog stage. The D-configuration of the penicillin isolated by mutasynthesis9) 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 C9).

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References

3) O'SULLIVAN, J.; R. C. BLEANEY, J. A. HUDLESTON & E. P. ABRAHAM: Incorporation of 3H

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zone diameter in mm</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ec¹</td>
<td>Mi</td>
</tr>
<tr>
<td>Product from LLD-ACVb</td>
<td>14.0</td>
<td>27.4</td>
</tr>
<tr>
<td>Synthetic isopenicillin N</td>
<td>10.1</td>
<td>24.7</td>
</tr>
<tr>
<td>Synthetic penicillin N</td>
<td>27.4</td>
<td>32.6</td>
</tr>
<tr>
<td>Product from LLD-CMC-CVb</td>
<td>10.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Synthetic S analog of isopenicillin N</td>
<td>13.1</td>
<td>27.6</td>
</tr>
<tr>
<td>Synthetic S analog of penicillin N</td>
<td>36.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

¹ Abbreviation: Ec; Escherichia coli ESS, Mi; Micrococcus luteus, Sp; Streptococcus pyogenes, St; Salmonella typhimurium.
² The products were prepared with a partially purified enzyme preparation of Cephalosporium acremonium C-10 as previously described9).
from $\delta$-(l-\(\alpha\)-amino[4,5-\(^3\)H]-adipyl)-l-cysteinyl-d-
[4,4-\(^3\)H]valine into isopenicillin N. Biochem. J. 184: 421 ~ 426, 1979


