Supplementary Material Available: A table of the positional and thermal parameters and their estimated standard deviations (2 pages). Ordering information is given on any current masthead page.

Cell-Free Biosynthesis of Penicillins. Conversion of Peptides into New β-Lactam Antibiotics

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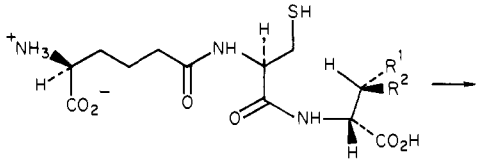
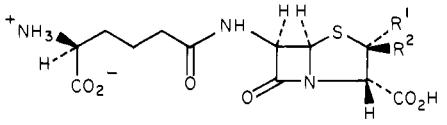
The efficient conversion of (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (**1**) to isopenicillin N (**2**) by a partially purified cell-free system from *Cephalosporium acremonium* has been studied in detail during investigations of the mechanism of formation of the pencillin ring system.[1,2] We now report the results of experiments with structural analogues[1] of tripeptide **1** designed to evaluate the substrate specificity of this conversion. The results of these experiments have shown, for the first time, that the cyclase enzyme system, which converts **1** to **2**, is able to accept and transform modified substrates into new penicillins. Furthermore, these substrates act as inhibitors of the conversion of the natural substrate **1** into isopenicillin N.



1, $R^1 = R^2 = CH_3$
3, $R^1 = CH_3$; $R^2 = CH_3CH_2$
4, $R^1 = CH_3CH_2$; $R^2 = CH_3$
5, $R^1 = H$; $R^2 = CH_3$



2, $R^1 = R^2 = CH_3$
11, $R^1 = CH_3$; $R^2 = CH_3CH_2$
12, $R^1 = CH_3CH_2$; $R^2 = CH_3$
14, $R^1 = H$; $R^2 = CH_3$

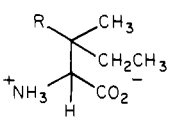
The L,L,D-tripeptides **3**, **4**, and **5** were synthesized by standard procedures[3,4] and separately incubated with a cell-free system from *C. acremonium* C-91.[1] The extent of conversion to bioactive products was assayed with *Staphylococcus aureus* NCTC 6571. Under these conditions each peptide was converted into a penicillinase-sensitive antibiotic, presumed to be an analogue of isopenicillin N. However, the extent of conversion under the conditions used was much less than the virtually quantitative yield from the natural substrate **1**. After 60-min incubation a significant proportion of these analogues remained unchanged and they behaved as inhibitors of the natural substrate (see Table I).

Table I. Conversion of Peptides to Penicillins in Extracts of *C. acremonium*

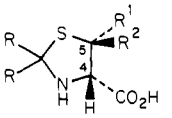
peptide	product	% yield[a]	% inhibition[b]
1	2	100	
3	11	36	40
4	12	4	75
5	14[c]	10	78

[a] Peptides (1 mM) and cell-free extract (4–10 mg/mL protein) were incubated at 27 °C. Yields after 60 min were estimated by bioassay (*S. aureus* NCTC 6571) on the assumption that penicillins **11**, **12** and **14** had about 50% of the specific antibacterial activity of isopenicillin N (**2**).[5] Since conversion of **1** to **2** was quantitative within 30 min. the yields shown may provide an overestimate of the relative rates of conversion of the peptide analogues of **1**. [b] Inhibition is defined as the decrease (%) in the bioactivity generated after 30 min when a peptide analogue (1 mM) was incubated with the cell extract (1–2 mg/mL protein) for 15 min at 27 °C before addition of the natural substrate (**1**) (0.4 mM). [c] Predominant product.

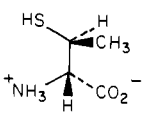
The nature of the biosynthesis products was determined as follows. Oxidation (performic acid, 4 °C, 5 h) of the incubation mixture from **3**, followed by electrophoresis on paper (pH 1.8), gave a sulfonic acid[6] (cadmium ninhydrin positive) which comigrated with the more mobile of a diastereomeric pair of sulfonic acids **6** produced by similar oxidation of a diastereomeric mixture



6, R = SO_3H
7, R = SH



8, $R = CH_3$; $R^1 = CH_3$; $R^2 = CH_3CH_2$
9, $R = CH_3$; $R^1 = CH_3CH_2$; $R^2 = CH_3$
10, $R = CD_3$; $R^1 = CH_3CH_2$; $R^2 = CH_3$



13

of β-mercapto-DL-isoleucine **7**[7]. This result, coupled with the antibiotic activity and penicillinase sensitivity, is consistent with a stereospecific conversion of **3** into a penicillin bearing a 2-ethyl substituent. The stereochemistry was determined as follows. Fractional crystallization (acetone, ethanol) of the thiazolidine mixture **8** and **9** prepared by reaction of **7** with acetone (reflux) gave one diasteromer, **8**, mp 182–184 °C (dec),[8] which was oxidized (performic acid) to a sulfonic acid which comigrated with that derived from the biosynthetic experiment (electrophoresis on paper, pH 1.8). The relative configuration of **8** was established by observation of an NOE (300 MHz, D_2O) on the C-5 ethyl group on irradiation of the C-4 (δ 4.38) hydrogen. The minor isomer **9**, corresponding to the less mobile sulfonic acid, gave an NOE (18%) on C-4 (δ 4.45) hydrogen upon irradiation of the C-5 (δ 1.47) methyl group. This NOE was also observed in **10**, the deuterated analogue, thereby removing the possibility of misassignments of the methyl groups. Thus the relative stereochemistry of **8** is as shown and hence that of the new penicillin corresponds to **11**.

Incubation of **4**, derived from D-alloisoleucine, gave a smaller yield (Table I) of a bioactive, penicillinase-sensitive product. The latter was oxidized to a sulfonic acid and shown (electrophoresis)

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0002-7863/81/1503-7650$01.25/0

to comigrate with the less mobile isomer derived from thiazolidine **9**. The stereochemical retentions observed in these biosynthetic ring closures are in accord with previous observations on the incorporation of chiral valines into penicillin with overall retention[9,10,11,12] and with the incorporation of valine into tripeptide without loss of chirality at C-3.[13,14] Hence the relative stereochemistry at C-3 of peptide-bound valine is retained during conversion to penicillin, in agreement with our results on peptide analogues.

The selectivity of ring closure onto a methylene group was assessed by incubating the peptide **5**, from D-α-aminobutyric acid. This yielded a bioactive, penicillinase-sensitive product (Table I). That this was a mixture of C-2 epimers of demethylisopenicillin N was demonstrated by oxidation to a mixture of sulfonic acids, corresponding in electrophoretic mobility to those obtained from the diastereoisomers of 2-amino-3-mercaptobutyric acid. The major, less mobile isomer corresponded to that derived from **13**,[15] whose configuration has already been assigned.[16] This result, i.e., preferential formation of **14**, together with those from peptides **3** and **4** suggests that cyclization is favored to a penicillin with the larger group at C-2 in the β configuration.

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Deuterium Isotope Effects on Methyl Transfer to Alcohols. Possible Asynchronous Solvent Repolarization and Internal Structural Changes

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Recently we reported[1] that the H_2O/D_2O kinetic isotope effect (KIE) on methyl transfers to water (reaction 1: L = H, D; R = L) vanishes when the water is a dilute solute in aprotic solvents.

$$ROL + CH_3X \rightarrow \underset{L}{RO^+CH_3} + X^- \qquad (1)$$

We now have observed that alkyl substitution on the nucleophilic oxygen (reaction 1, R = CH_3, t-C_4H_9) causes large changes in this KIE; these changes would not be expected for a conventional S_N2 mechanism but are consistent with our suggestion[1,2] that the activation process for methyl transfer to L_2O is predominantly a fluctuation in the structure of the solvent surrounding the L_2O,CH_3X reactant pair.

The spectrophotometric method for measuring rate constants and the preparations of *S*-methythiophenium ($MeTh^+$) hexafluorophosphate and methyl perchlorate ($MeOClO_3$) were as previously[1] described. Commercial CH_3OL and t-C_4H_9OL were purified by vacuum line distillation from $Mg(OCH_3)_2$ and freshly calcined CaO, respectively. In each run, the absorbance change rate was first order, and the pseudo-first-order rate constants (k_ψ) were linear functions of ROL concentration (eq 2) when [ROL] < 1 M in CH_3CN.[3] Table I summarizes the KIE's observed at different concentrations of ROL in CH_3CN.

$$k_\psi^L = k_0 + k_1^L[ROL] \qquad (2)$$

For both $MeTh^+$ and $MeOClO_3$, Table I shows that KIE for CH_3OL is greater than KIE for L_2O. This is the *reverse* of the order expected for displacements proceeding by conventional S_N2 mechanisms, since L_2O has twice as many O–L bonds as CH_3OL.[4]

Table I also shows that, except for t-C_4H_9OL + $MeOClO_3$, KIE in CH_3CN is less than KIE in neat ROL. The KIE in CH_3CN should be a measure of the $R(L)O\cdots CH_3$ bond order in the transition state ($n_{OC}^\ddagger$),[1,4] and if $n_{OC}^\ddagger$ does not greatly increase as the ROL/CH_3CN solvent ratio changes, then the increase in KIE when the solvent changes from CH_3CN to ROL can be attributed to the τ_D/τ_H factor[1,5] (τ = effective dielectric relaxation time of solvent) which is present when reorganization of isotopically substituted solvent structure contributes to motion along the reaction coordinate. Estimates of $n_{OC}^\ddagger$ based on the KIE's in CH_3CN are listed in Table II. The range of these estimated $n_{OC}^\ddagger$ values is larger than would be expected if these reactions had the same mechanism as most previously studied methyl transfers; structure–reactivity relationships and KIE's suggest that structural changes in the nucleophile (or equivalently in the leaving group) do not commonly induce such large changes in the internal structure of S_N2 activated complexes, particularly for methyl transfers.[6-14] However, the directions and large magnitudes of

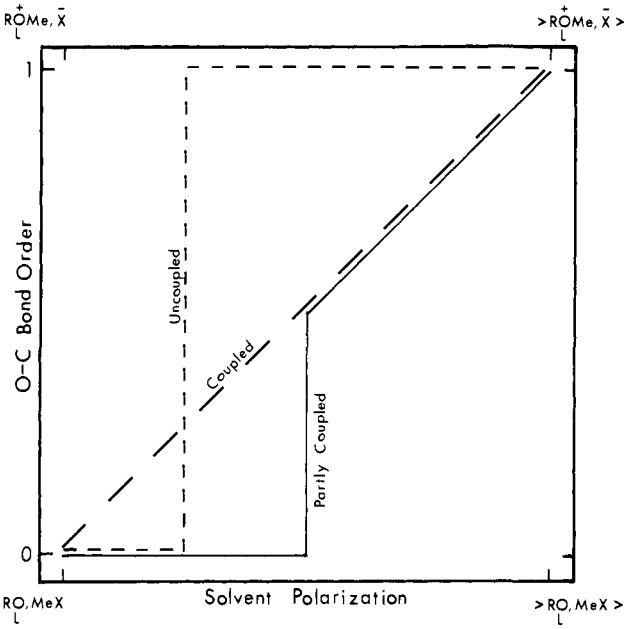

Figure 1. Example of possible reaction paths. The scale on the solvent polarization axis is defined by requiring that all structures along the diagonal path have solvent polarization in equilibrium with internal structure.

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