Stereochemical Investigation of the α-Ketoglutarate-dependent 3′-Hydroxylation in Cephalosporin Biosynthesis

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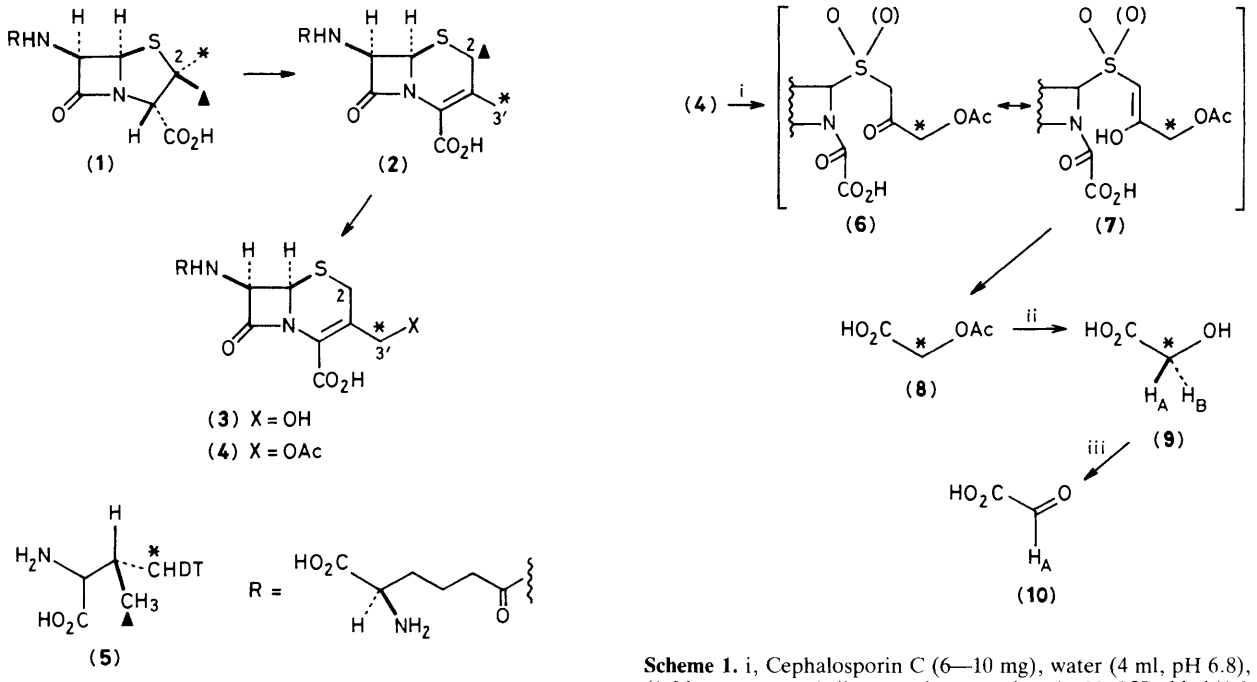
Using samples containing D,L-(3*S*,4*S*)- and (3*S*,4*R*)-[4-^{2}H,^{3}H]valine, the stereochemical course of the α-ketoglutarate-dependent 3′-hydroxylation of deacetoxycephalosporin C (**2**) to deacetylcephalosporin C (**3**) has been determined to be retention.

In their important respects the oxidative transformations responsible for penicillin N (**1**) and cephalosporin C (**4**) biosynthesis from a tripeptide precursor remain poorly understood.[1] Cell-free experiments indicate that both the ring expansion of penicillin N (**1**) to deacetoxycephalosporin C (**2**)[2] and the 3′-hydroxylation[3] of the latter to deacetylcephalosporin C (**3**) are carried out by α-ketoglutarate-dependent dioxygenases.[4] Reaction with acetyl CoA then gives cephalosporin C (**4**). It has been shown[5] in these laboratories that during the conversion of (**1**) into (**2**), the C-2 β-methyl (▲) of the former, labelled with a chiral methyl group from the 3-*pro*(*R*) position of valine (▲),[6] suffers epimerization upon

Table 1. Enzymic assay of tritium distribution in glycolates from chiral methyl valine incorporation into cephalosporin C.

Expt.	Glycolate origin	$^3H/^{14}C$ Glycolate[a]	$^3H/^{14}C$ Glyoxylate	% ^3H Retained	% ^3H in H_2O[d]
1	Racemic	3.80	2.08[b]	54	45
2	(3*S*,4*S*)-Valine				
	A	1.5	0.40[b]	27	—
	B	2.14	0.69[c]	32	82
3	(3*S*,4*R*)-Valine				
	A	4.74	3.57[c]	75	28
	B	4.65	3.43[c]	74	31

[a] Determined as the *p*-bromophenacyl ester.[24] [b] Determined as the oxime.[22] [c] Determined as the hydrogen sulphite addition product.[23] [d] Percent of total tritium activity present at the beginning of the glycolate oxidase assay measured by lyophilization of the incubation in a closed system and scintillation counting of a known fraction of the total water recovered.



Scheme 1. i, Cephalosporin C (6—10 mg), water (4 ml, pH 6.8), O_3 (1.0 h, room temp.); ii, to previous reaction, 1 M NaOH added (1.0 ml, 2 h, room temp.); iii, labelled glycolate (*ca.* 2 mg), Tris buffer (2.5 ml, 0.1 M, pH 8.3), glycolic acid oxidase (10 units, Sigma: contains FMN), catalase (15 000 units, Sigma, 30 °C, 5 h).

conversion into the C-2 methylene of the latter. In contrast, we record in this paper that the simple allylic hydroxylation of (**2**) to (**3**) proceeds with overall retention of configuration, paralleling results obtained at unactivated methyl centres with other classes of oxygenases.[7]

Syntheses of the required (3*S*,4*S*)- and (3*S*,4*R*)-[4-^{2}H,^{3}H]valines (**5**) were carried out by a previously described route[6] modified and improved in some details.[8] Cultures of *Acremonium strictum* (ATCC 36225, also known as *Cephalosporium acremonium* CW19[9]) were maintained on agar plates of a modified LePage and Campbell medium,[5,10] propagated in the seed medium of Caltrider and Niss,[11] and finally grown in Demain's fermentation medium[5,12] (40 ml/250 ml Erlenmeyer flask, 300 r.p.m., 27 °C). Chiral methyl valine (1 mg/flask) was administered five times to four such flasks between 72 and 120 h. After 6 days, the mycelia were harvested and the cephalosporin C (**4**) produced was isolated by chromatography on carbon and preparative h.p.l.c.[5,13]

Examination of the literature on chemical modification of the cephalosporin 3′-position[14] did not augur well for stereospecific conversion of the acetoxymethylene into a chiral methyl group for configurational assay[15] (after its excision as acetic acid). Expecting a significant, normal kinetic isotope effect[7,16] in the hydroxylation of (**2**) to (**3**) (*vide infra*), we chose to ozonolyse the labelled cephalosporin C with the hope that sulphoxide–sulphone formation would be competitive[17] with cleavage of the ceph-3-em double bond to generate an intermediate as (**6**). Presuming further that the preferred direction of enolization in (**6**) would be towards the sulphoxide–sulphone, as in (**7**), subsequent oxidation would yield acetylglycolate (**8**), which could be saponified to glycolic acid (**9**). A control experiment in deuterium oxide (99.8%, pH 7.4[18]) supported these expectations giving about a 20% yield of glycolate whose crystalline *p*-bromophenacyl ester showed a 4% incorporation of deuterium (by mass spectrometry). The labelled glycolate (**9**), after admixture of ^{14}C-internal standard, may be assayed for tritium distribution at the enantiotopic hydroxymethylene positions with glycolate oxidase, an enzyme known to remove stereospecifically the *pro*-(*R*) hydrogen, H_B, in the formation of glyoxylate (**10**).[19]

A sample of racemic [2-^{3}H]glycolate was prepared by the method of Stubbe and Abeles.[20] After addition of [2-^{14}C]-glycolate, enzymic assay under standard conditions (Scheme 1, iii), separation by Dowex-1 (OAc form), chromatography,[21,22] and derivatization[22] showed a 54% retention of tritium in the glyoxylate.† An approximately complementary fraction of the total tritium activity presented to the assay was released into the medium (Table 1, expt. 1). D,L-(3*S*,4*S*)- and (3*S*,4*R*)Valines (**5**) were separately incorporated into cephalosporin C in duplicate trials and the isolated samples of (**4**) (>95% pure) were ozonolysed. Hydrolysis of the acetylglycolates (**8**) obtained and purification by Dowex-1 chromatography[21,22] gave specimens of glycolate (**9**) which, after addition of the appropriate quantity of [2-^{14}C]glycolate, were assayed as above (Scheme 1). The results are summarized in Table 1 (expts. 2AB, 3AB).

For labelled glycolate originating from (*S*)- and (*R*)-methyl substrates (**5**), complementary results were obtained indicating a 3 or 4 to 1 ratio of tritium retention in the glyoxylates demonstrating, therefore, overall stereochemical *retention* in the cephalosporin 3′-hydroxylation. This finding correlates with ^{3}H n.m.r. data of Crout[25] where this allylic hydroxylation is seen to be at least stereoselective and accompanied by a sizeable intrinsic isotope effect. However, as n.m.r. spectral assignments of the acetoxymethylene hydrogens in (**4**) are not known, no determination of the absolute stereochemical sense of the 3′-oxidation was possible. Presuming a radical or cation (allylic) intermediate[4] in the oxidation, the observation of retention herein, as opposed to epimerization,[5] may reflect the barrier[26] to rotation in such species only or other mechanistic factors.

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† The values in Table 1 show slightly higher than expected tritium retentions owing presumably to a small degree of further oxidation (and partitioning according to an isotope effect) to oxalic acid by enzymic or chemical means.