Synthesis of α-Hydroxy-β-acetylenic Acids and their Oxidation by and Inactivation of Flavoprotein Oxidases

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Summary Five α-hydroxy-β-acetylenic acids have been prepared; all are active substrates for a mammalian flavoprotein oxidase, with only the C_4 molecule a particularly effective suicide substrate.

WE have previously reported the preparation of the four-carbon α-hydroxy-β-acetylenic acid (I)[1] by transesterification and aqueous acid hydrolysis of the known n-butyl ester[2] prepared from addition of ethynylmagnesium bromide to n-butyl glyoxalate. The acid (I) was found to be a specific suicide substrate for two bacterial flavoprotein oxidases, a soluble L-lactate[3] oxidase from *M. smegmatis* and a membrane-bound D-lactate dehydrogenase from *E. coli*.[4]

We have now synthesized the acetylenic hydroxy-acids (I)—(V) as a mixture of DL-isomers on a 10 mmol scale by addition of HCN to the precursor acetylenic aldehydes to form the β-acetylenic cyanohydrins. The aldehydes for the preparations (II)–(V) were obtained as acetals from Farchan Chemical Co., while but-4-ynal was used for (I). Controlled hydrolysis of the cyanohydrins in aqueous acid[6] at 60—80° for several hours gave the pure acids as oils after repeated vacuum distillation. Yields were not maximised. I.r. analysis revealed the presence of the acetylenic linkage in each product; n.m.r. spectra were consistent with assigned structures, and mass spectra revealed the expected molecular ions and fragmentation patterns. With $H^{14}CN$† (35 mCi/mmol) on a mmol scale 2-hydroxy[1-^{14}C]but-3-ynoic acid (I) was prepared with a specific radioactivity of 35 mCi/mmol. Use of acrolein instead of but-4-ynal likewise generated 2-hydroxy[1-^{14}C]but-3-enoic acid,[6] at specific radioactivities of 6—60 mCi/mmol. The radioactive hydroxy-acids were radiopure and identical with

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authentic samples on silica gel t.l.c. All the hydroxy-acids have water-soluble sodium salts and can be stored at $-20°$ for weeks without decomposition.

These synthetic acetylenic hydroxy-acids were then tested as oxidizable substrates with an L-amino-acid oxidase purified to homogeneity from rat kidney.[7] This flavoenzyme also oxidizes α-hydroxy acids, at considerably higher V_{max} values than amino-acid substrates. All five acetylenic hydroxy-acids are active substrates (Table) undergoing two-electron oxidation to the β-acetylenic-α-keto-acids, at V_{max} rates comparable to that of α-hydroxybutyrate, one of the better physiological hydroxy-acid substrates.

TABLE

$RC{\equiv}CCHOHCO_2H$

Substrate	R	Relative V_{max}[a]
(I)	H	1
(II)	Me	1·89
(III)	Et	0·57
(IV)	Pr	0·86
(V)	Bu	0·70

[a] These experiments were performed with the purified rat kidney oxidase. On this scale DL-α-hydroxybutyrate has a value of 0·9. Full kinetic details will be reported elsewhere.[7]

The C_4 acetylenic hydroxy-acid (I) irreversibly inactivates this flavoenzyme, after about 25 turnovers in air-saturated solution. (I) partitions to a species which covalently modifies the isoalloxazine coenzyme[7] (without modification of any amino-acid residues at the active site), reminiscent of the bacterial flavoenzymes.[1,4] With [1-^{14}C]-(I), a stoicheiometric labelling of the bound flavin coenzyme occurs. The C_5—C_8 molecules show much lessened inactivating propensities, turning over more than 2000 times each under incubation conditions. The pentynoate may cause some inactivation (*ca.* 50%) over a period of hours, compared with a half time of 5 min with the butynoate under similar assay conditions.

The failure of the longer acetylenic hydroxy-acids to produce rapid covalent modification of enzyme-bound flavin coenzyme could imply catalytic involvement of 4-H in inactivation, such as dissociation of this moderately acidic acetylenic hydrogen to generate an anionic species which could attack the isoalloxazine ring. Mitigating against this possibility is the observation[3] that [4-^{3}H]-(I) retained tritium quantitatively in the inactivated coenzyme-modified complex with this enzyme and the *M.smegmatis* L-lactate oxidase.

We have argued[1] that inactivation may occur by Michael addition between the isoalloxazine ring of the coenzyme and either an allenic species arising from rearrangement of a β-acetylenic α-carbanionic species or with the β-acetylenic-α-keto-acid product. The presence of alkyl substituents at C-4 may decrease such an addition inductively. Alternatively, steric restrictions may develop which either suppress enzyme-catalysed rearrangements to allenic species or slow Michael addition sufficiently for the β-acetylenic α-keto-acids to dissociate from the active site.

As a control for the necessity of the acetylenic functionality in inactivation, 2-hydroxybut-3-enoic acid was tested with the mammalian oxidase; it was a good substrate, producing thousands of product molecules without modification of the bound coenzyme. Eventual loss of catalytic activity resulted from slow alkylation of the enzyme (not the coenzyme) by the keto-acid product.[7]

Addition of the simple allenic acid, buta-2,3-dienoic acid, as a substrate analogue, to either the oxidized or the reduced coenzyme-enzyme complex did not result in detectable inactivation. However, the allenic acid was not bound well by the enzyme even as a competitive inhibitor, rendering its inertness for inactivation inconclusive.

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