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Stereochemistry of hydrogen removal from the 'unactivated' C-3 position of 4-hydroxybutyryl-CoA catalysed by 4-hydroxybutyryl-CoA dehydratase

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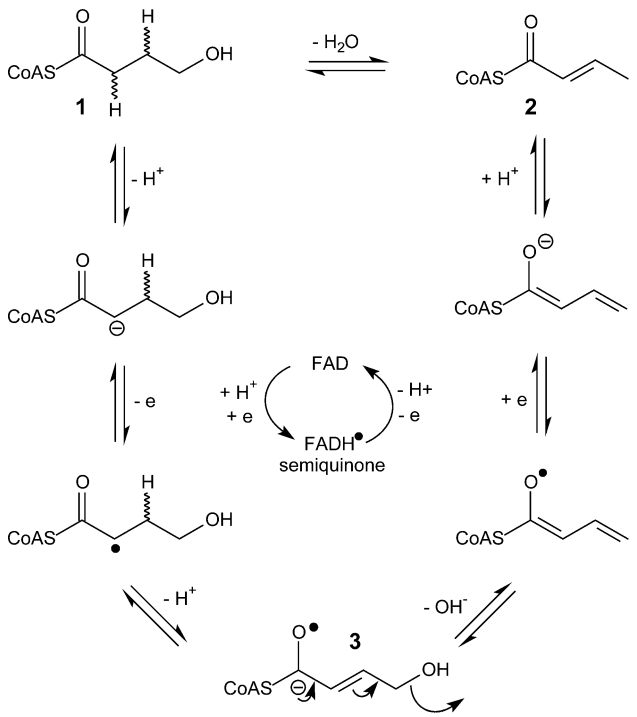
(*R*)- and (*S*)-γ-[3-$^2H_1$]butyrolactones have been synthesised from (*R*)- and (*S*)-glycidol, respectively, and used to demonstrate that it is the pro-(*S*) hydrogen atom that is stereospecifically abstracted from C-3 of 4-hydroxybutyryl-CoA by 4-hydroxybutyryl-CoA dehydratase, and that this atom is not returned to C-4.

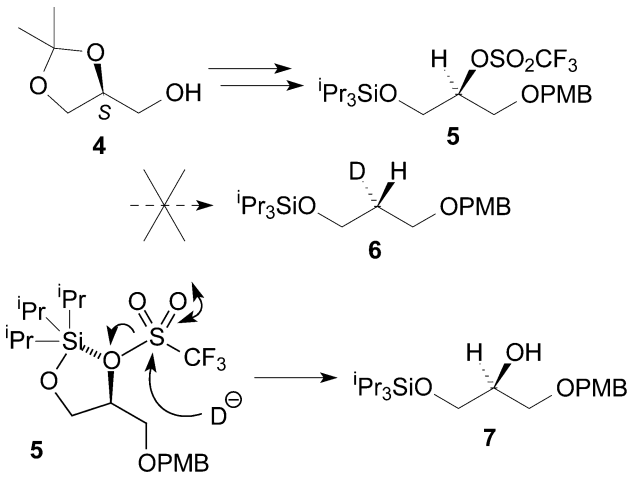
4-Hydroxybutyryl-CoA dehydratase catalyses the reversible dehydration of 4-hydroxybutyryl-CoA (**1**) to crotonyl-CoA (**2**). The enzyme is homotetrameric and contains one [4Fe-4S] cluster and one flavin adenine dinucleotide (FAD) per subunit.[1] The catalytic reaction requires the removal of a hydrogen atom from the least activated position of the butyryl chain of 4-hydroxybutyryl-CoA and is the mechanistically most demanding step in the fermentation of γ-aminobutyrate (GABA) by the anaerobic bacterium *Clostridium aminobutyricum*. The proposed mechanism[2] involves generation of ketyl radical anion **3** from the substrate, **1**, by a deprotonation at C-2, followed by one-electron oxidation of the enolate to give an enoxy radical that suffers deprotonation at C-3. The point of the initial deprotonation/oxidation at C-2 is therefore to generate a species that is sufficiently acidic at C-3 ($pK_a \sim 14$)[3] to be deprotonated. The intermediate **3** eliminates a hydroxyl group and yields **2** after one-electron reduction to a dienolate that undergoes protonation at C-4 (Scheme 1).



Scheme 1 Proposed mechanism for the dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA by *Clostridium aminobutyricum*.

The sequence of reactions described is highly unusual in its requirement for the mobilisation of a hydrogen atom at each carbon of a C_3 unit. To investigate the nature of the hydrogen removal at C-3, which is the most remarkable of all the steps described, and to aid the detection of intermediates, samples of 3-$^2H_1$-labelled 4-hydroxybutyric acid have been synthesised in the form of labelled γ-butyrolactones. Initially, an attempt was made to prepare both enantiomers of γ-[3-$^2H_1$]butyrolactone from (*S*)-solketal (**4**) *via* a single chiral intermediate (**5**), which was activated as a trifluoromethanesulfonate ester. Compound **5** was to be reduced with a deuteride reagent (or hydride reagent whilst the chemistry was being optimised) to **6**, which would be orthogonally deprotected for further transformations. However, it proved impossible to reduce intermediate **5** in the desired manner with either lithium aluminium hydride or lithium triethylborohydride[4] (Super Hydride™). This could be attributed to the oxygen atom on the leaving group coordinating to the adjacent silicon atom in the triisopropylsilyl protecting group and thus promoting nucleophilic attack of hydride on the sulfur atom with regeneration of alcohol **7** (Scheme 2). Isolation from the reaction mixture of alcohol **7**, which had been the immediate precursor to triflate **5**, supported this hypothesis. Alternatively, the silyl group could merely suppress the rate of substitution at the carbon bearing the triflate by steric shielding.

To avoid the problem described above, the silyloxy group of **5** was replaced by the non-coordinating vinyl group (Scheme 3). Accordingly, the hydroxyl group of (*R*)-glycidol (**8**) was protected as a *p*-methoxybenzyl ether (**9**). The epoxide ring of **9** was opened with vinyl magnesium bromide to afford alcohol **10**. The newly formed hydroxyl group was activated as a methanesulfonate ester, which was displaced with lithium aluminium deuteride to give compound **11**. It has been shown that hydride or deuteride reduction of sulfonate esters occurs with complete inversion of stereochemistry.[5] Direct oxidative cleavage (by *e.g.* ozonolysis or



Scheme 2 Attempted reduction of a solketal-derived trifluoromethanesulfonate.

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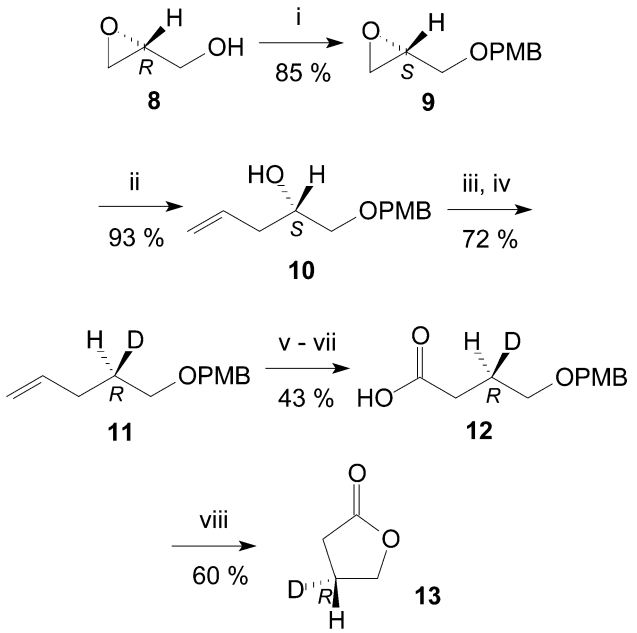

Scheme 3 Synthesis of (*R*)-γ-[3-$^2H_1$]butyrolactone (**13**). *Reagents and conditions*: (i) PMB–Cl, NaH, Bu_4NI, DMF, 0 → 25 °C, N_2, 2 h; (ii) $CH_2CHMgBr$, Li_2CuCl_4, THF, −78 → 25 °C, N_2, 18 h; (iii) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 0 °C, 30 min; (iv) $LiAlD_4$, THF, reflux, N_2, 48 h; (v) $Hg(OAc)_2$, THF, H_2O, 25 °C, 2 h, then $NaBH_4$, NaOH, H_2O, 5 → 25 °C, 1 h; (vi) Dess–Martin periodinane, CH_2Cl_2, 25 °C, 3 h; (vii) NaOH, Br_2, H_2O, dioxane, 15 °C, 2 h; (viii) DDQ, CH_2Cl_2, H_2O, 0 °C, 3 h.

perruthenate oxidation) of the vinyl group to acid **12** proved problematic in the presence of the *p*-methoxybenzyl group, but was eventually achieved efficiently by an indirect route involving oxymercuration,[6] Dess–Martin oxidation[7] and bromoform[8] reaction. On deprotection of the hydroxyl group, spontaneous cyclisation occurred to afford (*R*)-γ-[3-$^2H_1$]butyrolactone (**13**), which is a convenient form of 4-hydroxybutyric acid for purification and analysis. The (*S*)-enantiomer of γ-[3-$^2H_1$]butyrolactone was obtained by a similar route, starting with (*S*)-glycidol. For the enzymatic experiments, the γ-[3-$^2H_1$]butyrolactones were converted to 4-hydroxy[3-$^2H_1$]butyrates with 1 M NaOH solution. The synthesis of 4-hydroxybutyryl-CoA esters was performed using 100 mM labelled 4-hydroxybutyrate in 100 mM potassium phosphate pH 7.4, 2.5 mM acetyl-CoA and 4-hydroxybutyrate CoA-transferase (3 U ml^{-1}) in a total volume of 0.5 ml. After incubation at ambient temperature for 10 min, the completeness of the conversion was checked in an assay using 5,5′-dithiobis(2-nitrobenzoate) (DTNB), oxaloacetate and citrate synthase; subsequently, acetate and 4-hydroxybutyrate CoA-transferase were added.[9] The CoA esters were purified using a C_{18} cartridge, eluting with 50% aqueous acetonitrile containing 0.1% TFA. The CoA esters from (*R*)- and (*S*)-4-hydroxy[2-^2H]butyrates showed one major peak by MALDI-TOF mass spectrometry with the same mass of 855 Da.

The CoA esters were incubated with 4-hydroxybutyryl-CoA dehydratase for 15 min at 37 °C and the CoA-containing products separated with a C_{18} cartridge as described above. MALDI-TOF mass spectrometry was used to analyse the products, an equilibrated mixture of 4-hydroxybutyryl-CoA and crotonyl-CoA. The spectra show masses of 854 and 836, respectively, for the (*S*)-enantiomer and 855 and 837, respectively, for the (*R*)-enantiomer (Fig. 1). The (*R*)-isomer therefore lost its ^1H (proton at C-3), whereas the (*S*)-isomer lost its ^2H (deuteron at C-3).

It has thus been demonstrated that the dehydration of 4-hydroxybutyryl-CoA proceeds with the stereospecific removal of the pro-(*S*) hydrogen atom from the C-3 position of the substrate (Fig. 2). The hydrogen (deuterium) removed is not returned to C-4, indicating that the base that removes H(D) from C-3 either does not

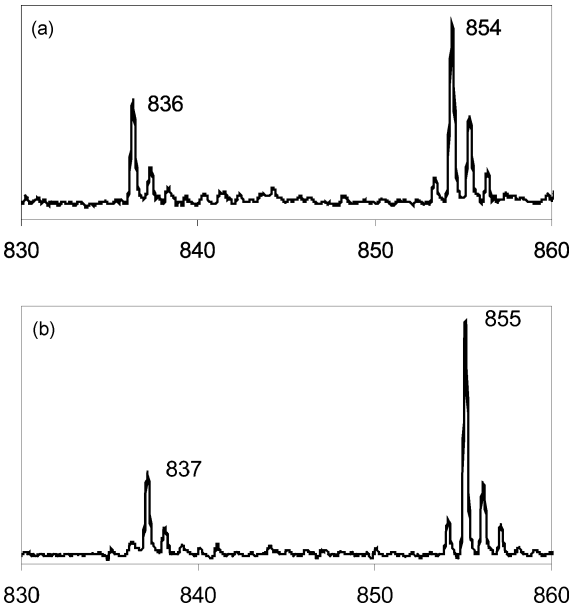

Fig. 1 MALDI-TOF mass spectra for the products from (*S*)- (a) and (*R*)-4-hydroxy[3-$^2H_1$]butyryl-CoA (b).

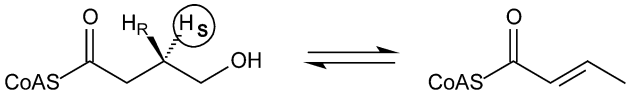

Fig. 2 Stereospecific loss of the pro-(*S*) hydrogen atom from 4-hydroxybutyryl-CoA.

return the abstracted atom to C-4 or only does so after exchange with solvent. Work is in progress to determine the stereochemistry of hydrogen removal/addition at C-2 and C-4. These results will aid the positioning of the substrate/product in the active site of 4-hydroxybutyryl-CoA dehydratase, whose crystal structure is currently under investigation.[10] Initial results show that the structure is very similar to that of medium chain acyl-CoA dehydrogenase,[11] even though there is only 16% sequence identity. Interestingly, acyl-CoA dehydrogenases show the same stereospecificity at C-3 as 4-hydroxybutyryl-CoA dehydratase.[12]

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