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A Mutase Mimic with Cobalamin Linked to Cyclodextrin

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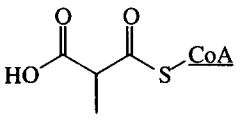
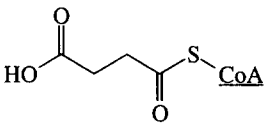
Abstract : Cobalamin covalently tethered to β-cyclodextrin was synthesized as a methylmalonyl-CoA mutase mimic. The artificial enzyme is capable of discriminating between two substrates containing different binding groups. © 1997 Elsevier Science Ltd.

The biochemical rearrangements catalyzed by various mutase enzymes that use cobalamin (vitamin B_{12}) as a cofactor remain major challenges for bioorganic chemistry. Enzyme mimics cannot yet perform reactions like the isomerization of methylmalonyl CoA (**1**) to succinyl-CoA (**2**) that is catalyzed by methylmalonyl-CoA mutase with coenzyme B_{12} as its cofactor. The enzyme promotes the cleavage of the carbon-cobalt bond of the coenzyme to form the Co(II) form of vitamin B-12.[1,2] The other product, a 5'-deoxyadenosyl radical, then abstracts a hydrogen atom from the methyl group of **1** to form the substrate radical. This rearranges to a product radical, which recovers a hydrogen atom from 5'-deoxyadenosine to form **2**, and the resulting adenosyl radical then couples with the Co(II) B-12 again to form the coenzyme. Other mutase enzymes perform related rearrangements, in which a hydrogen atom and a different group are transposed across a two-carbon fragment.



1



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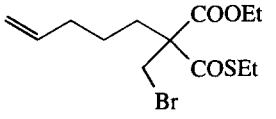


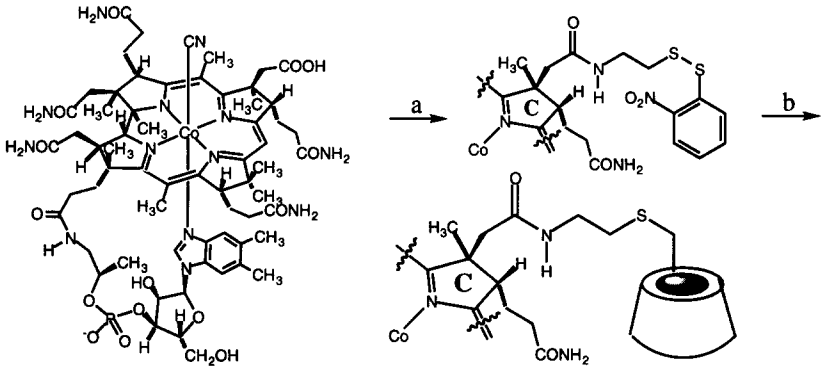
3

We have mimicked the hydrogen atom transfer and coupling of the radical with B-12 in an intramolecular model.[3] We have also shown that an enzyme mimic with B-12 attached to the primary carbon of β-cyclodextrin will dissociate easily to form a cyclodextrinyl radical that can form a substrate radical by group transfer, but not yet hydrogen transfer, from a substrate bound in the cyclodextrin cavity.[4] Other work on enzyme models has raised important questions about the nature of the rearrangement after the substrate hydrogen has been abstracted. Dowd has shown[5] that reaction of substrate **3** with Co(I) B-12 anion leads to extensive migration of the thioester group, while reaction of **3** with tributyltin hydride leads instead to cyclization of the intermediate radical, not to thioester migration. This and a series of related experiments suggest[6,7] that the cobalamin moiety plays a role in directing the rearrangement and is not simply a convenient source of adenosyl radical. On the other hand, other authors suggest that the rearrangement occurs at the level of substrate radical[8,9] and perhaps involves its fragmentation and recombination.[10] Regardless of the mechanism, the proximity of the cobalamin moiety and a substrate species has been suspected to be of importance since the pioneering study of Rétey.[11] Numerous studies tested this hypothesis using supramolecular complexes between the substrate and cobalamin,[12,13] or vesicles.[14,15]

The goal of our work was to construct an artificial cobalamin enzyme containing β-cyclodextrin as a hydrophobic binding site. When two substrates were allowed to compete for the cobalamin, the substrate containing a better binding group would compete more successfully.

In our synthetic scheme (Scheme I), we took advantage of the selective cleavage of a single propionamide side chain in B-12 by N-bromosuccinimide yielding the cobalamin-c-acid.[16] We then coupled the resulting unique carboxyl group with protected 2-aminoethanethiol, and linked this to β-cyclodextrin by thiol displacement on 6-deoxy-6-iodo-β-cyclodextrin.[17] Molecular models indicated that this flexible linkage permits the Co atom of attached B-12 to reach a substrate bound into the cyclodextrin cavity, and selectivity studies confirmed that this is so. The preparation of substrates and products is described in Scheme II.[18]

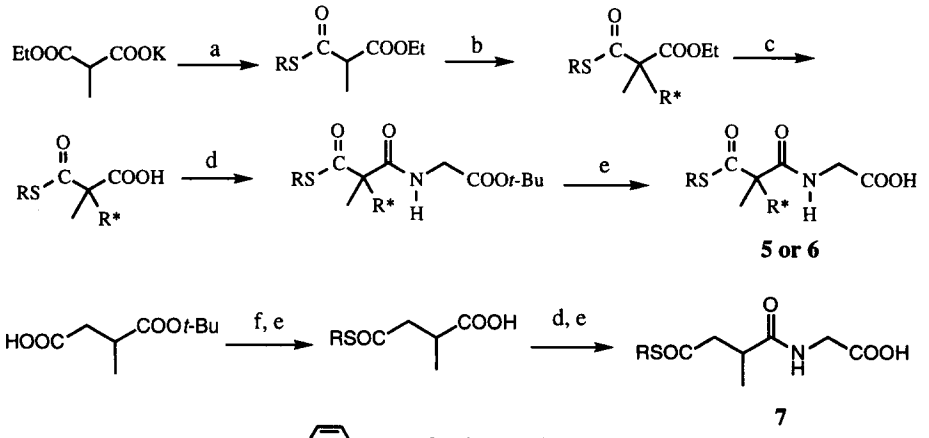
SCHEME I



cobalamin-c-acid **4**

a) EtOOCCl, DMF, Et_3N, then o-nitrophenyldithioethylammonium chloride, 59% yield; b) PBu_3, $NaHCO_3$, then β-cyclodextrinyl 6-iodide, 20% yield.

SCHEME II



R= Et for the **b** series; *t*-Bu-C$_6$H$_4$-CH$_2$ for the **a** series

R*= CH_2Br for **5**; CH_3 for **6**

a) $(COCl)_2$, CH_2Cl_2, then RSH; b) NaH, THF, then CH_3Br or CH_2Br_2, reflux; c) BBr_3, CH_2Cl_2; d) $(COCl)_2$, CH_2Cl_2, then glycine *t*-butyl ester; e) CF_3COOH; f) RSH, EDC, DMAP, CH_2Cl_2

We examined the reaction/rearrangement of substrate **5a**, with a *t*-butylphenyl group that will bind strongly into the cyclodextrin cavity in aqueous solution. In order to evaluate the effect of binding or proximity effects on the catalysis, **5a** was allowed to compete for the catalyst **4** (cat) with substrate **5b**, lacking a good binding group. As a control we examined the analogous process catalyzed by nonbinding cobalamins: simple aquacobalamin (cbl) or the enzyme mimic **4** unable to bind the substrates due to the presence of aminoadamantane hydrochloride (adm) as an inhibitor. The results are summarized in Table 1.

Table 1. Selectivity of the Catalyst.

	[7b]/[6b]	[7a]/[6a]	preference
cat	0.16	0.43	2.73
cbl	0.53	0.23	0.43
cat-adm	0.21	0.16	0.77
cbl-adm	0.46	0.22	0.48
cat : cbl	0.30	1.89	6.34
cat : cat-adm	0.77	2.74	3.56
cbl : cbl-adm	1.16	1.04	0.89

The values represent the ratio of products quantified after incubation of **5** with catalyst **4**, aquacobalamin, catalyst **4** with aminoadamantane hydrochloride and cobalamin with aminoadamantane hydrochloride. The three last rows show the respective ratios compared for different catalytic systems. The "preference" column is the ratio of the value in the [**7a**]/[**6a**] column to the value in the [**7b**]/[**6b**] column Each substrate is converted into twin products, the rearranged **7** (a rearrangement catalyzed by cobalamin) and the reduced **6**.

All experiments were carried out in a 2:8 mixture of ethylene glycol and 8% aqueous ammonium chloride. Equimolar mixture of both substrates (70 μ M each) was incubated with zinc and 90 μM catalyst **4** or aquacobalamin. After 4 minutes incubation period all of the starting materials were consumed. This is much faster than the typical previous cobalamin systems, which normally required hours. The mixture was filtered and the product composition analyzed by HPLC. All values are the average of four independent experiments, error ≤ 10%.

We observed that the preference for **5a** (see Table 1) increased 6.3 times when going from aquacobalamin to the catalyst **4**. The presence of 0.1M aminoadamantane hydrochloride has a dramatic effect on the catalyst **4** but almost no effect on aquacobalamin. That indicates that the high preference of **4** for **5a** is caused by binding of **5a** to the cyclodextrin cavity of **4**. Also, addition of an equimolar amount of β-cyclodextrin to aquacobalamin does not have any effect on its preference (data not shown), indicating that the preference of **4** is not caused by a non-specific effect of its cyclodextrin moiety. Finally, in the absence of either **4** or aquacobalamin only traces (<5%) of either substrate undergo rearrangement, which shows that the cobalamin moiety actively participates in directing the substrate species to the rearrangement path. Thus the enzyme mimic **4** preferentially reacts with a substrate bound into its cyclodextrin cavity, showing moderate substrate specificity.

In compound **4** we have the beginning of a potential mimic of mutases. More is needed before we or others can build on this to produce catalysts that reproduce the entire process that a mutase performs using coenzyme B-12. However, the processes catalyzed are so unusual, and so unattainable by any known non-enzymatic chemistry, that the goal is worth the effort.

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17. **Preparation of catalyst 4**. Cobalamin-c-acid (0.037 mmol) dried over phosphorus pentoxide in vacuo was dissolved in 5 ml of anhydrous DMF and dry triethylamine (2 mmol) was added. The solution was cooled under argon to 0 °C and treated with isobutyl chloroformate (0.37 mmol) for 15 minutes. Then a solution of 2-(2-nitrophenyldisulfido)ethylamine hydrochloride in 2 ml of DMF was added. The mixture was kept for 1 h in an ice bath and then evaporated. Chromatography on silica RP-18 with methanol-water gave, after lyophylization, 34 mg of cobalamin-c-acid-2-(2-nitrophenyldisulfido)ethylamide as a red fluffy solid (0.022 mmol, 59%). The NMR analysis is based on the region $\delta > 5$ ppm, where cobalamin has 5 singlets. ^{1}H-NMR (400 MHz, CD_3OD): 8.20 (d, 1H, J=8), 8.16 (d, 1H, J=8), 7.72 (broad s, 1H), 7.67 (t, 1H, J=8), 7.34 (t, 1H, J=8), 7.15 (s, 1H), 7.03 (s, 1H), 6.47 (s, 1H), 6.18 (d, 1H), 5.94 (s, 1H). ^{13}C-NMR (75 MHz, CD_3OD, d > 160 ppm): 181.5, 180.1, 177.6, 177.5, 177.3, 176.5, 175.4, 175.3, 174.2, 172.0, 167.0, 166.4. FAB-MS: 1568(M^+), 1541 (M-CN).

    Cobalamin-c-acid-2-(2-nitrophenyldisulfido)ethylamide (0.104 mmol) and a catalytic amount of NaCN in a mixture of 3 ml of DMA, 1.5 ml water and 0.5 ml of a saturated aqueous solution of $NaHCO_3$ were treated with tributylphosphine (0.30 mmol) for 2 h at room temperature. At this time RP-18 TLC showed transformation of the starting material into a more polar species. The whole mixture was cannulated into a solution of 6-iodo-6-deoxycyclodextrin (0.5 mmol) in DMA and the mixture was kept at 80 °C for 15 h. The compound was prepurified by chromatography on silica RP-18 and final purification was performed on Diol-RP silica of Merck. Chromatography (20% EtOAc/MeOH -> MeOH ->water) and lyophylization gave 55 mg of the title compound (0.02 mmol, 20% yield) as a red fluffy solid. ^{1}H-NMR (D_2O, $\delta > 4.7$ ppm): 7.20 (s, 1H), 7.04 (s, 1H), 6.40 (s, 1H), 6.30 (s, 1H), 6.08 (s, 1H), ~5.0 (apparent s, 8.8 H). ^{13}C-NMR (D_2O, d > 160 ppm): 180.4, 180.2, 179.9, 178.2, 177.9, 176.4, 175.9, 175.8, 175.6, 174.9, 167.8, 166.0, 163.8 (13 lines; expected: *13 lines in this region*). FAB-MS: 2532(M^+), 2505(M-27). Prior to use the compound **4** was converted to its aqua form.[19]
18. All compounds were characterized satisfactorily by ^{1}H and ^{13}C-NMR and mass spectrometry.
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