Vitamin B_{12} Model Studies. Acrylate Migration in the Model Carbon-skeleton Rearrangement Leading to α-Methyleneglutaric Acid

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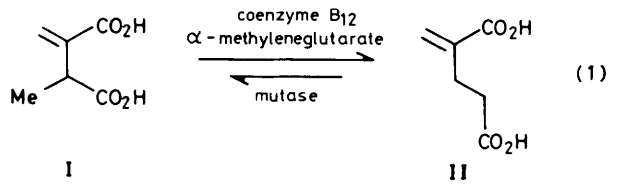
Rearrangement of the model vitamin B_{12}–methylitaconate adduct (**IV**) in D_2O leads to the uptake of one deuterium in each of two of the products: methylitaconic acid (**I**) and α-methyleneglutaric acid (**II**). The third product, butadiene-2,3-dicarboxylic acid (**V**), contained no deuterium. The deuterium in the methylitaconic acid [^{2}H]-(**I**) was incorporated in the methyl group. The deuterium in the α-methyleneglutaric acid [^{2}H]-(**II**) was located at the γ-carbon. The latter result demonstrates that the migrating group in the rearrangement is the acrylate group, as it is in the corresponding coenzyme B_{12}-dependent, enzyme-catalysed, carbon-skeleton rearrangement of methylitaconic acid (**I**) to α-methyleneglutaric acid (**IV**). The labelling result establishes a firm connection between the model and the enzyme-catalysed reactions.

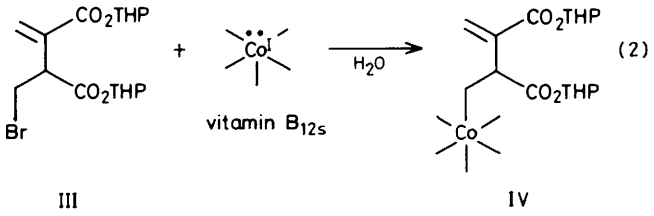
A nonenzymic model[1] for the coenzyme B_{12}-dependent, enzyme-catalysed, carbon-skeleton rearrangement interconverting methylitaconic acid (**I**) with α-methyleneglutaric acid (**II**)[2] [equation (1)] has been discovered.[1] Fabrication of the



(1)

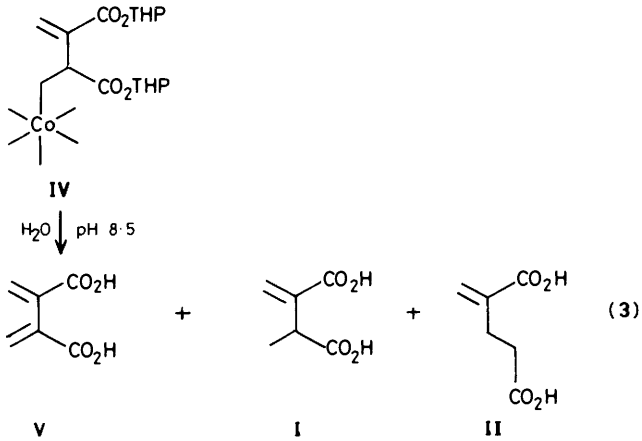
I II

model consisted of forming the carbon–cobalt bond to the methyl carbon of methylitaconic acid (**I**). That was accomplished by condensing vitamin B_{12s} with bromomethylitaconic ester, specifically, the bis(tetrahydropyranyl) (THP) ester (**III**), yielding the adduct (**IV**) [equation (2)]. The model cobalamin (**IV**) then underwent *spontaneous* conversion at room temperature, in the dark, in aqueous solution, at pH 8—10, conditions ideal for a biochemical model reaction, to the carbon-skeleton rearrangement product α-methyleneglutaric acid (**II**) [equation (3)].



(2)

III IV



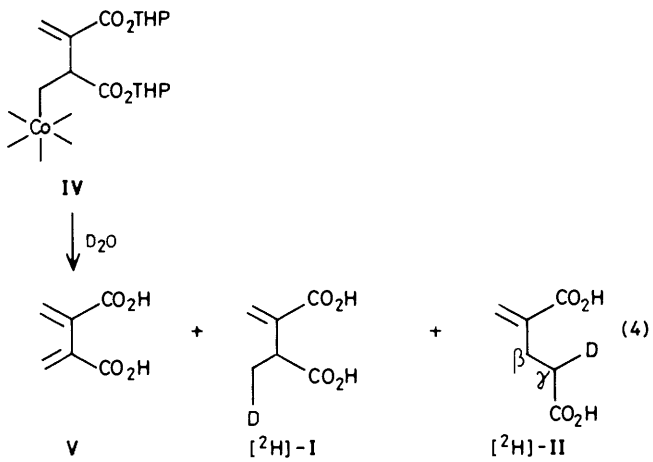
(3)

V I II

Since the reaction leading from (**III**) to (**I**) and (**II**) is a reduction, it was very important to learn the source of the hydrogen newly introduced into the products of rearrangement (**I**) and (**II**). It was also important to establish the regiochemical sense of the model rearrangement. In the enzyme-catalysed rearrangement, the acrylate fragment is the exclusive migrating group. If the model rearrangement [equation (3)] is a faithful reflection of the enzymic reaction, the same migratory preference should be displayed.

Results

When the rearrangement of the model alkylcobalamin (**IV**) was carried out in D_2O [equation (4)],[3] one atom of deuterium was



(4)

V [^{2}H]-I [^{2}H]-II

incorporated into each of the products (**I**) and (**II**). The third product, butadiene-2,3-dicarboxylic acid (**V**), contained no deuterium.

Methylitaconic acid, [^{2}H]-(**I**), was deuteriated only on the methyl group. The presence of a single deuterium was established by mass spectral peaks for the molecular ion at *m/z* 145 and 144 in the ratio 4.7:1 corresponding to 82% [^{2}H]methylitaconic acid. A pair of one-proton vinyl singlets at

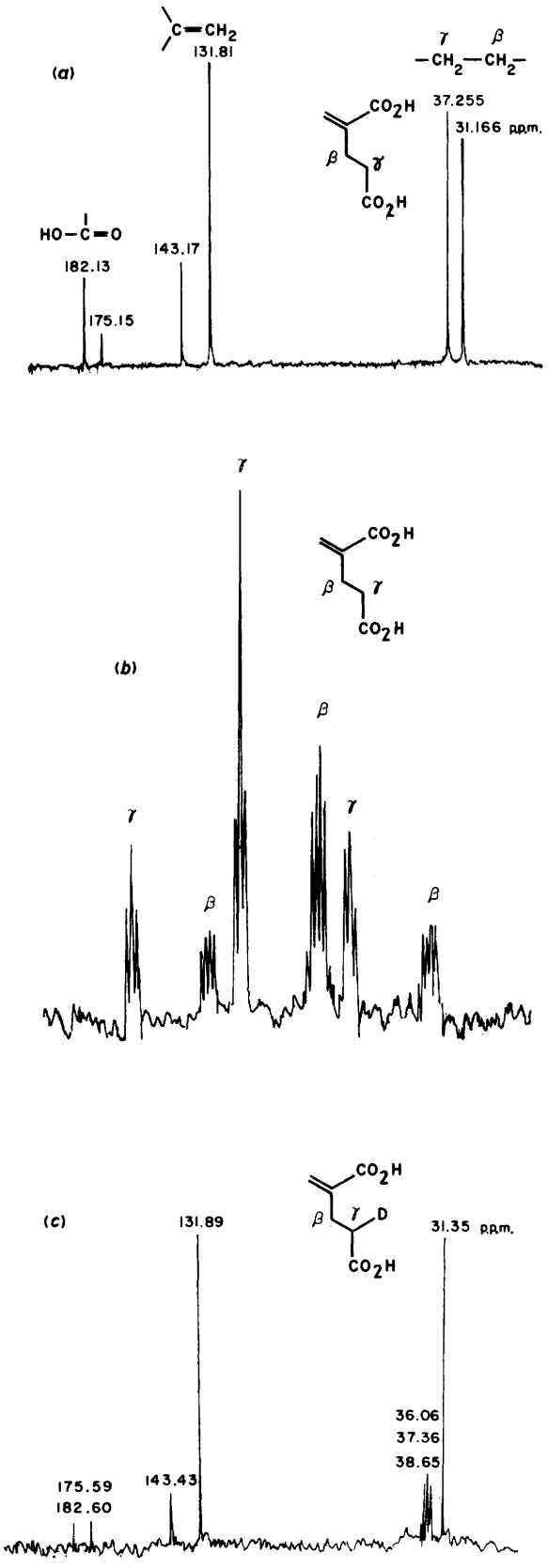
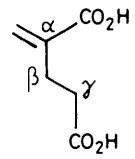


Figure 1. (*a*) Proton-decoupled carbon-13 spectrum of α-methyleneglutaric acid (**II**). (*b*) Proton-coupled carbon-13 spectrum showing the splitting of the β- and γ-carbons in α-methyleneglutaric acid (**II**). (*c*) Proton-decoupled spectrum of α-methylene [γ-^{2}H] glutaric acid [^{2}H]-(**II**)

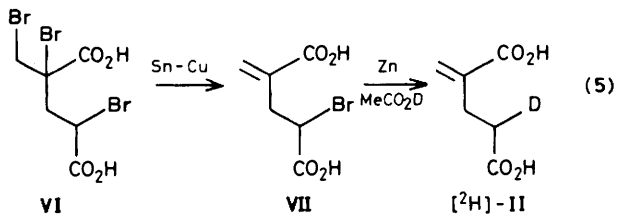
δ 6.3 and 5.8, a one-proton methine triplet (J = 6.5 Hz) at δ 3.59, and a two-proton methyl doublet (J = 6.5 Hz) at δ 1.37 established the position of the deuterium at the methyl carbon. The labelling result also has bearing on the mode of addition of the cobalamin to the bromomethylitaconate (**III**). Since the entire reaction, including the preparation of the alkyl cobalamin, was carried out in D_2O, the labelling pattern establishes that formation of the carbon–cobalt bond occurs, in this instance, by substitution rather than by an elimination–addition sequence. The latter path would have resulted in dideuteriation with incorporation of deuterium also at the methine carbon.



II

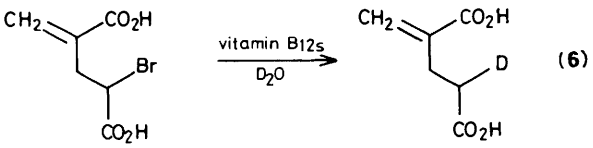
The product of major interest was the α-methyleneglutaric acid, [^{2}H]-(**II**). This substance was shown by two independent lines of evidence to be monodeuteriated on the γ-carbon. The proton n.m.r. spectrum of α-methyleneglutaric acid (**II**) shows a very small chemical shift difference between the β- and γ-methylene protons. At 60 MHz the two methylene groups appear as a reasonably sharp singlet; at 250 MHz the two methylenes are separated by 0.1 p.p.m. The carbon-13 n.m.r. spectrum (Figure 1) shows the β- and γ-carbons to be cleanly separated and distinguishable from one another. The proton-decoupled carbon-13 n.m.r. spectrum of the parent undeuteriated compound (**II**) shows a pair of sharp singlets at 35.35 and 29.17 p.p.m. corresponding to the γ- and β-carbon, respectively [Figure 1(*a*)]. The assignment was made on the basis of the splitting pattern in the undecoupled spectrum [Figure 1(*b*)] of the parent (**II**). The lower field peak at 35.35 p.p.m. appears as a clean triplet of triplets (^1J = 132 Hz, ^2J = 6 Hz) due to coupling of the γ- and β-protons with the γ-carbon. The higher field peak at 29.17 p.p.m. appears as a triplet (^1J = 132 Hz) resulting from coupling of the β-hydrogens to the β-carbon, but each component of the triplet is a complex multiplet because of coupling of the β-carbon to both the γ-hydrogens and the vinyl hydrogens. In the proton-decoupled spectrum of the rearrangement product [^{2}H]-(**II**) [Figure 1(*c*)], the peak at 35.35 p.p.m. appears as a 1 : 1 : 1 triplet, while the peak at 29.17 p.p.m. remains a sharp singlet. No satellite peaks on the 29.17 p.p.m. singlet, which might have indicated a small amount of deuterium attached to the β-carbon, were observed. Approximately 1% incorporation of deuterium at the β-carbon could have been detected.

That the rearrangement reaction of the cobalamin (**IV**) had resulted in exclusive deuterium incorporation at the γ-carbon was further guaranteed by comparison with an authentic sample of [^{2}H]-(**II**) prepared by reduction of γ-bromo-α-methyleneglutaric acid (**VII**) with zinc in CH_3CO_2D [equation (5)].[4] The bromide (**VII**) was prepared by Sn–Cu couple debromination of the tribromide (**VI**).[4]



VI **VII** [^{2}H]-**II** (5)

The γ-deuteriated-α-methyleneglutaric acid [^{2}H]-(II) was also produced in an attempt to attach the bromide (VII) to the cobalt atom of vitamin B_{12}. This was an experiment designed to explore the reverse rearrangement of α-methyleneglutarate (II) to methylitaconic acid (I), the enzymic rearrangement being a reversible reaction. Unfortunately, attempts to attach γ-bromo-α-methyleneglutarate (VII) to cobalt have been unsuccessful.



(6)

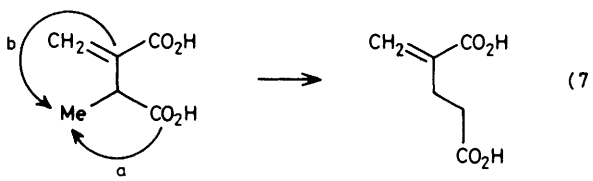
VII [2H]-II

When carried out in D_2O, α-methyleneglutaric acid [^{2}H]-(II) was isolated from these reactions with one atom of deuterium incorporated at the γ-position [equation (6)].

Formation of methylitaconic acid or butadiene-2,3-dicarboxylic acid was not observed in these experiments. This constitutes a negative result and should be treated as such; it may be worth exploring from other standpoints. However, there are two aspects of this approach which might be noted. Firstly, it is known to be difficult, because of steric hindrance, to attach secondary carbon to the cobalt atom of vitamin B_{12}. Secondly, in the enzymic equilibrium, α-methyleneglutarate (II) is favoured over methylitaconate (I) by a factor of approximately four.[1c] If part of this preference is reflected in the model rearrangement, it may be difficult to observe the desired methylitaconic acid in the model rearrangement.

Discussion

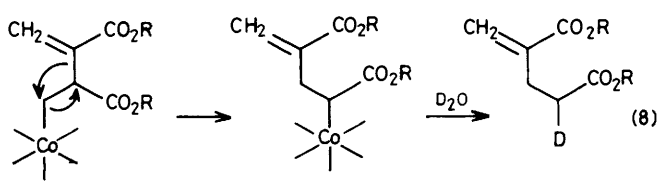
The principal issue at this stage in the exploration of the B_{12} model was the regiochemical sense of the model rearrangement in comparison to the enzymic one. There are two possible paths leading to α-methyleneglutaric acid (II). Either (a) the carboxy group or (b) the acrylate group can migrate [equation (7)].



(7)

Acrylate migration is the course followed in the enzymic reaction. This conclusion was inferred from labelling studies[2] carried out on ^{14}C-labelled nicotinic acid, which is degraded by the cell-free system which uses the coenzyme B_{12}-dependent carbon-skeleton rearrangement [equation (1)] as one of a number of steps in the catabolic transformation of nicotinic acid to acetic acid, propionic acid, carbon dioxide, and ammonia.

The experiment carried out in D_2O [equation (8)] demonstrates that acrylate is also the migrating group in the model rearrangement [equation (3)]. The argument requires



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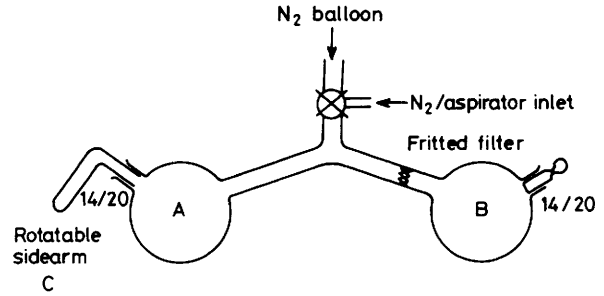

Figure 2. Diagram of double flask used in reaction

that the position of the deuterium reflects the position of the cobalt following rearrangement.

It is possible that the carboxy group is the migrating group and that primary rearrangement is followed by one in which a γ-hydrogen and β-cobalt undergo an interchange reaction. This would lead to the same labelling result following hydrolysis of the carbon–cobalt bond with D_2O. We regard this possibility as remote. However, it can and will be tested with a carbon-13 label.

It should be borne in mind that when the enzymic carbon-skeleton rearrangement reactions are carried out in D_2O, no label is incorporated into either substrate. In this important sense the model above is not sufficiently sophisticated; it contains no means of transferring hydrogen from substrate to substrate, the role played in the enzymatic reaction by the 5′-deoxyadenosine.

In every other respect, the model is a faithful nonenzymic representative of the enzyme-catalysed rearrangement reaction, the position of the deuterium at the γ-carbon having been demonstrated.

Experimental

Reaction of bis(tetrahydropyranyl) bromomethylitaconate (III) *with Vitamin* B_{12s} *in* D_2O.—A solution of 2.0 g (1.44 mmol) of hydroxocobalamin (Merck) in 120 ml of D_2O was placed in the left side (A) of the double flask (Figure 2). Bis(tetrahydropyranyl) bromomethylitaconate (III) (3.5 g, 9.3 mmol) was placed in the right side (B). The left side was fitted with a bent tube (C) in which 1.16 g (29.6 mmol) of $NaBH_4$ in 5 ml of D_2O was placed. The system was deoxygenated by evacuating and flushing the system ten times with nitrogen. The sodium borohydride was then added to the hydroxocobalamin by rotating sidearm C, and the resulting reaction mixture became grey-green after 10—15 min. The apparatus was then removed to a darkroom and the vitamin B_{12s} formed was added to the bromide (III) by tilting the apparatus.

After 30 min, a visible spectrum of an aliquot from the reaction showed maxima at 525, 440, 372, and 312 nm, and a minimum at 412 nm indicating formation of the alkyl cobalamin (IV). Upon exposure of the u.v. sample to light, a new band was formed at 352 nm indicative of the conversion of the adduct into hydroxocobalamin. The alkyl cobalamin (IV) is a sensitive substance and cannot be purified by extraction with phenol. Accordingly, the product was precipitated from the reaction mixture with ice-cold acetone. The precipitate was filtered, washed once with 200 ml of cold acetone and dried yielding 3.92 g of red solid (IV). A visible spectrum of this product was identical with that described above.

The alkyl cobalamin (IV) was dissolved in 180 ml of D_2O and allowed to stand for 400 h in the dark under an atmosphere of nitrogen at pH 9.3 and 24 °C. The slow formation of hydroxocobalamin was signalled by the development of the

characteristic band at 352 nm in the visible spectra of aliquots removed from the reaction mixture at 24 h intervals.

When the reaction was complete, the mixture was made acidic with 10% DCl, then extracted continuously overnight with ether. The ether extract was dried (Na_2SO_4), filtered, and the solvent was evaporated yielding 0.24 g of a crude solid product. An n.m.r. spectrum of this substance revealed the presence of three products derived from the organic ligand: β-methylitaconic acid (**I**), butadiene-2,3-dicarboxylic acid (**V**), and α-methyleneglutaric acid (**II**). The mixture was passed through a silica gel column (9 g, 10 cm × 11 mm) with 400 ml of 50:50 ethyl acetate–hexane. The resulting oil (0.146 g) was re-chromatographed on a silica gel column (12 g, 30 cm × 11 mm). Fractions (10 ml each) 6—28 (0.055 g) eluted with 20 : 80 ethyl acetate–hexane contained a mixture of α-methyleneglutaric acid (**II**) and methylitaconic acid (**I**). Fractions 31—36 contained butadiene-2,3-dicarboxylic acid (**V**) (0.013 g). The crude butadiene-2,3-dicarboxylic acid was crystallized from ether–CCl_4 yielding 8 mg of white crystals (2.9%), m.p. 182—185 °C; reported m.p. 185—187 °C. The proton n.m.r. spectrum ([$^2H_6$]acetone) showed vinyl proton doublets at δ 6.2 (J = 1.5 Hz) and δ 5.83 (J = 1.5 Hz). The mass spectrum showed the molecular ion at 142 with an M + 1 peak of normal intensity (6% of M^+).

Fractions 6—28 were combined and chromatographed again on 10 g of silica gel with 20:80 ethyl acetate–hexane yielding fractions containing pure α-methyleneglutaric acid [^{2}H]-(**II**) and pure methylitaconic acid [^{2}H]-(**I**). The α-methyleneglutaric acid [^{2}H]-(**II**) was crystallized from ether–chloroform yielding 6 mg (2.5%) with m.p. 130—132 °C. The proton n.m.r. spectrum (60 MHz, [$^2H_6$]acetone) showed vinyl proton multiplets at δ 6.24 and 5.73 and a three-proton singlet at δ 2.56. In the 250 MHz n.m.r. spectrum, the apparent singlet (at 60 MHz) at δ 2.56 is split into a multiplet, the high-field portion of which is reduced in intensity by half in comparison with that of the undeuteriated α-methyleneglutaric acid (**II**). In the mass spectrum, the molecular ion is extremely weak in both the 15 and 70 eV spectra; however, the extent of deuterium incorporation can be judged using the base peak at m/z 99 ($M^+ - H_2O - CO$); exact mass calculated for $C_5H_6DO_2$, 99.043 06; Found, 99.043 06, and a peak at m/z 127 ($M^+ - H_2O$). These peaks occur at 98 ($M^+ - H_2O - CO$); exact mass: calculated for $C_5H_6O_2$, 98.0368; Found, 98.0352, and 126, respectively, in the undeuteriated sample. By this means, it was estimated that the product contained 88% α-methylene[^{2}H]glutaric acid. The position of incorporation of deuterium in [^{2}H]-(**II**) was established by the proton-decoupled carbon-13 n.m.r. spectrum in D_2O, which showed carboxy carbons at δ 180.23 and 173.28, vinyl carbons at δ 141.14 and 129.88, the γ-methylene triplet (1:1:1, $J_{^{13}C-D}$ = 19.5 Hz) at δ 35.35, and the β-methylene singlet at δ 29.17 (see Figure 1).

[^{2}H]Methylitaconic acid (**I**) from the chromatography was crystallized from ether–chloroform yielding 4 mg (2%), m.p. 150—152 °C. The proton n.m.r. spectrum ([$^2H_6$]acetone) showed vinyl proton singlets at δ 6.3 and 5.78, a one-proton methine triplet at δ 3.59 (J = 6.5 Hz), and a two-proton deuteriomethyl doublet at δ 1.33 (J = 6.5 Hz). Mass spectrum (15 eV): m/z (relative intensity) 145 (5.6, M^+), 129 (100, $M^+ - CH_2D$). Molecular ion peaks at m/z 145 and 144 in the ratio of 4.67:1 corresponded to 82% [^{2}H]methylitaconic acid [^{2}H]-(**I**).

Authentic Sample of γ-Deuterio-α-methyleneglutaric Acid.—(*a*) A mixture of 0.360 g (1.61 mmol) of γ-bromo-α-methyleneglutaric acid[4] and 0.5 g (7.65 mmol) of freshly prepared Zn–Cu couple[5] in 10 ml of CH_3CO_2D, freshly prepared from acetic anhydride and D_2O, was stirred at room temperature for 5 h. The excess of acetic acid was removed under vacuum. The residue was treated with 1 ml of 10% HCl then continuously extracted for 15 h with ether. Evaporation of the ether extract, after drying ($MgSO_4$), yielded 0.221 g of crude product. Chromatography on a 1 × 30 cm silica gel column and elution with 15% ethyl acetate in hexane yielded 0.125 g of crystalline γ-deuterio-α-methyleneglutaric acid, m.p. 131—132 °C. The proton and carbon-13 n.m.r. spectra of the product were identical with those of the rearrangement product [^{2}H]-(**II**), described above.

(*b*) A solution of 0.935 g (0.69 mmol) of hydroxocobalamin in 60 ml of D_2O was reduced under an inert atmosphere to vitamin B_{12s} by the addition of 0.560 g (14.8 mmol) of sodium borohydride in 2 ml of D_2O. To this solution was added 1.409 g (4.511 mmol) of bis(tetrahydropyranyl) γ-bromo-α-methyleneglutaric acid. The mixture was allowed to stand in the dark for 72 h. The solution was made acidic to pH 1 by the addition of DCl. The resulting solution was then extracted continuously overnight with ether yielding 1.945 g of a thick oil. Multiple chromatography on silica gel yielded 0.03 g of pure γ-deuterio-α-methyleneglutaric acid (30% based on hydroxocobalamin). The mass spectrum showed this material to be deuteriated to the extent of 86%. The proton and carbon-13 n.m.r. spectra were identical with those obtained with the rearrangement product [^{2}H]-(**II**) above.

Acknowledgements

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