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Mechanism-Based Inactivation of Coenzyme B_{12}-Dependent 2-Methyleneglutarate Mutase by (*Z*)-Glutaconate and Buta-1,3-diene-2,3-dicarboxylate

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In the presence of *holo* 2-methyleneglutarate mutase, buta-1,3-diene-2,3-dicarboxylate and (*Z*)-glutaconate [(*Z*)-pent-2-ene-1,5-dicarboxylate], but not (*E*)-glutaconate, each induced homolysis of the Co–C bond of coenzyme B_{12} to afford cob(II)alamin and the 5′-deoxyadenosyl radical. The latter probably added to the double bond in (*Z*)-glutaconate and one of the double bonds in buta-1,3-diene-2,3-dicarboxylate to afford a corresponding "radical adduct". The formation of new radicals and cob(II)alamin was diagnosed by UV/Visible and EPR spectroscopy. (*Z*)-Glutaconate rapidly inactivated the mutase with formation of aquocobalamin, which was possibly derived by electron transfer from cob(II)alamin to the radical adduct. In contrast, buta-1,3-diene-2,3-dicarboxylate was a much slower inactivator. In this case, the spectroscopic data revealed a relatively stable complex of the radical adduct with cob(II)alamin in the active site of the enzyme.

Introduction

Coenzyme B_{12}-dependent enzymes comprise eliminases and mutases, both of which catalyse molecular rearrangements, whereby a functional group undergoes a 1,2-shift enabling conversion of substrate to product. In the eliminases, the migrating group is amino or hydroxy, whereas in the mutases it is either amino or a carbon-based group. The mechanisms of these reactions have been extensively discussed and reviewed.[1–3] For the mutases, experimental and theoretical data have recently led to the proposal that in the mutases cob(II)alamin assists the group migration by stabilising intermediate methylene radicals.[3]

2-Methyleneglutarate mutase from the strict anaerobic bacterium *Eubacterium barkeri*, formerly called *Clostridium barkeri*, catalyses the reversible carbon-skeleton rearrangement of 2-methyleneglutarate (**1a**) to (*R*)-3-methylitaconate (**2a**, 2-methylene-3-methylsuccinate) in the pathway of nicotinate fermentation.[4] Binding of the substrate **1a** to the enzyme induces homolytic cleavage of the Co–C σ bond in the prosthetic group coenzyme B_{12} to afford cob(II)alamin and the 5′-deoxyadenosyl radical. This highly reactive methylene radical abstracts the 4-*Re*-hydrogen atom from 2-methyleneglutarate to yield the substrate-derived 2-methylene-4-ylglutarate radical (**1b**) and 5′-deoxyadenosine. Cob(II)alamin coupled to an organic radical, possibly 2-methylene-4-ylglutarate (**1b**), can be observed by EPR spectroscopy.[5] It has been proposed that the substrate radical either fragments into acrylate and the 2-acrylate radical ($CH_2{=}C^{\cdot}HCO_2^-$), which recombine to the product-related (*R*)-3-methylene-itaconate radical (**2b**), or the latter radical is derived via an intermediate 1,2-dicarboxycyclopropylmethyl radical (**3**) (see Scheme 1).[5] Recently, Newcomb and Miranda have proposed a variant ("polar/heterolytic") of fragmentation–recombination in which the intermediates are the 2-carbanion of acrylate ($CH_2{=}C^-CO_2^-$) and the carboxyl radical of acrylate ($CH_2{=}CHCO_2^{\cdot}$).[6] The reaction cycle is completed by a H-atom transfer from 5′-deoxyadenosine to the 3-methylene-itaconate radical (**2b**) yielding the product **2a**. Finally, the coenzyme is regenerated by combining cob(II)alamin and the 5′-deoxyadenosyl radical, thereby reforming the Co–C σ-bond. In this paper we report the use of stable analogues of the proposed intermediate radicals with π bonds at those carbon atoms that during catalysis are converted into the sp^2 centres of radicals. In principle, these compounds could either competitively inhibit the enzyme or the 5′-deoxyadenosyl radical might add at a double bond to form an adduct radical and cob(II)alamin, thus leading to inactive enzyme. We synthesised (*Z*)-glutaconic acid [**4a**, (*Z*)-pent-2-ene-1,5-dicarboxylic acid] as a potential analogue of the 2-methylene-4-ylglutar-

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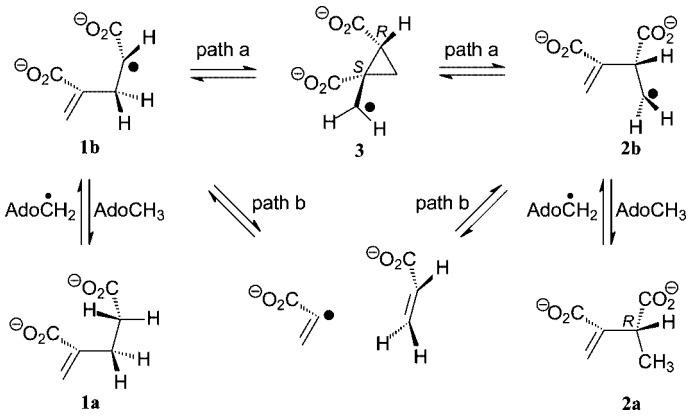
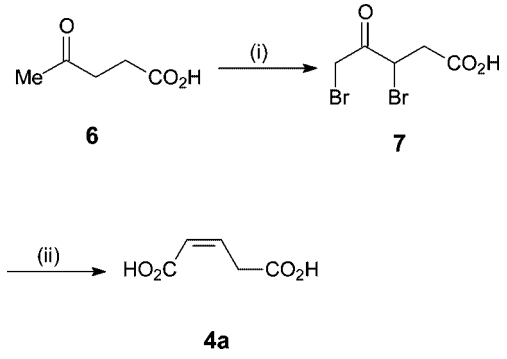


Scheme 1. Proposed reaction pathways for 2-methyleneglutarate mutase. (Path a) Addition-elimination mechanism with the participation of an intermediate 1-methylene-1,2-cyclopropanedicarboxylate radical **3**, for which the (1*S*,2*R*)-isomer is shown {$AdoCH_2$ = 5′-deoxyadenosyl; $AdoCH_3$ = 5′-deoxyadenosine}. (Path b) Fragmentation–recombination mechanism for 2-methyleneglutarate mutase involving an acrylate molecule and a 2-acrylate radical.

ate radical (**1b**), as well as buta-1,3-diene-2,3-dicarboxylic acid (**5**) as an analogue of the (*R*)-3-methyleneitaconate radical (**2b**). The commercially available (*E*)-glutaconic acid (**4b**) was also studied. Interestingly, compounds **4a** and **5** were inactivators of 2-methyleneglutarate mutase, whereas **4b** had no effect on the enzyme.

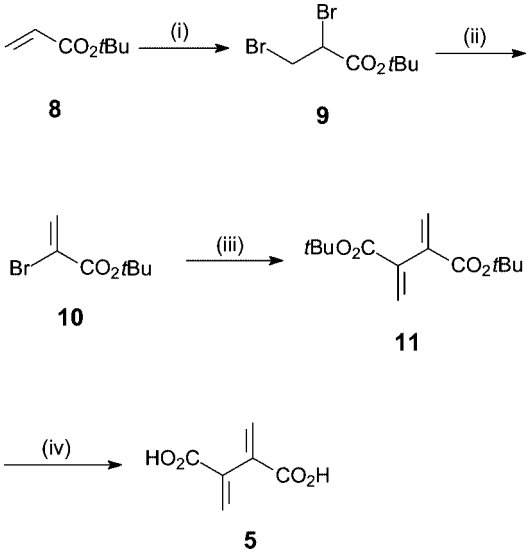
Synthesis of Inhibitors

The synthesis of (*Z*)-glutaconic acid (**4a**, Scheme 2) was based on the procedure described in ref.[7] (Scheme 2). Thus, laevulinic acid (**6**) was cleanly dibrominated to 3,5-dibromolaevulinic acid (**7**), which underwent a Favorski rearrangement on treatment with potassium hydrogen carbonate to give exclusively (*Z*)-glutaconic acid with no trace of the (*E*)-isomer **4b** according to ^{1}H NMR analysis.



Scheme 2. Synthesis of (*Z*)-glutaconic acid (**4a**) [reagents: (i) Br_2, aq. 48% HBr; (ii) aq. $KHCO_3$].

The synthesis of buta-1,3-diene-2,3-dicarboxylic acid (**5**, Scheme 3) was performed via the corresponding di-*tert*-butyl ester, the preparation of which was closely modelled on that of dimethyl buta-1,3-diene-2,3-dicarboxylate.[8] Thus, *tert*-butyl acrylate (**8**) was treated with bromine to give *tert*-butyl 2,3-dibromopropionate (**9**), which was dehydrobrominated to *tert*-butyl 2-bromoacrylate (**10**) using triethylamine as base. The *tert*-butyl 2-bromoacrylate was subjected to nickel(0)-induced coupling to afford di-*tert*-butyl buta-1,3-diene-2,3-dicarboxylate (**11**), which was deprotected with trifluoroacetic acid to give buta-1,3-diene-2,3-dicarboxylic acid (**5**).



Scheme 3. Synthesis of buta-1,3-diene-2,3-dicarboxylic acid (**5**) [reagents and conditions: (i) Br_2, DCM, <10 °C; (ii) Et_3N, diethyl ether, 0 °C to room temperature; (iii) $(cycloocta\text{-}1,5\text{-}diene)_2Ni$, Ph_3P, diethyl ether, 25 °C, 4.5 h; (iv) TFA, room temperature].

Enzyme Inhibition Studies

The activity of 2-methyleneglutarate mutase was determined in a coupled assay using 3-methylitaconate Δ-isomerase as an auxiliary enzyme.[5] The formation of 2,3-dimethylmaleate was followed spectrophotometrically at λ = 256 nm. At first all three compounds were checked for their ability to inhibit or inactivate the isomerase. Whereas the glutaconates (**4a**, **4b**) did not affect the enzyme at all, 1 mM buta-1,3-diene-2,3-dicarboxylate (**5**) caused complete inactivation within 3 min under the conditions of the assay. Possibly a cysteine residue at the active site of the isomerase

added to one of the conjugated double bonds of this substrate analogue. This interesting result was not pursued further for the time being.

Because both glutaconates did not inhibit the isomerase, their action on the mutase could be studied directly in the coupled assay. It was found that 0.2 mM (*Z*)-glutaconate (**4a**) already inactivated the mutase by 60% within 1 min; after 5 min the inactivation was complete. Furthermore, whereas 1 mM (*Z*) isomer **4a** completely inactivated the mutase within 1 min, 1 mM (*E*) isomer **4b** exhibited no effect during this time. Only with 4 mM **4b** was there a slight deviation from the control observed after the first minute. ^{1}H NMR spectroscopy revealed that the commercial **4b** used in this study contained ca. 2% **4a**. This was deduced from the integrated signal at δ = 6.55 ppm (β-H of **4a**) as compared to 7.03 ppm (**4b**). Hence the observed inactivation is completely specific for the (*Z*) isomer **4a**. In order to show that inactivation of 2-methyleneglutarate mutase only occurred with the *holo*-enzyme, coenzyme B_{12}-free *apo*-2-methyleneglutarate mutase was incubated for 10 min with **4a**, which was then removed by gel filtration through Sephadex G25. Following the addition of coenzyme B_{12} to the recovered *apo*-enzyme, the reconstituted *holo*-enzyme was found to be fully active towards 2-methyleneglutarate.

The effect of **5** on the enzyme was assessed with 15 μM *apo*-2-methyleneglutarate mutase (subunit m = 67 kDa), 100 μM coenzyme B_{12}, 2 mM dithiothreitol and 1 mM **5** in 100 mM potassium phosphate (pH 7.4) which were incubated for 30 min at 25 °C. Afterwards, the inhibitor was removed by gel filtration and the activity of the recovered enzyme was measured in the coupled assay. The results showed that incubation of the *holo*-enzyme with **5** decreased the activity of the enzyme by 45 ± 2% as compared to that of a control without **5**. We also studied the mutase under steady-state conditions and found that in the presence of excess of coenzyme B_{12} and 10 mM 2-methyleneglutarate the *apo*-mutase lost 80% of its activity after 20 min (50% after 10 min) or about 6 million turnovers; within the next 20 min the decrease was much slower, leading to 15% residual activity. In the absence of either coenzyme B_{12} or substrate the enzyme remained stable during this time.

UV/Visible spectroscopy of the reconstituted (*holo*) 2-methyleneglutarate mutase, from which the excess of coenzyme B_{12} had been removed by gel filtration, exhibited a broad peak at 535 nm and a shoulder at around 570 nm (Figure 1). Upon addition of 25 mM 2-methyleneglutarate the absorbance of both features decreased, and the peak shifted by about 5 nm to 530 nm. A new peak arose at 470 nm indicating the formation of cob(II)alamin. These spectral changes were already observed after 1 min; incubation for a further 5 min revealed only a slight increase in the intensities of the new peaks. Addition of 10 mM **5** to *holo*-mutase resulted in the same changes but with a twofold increase in intensity. An almost identical spectrum was obtained after 30 min incubation of the apoenzyme with 1 mM **5** and an excess of coenzyme B_{12}, followed by removal of the unbound small molecules through gel filtration. Different spectral changes were observed by addition of 10 mM **4a** to the *holo*-enzyme. The peak at 535 nm shifted to 521 nm and a new peak arose at 495 nm. The same concentration of the (*E*) isomer **4b** modified the spectrum in a similar manner but to a lesser extent, and is ascribed to 2% **4a** as an impurity. The new peaks revealed the formation of aquocobalamin, the oxidation product of cob(II)alamin.

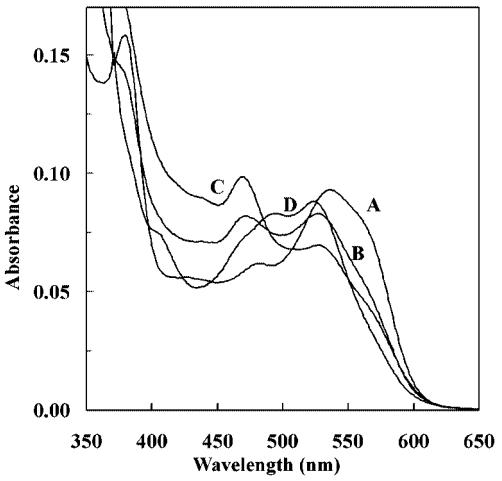


Figure 1. Visible spectra of 20 μM reconstituted *holo*-2-methyleneglutarate mutase (A) in the presence of 25 mM 2-methyleneglutarate (B) or 10 mM buta-1,3-diene-2,3-dicarboxylate (**5**, C) or 10 mM (*Z*)-glutaconate (**4a**, D). The addition of the dicarboxylate caused 10% dilution. The spectra were run 6 min after the additions.

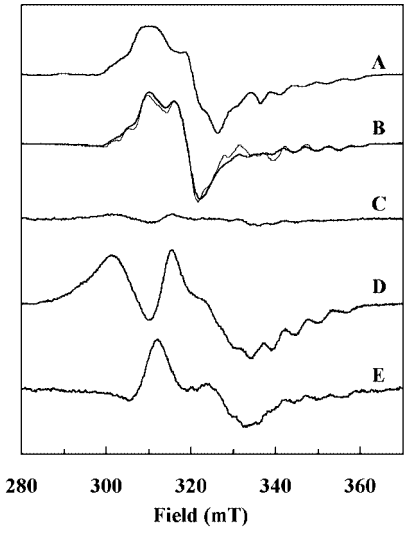


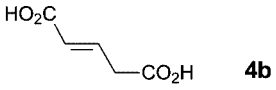
Figure 2. EPR spectra of 270 μM 2-methyleneglutarate mutase incubated in the presence of 1.6 mM coenzyme B_{12} and 40 mM of 2-methyleneglutarate (A) or acrylate (B) or [2,3,3-$^2H_3$]acrylate (B, sharper trace) or (*E*)-glutaconate (**4b**, C) or (*Z*)-glutaconate (**4a**, D) or buta-1,3-diene-2,3-dicarboxylate (**5**, E). EPR conditions: temperature, 40 K for A, 77 K for B–E; microwave power 2 mW (A), 1.3 mW (B), 10 mW for C–E; microwave frequency 9463 ± 1 MHz (A) or 9435 ± 2 MHz (B–E); modulation amplitude 0.5 mT (A), 1.25 mT (B–E). Spectra were normalised to identical amplitudes, except for (*E*)-glutaconate. Thus, the spectra of the glutaconates can be directly compared.

EPR spectra were obtained by mixing 270 μM *apo*-methyleneglutarate mutase (final concentration; 18 mg protein mL^{-1}) in 50 mM Tris/HCl (pH 7.5) and 75 mM NaCl with 1.6 mM coenzyme B_{12}, and 40 mM substrate or inactivator, followed by freezing in liquid pentane/N_2 within 30 s. The results are shown in Figure 2. The spectra revealed that 2-methyleneglutarate, as well as the inactivators **4a** and **5**, led to the formation of stable enzyme-bound organic radicals, which interact with cob(II)alamin.[5] In the case of **4a** the concentration dependence of the EPR signal was studied. Saturation was observed with an apparent K_i of 0.9 ± 0.1 mM. The determination of the nature of the radicals (see below) has to await the syntheses of ^2H- and ^{13}C-labelled inactivators. Interestingly, acrylate also induced the formation of radicals even though it was recently shown not to act as an inhibitor.[5] This result was confirmed with perdeuterated acrylate, which sharpened the signal.

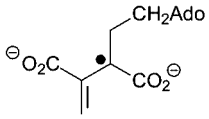
Discussion

In this paper we describe two mechanism-based inactivators of 2-methyleneglutarate mutase. Buta-1,3-diene-2,3-dicarboxylate (**5**) can be regarded as a close analogue of the product (*R*)-3-methylitaconate (**2a**): just the methyl group is converted to a second methylene group. Unfortunately we were not able to perform steady-state kinetics with **5**, because it reacted rapidly and irreversibly with the auxiliary enzyme, the isomerase. However, incubation of **5** with the mutase resulted in partial inactivation, formation of oxygen-insensitive cob(II)alamin and induction of an organic radical interacting with cob(II)alamin. Interestingly the same features were observed with the substrate 2-methyleneglutarate (**1a**) itself; only the EPR spectrum was somewhat different. Hence **5** behaved superficially like 2-methyleneglutarate. However, whereas the 5′-deoxyadenosyl radical abstracts a hydrogen atom from C-4 of 2-methyleneglutarate, with **5** the 5′-deoxyadenosyl radical most likely added at one of its methylene groups leading to an allyl radical **12**. A similar proposal has been made for the inactivation of glutamate mutase induced by 2-methyleneglutarate.[9] The partial inactivation of 2-methyleneglutarate mutase by **5** suggests that the proposed radical addition may be reversible. The substrate analogue **5** may fit sufficiently well into the enzyme active site that the adduct produced by the interaction of **5** with the 5′-deoxyadenosyl radical protects cob(II)alamin from interaction with oxygen.

The inactivator **4a** is not so closely related to the substrate 2-methyleneglutarate because it lacks the *exo*-methylene group. However, it apparently fits well into the active site, possibly because the distance between the two carboxylates is similar to that in the enzyme-bound conformation of (*R*)-3-methylitaconate. This could be the explanation for the inability of the (*E*) isomer **4b** to inactivate the enzyme. The (*Z*) isomer reacts rapidly with the mutase as shown by activity measurements and EPR spectroscopy. Already 60 s after mixing under anoxic conditions an organic radical interacting with cob(II)alamin was detected. In the visible spectra, however, which were taken after 1 and 6 min (Figure 1), only aquocob(III)alamin rather than cob(II)alamin could be seen. This could be due to an electron transfer from cob(II)alamin to the initially derived adduct radical from **4a** and the 5′-deoxyadenosyl radical.



4b



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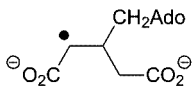

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(AdoCH$_2$ = 5'-deoxyadenosyl)

The adduct radical is possibly **13**, arising from addition of the 5′-deoxyadenosyl radical to C-3 of **4a**. Similar electron transfers have been observed from cob(II)alamin to substrate-derived radicals in β-lysine 5,6-aminomutase[10] and in variants of methylmalonyl-CoA mutase.[11]

Experimental Section

Enzymology: The preparation and assay of 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase, as well as the method of recording the EPR spectra, have been described previously.[5] In the standard assay, 2-methyleneglutarate mutase activity (0.05–0.1 unit) was measured with 10 mM 2-methyleneglutarate as substrate in 100 mM potassium phosphate (pH 7.4, 25 °C) using 3-methylitaconate isomerase (10–30 units/mL) as auxiliary enzyme. The 2,3-dimethylmaleate formed was measured by its UV absorbance (ε_{256} = 0.66 $mM^{-1} cm^{-1}$). For the inactivation studies (*E*)- and (*Z*)-glutaconate and buta-1,3-diene-2,3-dicarboxylate were dissolved in aqueous KOH to give 0.1 M solutions (pH ca. 7). In order to remove small molecules from the enzymes, PD-10-desalting columns containing Sephadex® G-25 Medium from GE Healthcare (Amersham Biosciences) were used. Concentrations (mM or μM) given in this paper are the final concentration for a particular component in the reaction mixture.

3,5-Dibromolaevulinic Acid (7): Bromine (13.7 g, 86 mmol) was added dropwise to a mixture of laevulinic acid (5.0 g, 43 mmol) and 48% aqueous HBr (4.3 mL, 38 mmol) at 0 °C over 30 min. The mixture was stirred at 0 °C for 4 h. The excess of bromine was removed using a nitrogen flow. The resulting solution was stirred overnight at 0 °C to give a solid, which was taken up in ethyl acetate (40 mL) and washed with saturated aqueous sodium metabisulfite (3 × 25 mL). After drying ($MgSO_4$), the solvent was removed to afford crude product as a pale yellow solid. Recrystallisation from ethyl acetate/petroleum ether afforded the title compound as a white solid (7.83 g, 66% yield). M.p. 110–112 °C (lit.[7] m.p. 111–113). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.97 (dd, J = 5.6 and 17.6 Hz, 1 H, C*H*HCO_2H), 3.29 (dd, J = 8.1 and 17.4 Hz, 1 H, CH*H*CO_2H), 4.07 (d, J = 13.2 Hz, 1 H, C*H*HBr), 4.28 (d, J = 12.9 Hz, 1 H, CH*H*Br), 4.94 (br. t, J = 6.0 Hz, 1 H, CHBr) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 30.9 (C-2), 38.4 (C-5), 41.1 (C-3), 174.5 (C-1), 194.4 (C-4) ppm. $C_5H_6O_3Br_2$: calcd. C 21.93, H 2.21, found C 22.02, H 2.27. IR (KBr disc): $\tilde{\nu}$ = 3000 (m), 1724 (s), 1700 (s), 611 (m) cm^{-1}.

(*Z*)-Glutaconic Acid (4a): $KHCO_3$ (17.0 g, 169 mmol) in water (210 mL) was added to 3,5-dibromolaevulinic acid (5.5 g, 21 mmol). The mixture was vigorously stirred for 19 h. When constant titration values against methyl orange were obtained, the reaction was quenched by acidification (aq. HCl) and extracted with diethyl ether (10 × 30 mL). After drying the combined organic extracts (Na_2SO_4) the solvent was removed. The resulting yellow solid was recrystallised from acetonitrile (decolourisation by charcoal) to give the title compound as a white solid (0.21 g, 8% yield). M.p. 130–132 °C (lit.[7] m.p. 136–136.5, lit.[12] 136–136.5). ^{1}H NMR (300 MHz, CD_3OD): δ = 3.71 (dd, J = 3.0 and 7.0 Hz, 2 H, CH_2CO_2H), 5.93 (dt, J = 2.0 and 11.5 Hz, 1 H, CH), 6.52 (dt, J = 7.0 and 11.6 Hz, 1 H, CH) ppm. ^{13}C NMR [δ_C (75 MHz, CD_3OD)]: δ = 35.2 (C-4), 123.5 (C-3), 142.2 (C-2), 169.5 (C-1), 174.7 (C-5) ppm. $C_5H_6O_4$: calcd. C 46.16, H 4.65; found C 45.99, H 4.62. IR (KBr disc): $\tilde{\nu}$ = 3066 (m), 1686 (s), 1649 (s) cm^{-1}.

***tert*-Butyl 2,3-Dibromopropionate (9):** Bromine (2.9 ᴍ) in DCM (10 mL) was added dropwise to *tert*-butyl acrylate (2.0 g, 15.6 mmol) in DCM (20 mL), cooled below 10 °C over 20 min. The mixture was stirred at ca. 5 °C for 2 h at which stage an orange colour persisted. The solvent was removed to afford the title compound as a colourless oil (4.41 g, 90% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.43 [s, 9 H, $C(CH_3)_3$], 3.57 (dd, J = 4.4 and 9.8 Hz 1 H, CH*H*Br), 3.81 (dd, J = 9.8 and 11.3 Hz, 1 H, C*H*HBr), 4.27 (dd, J = 4.4 and 11.3 Hz, 1 H, $CHBr_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.1 [$C(CH_3)_3$], 41.0 (C-3), 43.3 (C-2), 83.9 [$C(CH_3)_3$], 166.8 (C-1) ppm.

***tert*-Butyl 2-Bromoacrylate (10):** Triethylamine (0.77 g, 0.80 mL, 7.7 mmol) in diethyl ether (5 mL) was added dropwise to *tert*-butyl 2,3-dibromopropionate (3.0 g, 7.0 mmol) in dry diethyl ether (15 mL), cooled to 0 °C . The mixture was stirred overnight at room temperature and then filtered. The filtrate was washed with water (3 × 10 mL), dried (Na_2SO_4) and the solvent was removed. The title compound was obtained as a pale yellow oil for which no further purification was needed (1.72 g, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 [s, 9 H, $C(CH_3)_3$], 6.13 (d, J = 1.5 Hz, 1 H, CH*H*), 6.77 (d, J = 1.5 Hz, 1 H, C*H*H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.3 [$C(CH_3)_3$], 83.4 [$C(CH_3)_3$], 123.8 (C-3), 129.2 (C-2), 161.1 (C-1) ppm.

Di-*tert*-butyl Buta-1,3-diene-2,3-dicarboxylate (11): Bis(1,5-cyclooctadiene)nickel(0) (1.5 g, 5.3 mmol) and triphenylphosphane (2.8 g, 10.6 mmol) were suspended in dry diethyl ether (20 mL) at 25 °C under nitrogen in a flame-dried Schlenk tube. Neat *tert*-butyl 2-bromoacrylate (1.87 g, 8.9 mmol) was added rapidly via syringe and the reaction mixture was stirred at 25 °C for 4.5 h. The mixture was diluted with diethyl ether (20 mL), washed with water (2 × 25 mL), dried ($MgSO_4$) and the solvent was removed. The crude product was purified by medium pressure chromatography (silica, elution with 5% ethyl acetate in petrol). The product containing fractions (R_f = 0.36 on silica, same solvent system) were collected and the solvent was removed to afford the title compound as a colourless oil (0.48 g, 47% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.42 [s, 18 H, $C(CH_3)_3$], 5.60 (d, J = 1.7 Hz, 2 H, CH*H*), 6.06 (d, J = 1.7 Hz, 2 H, C*H*H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.4 [$C(CH_3)_3$], 81.3 [$C(CH_3)_3$], 116.6 (CH_2), 126.0 (C-2, C-3), 165.3 (C-1) ppm.

Buta-1,3-diene-2,3-dicarboxylic Acid (5): Trifluoroacetic acid (TFA) (1.0 mL) was added to di-*tert*-butyl buta-1,3-diene-2,3-dicarboxylate (0.16 g, 0.63 mmol) in DCM (2 mL). The resulting solution was stirred at room temperature for 20 min. The solvent was removed and the title compound was obtained as a white solid (0.08 g, 90% yield). Recrystallisation from acetonitrile gave an analytically pure sample. M.p. 184–186 °C (lit.[13] m.p. 186–188, lit.[8] 192–192.5). ^{1}H NMR (300 MHz, CD_3OD): δ = 5.85 (d, J = 1.5 Hz, 2 H, C*H*H), 6.22 (d, J = 1.5 Hz, 2 H, CH*H*) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ = 128.1 (CH_2), 140.1 (C-2, C-3), 167.1 (C-1) ppm. IR (KBr disc): $\tilde{\nu}$ = 3066 (m), 1675 (s), 1614 (s) cm^{-1}.

Acknowledgments

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