Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic Chemistry 33 (2005) 53–66
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Synthesis of ^{13}C-labeled γ-hydroxybutyrates for EPR studies with 4-hydroxybutyryl-CoA dehydratase

Ulrike Näser[a], Antonio J. Pierik[b], Richard Scott[a], Irfan Çinkaya[b], Wolfgang Buckel[b], Bernard T. Golding[a,*]

[a] *School of Natural Sciences—Chemistry, University of Newcastle upon Tyne, Bedson Building, Newcastle upon Tyne, NE1 7RU, United Kingdom*
[b] *Laboratorium für Mikrobiologie, Fachbereich Biologie, Phillips-Universität, Marburg, D-35032, Germany*

Received 11 August 2004
Available online 13 October 2004

Abstract

4-Hydroxybutyryl-CoA dehydratase from *Clostridium aminobutyricum* catalyses the reversible dehydration of its substrate 4-hydroxybutyryl-CoA (4-HB-CoA) to crotonyl CoA. The enzyme contains one $[4Fe–4S]^{2+}$ cluster and one flavin adenine dinucleotide (FAD) molecule per homotetramer. Incubation of the enzyme with its substrate under equilibrium conditions followed by freezing at 77 K induced the EPR-spectrum of a neutral flavin semiquinone ($g = 2.005$, linewidth 2.1 mT), while at 10 K additional signals were detected. In an attempt to characterize these signals, 4-HB-CoA molecules specifically labeled with ^{13}C have been synthesized. This was achieved via ^{13}C-labeled γ-butyrolactones, which were obtained from ^{13}C-labeled bromoacetic acids by efficient synthetic routes. Incubation of the ^{13}C-labeled 4-hydroxybutyrate-CoA molecules with 4-hydroxybutyryl-CoA dehydratase did not lead to marked broadening of the signals.

Keywords: Isotopic labeling; γ-Butyrolactone; 4-Hydroxybutyryl-CoA dehydratase; Flavin adenine dinucleotide semiquinone; EPR spectroscopy

* Corresponding author. Fax: +44 191 222 6929.
E-mail address: B.T.Golding@ncl.ac.uk (B.T. Golding).

0045-2068/$ - see front matter
doi:10.1016/j.bioorg.2004.09.001

1. Introduction

Clostridium aminobutyricum ferments γ-aminobutyrate (GABA, 4-aminobutyrate) to ammonia, acetate, and butyrate [1]. γ-Aminobutyrate is converted into 4-hydroxybutyrate via amino transfer to 2-oxoglutarate giving glutamate and succinate semialdehyde, which is reduced by NADH [2,3]. Reaction of 4-hydroxybutyrate with acetyl-CoA mediated by 4-hydroxybutyrate CoA-transferase yields 4-hydroxybutyryl-CoA [4], which is reversibly dehydrated to crotonyl-CoA [3,5]. In the final step, disproportionation of crotonyl-CoA leads to acetate and butyrate. 4-Hydroxybutyryl-CoA dehydratase has been characterized as a homotetramer (260 kDa) containing one FAD and one $[4Fe–4S]^{2+}$ cluster per subunit [5,6]. The crystal structure has recently been solved and shown to be related to that of medium chain acyl-CoA dehydrogenase. Modeling of 4-hydroxybutyryl-CoA into the putative active site revealed an interaction of the hydroxyl group with the [4Fe–4S] cluster, which is coordinated by three cysteine and one histidine side chains [7]. The dehydratase catalyses a mechanistically most intriguing transformation, because the C-3 hydrogen that needs to be eliminated is not activated ($pK_a = 40$) [8]. The proposed catalytic mechanism starts with the abstraction of the C-2 proton from 4-hydroxybutyryl-CoA, followed by one-electron transfer to FAD [9,10]. The resulting semiquinone radical anion removes the pro-*S* hydrogen from C-3 of the accompanying enoxy radical, [11] the pK_a of which is estimated to be ca. 14 [12]. This process yields a ketyl radical anion and a neutral FAD semiquinone radical. The ketyl radical anion eliminates the hydroxyl group to give a dienoxy radical, which is reduced by the FAD semiquinone radical to a dienolate. Finally, this species is protonated at the C-4 position to afford the product crotonyl-CoA (Scheme 1).

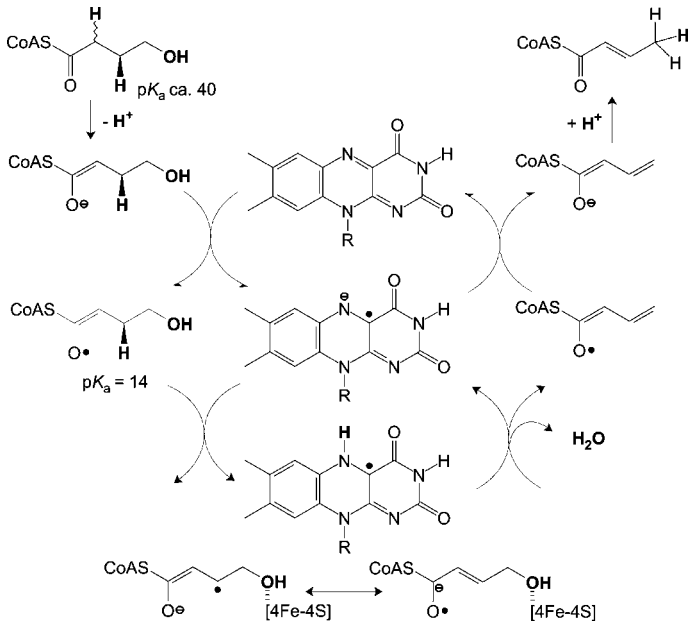
When isolated under strictly anaerobic conditions, about 20% of the FAD of 4-hydroxybutyryl-CoA dehydratase is in the neutral semiquinone form, indicating that the enzyme is able to stabilize this radical. Full enzymatic activity is achieved after oxidation of the FAD semiquinone to FAD quinone with ferricyanide [13].

We have found that upon addition of substrate, part of the FAD is reduced to the semiquinone as revealed by EPR spectroscopy at 10 and 77 K. At the lower temperature, additional signals were obtained which could be due to substrate-derived radicals interacting with the $[4Fe–4S]^{2+}$ cluster. To characterize these signals we have synthesized specifically ^{13}C-labeled γ-butyrolactones (Fig. 1), which were hydrolyzed with aq. sodium hydroxide to 4-hydroxybutyrates and converted enzymatically into 4-hydroxybutyryl-CoA species using 4-hydroxybutyrate CoA-transferase [4].

2. Results and discussion

2.1. Chemistry

All the syntheses were optimized on unlabeled starting material. For ^{13}C-labeled γ-butyrolactone, a synthesis was devised starting from commercially available ^{13}C-labeled bromoacetic acid. The acid was esterified with diazomethane, and then either



Scheme 1. Proposed mechanism for the dehydration of 4-hydroxybutyryl-CoA mediated by 4-hydroxybutyryl-CoA dehydratase.

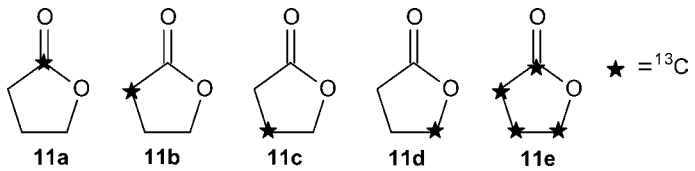


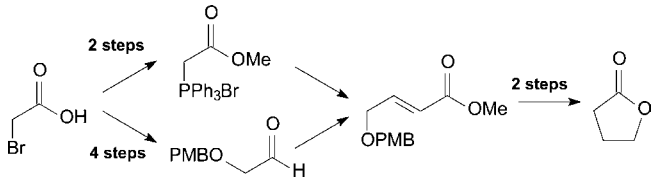
Fig. 1. ^{13}C-labeled γ-butyrolactones synthesized and used in the present study: **11a** γ-[2-^{13}C]butyrolactone, **11b** γ-[3-^{13}C]butyrolactone, **11c** γ-[4-^{13}C]butyrolactone, **11d** γ-[5-^{13}C]butyrolactone, **11e** γ-[2,3,4,5-^{13}C]butyrolactone.

converted to a Wittig salt or an aldehyde. Combining the Wittig salt with the aldehyde yielded an alkene which upon reduction and deprotection afforded the appropriate γ-butyrolactone (Scheme 2).

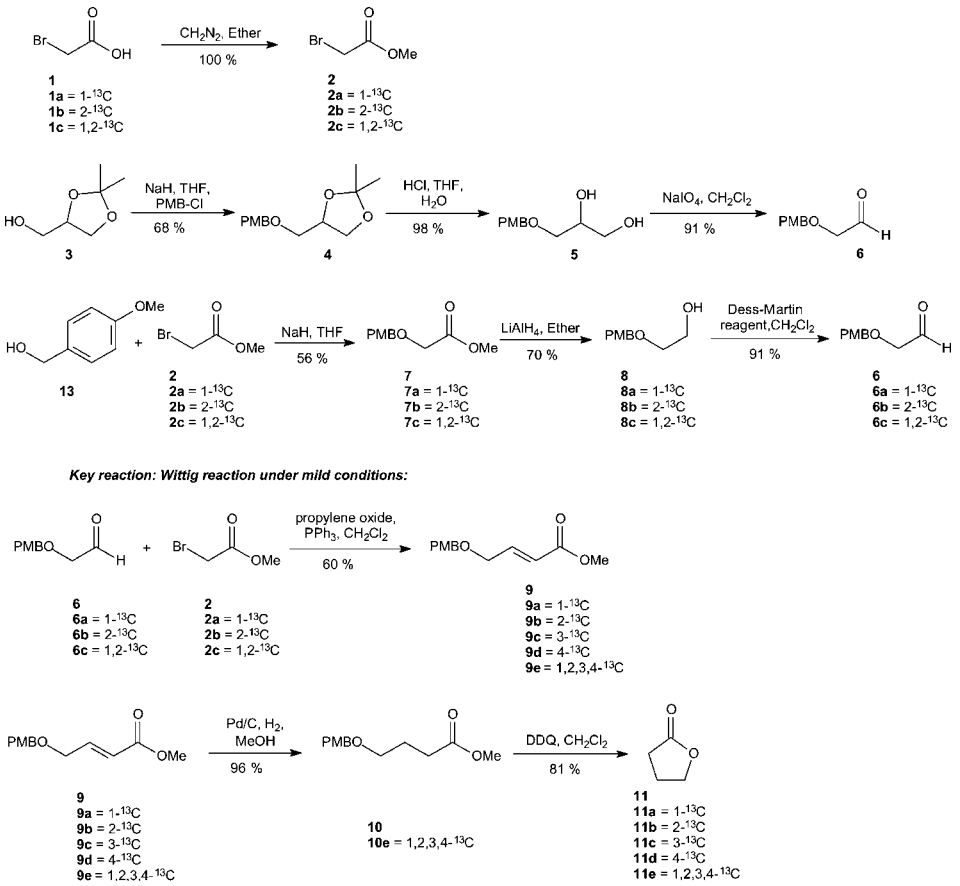
2.2. Labeling

Starting from [1-^{13}C]-, [2-^{13}C]-, or [1,2-^{13}C]-labeled bromoacetic acid and employing either labeled or unlabeled starting material in each branch of the synthesis, enabled the introduction of ^{13}C into all individual positions of the target γ-butyrolactone (Scheme 3). $^{13}C_4$-labeled γ-butyrolactone was also synthesized.

Bromoacetic acid (**1**) was converted to the corresponding methyl ester (**2**) using diazomethane, as the method is clean and quantitative [14]. The synthesis of



Scheme 2. Synthetic route to γ-butyrolactone.



Scheme 3. Syntheses of the labeled γ-butyrolactones.

unlabeled (4-methoxybenzyloxy)acetaldehyde (**6**) was achieved in three steps starting from *rac.*-1,2-di-*O*-isopropylideneglycerol, Solketal (**3**), to give the desired unlabeled aldehyde in an overall yield of 61% [15]. The synthesis of labeled (4-methoxybenzyloxy)acetaldehyde (**6**) was achieved in three steps: reaction of (**2**) with the sodium salt of 4-methoxybenzyl alcohol using the method of Lal et al. [16] followed by a

reduction of (4-methoxybenzyloxy)acetic acid methyl ester (**7**) with lithium aluminium hydride to give 2-(4-methoxybenzyloxy)ethanol (**8**). Oxidation of alcohol (**8**) to the corresponding aldehyde (**6**) was performed using Dess–Martin periodinane (1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)) [17,18]. The key step in the synthetic pathway is the Wittig reaction of (**6**) with labeled or unlabeled bromoacetic acid methyl ester (**2**) under mild conditions [19]. The alkene 4-(4-methoxybenzyloxy)but-2-enoic acid methyl ester (**9**) was reduced to 4-(4-methoxybenzyloxy)butanoic acid methyl ester (**10**) and finally deprotection, using 2,3-dichloro-5, 6-dicyano-1,4-benzoquinone (DDQ), gave γ-butyrolactone (**11**) [20]. The crude ^{13}C-labeled γ-butyrolactones (**11a–e**) were purified by preparative gas chromatography using dichloromethane as the trapping solvent. The dichloromethane was removed in vacuo to give a γ-butyrolactone suitable for conversion into 4-hydroxybutyrate.

2.3. Biochemistry and EPR-spectroscopy

The γ-butyrolactones (**11a–e**) were each hydrolyzed with one equivalent of sodium hydroxide to yield the corresponding 4-hydroxybutyrate, which was subsequently converted to its CoA-ester by incubation with acetyl-CoA and 4-hydroxybutyrate CoA-transferase [4]. The integrity and ^{13}C-labeling of the CoA-esters was confirmed by MALDI-TOF mass spectrometry. The 4-hydroxybutyryl-CoA dehydratase was incubated anoxically with ferricyanide and desalted by gel filtration. The oxidized enzyme and the CoA-thiol esters were combined and frozen at 77 K.

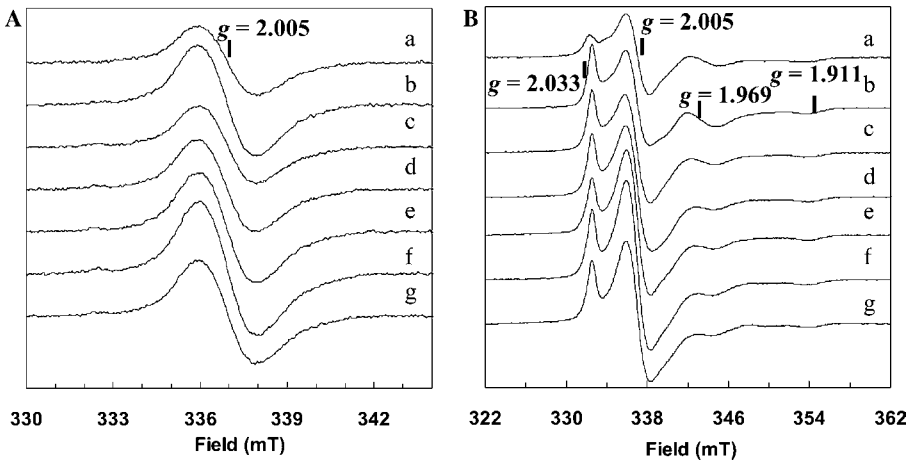


Fig. 2. EPR spectra of the neutral FAD semiquinone radical (A) and neutral FAD semiquinone radical with superimposed additional signals (B) of 9.9 μM tetrameric 4-hydroxybutyryl-CoA dehydratase in 100 mM potassium phosphate (pH 7.4) incubated in the presence of (a) 2 mM CoA, (b) unlabeled and [1-^{13}C]-, [2-^{13}C]-, [3-^{13}C]-, [4-^{13}C]-, [1,2,3,4-^{13}C]-labeled 4-hydroxybutyryl-CoA (trace c–g, respectively). 4-Hydroxybutyryl-CoA dehydratase was oxidized with potassium ferricyanide and desalted, as described in materials and methods. EPR conditions: microwave frequency 9460 ± 1 MHz; microwave power 2 μW (A) or 20 mW (B); modulation amplitude 0.55 mT; modulation frequency 100 kHz; temperature 77 K (A), 10 K (B).

The EPR signal of the oxidized enzyme markedly increased upon addition of substrate, regardless of whether unlabeled substrate or the five differently ^{13}C-labeled 4-hydroxybutyryl-CoA species were used. The EPR spectra at 77 K and at 10 K (Fig. 2) clearly revealed an intense isotropic signal of the neutral FAD semiquinone radical (line width 2.1 ± 0.1 mT $g = 2.005$, cf. [13]). At the temperature of 10 K additional signals above and below $g = 2.005$ were detected (g values 2.033, 1.969, and 1.911), and probably arise from a partner radical of the FAD semiquinone radical. All spectra with the unlabeled and labeled substrates were standardized to equal intensity at the sharpest signal at $g = 2.033$ (linewidth 1.2 mT). The small variations observed at the broader signals ($g = 1.969$ and 1.911, with linewidths of 2.8 and 3.2 mT, respectively) can be attributed to unavoidable variations of the experimental conditions. However, the interaction of the substrate-derived radicals with excited states of the $[4Fe–4S]^{2+}$ cluster could have caused broadening of the signals in such a way that ^{13}C-induced coupling could no longer be observed. The low temperature (10 K) required for visualization of the additional signals is consistent with such an interaction.

3. Conclusion

We have shown that the synthesis of ^{13}C-labeled γ-butyrolactones can be readily achieved from commercially available starting materials. Using the methods described, it is possible to insert the ^{13}C-label in any one or more of the carbon positions. All of the steps proceeded in good to excellent yield, therefore avoiding significant loss of ^{13}C-label. The use of diazomethane and Dess–Martin periodinane, although potentially hazardous, posed no problem in our hands, and were milder and cleaner than the alternatives. Alkaline hydrolysis of the γ-butyrolactones obtained, followed by enzymatic thioesterification, afforded the corresponding labeled 4-hydroxybutyryl-CoA.

Incubation of 4-hydroxybutyryl-CoA dehydratase with 4-hydroxybutyryl-CoA led to the formation of radicals. Whereas one radical could be identified as the neutral flavin semiquinone radical, the identity of the other species remains elusive. Owing to a possible interaction with excited states of the $[4Fe–4S]^{2+}$ cluster, the additional signals observed at 10 K could have arisen from substrate-derived radicals, despite the lack of ^{13}C-induced line broadening. This is the first indication of the involvement of substrate-derived radicals in the reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA and is consistent with the proposed mechanism (Scheme 1).

4. Experimental

4.1. Caution

Dess–Martin periodinane [17,18] must be handled with caution as it can hydrolyze to iodoxybenzoic acid, which is shock sensitive. Diazomethane is a potent

carcinogen and is also shock sensitive, and therefore must be handled in an efficient hood behind a safety screen.

4.2. General

All chemicals were obtained from either Aldrich (Gillingham, Dorset, UK) or Lancaster (Newgate, Lancashire, UK) and were used without further purification. Solvents were dried by standard techniques. ^{1}H NMR spectra were run at 200, 300, or 500 MHz and ^{13}C NMR at 50.3, 75.45, or 125.75 MHz, using residual signals from the deuterated solvent as references; D_2O (4.81 ppm), d_6-DMSO (2.50 ppm), chloroform (7.25 ppm) for ^{1}H spectra; d_6-DMSO (39.7 ppm), chloroform (77.0 ppm) for ^{13}C spectra. All coupling constants were measured in Hertz. Sep-Pak C_{18}-cartridges were supplied by Whatman International (Maidstone, Kent, UK). Other chromatographic materials were obtained from VWR International (Poole, Dorset, UK).

4.3. Enzyme experiments and EPR spectroscopy

γ-Butyrolactone was converted into the corresponding 4-hydroxybutyrate using 1.1 eq. of 1 M NaOH solution. The formation of the sodium salt was quantitative. The 1 M solution was neutralized to pH 7 using 0.5 M HCl. The synthesis of 4-hydroxybutyryl-CoA esters was performed using 1 mM labeled 4-hydroxybutyrate (50 μl) in 1 M potassium phosphate (50 μl) pH 7.4; 32 mM acetyl-CoA (16 μl), transferase (15 μl, 53 U/ml), total sample volume of 0.5 ml. After incubation at rt for 10 min, a DTNB-assay [5,5′-dithiobis(2-nitrobenzoate)] [4] was carried out to determine whether the conversion of 4-hydroxybutyrate into its corresponding CoA-ester was complete. The synthesized 4-hydroxybutyryl-CoA esters were purified using a C_{18}-cartridge. The column was initially washed with 1 column volume (cv) of methanol and with 10 cv's of 0.1% TFA to equilibrate. The CoA-ester was loaded and washed with 10 cv's of 0.1% TFA, which were discarded. The column was dried by purging with pressurized air, eluted with 1 cv of 0.1% TFA/50% MeCN, and further rinsed with 5 cv's of 0.1% TFA/50% MeCN. The fractions containing the CoA ester were collected, the solutions were concentrated (speed vac) and the aqueous residue was freeze dried. MALDI-TOF mass spectrometry of the CoA-esters was performed with an Applied Biosystems Voyager System 2048 using α-cyano-4-hydroxycinnamic acid as the matrix [21].

The 4-hydroxybutyryl-CoA dehydratase was treated in an anaerobic chamber with 10 mM ferricyanide at pH 7.0 for 30 min. Low molecular mass compounds were separated from the enzyme by gel filtration on Sephadex G25. Labeled or unlabeled 6 mM hydroxybutyryl-CoA ester (59 μl) in 500 mM potassium phosphate (35 μl, pH 7.4), 396 μM 4-hydroxybutyryl-CoA dehydratase (17.5 μl, 21.4 mg ml^{-1}) and water (63.5 μl) were mixed and transferred to an EPR tube, which was immediately cooled in liquid nitrogen. The EPR spectra of these samples were recorded using an *X*-band Bruker EPR spectrometer equipped with an Oxford Instruments flow cryostat using liquid nitrogen or helium as coolant.

4.4. Synthesis

4.4.1. Conversion of the ^{13}C-labeled bromoacetic acid (**1**) to ^{13}C-labeled methyl bromoacetate (**2**)

4.4.1.1. This reaction must be performed in an efficient hood and behind a safety screen. To a labeled bromoacetic acid (**1**) (1.0 g, 7.1 mmol) in diethyl ether (20 mL) was added a 0.3–0.4 M solution of diazomethane in diethyl ether [14] until the reaction mixture remained yellow. The excess of diazomethane was removed by passing N_2 through the ethereal mixture and into a trap containing dilute acetic acid. The ether was removed to yield labeled methyl bromoacetate (**2**) as a pale yellow liquid (1.1 g, 7.2 mmol, 100%). ^{1}H NMR (200 MHz, $CDCl_3$) methyl bromo[1-^{13}C]acetate (**2a**): δ 3.78 (2 H, d, J 4.7 Hz, CH_2), 3.73 (3H, d, J 3.9 Hz, OCH_3); methyl bromo[2-^{13}C]acetate (**2b**): δ 3.78 (2H, d, J 153.4 Hz, CH_2), 3.73 (3H, s, OCH_3); methyl bromo[1,2-^{13}C]acetate (**2c**): δ 3.79 (2H, dd, J 4.7 and 153.3 Hz, CH_2), 3.74 (3H, d, J 3.9 Hz, OCH_3).

4.5. Syntheses of (4-methoxybenzyloxy)acetaldehyde (**6**)

4.5.1. Method A

4.5.1.1. Rac.-3-O-4′-methoxybenzyl-1,2-di-O-isopropylideneglycerol (4). *Rac.*-1,2-di-*O*-isopropylideneglycerol (**3**) (8.9 g, 67 mmol) in THF (30 mL) was added dropwise to a stirred suspension of sodium hydride (60% oil dispersion, 3.0 g, 80 mmol, prewashed with petrol) in dry THF (30 mL) under N_2. After stirring for 15 min, *p*-methoxybenzyl chloride (10.6 g, 67 mmol) in dry THF (30 mL) was added dropwise. The mixture was heated at reflux overnight and the progress of reaction was monitored by TLC (petrol/ethyl acetate, 4:1, starting material $R_f = 0.12$ and product $R_f = 0.56$). After 20 h, the mixture was cooled in an ice-bath and water (25 mL) was added. The volatile solvents were removed and the orange residue was partitioned between water (30 mL) and ethyl acetate (30 mL). The aqueous phase was further extracted with ethyl acetate (4×20 mL). The combined organic fractions were washed with water (3×20 mL), sat. $NaHCO_3$ (2×20 mL), dried ($MgSO_4$), filtered, and concentrated to give a yellow liquid (15.1 g). The crude product was purified by medium pressure chromatography (MPC) on silica eluting with petrol/ethyl acetate (4:1) to afford (**4**) as a colorless liquid (11.6 g, 68%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (2H, d, J 8.5 Hz, H-2′ and H-6′), 6.82 (2H, d, J 8.6 Hz, H-3′ and H-5′), 4.44 (2H, s, pmb-OCH_2), 4.21 (1H, m, H-2), 3.97 (2H, m, 2× H-1), 3.73 (3H, s, OCH_3), 3.39 (2H, m, 2× H-3), 1.34 (3H, s, CH_3), 1.29 (3H, s, CH_3). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 159.66, 130.41, 129.79, 114.18, 109.77, 75.14, 73.56, 71.16, 67.32, 55.66, 27.17, and 25.77.

4.5.1.2. 3-O-4′-methoxybenzylglycerol (5). Aqueous hydrochloric acid (1 M, 100 mL) was added to a solution of (4) (11.5 g, 45.6 mmol) in THF (100 mL) at room temperature. The reaction was monitored by TLC (ether, starting material $R_f = 0.95$ and product $R_f = 0.21$) until the starting material had disappeared. After 6 h the solution was neutralized with sat. $NaHCO_3$ and the volatiles were removed under reduced pressure. The resulting solution was extracted with ethyl acetate (4×50 mL), the combined organic layers were dried ($MgSO_4$), filtered, and the

solvent was removed. The residue was taken up in ether and treated dropwise with petrol until precipitation persisted and a two-phase system was formed. The mixture was stirred vigorously and cooled in an ice bath to give a white solid that was dried in vacuo to give crystalline (5) (9.54 g, 98%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.28 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.91 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.51 (2H, s, pmb-OCH_2), 3.89 (1H, m, H-2), 3.84 (3H, s, OCH_3), 3.67 (2H, m, 2× H-1), 3.56 (2H, m, H-3), 2.96 (1H, s, HOCH), 2.54 (1H, s, HOCH_2). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 159.54 (C-4′), 129.92 (C-1′), 129.63 (C-2′ and C-6′), 114.05 (C-3′ and C-5′), 73.39 (pmb-OCH$_2$), 71.65 (C-2), 70.78 (C-1), 64.24 (OCH_3), 55.45 (C-3).

*4.5.1.3. (4-Methoxybenzyloxy)acetaldehyde (**6**).* Sodium(meta)periodate (2.1 g, 9.9 mmol) and water (20 μL) were added to (**5**) (2.0 g, 9.0 mmol) in dichloromethane (20 mL) and the solution was vigorously stirred overnight. After the starting material had disappeared (TLC ethyl acetate, starting material $R_f = 0.32$ and product $R_f = 0.78$), the mixture was filtered and concentrated, and the residue was purified by medium pressure chromatography (MPC) on silica with petrol/ethyl acetate (2:3) as eluent to give (**6**) as a colorless oil (1.47 g, 91%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.78 (1H, s, CHO), 7.23 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.49 (2H, s, pmb-OCH_2), 4.00 (2H, s, 2× H-2), 3.74 (3H, s, OCH_3). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 200.72 (CHO), 159.73 (C-4′), 129.85 (C-2′ and C-6′), 128.93 (C-1′), 114.08 (C-3′ and C-5′), 75.08 (pmb-OC H$_2$ or C-2), 73.43 (pmb-OC H$_2$ or C-2), 55.38 (OCH_3).

4.5.2. Method B

*4.5.2.1. (4-Methoxybenzyloxy)acetic acid methyl ester (**7**).* Sodium hydride (60% oil dispersion, 0.60 g, 16 mmol, pre-washed with petrol) was suspended in dry THF (10 mL) under N_2. 4-Methoxybenzyl alcohol (1.9 g, 14 mmol) was added portionwise (1 h) with stirring at room temperature until the evolution of H_2 ceased. Methyl bromoacetate (**2**) (2.1 g, 14 mmol) was added dropwise at 0 °C (1 h). The reaction mixture was slowly brought to room temperature and stirring was continued for 20 h. After the starting material had disappeared (TLC petrol/ethyl acetate, 3:2, starting material $R_f = 0.51$ and product $R_f = 0.74$) the reaction mixture was diluted with ethyl acetate (42 mL) and slowly poured over crushed ice (56 g) containing ammonium chloride (1.4 g). The ethyl acetate layer was separated, washed with sat. brine (100 mL) and dried ($MgSO_4$). The solvent was removed and the residual oil was purified by MPC on silica with petrol/ethyl acetate (2:3) as eluent to yield (**7**) as a pale yellow liquid (1.60 g, 56%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.81 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.50 (2H, s, pmb-OCH_2), 4.01 (2H, s, 2× H-2), 3.74 (3H, s, OCH_3), 3.69 (3H, s, $COOCH_3$). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 171.52 (C $OOCH_3$), 159.82 (C-4′), 129.94 (C-2′ and C-6′), 129.22 (C-1′), 114.04 (C-3′ and C-5′), 73.13 (pmb-OCH$_2$), 66.92 (CH_2), 55.43 (OCH_3), 51.98 (COOCH$_3$).

*4.5.2.2. 2-(4-Methoxybenzyloxy)ethanol (**8**).* Compound (**7**) (0.50 g, 2.4 mmol) was added to a solution of $LiAlH_4$ (0.10 g, 2.6 mmol) in ether (20 mL) under N_2

in a way that the reaction mixture gently boiled at reflux. After the addition was complete, the mixture was further heated at reflux for 2 h until the starting material had disappeared (TLC petrol/ethyl acetate, 3:2, starting material $R_f = 0.74$ and product $R_f = 0.21$). Ethyl acetate (500 μL) and water (250 μL) were added and the mixture was filtered. The solvent was removed and the residual oil was purified by MPC on silica with petrol/ethyl acetate (3:2) as eluent to yield (**8**) as a colorless liquid (0.32 g, 70%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.82 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.42 (2H, s, pmb-OCH_2), 3.74 (3H, s, OCH_3), 3.67 (2H, m, 2× H-1), 3.49 (2H, t, J 4.5 Hz, 2× H-2), 2.03 (1H, br s, OH). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 159.85 (C-4′), 130.36 (C-1′), 129.74 (C-2′ and C-6′), 114.19 (C-3′ and C-5′), 73.36 (pmb-OCH_2), 71.37 (C-1), 62.29 (C-2), 55.60 (OCH_3).

*4.5.2.3. (4-Methoxybenzyloxy)acetaldehyde (**6**).* Compound (**8**) (0.30 g, 1.6 mmol) was added to a solution of Dess–Martin periodinane (0.80 g, 1.9 mmol) in dry dichloromethane (20 mL) in the dark at room temperature. The reaction was vigorously stirred and monitored by TLC (petrol/ethyl acetate, 3:2, starting material $R_f = 0.22$ and product $R_f = 0.5$) until no starting material remained. The reaction was poured onto a silica column and eluted with petrol/ethyl acetate (2:3) to give (**6**) as a colorless oil (0.27 g, 91%). NMR data as quoted before.

*4.6. Syntheses leading to γ-butyrolactone (**11**)*

*4.6.1. 4-(4-Methoxybenzyloxy)but-2-enoic acid methyl ester (**9**)*

A solution of (4-methoxybenzyloxy)acetaldehyde (0.50 g, 2.8 mmol), triphenylphosphine (1.9 g, 7.3 mmol) and methyl bromoacetate (0.65 g, 4.3 mmol) in dry DCM (20 mL) was cooled to 0 °C. Propylene oxide (2.44 g, 42.1 mmol) was added under N_2 and the resulting mixture was stirred at room temperature for 4 days (monitored by TLC, petrol/ethyl acetate, 3:2, starting material $R_f = 0.45$ and product $R_f = 0.72$). Removal of the solvent gave an oil, which was triturated with petrol (3 × 10 mL). After the last addition the mixture was left overnight. Filtration and removal of the solvent gave a crude product that was purified by MPC on silica, eluting with petrol/ethyl acetate (5:1) to give (**9**) as a colorless liquid (0.39 g, 60%), which comprised of a mixture of two alkene isomers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.92 (1H, dt, J 4.2 and 15.5 Hz, H-2_{trans}), 6.82 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.36 (1H, dt, J 4.9 and 11.9 Hz, H-2_{cis}), 6.05 (1H, dt, J 2.1 and 15.9 Hz, H-3_{trans}), 5.75 (1H, dt, J 2.1 and 11.6 Hz, H-3_{cis}), 4.42 (2H, s, pmb-OCH_2), 4.08 (2H, dd, J 2.2 and 4.6 Hz, CH_2), 3.74 (3H, s, OCH_3), 3.68 (3H, s, $COOCH_3$). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 166.78 (*C*$OOCH_3$), 159.36 (C-4′), 148.73 (*C*H = CH_{cis}), 144.72 (*C*H = CH_{trans}), 129.77 (C-1′), 129.32 (C-2′ and C-6′), 120.91 (CH = *C*H_{trans}), 119.01 (CH = *C*H_{cis}), 113.87 (C-3′ and C-5′), 72.44 (pmb-O*C*H_2), 68.18 (CH_2), 55.38 (OCH_3), 51.31 ($COOCH_3$).

*4.6.2. 4-(4-Methoxybenzyloxy)butanoic acid methyl ester (**10**)*

Compound (**9**) (0.10 g, 0.42 mmol) and palladium catalyst (10 wt. % on activated carbon, 0.08 g) were stirred in dry methanol (25 mL) under a hydrogen atmosphere.

The reaction was monitored by TLC (petrol/ethyl acetate, 3:2, starting material $R_f = 0.74$ and product $R_f = 0.65$). After 30 h the catalyst was removed by filtration (Celite) and the filtrate was concentrated to yield (**10**) as a colorless liquid (0.90 g, 96%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.80 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.37 (2H, s, pmb-OCH_2), 3.73 (3H, s, OCH_3), 3.58 (3H, s, $COOCH_3$), 3.41 (2H, t, J 6.1 Hz, 2× H-4), 2.36 (2H, t, J 7.3 Hz, 2× H-2), 1.87 (2H, quint, J 6.4 Hz, 2× H-3). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 166.58 (COOCH$_3$), 159.26 (C-4′), 129.41 (C-1′), 129.15 (C-2′ and C-6′), 113.97 (C-3′ and C-5′), 72.34 (pmb-OCH$_2$), 69.03 (C-4), 55.47 (OCH_3), 51.41 (COOC H$_3$), 31.08 (C-2), 25.26 (C-3).

*4.6.3. γ-Butyrolactone (**11**)*

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (70 mg, 0.34 mmol) was added to a solution of (**10**) (70 mg, 0.29 mmol) in dichloromethane (10 mL) and water (0.5 mL) at 0 °C. The mixture was stirred until TLC showed disappearance of the starting material (petrol/ethyl acetate, 3:2, starting material $R_f = 0.68$ and product $R_f = 0.45$). The resulting solution was stirred with aq. NaOH (1 M, 10 mL) overnight. The aqueous layer was washed with DCM (4 × 20 mL) and the washings discarded. After acidification of the aqueous layer with aq. HCl (3 M, 20 mL), DCM (20 mL) was added and the mixture was stirred for 2 h. The layers were separated and the aqueous phase was further extracted with DCM (4 × 20 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent was removed to give crude γ-butyrolactone (**11**) (30 mg, 81%). Further purification was achieved by preparative GC (23 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.32 (2H, t, J 7.1 Hz, 2× H-5), 2.48 (2H, t, J 7.6 Hz, 2× H-3), 2.24 (2H, quint, J 2.4 Hz, 2× H-4). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.76 (C-2), 68.59 (C-5), 27.89 (C-3), 22.29 (C-4).

4.7. NMR-data of the labeled compounds

*4.7.1. 4-(4-Methoxybenzyloxy)but-2-enoic[1-^{13}C]acid methyl ester (**9a**)*

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.21 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.97 (1H, m, H-2$_{trans}$), 6.82 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.37 (1H, m, H-2$_{cis}$), 6.02 (1H, m, H-3$_{trans}$), 5.77 (1H, m, H-3$_{cis}$), 4.41 (2H, s, pmb-OCH_2), 4.05 (2H, dd, J 2.4 and 4.8 Hz, CH_2), 3.74 (3H, s, OCH_3), 3.64 (3H, d, J 3.9 Hz, $^{13}COOCH_3$).

*4.7.2. γ-[2-^{13}C]Butyrolactone (**11a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.29 (2H, dt, J 3.1 and 7.0 Hz, 2× H-5), 2.43 (2H, dt, J 5.8 and 8.3 Hz, 2× H-3), 2.20 (2H, m, 2× H-4). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.67 (s, ^{13}C-2, enhanced), 68.45 (d, J 3.0 Hz, C-5), 27.74 (d, J 49.0 Hz, C-3), 22.14 (s, C-4).

*4.7.3. 4-(4-Methoxybenzyloxy)but-2-enoic[2-^{13}C]acid methyl ester (**9b**)*

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.96 (1H, m, H-2$_{trans}$), 6.82 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.39 (1H, m, H-2$_{cis}$), 6.19 (1H, m,

H-3_{trans}), 5.67 (1H, m, H-3_{cis}), 4.42 (2H, s, pmb-OCH_2), 4.54 (2H, m, CH_2), 3.74 (3H, s, OCH_3), 3.67 (3H, s, $COOCH_3$).

*4.7.4. γ-[3-^{13}C]Butyrolactone (**11b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.29 (2H, dt, J 2.5 and 7.0 Hz, 2× H-5), 2.43 (2H, dm, J 81.0 Hz, 3-$^{13}CH_2$), 2.20 (2H, m, 2× H-4). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.75 (d, J 50.0 Hz, C-2), 68.47 (d, J 2.0 Hz, C-5), 27.96 (s, ^{13}C-3, enhanced), 22.19 (d, J, 34.0 Hz, C-4).

*4.7.5. (4-Methoxybenzyloxy)acetic[1-^{13}C]acid methyl ester (**7a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.81 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.50 (2H, s, pmb-OCH_2), 4.05 (2H, dd, J 7.1 and 14.4 Hz, 2× H-2), 3.83 (3H, s, OCH_3), 3.75 (3H, d, J 1.3 Hz, $^{13}COOCH_3$).

*4.7.6. 2-(4-Methoxybenzyloxy)[1-^{13}C]ethanol (**8a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.41 (2H, s, pmb-OCH_2), 3.75 (3H, s, OCH_3), 3.69 (2H, m, 2× H-1), 3.29 (2H, m, 2× H-2), 2.01 (1H, br s, OH).

*4.7.7. (4-Methoxybenzyloxy)[1-^{13}C]acetaldehyde (**6a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (1H, d, J 175.5 Hz, ^{13}CHO), 7.29 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.88 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.55 (2H, s, pmb-OCH_2), 4.05 (2H, dd, J 0.6 and 4.0 Hz, CH_2), 3.79 (3H, s, OCH_3).

*4.7.8. 4-(4-Methoxybenzyloxy)but-2-enoic[3-^{13}C]acid methyl ester (**9c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.92 (1H, dt, J 4.3 and 15.9 Hz, H-2_{trans}), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.75 (1H, dt, J 4.3 and 15.6 Hz, H-2_{cis}), 6.05 (1H, m, H-3_{trans}), 5.75 (1H, m, H-3_{cis}), 4.43 (2H, s, pmb-OCH_2), 4.09 (2H, m, CH_2), 3.75 (3H, s, OCH_3), 3.68 (3H, s, $COOCH_3$).

*4.7.9. γ-[4-^{13}C]Butyrolactone (**11c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.27 (2H, dt, J 2.2 and 7.0 Hz, 2× H-5), 2.43 (2H, dt, J 2.7 and 8.3 Hz, 2× H-3), 2.20 (2H, dm, J 81.0 Hz, 4-$^{13}CH_2$). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.43 (s, C-2), 68.26 (d, J 31.0 Hz, C-5), 27.53 (d, J 33.0 Hz, C-3), 22.19 (s, ^{13}C-4, enhanced).

*4.7.10. (4-Methoxybenzyloxy)acetic[2-^{13}C]acid methyl ester (**7b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.50 (2H, d, J 4.0 Hz, pmb-OCH_2), 4.05 (2H, dd, J 7.1 and 14.4 Hz, 2× H-2), 3.82 (3H, s, OCH_3), 3.75 (3H, s, $COOCH_3$).

*4.7.11. 2-(4-Methoxybenzyloxy)[2-^{13}C]ethanol (**8b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.84 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.39 (2H, d, J 4.0 Hz, pmb-OCH_2), 3.75 (3H, s, OCH_3), 3.69 (2H, m, 2× H-1), 3.45 (2H, m, 2× H-2), 2.01 (1H, br s, OH).

*4.7.12. (4-Methoxybenzyloxy)[2-^{13}C]acetaldehyde (**6b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (1H, d, J 26.0 Hz, CHO), 7.28 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.87 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.55 (2H, d, J 4.0 Hz, pmb-OCH_2), 4.05 (2H, d, J 141.0, CH_2), 3.79 (3H, s, OCH_3).

*4.7.13. 4-(4-Methoxybenzyloxy)but-2-enoic[4-^{13}C]acid methyl ester (**9d**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.93 (1H, dt, J 4.3 and 15.9 Hz, H-2$_{trans}$), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.75 (1H, dt, J 4.3 and 15.6 Hz, H-2$_{cis}$), 6.05 (1H, m, H-3$_{trans}$), 5.75 (1H, m, H-3$_{cis}$), 4.23 (2H, d, J 4.0 Hz, pmb-OCH_2), 3.94 (2H, m, CH_2), 3.72 (3H, s, OCH_3), 3.68 (3H, s, $COOCH_3$).

*4.7.14. γ-[5-^{13}C]butyrolactone (**11d**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.28 (2H, dt, J 151.9 and 7.0 Hz, 5-$^{13}CH_2$), 2.43 (2H, m, 2× H-3), 2.21 (2H, m, 2× H-4). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.42 (s, C-2), 68.88 (s, ^{13}C-5, enhanced), 28.19 (s, C-3), 22.16 (d, J 31.0 Hz, C-4).

*4.7.15. (4-Methoxybenzyloxy)acetic[1,2-^{13}C]acid methyl ester (**7c**)*

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.81 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.50 (2H, d, J 4.0 Hz, pmb-OCH_2), 4.02 (2H, m, 2× H-2), 3.76 (3H, s, OCH_3), 3.69 (3H, d, J 4.0 Hz, $COOCH_3$).

*4.7.16. 2-(4-Methoxybenzyloxy)[1,2-^{13}C]ethanol (**8c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.84 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.47 (2H, d, J 4.0 Hz, pmb-OCH_2), 3.79 (3H, s, OCH_3), 3.69 (2H, m, 2× H-2), 3.44 (2H, m, 2× H-1), 2.03 (1H, br s, OH).

*4.7.17. (4-Methoxybenzyloxy)[1,2-^{13}C]acetaldehyde (**6c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (1H, dd, J 26.0 and 175.5 Hz, CHO), 7.28 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.87 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.51 (2H, d, J 4.0 Hz, pmb-OCH_2), 4.05 (2H, dd, J 4.0 and 141.0, CH_2), 3.79 (3H, s, OCH_3).

*4.7.18. 4-(4-Methoxybenzyloxy)[1,2,3,4-^{13}C]but-2-enoic acid methyl ester (**9e**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (2H, d, J 8.6 Hz, H-2′ and H-6′), 7.05 (1H, m, H-2$_{trans}$), 6.83 (2H, d, J 8.7 Hz, H-3′ and H-5′), 6.72 (1H, m, H-2$_{cis}$), 6.22 (1H, m, H-3$_{trans}$), 5.88 (1H, m, H-3$_{cis}$), 4.43 (2H, d, J 4.3 Hz, pmb-OCH_2), 4.21 (2H, m, CH_2), 3.74 (3H, s, OCH_3), 3.69 (3H, d, J 4.0 Hz, $COOCH_3$).

*4.7.19. 4-(4-Methoxybenzyloxy)butanoic-[1,2,3,4-^{13}C] acid methyl ester (**10e**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.18 (2H, d, J 8.6 Hz, H-2′ and H-6′), 6.81 (2H, d, J 8.7 Hz, H-3′ and H-5′), 4.37 (2H, d, J 4.0, pmb-OCH_2), 3.76 (3H, s, OCH_3), 3.59 (3H, dd, J 4.0 and 10.1 Hz, $COOCH_3$), 3.41 (2H, m, 2× H-2), 2.47 (2H, t, J 7.3 Hz, 2× H-4), 1.72 (2H, m, 2× H-3).

*4.7.20. γ-[2,3,4,5^{13}C]Butyrolactone (**11e**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.28 (2H, dm, J 150.0 Hz, 2× H-5), 2.41 (2H, m, 2× H-3), 2.23 (2H, dm, J 255.0 Hz, 2× H-4). ^{13}C NMR (125.75 MHz, $CDCl_3$) δ 177.77 (dd, J 4.0 and 50.0 Hz, ^{13}C-2), 68.47 (dt, J 4.0 and 31 Hz, ^{13}C-5), 28.02 (ddd, J 2.0, 33.0 and 50.0 Hz ^{13}C-3), 22.49 (t, J 31.0 Hz, ^{13}C-4).

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft and the European Commission (Contract No HPRN-CT-2002-00195). We thank Professor Rolf K Thauer for access to the EPR spectrometer in the Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Straße, Marburg, Germany.

References

[1] J.K. Hardman, T.C. Stadtman, J. Bacteriol. 79 (1960) 544.
[2] J.K. Hardman, T.C. Stadtman, J. Biol. Chem. 238 (1963) 2081.
[3] P. Willadsen, W. Buckel, FEMS Microbiol. Lett. 70 (1990) 187.
[4] U. Scherf, W. Buckel, Appl. Environ. Microbiol. 57 (1991) 2699.
[5] U. Scherf, W. Buckel, Eur. J. Biochem. 215 (1993) 421.
[6] U. Müh, I. Çinkaya, S.P.J. Albracht, W. Buckel, Biochemistry 35 (1996) 11710.
[7] B.M. Martins, H. Dobbek, I. Çinkaya, W. Buckel, A. Messerschmidt, submitted to Proc. Natl. Acad. Sci. USA 2004 (in press).
[8] T.L. Amyes, J.P. Richard, J. Am. Chem. Soc. 114 (1992) 10297.
[9] W. Buckel, FEBS Lett. 389 (1996) 20.
[10] W. Buckel, B.T. Golding, FEMS Microbiol. Rev. 22 (1999) 523.
[11] R. Scott, U. Näser, P. Friedrich, T. Selmer, W. Buckel, B.T. Golding, Chem. Commun. 10 (2004) 1210.
[12] D.M. Smith, W. Buckel, H. Zipse, Angew. Chem. Int. Edn. Engl. 42 (2003) 1867.
[13] I. Çinkaya, W. Buckel, M. Medina, C. Gomez-Moreno, R. Cammack, Biol. Chem. 378 (1997) 843.
[14] B.S. Furniss, A.J. Hannaford, V. Rogers, P.W.G. Smith, A.R. Tatchell, Vogel's Textbook of Practical Organic Chemistry, fourth ed., Longman, London and New York, 1978, 291.
[15] B.T. Golding, L. Cottrell, D. Mackay, D. Zhang, W.P. Watson, Chem. Res. Toxicol. 16 (2003) 933.
[16] B. Lal, A.K. Gangopadhyay, R. Rajagopalan, A.V. Ghate, Bioorg. Med. Chem. 6 (1998) 2061.
[17] D.B. Dess, J.C. Martin, J. Org. Chem. 48 (1983) 4155.
[18] D.B. Dess, J.C. Martin, J. Am. Chem. Soc. 113 (1991) 7277.
[19] M. Bennet, G.B. Gill, G. Pattenden, A.J. Shuker, A. Stapleton, J. Chem. Perkin Trans. 1 (1991) 929–937.
[20] T.W. Green, P.G.M. Wuts, Protective Groups in Organic Synthesis, John Wiley, New York, 1999.
[21] R.C. Beavis, T. Chaughary, B.T. Chait, Org. Mass. Spectrom. 27 (1992) 156.