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A highly efficient synthesis of [1-^{13}C, ^{18}O]- and [1-^{13}C, $^{2}H_2$]-glycerol for the elucidation of biosynthetic pathways

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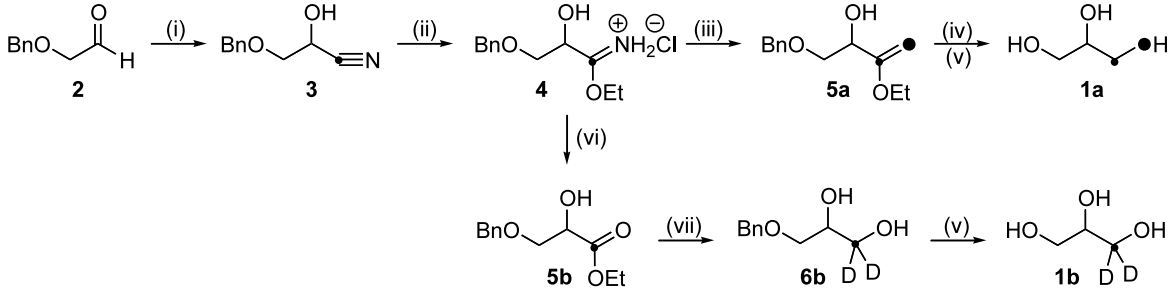
Abstract—Labeled glycerol is a widely used biochemical probe to investigate biosynthetic pathways. A highly efficient synthesis of [1-^{13}C, ^{18}O]- and [1-^{13}C, $^{2}H_2$]-glycerol is described in which the ^{13}C label is introduced using cyanide. The ^{18}O label was introduced by a Pinner synthesis and reduction of the ester **5** allowed incorporation of the ^{2}H labels.

Glycerol is an ubiquitous primary metabolite with numerous metabolic sources and is an important intermediate of energy metabolism.[1] Glycerol and glycerate are often efficiently incorporated into primary and secondary metabolites via a number of biosynthetically distinct pathways and isotopically labeled glycerols and glycerates have been used to investigate numerous biosynthetic pathways[2–5] and in metabolism studies.[6–9] Doubly isotopically labeled compounds enable bond-breaking, bond-forming and rearrangement processes in biosynthetic pathways to be investigated using NMR spectroscopy.[10] Detailed information on the oxidation levels of intermediates and oxidative and reductive processing steps of the metabolite can be obtained by using precursors labeled with ^{13}C in conjunction with ^{2}H and ^{18}O. We were particularly interested in the incorporation of glycerol into the polyketide metabolite soraphen A where we had observed incorporation of ^{13}C and deuterium from [1,3-$^{13}C_2$]- and [1-$^{2}H_2$]-glycerol, respectively.[11] To ascertain whether intact incorporation of the 1-CH bond of glycerol into the 11-CH bond of soraphen A was taking place, we needed to prepare [1-^{13}C, $^{2}H_2$]-glycerol **1b**.[2] We were also interested in the origin of the oxygen atoms in soraphen A and in particular whether the 1-CO bond of glycerol was incorporated intact into soraphen A and consequently required [1-^{13}C, ^{18}O]-glycerol **1a**.[2] In this paper we describe a highly efficient sequence to produce both of these doubly isotopically labeled glycerols.

Benzyloxyacetaldehyde **2** was prepared from ozonolysis of *Z*-dibenzyloxybut-2-ene.[12] Introduction of the ^{13}C label was achieved by reaction of **2** with potassium ^{13}C-cyanide to give the cyanohydrin **3** (Scheme 1).[4,13,14]



Scheme 1. Synthesis of [1-^{13}C, ^{18}O]- and [1-^{13}C, $^{2}H_2$]-glycerol. *Reagents and conditions*: (i) K^{13}CN, H_2O, 95%; (ii) AcCl, EtOH; (iii) H_2●, THF, 92% (over two steps); (iv) $LiAlH_4$, ether, 79%; (v) H_2, Pd, EtOH, 95–98%; (vi) H_2O, THF, 89% (over two steps); (vii) $LiAlD_4$, ether, 93%. Key: $^{\bullet}$ = ^{13}C; ● = ^{18}O.

Keywords: glycerol; labeling; cyanohydrin; imidic acids and derivatives; biosynthesis.

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Excess aldehyde (1.4 equiv. or more) was critical for the complete consumption of the cyanide (monitored using the cyanide sedimentation test with silver nitrate).[13] Workup of the reaction gave a mixture of the cyanohydrin **3** (78%) and aldehyde (22%) which could be used without any purification in the next step; separation over silica gave the pure product if required. We wanted to introduce the ^{18}O label using $H_2^{18}O$ which is very expensive and so we required a method which utilised the minimum amount of labeled water while simultaneously giving a product that was isotopically enriched. Conversion of **3** into the corresponding acid was not straightforward due to its reactive nature. Hydrolysis under basic and acidic reaction conditions failed to give a product. Hence, a different strategy was adopted: the cyanohydrin **3** was converted to the ester **5** using the Pinner synthesis.[15] Treatment of **3** with acetyl chloride and ethanol gave the imino ethyl ester **4** as its hydrochloride salt which was unstable and hygroscopic. Consequently, it was important to use the imidate salt **4** immediately in the next reaction, especially when ^{18}O was being introduced. The advantage of using this method is that introduction of the ^{18}O label is highly efficient as only the carboxylate oxygen atom is labeled (which is retained in the subsequent reduction step) and hence only 4.5 equiv. of $H_2^{18}O$ were required to give the ethyl ester **5a** in 92% yield over two steps. Reduction and deprotection gave the [1-^{13}C, ^{18}O]-glycerol **1a**. To prepare [1-^{13}C, $^{2}H_2$]-glycerol **1b**, the imidate salt **4** was converted to the ethyl ester **5b** and the deuterium atoms were introduced using $LiAlD_4$ in the reduction step. Overall, the five step sequence gave a 66 and 77% yield of the doubly isotopically labeled glycerols **1b** and **1a**, respectively, based on $K^{13}CN$.[16]

The synthetic route is highly versatile allowing the incorporation of any one, two or three of the isotopic labels to be introduced at position 1 of glycerol. The route could also be adapted to prepare glycerate with one or both of the carbonyl atoms isotopically labeled. The incorporation of ^{18}O is highly efficient as only 4.5 equiv. of ^{18}O water were used; this compares favourably with 10 equiv. used to prepare [1-^{13}C, $^{18}O_2$]-labeled glycerate.[4,13] The synthesis of small molecules such as glycerol is difficult as they partition into organic and aqueous solutions making isolation difficult. The use of a benzyl protecting group for the 3-hydroxyl moiety made the intermediates UV active and organic soluble which made isolation and handling of the intermediates straightforward. The benzyl group was removed efficiently and cleanly by hydrogenolysis and the labeled glycerol **1** could be isolated by a simple procedure of filtration and removal of the solvent thereby eliminating the need for an extraction step. In conclusion, a highly efficient five-step procedure has been developed to produce doubly labeled glycerol.

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16. **Experimental data**. Unless otherwise indicated all reactions were carried out under argon or nitrogen. THF and ether were dried and freshly distilled from sodium/benzophenone. Dichloromethane was freshly distilled over CaH_2. Flash chromatography was conducted on Merck silica gel 60 (40–63 μm). ^{1}H, ^{2}H and ^{13}C NMR spectra were recorded on a Bruker AM360 or AMX400 spectrometers. Chemical shifts were reported in ppm with reference to TMS (0.0 ppm) for ^{1}H NMR, natural abundance signal of CH_2Cl_2 (5.30 ppm) or H_2O (4.79 ppm) for ^{2}H NMR and to dideuteriomethane (53.52 ppm) for ^{13}C NMR. Mass spectra were recorded on a Jeol AX505W and a Kratos MS890MS spectrometer.
[1-^{13}C]-3-Benzyloxy-2-hydroxy-propionitrile 3. To a solution of potassium [^{13}C]cyanide (2.00 g, 30.3 mmol, CIL, 99% ^{13}C) in water (140 mL) at pH 8.5, a solution of benzyloxyacetaldehyde **2** (6.36 g, 42.3 mmol, 1.4 equiv.) in THF (90 mL) was added dropwise over 1 h and the resultant solution stirred for a further 1 h at room temperature. The pH of the reaction was continuously monitored and maintained between pH 7–9 using sodium hydroxide (3 M) and hydrochloric acid (3 M). The reaction was monitored by TLC (R_f aldehyde **2** 0.56, nitrile **3**

0.72, ether) and with the cyanide sedimentation test with silver nitrate.[13] Once all the cyanide had been consumed, the pH was lowered to 4–5 and nitrogen was passed through the reaction mixture for 10 min; the gas outlet stream was connected to a solution of potassium hydroxide (8 M) to remove any remaining hydrogen cyanide. The reaction mixture was then extracted with dichloromethane (6×25 mL) and the organic extracts were washed with brine and dried (Na_2SO_4 and $MgSO_4$). The solvent was removed in vacuo to leave a pale yellow oil (6.40 g) which was a mixture of the nitrile **3** (78%, 29.0 mmol, 95% based on $K^{13}CN$, 69% based on benzyloxyacetaldehyde) and the aldehyde (22%, 8.3 mmol). The nitrile **3** was used for the next step without any further purification but can be purified by flash chromatography on silica gel (hexane:ether, 1:2) to give pure nitrile **3**. Found M^+ 178.0827, calculated for $C_9{}^{13}CH_{11}O_2N$ 178.0823.

^1H NMR (CD_2Cl_2, 400 MHz) 3.30 (d, 1H, *J* 6.1 Hz, OH), 3.69 (m, 2H, CH_2OBn), 4.56 (m, 1H, CH), 4.62 (s, 2H, CH_2Ph), 7.30–7.39 (m, 5H, ArH); ^{13}C NMR (CD_2Cl_2, 100 MHz) 61.3 (d, *J* 61.8 Hz, CH), 71.0 (CH_2), 74.1 (CH_2), 118.7 (CN), 128.4, 128.5 (C4′), 129.9, 137.4 (C1′); ν_{max}/cm^{-1} 3424, 2922, 2869; *m*/*z* (EI) 178 (M^+, 12%), 148 (15), 107 (14), 91 (100).

Ethyl [1-^{13}C,^{18}O]-3-benzyloxy-2-hydroxypropionate 5a. A solution of the nitrile **3** (2.549 g, 14.3 mmol) in dry ethanol (40 mL) was cooled to 0°C. Acetyl chloride (162 mmol, 11.3 equiv.) was added dropwise over 45 min and the solution stirred for 12 h. An additional aliquot of acetyl chloride was added (18 mmol, 1.25 equiv.) and the reaction stirred for a further 2 h. The volatiles were removed in vacuo to yield a white solid which was suspended in dry hexane. The crude imidate salt **4** (4.524 g) was obtained by removal of the hexane in vacuo and was used immediately for the next step. {^{13}C NMR (DMSO-d_6, 90 MHz) of the unlabeled imidate salt **4**: 14.1 (CH_3), 60.1 (CH_2CH_3), 70.0 (CH), 71.8 (CH_2), 72.3 (CH_2), 127.4 (C4′), 127.4, 128.1, 132.2 (C1′), 172.2 ($C{=}NH_2^+$)}. The imidate salt was mixed with ^{18}O-water (1.3 g, 65.0 mmol, 4.5 equiv., Goss, 97.26% atom% ^{18}O, 0.96 atom% ^{17}O) and dry THF (45 mL) and stirred for 15 h. Water (30 mL) was added and the ester **5a** extracted into dichloromethane (5×30 mL), dried (Na_2SO_4) and the solvent evacuated in vacuo to give the crude ester **5a** as a brown oil. Silica chromatography (petrol 60–80°C/ethyl acetate, 2:1) gave the ester **5a** as a pale yellow oil (2.975 g, 13.1 mmol, 92% over two steps). ^{18}O enrichment is 89% by ^{13}C NMR. Found M^+ 227.1123, calculated for $C_{11}{}^{13}CH_{16}O_3{}^{18}O$ 227.1124.

^1H NMR (CD_2Cl_2, 360 MHz) 1.25 (t, 3H, *J* 7.2 Hz, CH_3), 3.12 (br s, 1H, OH), 3.73 (m, 2H, CH_2), 4.21 (q, 2H, *J* 7.2 Hz, CH_2CH_3), 4.27 (m, 1H, CH), 4.53 (s, 2H, CH_2Ph), 7.28–7.37 (m, 5H, ArH); ^{13}C NMR (CD_2Cl_2, 90 MHz) 14.3 (d, CH_3, ^3J 2.0 Hz), 62.1 (d, CH_2CH_3, ^2J 2.7 Hz), 71.1 (d, *J* 59.9 Hz, CH), 72.0 (CH_2), 73.7 (CH_2), 128.0, 128.0 (C4′), 128.7, 138.3 (C1′), 173.02 ($^{13}C{=}{}^{18}O$, 89%), 173.06 ($^{13}C{=}{}^{16}O$, 11%); ν_{max}/cm^{-1} 3452, 2980, 2869, 1664; *m*/*z* (EI) 227 (M^+, 20%), 179 (24), 166 (11), 121 (34), 91 (84), 84 (100).

Ethyl [1-^{13}C]-3-benzyloxy-2-hydroxypropionate 5b. Method as for **5a**. Nitrile **3** (2.468 g, 13.9 mmol) in dry ethanol (40 mL) and acetyl chloride (total of 12.2 equiv.) gave the crude imidate salt **4** (4.378 g) which was dissolved in water (35 mL) and THF (25 mL). The title compound was obtained as a pale yellow oil (2.791 g, 12.4 mmol, 89% over two steps). Found M^+ 225.1085, calculated for $C_{11}{}^{13}CH_{16}O_4$ 225.1082.

^1H NMR (CD_2Cl_2, 360 MHz) 1.25 (t, 3H, *J* 7.1 Hz, CH_3), 3.12 (br s, 1H, OH), 3.73 (m, 2H, $^{13}CH_2$), 4.21 (q, 2H, *J* 7.1 Hz, CH_2CH_3), 4.28 (m, 1H, CH), 4.53 (m, 2H, CH_2Ph), 7.28–7.37 (m, 5H, ArH); ^{13}C NMR (CD_2Cl_2, 90 MHz) 14.3 (d, CH_3, ^3J 21.9 Hz), 62.1 (d, CH_2CH_3, ^2J 2.5 Hz), 71.1 (d, *J* 59.6 Hz, CH), 72.0 (CH_2), 73.7 (CH_2), 128.0, 128.0 (C4′), 128.7, 138.3 (C1′), 173.1 ($^{13}C{=}O$); ν_{max}/cm^{-1} 3472, 2990, 2867, 1697; *m*/*z* (EI) 225 (M^+, 42%), 177 (53), 164 (22), 119 (45), 91 (75), 84 (100).

[1-^{13}C,^{18}O]-3-Benzyloxy-2-hydroxypropanol 6a. A solution of the ester **5a** (2.935 g, 12.9 mmol) in dry ether (20 mL) was added slowly to a solution of lithium aluminium hydride in ether (1 M, 27.2 mL, 27.2 mmol, 2.1 equiv.) and the mixture stirred for 1.5 h. The reaction was quenched with 50% aqueous THF (10 mL), filtered and washed with dichloromethane (100 mL). The solid was treated with a saturated solution of ammonium chloride (60 mL), filtered and the filtrate extracted with dichloromethane (6×10 mL). The organic extracts were combined and dried (Na_2SO_4). The solvent was removed in vacuo to give the crude alcohol which was purified by flash silica chromatography (petrol 60–80°C/ethyl acetate, 1:6) to give the alcohol **6a** as an almost colourless oil (1.88 g, 10.2 mmol, 79%). Found M^+ 185.1026, calculated for $C_9{}^{13}CH_{14}O_2{}^{18}O$ 185.1019.

^1H NMR (CD_2Cl_2, 360 MHz) 2.70 (br s, 1H, OH), 3.10 (br s, OH), 3.31–3.45 and 3.71–3.83 (m, 2H, $^{13}CH_2{}^{18}OH$), 3.45–3.54 (m, 2H, CH_2OBn), 3.76–3.83 (m, 1H, CH), 4.52 (s, 2H, CH_2Ph), 7.26–7.37 (m, 5H, ArH); ^{13}C NMR (CD_2Cl_2, 90 MHz) 64.3 ($^{13}CH_2^{18}OH$), 71.1 (d, *J* 40.2 Hz, CH), 72.1 (CH_2OBn), 73.7 (CH_2Ph), 128.1, 128.1 (C4′), 128.7, 138.4 (C1′); ν_{max}/cm^{-1} 3380, 2918, 2865, 1664; *m*/*z* (EI) 185 (M^+, 19%), 107 (53), 91 (100).

[1-^{13}C, $^2H_2$]-3-Benzyloxy-2-hydroxypropanol 6b. Method as for **6a**. A solution of the ester **5b** (2.737 g, 12.2 mmol) in dry ether (20 mL) was added slowly to a solution of lithium aluminium deuteride in ether (1 M, 23.0 mL, 23.0 mmol, 1.9 equiv.). The alcohol **6b** was obtained as an almost colourless oil (2.09 g, 11.3 mmol, 93%). ^2H isotopic enrichment was 98% by ^1H NMR. Found M^+ 185.1100, calculated for $C_9{}^{13}CH_{12}{}^2H_2O_3$ 185.1102.

^1H NMR (CD_2Cl_2, 360 MHz) 3.08 (br s, OH), 3.47 (m, 2H, CH_2OBn), 3.81 (m, 1H, CH), 4.50 (s, 2H, CH_2Ph), 7.26–7.37 (m, 5H, ArH); ^2H NMR (CH_2Cl_2, 61 MHz) 3.51 (d, J_{CD} 22.5 Hz, C*D*D), 3.58 (d, J_{CD} 21.8 Hz, CD*D*); ^{13}C NMR (CD_2Cl_2, 90 MHz) 63.6 (quin., J_{CD} 21.6 Hz, $^{13}CD_2OH$), [63.8 (t, J_{CD} 22.5 Hz, $^{13}CDHOH$), 63.9 (t, J_{CD} 21.6 Hz, $^{13}CHDOH$), 64.2 (s, $^{13}CH_2OH$)], 71.1 (d, *J* 40.2 Hz, CH), 72.1 (d, *J* 2.0 Hz, CH_2OBn), 73.7 (CH_2Ph), 128.1, 128.1 (C4′), 128.7, 138.4 (C1′); ν_{max}/cm^{-1} 3379, 2864; *m*/*z* (EI+) 185 (M^+, 26%), 107 (58), 91 (100).

[1-^{13}C,^{18}O]-Glycerol 1a. A mixture of benzyl ether **6a** (1.85 g, 10.0 mmol) and 10% palladium on carbon (718 mg) in ethanol (50 mL) was stirred vigorously at room temperature under a hydrogen atmosphere of initial pressure 6 atm. The reaction was completed after 16 h when one equivalent of hydrogen had been consumed. The reaction mixture was filtered through Celite, washed with

ethanol and the filtrate evaporated in vacuo. The crude oil was redissolved in ethanol and re-evaporated (bath temperature $\leq 60°C$) to remove any remaining toluene. The title compound was obtained as an almost colourless oil (908 mg, 95%). ^{18}O enrichment is 89% by ^{13}C NMR. Found $[M+1]^+$ 96.0622, calculated for $C_2{}^{13}CH_9O_2{}^{18}O$ 96.0628.

^1H NMR (D_2O, 360 MHz) 3.29–3.82 (m, 5H, $2CH_2$, CH); ^{13}C NMR (D_2O, 90 MHz) 63.28 ($^{13}CH_2{}^{18}OH$, 89%), 63.30 (CH_2OH); 72.9 (d, J 41.2 Hz); ν_{max}/cm^{-1} 3332; m/z (EI+) 96 ($[M+1]^+$, 25%), 77 (46), 61 (100).

[1-^{13}C,^{2}H$_2$]-Glycerol 1b. Method as for **1a**. A mixture of benzyl ether **6b** (2.04 g, 11.0 mmol) and 10% palladium on carbon (730 mg) in ethanol (55 mL). The title compound was obtained as an almost colourless oil (1.03 g, 98%). Found $[M+1]^+$ 96.0661, calculated for $C_2{}^{13}CH_9O_2{}^{18}O$ 96.0709.

^1H NMR (D_2O, 360 MHz) 3.48–3.53 (m, 1H, C*H*HOH), 3.58–3.64 (m, 1H, CH*H*OH), 3.71–3.75 (m, 1H, CH); ^2H NMR (H_2O, 61 MHz) 3.49 (d, J_{CD} 21.8 Hz, C*D*D), 3.58 (d, J_{CD} 21.7 Hz, CD*D*); ^{13}C NMR (D_2O, 90 MHz) 62.6 (quin., J_{CD} 21.7 Hz, $^{13}CD_2OH$), 63.3 (CH_2OH); 72.7 (d, J 41.1 Hz); ν_{max}/cm^{-1} 3370; m/z (EI+) 96 ($[M+1]^+$, 12%), 77 (61), 64 (100).