Synthesis of aromatic $^{13}\text{C}/^{2}\text{H}-\alpha$-ketoacid precursors to be used in selective phenylalanine and tyrosine protein labelling†

R. J. Lichtenecker

Recent progress in protein NMR spectroscopy revealed aromatic residues to be valuable information sources for performing structure and motion analysis of high molecular weight proteins. However, the applied NMR experiments require tailored isotope labelling patterns in order to regulate spin-relaxation pathways and optimize magnetization transfer. We introduced a methodology to use $\alpha$-ketoacids as metabolomic amino acid precursors in cell-based overexpression of phenylalanine and/or tyrosine labelled proteins in a recent publication, which we have now developed further by providing synthetic routes to access the corresponding side-chain labelled precursors. The target compounds allow for selective introduction of $^{13}\text{C}/^{2}\text{H}$ spin systems in a highly deuterated chemical environment and feature alternating $^{12}\text{C}/^{13}\text{C}-^{12}\text{C}$ ring-patterns. The resulting isotope distribution is especially suited to render straightforward $^{13}\text{C}$ spin relaxation experiments possible, which provide insight into the dynamic properties of the corresponding labelled proteins.

Introduction

Aromatic amino-acids represent a sensitive source of structural and dynamic parameters in high-molecular weight protein NMR spectroscopy. Phenylalanines and tyrosines are substantially overrepresented at protein binding interfaces due to their ability to contribute to hydrophobic, as well as to electrostatic interactions. Examples from the literature have proven the importance of aromatic residue based NOE data to complement the set of methyl-group derived distance restraints for structure calculation. Moreover, aromatic side chains display a remarkable flexibility in dynamic motion, which can be sensitively probed by $^{13}\text{C}-^{2}\text{H}$ spin pair relaxation. Insufficient chemical shift dispersion, extensive $^{13}\text{C}-^{13}\text{C}$ spin coupling and retarded side-chain motion strongly affect the signal assignment and analysis in the aromatic spectral region.

Selective stable-isotope patterns are required to enable effective magnetisation transfer and well defined spin relaxation, which is both necessary to decrypt the structural information buried in these residues. Alternating $^{12}\text{C}-^{13}\text{C}-^{12}\text{C}$ and/or $^{2}\text{H}-^{1}\text{H}$ arrangements in the aromatic ring systems have been shown to result in well resolved NMR signals due to significant reduction of scalar and dipolar couplings. Isolated $^{13}\text{C}-^{1}\text{H}$ spin systems in an otherwise $^{2}\text{H}$-containing aromatic ring have additionally been used as valuable tools to elucidate the aromatic side chain motion by erasing unwanted relaxation pathways. Reports on labelling phenylalanine and tyrosine residues with stable isotopes include cell-free (CF) protein synthesis, as well as cell-based expression systems. CF-approaches require the sophisticated synthesis of $^{15}\text{N}$-labelled amino acids, but display highly selective isotope composition in the target proteins. Cell-based overexpression, on the other hand, makes use of amino acid precursor compounds, which are introduced to the metabolism of a protein expressing organism.

Although economically preferred, cell-based methods often suffer from low incorporation rates and selectivity due to the loss of heavy isotopes at metabolic crossroads. In order to expand the methodology of introducing stable isotopes at distinct positions of a target protein, we recently presented highly selective phenylalanine- and tyrosine-residue labelling based on the corresponding metabolic $\alpha$-ketoacid precursors sodium phenylpyruvate and sodium 4-hydroxyphenylpyruvate (Scheme 1).

Protein synthesis using an *E. coli* overexpression host in the presence of the labelled aromatic $\alpha$-ketoacids thus resulted in the incorporation of $^{13}\text{C}$ without any cross-labelling to other residues. This new methodology combines the robustness and versatility of in-cell overexpression with high incorporation selectivity, which is usually the domain of cell-free protein
Results and discussion

The approach to access the target compounds 1–3 (Scheme 2) is based on the synthesis of the aromatic ring by the reaction of labelled acetone with nitromalonaldehyde in basic aqueous solution. Selective deuteration at activated ring-positions was planned in acidic D₂O using aniline or 4-aminophenol as electron rich substrates at elevated temperatures. On the other hand, this synthetic concept was designed as an economically practicable way of synthesizing enough material to be used in cell-based protein overexpression (quantitative isotope incorporation at 100–200 mg L⁻¹ minimal medium) due to the relatively cheap sources of stable isotopes and robust reaction steps. On the other hand, the routes should be flexible enough to access alternative isotope patterns by simply switching to commercially available starting compounds with different stable isotope composition (e.g. various patterns of labelled acetone for side-chain-, or glycine as a 13C-source for backbone labelling).

The synthesis of sodium 3,3-dideuterio[3,5-13C₂]2,4,6-trideuteriophenyl]pyruvate 1 was performed as outlined in Scheme 3. Initially, a straightforward way to access the aromatic ring system in one step was applied by the reaction of commercially available [1,3-13C₂]acetone 4 with sodium nitromalonaldehyde 5. Compound 5 can be prepared from muconic acid as a stable solid. Subsequent deoxygenation of [2,6-13C₂]4-nitrophenol 6 was performed in a two-step reaction sequence via the 1-phenyl-1H-tetrazolyether. Compound 7 was prepared by reaction of the phenolic hydroxy group with 5-chloro-1-phenyl-1H-tetrazole in the presence of KOtBu. Hydrogenation using palladium on charcoal at room temperature and a pressure of 4 bar removed the oxygen from the aromatic ring, while at the same time the nitro-group was reduced yielding [3,5-13C₂]aniline. At this stage, the deuterium pattern at the aromatic ring was installed, as compound 8 shows highly selective ¹H/²H exchange at the electron-rich ortho/para positions in the presence of D₂O and HCl under microwav irradiation. Subsequent formation of [3,5-13C₂]2,4,6-trideuteriobenzonitrile 10 was achieved by using potassium tetracyanonickelate in ammonium chloride buffer. Reduction of compound 10 using diisopropylaluminium hydride yielded [3,5-13C₂]2,4,6-trideuteriobenzaldehyde 11 which was then used in the subsequent condensation step with hydantoin. The preparation of labelled benzalhydantoin 12 was done in the presence of ammonium acetate, which provided higher and more reproducible yields than the use of sodium acetate reported in the literature. Finally, the hydan- toin ring of compound 12 was hydrolysed using 20% NaOD.

synthesis. In order to further develop our α-ketoacid precursor based approaches towards selective side-chain labelling, we developed a synthetic route to sodium phenylpyruvate containing 13C⁻²H at meta-positions in an otherwise perdeuterated chemical environment. We could already demonstrate that this side-chain labelled precursor is selectively converted to Phe residues in an E. coli expression medium. This article describes the synthetic details to obtain the 13C/²H aromatic α-ketoacids illustrated in Scheme 2. In addition to the already mentioned precursor 1, synthetic approaches to access para 13C⁻²H labelled phenylalanine precursor 2, as well as the meta 13C⁻²H tyrosine precursor 3 are presented. The routes feature acetone and heavy water as 13C and ²H sources, respectively. Labelling of backbone positions is feasible by application of 13C-glycine as shown previously.
solution, which simultaneously introduced $^2$H at the C3-position. Labelled sodium phenylpyruvate 1 was obtained by lyophilisation from aqueous solution as a stable white powder in an overall yield of $\sim 16\%$ in 8 steps from $[1,3-^{13}C]$acetone 4.

In order to access compounds 2 and 3, the deuteration of 4-aminophenol upon microwave irradiation was thoroughly studied (Scheme 4). A nearly quantitative deuteration at positions 3 and 5 was achieved within 30 minutes at $180\,^\circ C$ in the presence of D$_2$O and HClconc. (1.25% v/v). Additional incorporation of $^2$H at positions 2 and 6 was performed at a much slower rate with $>95\%$ deuteration after 8 hours and only minimal aminophenol degradation. The side-chain deuteration patterns for compounds 2 and 3 could thus be installed by varying the reaction time of the microwave mediated deuteration. Sodium 3,3-dideuterio$([4-{^{13}C}]$-2,3,5,6-tetradeterio-phenyl)pyruvate 2 was prepared by reducing $[1-{^{13}C}]$4-nitrophenol 14 to $[1-{^{13}C}]$4-nitrophenol 15 using the continuous-flow hydrogenation reactor H-cube® (Scheme 5). After microwave induced deuteration at positions 2, 3, 5 and 6, deoxygenation was again performed via the corresponding 1-phenyl-1-$H$-tetrazoleether 17.

In this case, the Pd/C-mediated hydrogenation was again conducted in the continuous-flow hydrogenation reactor, leading to an isolated hydrogen atom in the para position of the resulting labelled aniline 18. The following reaction steps were performed analogously to the reaction sequence reported for the preparation of the labelled sodium phenylpyruvate 1 leading to the target compound sodium 3,3-dideuterio$([4-{^{13}C}]$-2,3,5,6-tetradeterio-phenyl)pyruvate 2 in 9 steps and an overall yield of $\sim 11\%$.

To achieve straightforward labelling at the aromatic side chain of tyrosine residues, a route to sodium 3,3-dideuterio$([3,5-{^{13}C}_2]$2,6-dideuterio-4-hydroxyphenyl)pyruvate 3 was developed as outlined in Scheme 6. After formation of the aromatic system, $[2,6-^{13}C]$4-nitrophenol 6 was converted to $[2,6-{^{13}C}_2]$4-nitrophenol 22 as described in the synthesis of compound 2. Deuteration in the ring positions 3 and 5 was then conducted in D$_2$O–HCl at $180\,^\circ C$ for 37 minutes, followed by formation of labelled 4-hydroxybenzonitrile 24 using K$_2$Ni(CN)$_4$. Diisobutylaluminium hydride reduction gave 4-hydroxybenzaldehyde 25, which subsequently underwent condensation with hydantoin in the presence of piperidine. $^1$H Hydrolysis of the hydantoin ring in NaOD–D$_2$O finally gave sodium 3,3-dideuterio$([3,5-{^{13}C}_2]$2,6-dideuterio-4-hydroxy-phenyl)pyruvate 3 as a stable white solid. This 7-step sequence yielded the target compound 3 in a total yield of $\sim 28\%$, which contains $\sim 23\%$ deuterium in positions 3 and 5 of the aromatic ring (determined by NMR signal integration).

A more selective deuteration pattern can be achieved, if required, as shown in Scheme 7. Methylation of 4-nitrophenol$^{19}$ and subsequent reduction of the nitro group yielded $p$-anisidine 28, which showed no reactivity in the deuteration step meta to the amino group (28 $\rightarrow$ 29).$^{21}$ Demethylation using HBr in the presence of a phase transfer catalyst (Aliquat-
336®) gave selectively deuterated aminophenol 30.22 This sequence, which was verified using unlabelled 4-nitrophenol as a starting material, increases the number of reactions in the route to prepare sodium 3,3-dideuterio([3,5-13C2]2,6-dideuterio-4-hydroxyphenyl)pyruvate by two steps, but represents an effective approach to avoid partial deuteration at the 13C labelled aromatic positions in the target compound 3. The aromatic α-ketoacids 1–3 display high stability in their lyophilized forms as sodium salts, but undergo oxidative degradation in basic solution in the presence of atmospheric oxygen.18 NMR spectra of compounds 1–3 in D2O show mainly the keto forms, whereas in DMSO-d6 the enol forms predominate, which is in accordance with literature data.23

Conclusions

An efficient synthetic concept is presented to access labelled metabolic precursor compounds of phenylalanine and tyrosine based on the low-cost isotope sources 13C-acetone and D2O. The routes enable the construction of specific labelling patterns in the aromatic side chains with special focus on alternating 12C–13C–12C ring sequences and isolated 13C–1H spin systems in an otherwise deuterated chemical surrounding. Highly selective aromatic side-chain labelling is thus feasible in cell-based overexpression systems without the need for chiral labelled amino acid additives. The resulting isotope arrangements facilitate the interpretation of Carr–Purcell–Meiboom–Gill (CPMG) based spin-relaxation experiments,6b improve the quality of aromatic proton NOE derived distance restraints10 and enable the unambiguous assignment of aromatic ring signals even in very large proteins. The precursors presented constitute valuable reporters of motional dynamics in complex molecular processes, such as protein folding.
allostery and enzymatic catalysis. The straightforward and economic synthetic protocols shown below will further promote the efforts to turn aromatic residue labelling into a routinely used concept and complement the techniques of NMR-based analysis of protein dynamics, which traditionally rely on the interpretation of spin relaxation residing at the backbone or $^{13}$C and $^2$H methyl bearing side-chains.²⁴

**Experimental section**

**General methods**

All solvents were distilled prior to use. Anhydrous tetrahydrofuran and dimethylformamide were purchased from commercial suppliers. Dichloromethane was dried by elution over an aluminium oxide column. Isotope labelled reagents were purchased from Sigma-Aldrich ISOTEC with the following purity grades: $[1,3-^{13}$C]acetone (99% $^{13}$C), $[2-^{13}$C]acetone (99% $^{13}$C) and D$_2$O (99.9% $^2$H). Column chromatography was performed using silica gel 60 (0.040–0.063 mm, 240–400 mesh) from Merck. Thin layer chromatography (TLC) was done on precoated silica gel (Merck 60 F254) glass plates. TLC detection was carried out using a UVAC-60 neolab ultraviolet lamp, an iodine chamber, or by application of a Mo–Ce(SO$_4$)$_2$ complex solution (48 g (NH$_4$)$_6$Mo$_7$O$_24·4$H$_2$O and 2 g Ce(SO$_4$)$_2$ in 100 mL mixture was slightly warmed to dissolve all of the NaNO$_2$. A nitrite (30 g) was dissolved in water (30 mL) using a three-neck flask, a dropping funnel and a tube to drain the evolved gases. The reaction yielded 2.3 g of a crude product, which was precipitated by pouring the mixture in ice water (100 mL) and filtration. The resulting solid was filtered and the two combined filtrates were extracted with diethyl ether (6 × 100 mL). Subsequent drying of the combined organic phases over MgSO$_4$ and evaporation of the diethyl ether under reduced pressure yielded a yellow solid. The crude product was purified over a silica gel chromatography column by elution with hexane–ethyl acetate (6 : 4 v/v). The reaction yielded 1.47 g (63%) of $[2,6-^{13}$C$_2$]4-nitrophenol 6. $^1$H NMR (400 MHz, CDCl$_3$): 8.18 (d, $J = 8.7$ Hz, 2H, CH$_{arom}$), 7.67 (m, 3H, 2H, CH$_{phenyl}$), 7.67–7.54 (m, 3H, CH$_{phenyl}$), 7.69 (dm, $J = 164.6$ Hz, 2H, $^{13}$CH$_{arom}$); $^{13}$C NMR (100.6 MHz, CDCl$_3$): 116.10 (1$^{13}$CH), HRMS (ESI): calc for C$_{7}$H$_{10}$N$_{5}$O$_{3}$ [M + H]$^+$ 284.0415, found 284.030.

5-[2,6-^{13}C$_2$H$_2$N$_2$O$_3$]-1-phenyl-1H-tetrazole 7. A solution of $[2,6-^{13}$C$_2$]4-nitrophenol 6 (1.4 g) in dry dimethylformamide (18.4 mL) was stirred at room temperature, while potassium tert-butoxide (1.31 g) was added within 5 minutes in small aliquots under a constant stream of argon. After 1 h of vigorous stirring under an argon atmosphere, a solution of 5-chloro-1-phenyl-1H-tetrazole (1.9 g) in dry dimethylformamide (8 mL) was added and the reaction mixture was stirred for further 3 h. The solution was warmed to 65 °C and stirring continued overnight. Precipitation of the crude product was induced by pouring the mixture in ice water (100 mL) and completing at 4 °C in 12 h. The resulting precipitate was separated by filtration and washed with small portions of ice water. The reaction yielded 2.3 g of a crude product, which was further purified by column chromatography. Elution with hexane–ethyl acetate (8 : 2) gave 5-[2,6-^{13}C$_2$H$_2$N$_2$O$_3$]-1-phenyl-1H-tetrazole 7 (2.07 g, 74%) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$): 8.35 (d, $J = 9.3$ Hz, 2H, CH$_{arom}$), 7.76 (dd, $J = 8.2$ Hz, $J = 1.7$ Hz, 2H, CH$_{phenyl}$), 7.67–7.54 (m, 3H, CH$_{phenyl}$), 7.69 (dm, $J = 164.6$ Hz, 2H, $^{13}$CH$_{arom}$); $^{13}$C NMR (100.6 MHz, CDCl$_3$): 130.34 (CH), 122.90 (CH), 116.06 (CH); MS (EI): ccalc for C$_{11}$H$_{10}$N$_{5}$O$_{3}$ $[^{13}$C$_2$H$_2$N$_2$O$_3$] $M^+$ 285.08; found 284.9.

[3,5-$^{13}$C$_2$]6-aniline 8. Palladium on charcoal (10%, 1.04 g) was added to a solution of 5-[2,6-^{13}C$_2$H$_2$N$_2$O$_3$]-1-phenyl-1H-tetrazole 7 (1.04 g) in dry toluene (150 mL) in a thick walled hydrogenation flask. The flask was mounted on a hydrogenation Parr-apparatus and a pressure of 4 bar of hydrogen was applied. After 12 h of agitation, the pressure was released, the flask flushed with argon and the black solid palladium catalyst separated from the solution by filtration. The catalyst was washed with toluene (30 mL) and the combined filtrates poured on a 0.5 N NaOH solution (150 mL). After separation of the two layers, the aqueous phase was extracted with toluene (3 × 100 mL). The combined organic phases were then
extracted using 0.5 N HCl (3 × 100 mL). Addition of concentrated HCl (0.5 mL) to the combined aqueous phases was followed by reducing the volume of the resulting solution by half under reduced pressure at 50 °C. NaOH (1 N) was added until the solution showed a pH of ~10. The product was extracted from the solution with dichloromethane (3 × 100 mL). Drying of the combined organic phases over MgSO4 and subsequent careful evaporation of the solvents under reduced pressure (>100 mbar) gave 394 mg of a product/dichloromethane mixture which was used for further conversion. The reaction yield was determined by integrating the corresponding NMR signals to be 338 mg (97%).

1H NMR (400 MHz, CDCl₃): 7.16 (dd, J = 8.4 Hz, J = 7.4 Hz, J = 156.7 Hz, 2H, 13CHarom.), 6.80–6.62 (m, 3H, CHarom.), 3.63 (bs, 2H, NH₂); 13C NMR (100.6 MHz, CDCl₃): 129.69 (13CH); MS (EI): calcld for C₇H₁₀N₂O [M] 131.04; found 131.0.

[3,5-13C₂]2,4,6-trideuteriobenzonitrile. A solution of 13C₂H₇N [M] 95.06; found 94.9.

[3,5-13C₂]2,4,6-trideuterioaniline. A microwave vessel was charged with [3,5-13C₂]aniline (338 mg, D₂O (1.5 mL) and 10 drops of HCl conc.). The vessel was tightly closed and again irradiated for 10 minutes (150 °C). The procedure of evaporation, addition of D₂O (1.5 mL) and application of microwave irradiation was performed two more times. The solution was then brought to a neutral pH by addition of 1 N NaOH and the product extracted with diethyl ether (3 × 60 mL). Drying of the organic phases over MgSO₄ and careful evaporation of the solvents under reduced pressure gave 280 mg of a dark crude product. The same reaction procedure was applied to 250 mg of the sub-stance, which gave 230 mg of the crude product. Two batches were combined and purified using bulb-to-bulb distillation (50 mbar; up to 120 °C), which yielded [3,5-13C₂]2,4,6-trideuterioaniline (430 mg, 71%) as a light yellow liquid.

1H-NMR spectroscopy analysis showed quantitative deuterium incorporation at positions 2, 4 and 6. 1H NMR (400 MHz, CDCl₃): 7.15 (dd, J = 8.5 Hz, J = 158.3 Hz, 2H, 13CHarom.), 3.62 (bs, 2H, NH₂); 13C NMR (100.6 MHz, CDCl₃): 128.46 (13C); MS (EI): calcld for C₁₃H₁₂N₂ [M] 198.08; found 198.0.

[3,5-13C₂]2,4,6-trideuteriobenzonitrile. A solution of sodium nitrite (380 mg) in water (25 mL) was slowly added to a stirred mixture of [3,5-13C₂]aniline (420 mg) in HCl (0.4%, 160 mL) at 0 °C using a dropping funnel. After 2 h of stirring at 0 °C, the reaction mixture was brought to pH < 2 by slow addition of HCl conc. at 0 °C. Stirring was continued for 15 min at 60 °C. The solution was then filtered and the solid residue was washed with small aliquots of water. The combined filtrates were extracted with diethyl ether (4 × 100 mL) and the combined organic phases were dried over MgSO₄. Evaporation of the solvents under reduced pressure gave a crude product, which was further purified by bulb-to-bulb distillation (30 mbar; up to 120 °C) to yield [3,5-13C₂]2,4,6-trideuteriobenzonitrile (387 mg, 84%) as a slightly yellow liquid. 1H NMR (400 MHz, CDCl₃): 7.47 (dd, J = 8.1 Hz, J = 164.0 Hz, 2H, 13CHarom.); 13C NMR (100.6 MHz, CDCl₃): 129.29 (13C); MS (EI): calcld for C₁₃H₁₂N₂D₃ [M] 108.07; found 108.0.

[3,5-13C₂]2,4,6-trideuteriobenzaldehyde. A solution of [3,5-13C₂]benzylidene)hydantoin (332 mg; 86%) as a colorless liquid. 1H NMR (400 MHz, CDCl₃): 10.04 (s, 1H, CHO), 7.54 (dd, J = 7.8 Hz, J = 162.5 Hz, 2H, 13CHarom.); 13C NMR (100.6 MHz, CDCl₃): 129.17 (13C); MS (EI): calcld for C₁₃H₁₀N₂ [M] 111.07; found 111.0.

[3,5-13C₂]2,4,6-trideuteriobenzonitrile. A solution of [3,5-13C₂]benzylidene)hydantoin (225 mg), hydantoin (300 mg) and ammonium acetate (226 mg) was stirred in acetic acid (0.7 mL) using a round bottomed flask, equipped with a short reflux condenser. The mixture was heated to 120 °C for 4 h. The hot solution was cooled in an ice bath, leading to the precipitation of a yellow solid, which was separated by filtration. Drying in vacuo yielded 5-[3,5-13C₂]2,4,6-trideuteriobenzaldehyde (488 mg, 84%). 1H NMR (400 MHz, CDOD): 7.42 (dd, J = 7.9 Hz, J = 160.4 Hz, 2H, 13CHarom.), 6.57 (s, 1H, CH₃); 13C NMR (100.6 MHz, CDOD): 128.77 (13C); MS (EI): calcld for C₁₃H₁₀D₂ [M] 193.08; found 193.1.

Sodium 3,3-dideuterio[3,5-13C₂]2,4,6-trideuteriophenylpyruvate. A two necked round bottomed flask, equipped with a reflux condenser, was loaded with 5-[3,5-13C₂]2,4,6-trideuteriophenylpyruvate (176 mg, 82%) as a white powder. NMR analysis showed residual 1H at C₃ (<5%). 1H NMR (400 MHz, D₂O): 7.45 (dd, 1J = 8.1 Hz, 2H, 13CHarom.); 4.12 (s, 0.06 H, residual CH₂); 13C NMR (100.6 MHz, D₂O): 129.05 (13C); HRMS
(ESI): calcd for C_{12}H_{16}D_{2}ON [M − Na]^− 170.0777; found 170.0781.

[1-13C]4-nitrophenol 14. The synthesis was performed according to the preparation of [2,6-13C2]4-nitrophenol 6 using [2-13C]acetone as a reagent. Purification of the raw product by column chromatography eluting with hexane-ethyl acetate (6:4 v/v) yielded [1-13C]4-nitrophenol 14 (1.28 g, 54%) as a yellow solid. 1H NMR (400 MHz, DMSO-d_{6}): δ = 11.02 (s, 1 H), 8.12 (dd, J = 9.2 Hz, J = 9.2 Hz, 2H, m-CHarom.), 6.93 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, o-CHarom.), 13C NMR (100.6 MHz, DMSO-d_{6}): 164.37 (13C), 126.65 (m-CHarom.), 116.24 (d, J = 63.0 Hz, o-CHarom.); HRMS (ESI): calcd for C_{13}H_{18}D_{4}NO [M − H]^− 139.0225; found 139.0234.

[1-13C]4-amino phenol 15. [1-13C]4-nitrophenol 14 (1.28 g) was dissolved in MeOH (90 mL). This solution was loaded onto a Pd/C (10%) catalyst cartridge in a continuous-flow hydrogen reactor (H-cube® – Thalesnano) at a flow-rate of 1 mL min^{-1} and at room temperature. The hydrogen generator was set to full hydrogen mode. Evaporation of methanol under reduced pressure gave [1-13C]4-amino phenol 15 (986 mg, 98%). 1H NMR (400 MHz, DMSO-d_{6}): 8.30 (d, J = 2.1 Hz, 1H, OH), 6.47 (dd, J = 8.8 Hz, J = 8.8 Hz, 2H, o-CHarom.), 6.42 (dd, J = 8.8 Hz, J = 2.1 Hz, 2H, m-CHarom.), 4.36 (s, 2H, NH_{2}); 13C NMR (100.6 MHz, DMSO-d_{6}): 148.68 (13C), 141.12 (d, J = 8.8 Hz, CNH_{2}), 115.97 (d, J = 66.0 Hz, o-CHarom.), 115.69 (m-CHarom.); HRMS (ESI): calcd for C_{7}H_{15}DNO [M + H]^+ 111.0639; found 111.0636.

[1-13C]2,3,5,6-tetradecarboxy-4-amino phenol 16. [1-13C]4-amino phenol 15 (980 mg) was heated to 180 °C, together with MgSO_{4} (1.1 g) was loaded in a tert butoxide (1.1 g) was loaded in a syringe through a septum and the KOBu was slowly added within 15 min. The mixture was then stirred for 90 min before a solution of 5-chloro-1-phenyl-1H-tetrazole (1.27 g in 6 mL dry DMF) was added via a syringe. Stirring was continued at room temperature for another 90 min. The reaction was quenched by pouring the mixture on ice-water (150 mL). The precipitated solid was filtered off and dissolved again in dichloromethane (200 mL). This solution was washed with water until the aqueous phase remained colorless. The organic phase was dried over MgSO_{4} and the solvents removed under reduced pressure to yield 5-[1-13C]2,3,5,6-tetradecarboxy-4-amino phenyl-1H-tetrazole 17 (1.48 g, 84%). 1H NMR (400 MHz, DMSO-d_{6}): 7.85–7.80 (m, 2H, CH_{phenyl}), 7.69–7.56 (m, 3H, CH_{phenyl}), 7.11 (d, J = 4.6 Hz, 0.08 H, residual CH_{arom.}), 5.17 (s, 2H, NH_{2}); 13C NMR (100.6 MHz, DMSO-d_{6}): 161.60, 155.68, 154.78, 147.62 (d, J = 10.2 Hz), 144.35 (13C), 133.14, 130.28 (CH), 130.14 (CH), 123.55 (CH); HRMS (ESI): calcd for C_{13}H_{13}CH_{2}D_{4}NO [M + H]^+ 259.1327; found 259.1326.

[4-13C]2,3,5,6-tetradecarboxy-4-amino phenyl-1H-tetrazole 17. Purification of the raw product using bulb-to-bulb distillation gave [4-13C]2,3,5,6-tetradecarboxy-4-amino phenyl-1H-tetrazole 17 (390 mg, 75%) as a colourless liquid. 1H NMR (400 MHz, CDCl_{3}): 7.61 (d, J = 159.8 Hz, 1H, 13CH), 4.96 (s, 2H); 13C NMR (100.6 MHz, DMSO-d_{6}): 116.23 (13C); HRMS (ESI): calcd for C_{7}H_{15}D_{4}N_{5}O [M + H]^+ 259.0941; found 259.0940.

[4-13C]2,3,5,6-tetradecarboxy-4-amino phenyl-1H-tetrazole 17. A three necked round bottomed flask was charged with [1-13C]2,3,5,6-tetradecarboxy-4-amino phenol 16 (760 mg). Potassium tert-butoxide (1.1 g) was loaded in a slightly bent round bottomed flask attached to one neck and the apparatus set under an argon atmosphere. The addition of dry DMF (18 mL) was conducted via a syringe through a reduction pressure (<200 mbar) gave [4-13C]2,3,5,6-tetradecarboxy-4-amino phenyl-1H-tetrazole 18 in residual dichloromethane, which was used without further purification. NMR signal integration revealed a yield of 490 mg (86%). 1H NMR (400 MHz, DMSO-d_{6}): 6.99 (d, J = 7.5 Hz, 0.08 H, residual m-CH_{arom.}), 6.47 (d, J = 159.8 Hz, 1H, 13CH), 4.96 (s, 2H); 13C NMR (100.6 MHz, DMSO-d_{6}): 116.23 (13C); HRMS (ESI): calcd for C_{13}H_{13}CH_{2}D_{4}N [M + H]^+ 259.0941; found 259.0940.

[4-13C]2,3,5,6-tetradecarboxybenzonitrile 19. The synthesis was conducted similar to the conversion of compound 9 to [3,5-13C2]2,4,6-trideuteriobenzonitrile 10. Purification of the crude product using bulb-to-bulb distillation gave [4-13C]2,3,5,6-tetradecarboxybenzonitrile 19 (390 mg, 75%) as a colourless liquid. 1H NMR (400 MHz, CDCl_{3}): 7.61 (d, J = 159.8 Hz, 1H, 13CH), 4.96 (s, 2H); 13C NMR (100.6 MHz, CDCl_{3}): 123.53 (13C); HRMS (EI): calcd for C_{13}H_{13}CH_{2}D_{4}NO [M + H]^+ 259.0941; found 259.0940.
residual funnel. After 2 h of stirring at 0 °C, the solution was brought (900 mg) in HCl (0.4%; 325 mL) at 0 °C using a dropping titrations 2 and 6 revealed a deuteration grade of 23%. 1H NMR deuteration grade of 92% in positions 3 and 5 whereas positions 12 and 5 gave 915 mg of a brown solid, which was purified by column chromatography (eluent: hexane–ethyl acetate 7:3). The reaction yielded 850 mg (87%) of [3,5-13C2]2,6-dideuteriohydroxybenzonitrile 24 as a yellow solid. 1H NMR (400 MHz, DMSO-d6): 10.58 (s, 1H, OH); 7.65–7.61 (m, 0.14 H, residual 13C-arom.); 6.89 (d, J = 162.0 Hz, 1H, 13C-arom.); 13C NMR (100.6 MHz, DMSO-d6): 116.53 (13C); HRMS (ESI): calcd for C515CH2D3NO [M + H]+ 123.0564, found 123.0559.

Sodium 3,3-dieuteuro[14,13C2]2,3,5,6-tetra-deuteriophenyl]pyruvate 2. The synthesis of compound 2 was performed according to the preparation of sodium 3,3-dieuterio-[3,5,13C2]2,4,6-trideuteriophenyl]pyruvate 1, but using 5-[4,13C]2,3,5,6-tetra-deuteriobenzylidene]hydantoin 21 (85 mg) as a substrate. The reaction yielded sodium 3,3-dieuterio-[14,13C2]2,3,5,6-tetradereuteriophenyl]pyruvate 2 (76 g, 89%) as a colourless lyophilisate. NMR analysis showed residual 1H at C3 (<6%). 1H NMR (400 MHz, D2O): 7.40 (d, J = 160.9 Hz, 1H, 13C-arom.); 4.13 (s, 0.11 H, residual m-CH-arom.); 13C NMR (100.6 MHz, DMSO-d6): 127.48 (13C); HRMS (ESI): calcd for C1513CH2D3NO [M + Na]+ 170.0805; found 170.0803.

[2,6,13C2]2,3,5,6-tetra-deuteriophenyl]pyruvate 23. [2,6-13C2]3,5-dieuterio-4-amino-phenol 22 (460 mg) was treated with D2O (4.6 mL) and HCl conc. (57 µL) at 180 °C for 37 min in a microwave reactor. The solvents were removed in vacuo and the residual black solid dissolved in methanol (10 mL). Evaporation of the solvent gave [2,6,13C2]3,5-dieuterio-4-amino-phenol 23 (464 mg, 99%) as a dark solid. NMR spectroscopy showed a deuteration grade of 92% in positions 3 and 5 whereas positions 2 and 6 revealed a deuteration grade of 23%. 1H NMR (400 MHz, DMSO-d6): 8.29 (t, J = 4.1 Hz, 1H, OH), 6.45 (d, J = 153.2 Hz, 2H, 13C-arom.); 6.41 (d, J = 8.6 Hz, 2H, 13C-arom.); 4.35 (s, 2H, NH2); 13C NMR (100.6 MHz, DMSO-d6): 116.00 (13C); HRMS (ESI): calcd for C1513CH2D3NO [M + H]+ 112.0673; found 112.0668.

[2,6-13C2]3,5-dieuterio-4-amino-phenol 26. The reagents [3,5-13C2]2,6-dieuteriohydroxybenzaldehyde 25 (474 mg), hydantoin (423 mg) and piperidine (575 mg) were stirred in a 10 mL round bottomed flask, equipped with a reflux condenser at 130 °C for 30 min. Addition of warm water (8 mL) was followed by homogenization of the resulting mixture in an ultrasonic bath. Precipitation of a solid was induced by adding HCl conc. (0.5 mL). The crude product was separated by filtration and recrystallized from methanol, yielding 5-[3,5-13C2]2,6-dieuterio-4-hydroxybenzylidene]hydantoin 26 (742 mg, 74%) as a yellow solid. 1H NMR (400 MHz, DMSO-d6): 11.09 (s, 1H, NH), 10.30 (s, 1H, NH), 9.83 (s, 1H, CH), 7.50–7.43 (m, 0.12H, residual 13C-arom.); 6.78 (d, J = 159.2 Hz, 1H, 13C-arom.); 13C NMR (100.6 Hz, DMSO-d6): 116.07 (13C); HRMS (ESI): calcd for C1513CH2D3NO [M + Na]+ 126.0555; found 126.0549.

Sodium 3,3-dieuterio[13,13C2]2,6-dieuteriohydroxybenzaldehyde 25. A solution of [3,5-13C2]2,6-dieuteriohydroxybenzaldehyde 24 in dry dichloromethane (150 mL) was set under an argon atmosphere and cooled to −78 °C. After the addition of diisobutylaluminium hydride (11.4 mL, 1 M in dichloromethane) was accomplished by using a syringe, the mixture was allowed to warm to −40 °C for a period of 2 h. The reaction was quenched by addition of silica gel (5 g) and water (3 mL) in small portions and the resulting mixture was stirred at 0 °C for 1 h; then, the solution was transferred into an Erlenmeyer flask and a spatula of K2CO3 was added. After drying over MgSO4, the solid was separated off by filtration and rinsed with dichloromethane until no more product was washed out of the silica gel/MgSO4 mixture (control of TLC spots under UV light). Evaporation of the combined organic phases under reduced pressure gave 504 mg (90%) of [3,5-13C2]2,6-dieuteriohydroxybenzaldehyde 25 as a yellow solid. 1H NMR (400 MHz, DMSO-d6): 10.57 (s, 1H, OH), 9.79 (s, 1H, CHO), 7.78–7.73 (m, 0.15H, residual 13C-arom.); 6.92 (d, J = 160.8 Hz, 1.55 H, 13C-arom.); 13C NMR (100.6 MHz, DMSO-d6): 115.69 (13C); HRMS (ESI): calcd for C1513CH2D3NO [M + Na]+ 126.0555; found 126.0549.
syringe. Throughout the reaction a constant stream of argon was purged through the reaction mixture via a syringe and needle to prevent oxidative degradation of the product. The mixture was stirred at 110 °C for 4 h. After the reaction was allowed to cool to room temperature, the mixture was extracted with diethylether (2 × 20 mL). Subsequent addition of HClconc. (2.5 mL) to the aqueous phase was followed by extraction with diethylether (5 × 30 mL). The organic phases were combined and dried over MgSO4. Evaporation of the solvents yielded 4-nitroanisole (310 µL) were added and stirring was continued for 1 h. The iodomethane (934 µL) was added dropwise. After stirring the mixture was stirred at room temperature for 10 min before 4-nitrophenol (700 mg) in acetone (25 mL). The reaction mixture was stirred at 110 °C for 4 h. After the reaction was cooled and the solvents had been removed under reduced pressure, the residue was dissolved in MeOH (40 mL) and conducted over a Pd/C (10%) catalyst cartridge in a continuous-flow hydrogen reactor (H-cube® Thalesnano) at a flow-rate of 1 mL min−1 and room temperature. The hydrogen generator was set to full hydrogen mode. Evaporation of methanol under reduced pressure gave p-anisidine 28 (392 mg, 89%). 1H NMR (400 MHz, DMSO-d6): 6.74 (dm, J = 8.9 Hz, 2H, m-CHarom.), 6.65 (dm, J = 8.9 Hz, 2H, o-CHarom.); 13C NMR (100.6 Hz, DMSO-d6): 139.94 (C arom.NH2), 113.42 (C arom.CH3), 114.42 (CH3), 55.76 (CH3). A microwave vessel was charged with anisidine (272 mg), D2O (2.5 mL) and HClconc. (50 µL) and heated in the microwave reactor at 180 °C for 40 min. After the solvents had been removed under reduced pressure, the residue was dissolved in methanol (10 mL) and concentrated again to yield 2,6-dideuterio-p-anisidine 29 (245 mg, 90%). 1H NMR (400 MHz, DMSO-d6): 6.73 (s, 2H, CHarom.), 6.37 (bs, 2H, NH2), 3.65 (s, 3H, CH3); 13C NMR (100.6 Hz, DMSO-d6): 153.14 (C arom.), 138.15 (C arom.NH2), 114.95 (CHarom.), 55.81 (CH3). Drying of the organic phase over MgSO4 and evaporation of residue was dissolved in ethyl acetate (100 mL). This solution (686 mg, 90%).1H NMR (400 MHz, D2O): 6.37 (bs, 2H, NH2); 13C NMR (100.6 Hz, D2O): 115.69 (13C). HRMS (EI): C7H2OND2 125.0804; found 125.0803. 2,6-Dideuterio-4-aminophenol 30 (63 mg, 73%). 1H NMR (400 MHz, DMSO-d6): 8.30 (s, 1H, OH), 6.46 (s, 2H, o-CHarom.), 4.34 (s, 2H, NH2); 13C NMR (100.6 Hz, DMSO-d6): 148.16 (C arom.OH), 140.52 (C arom.NH2), 115.07 (o-CHarom.).

Notes and references


