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Synthesis of [3,4,8-$^{13}C_3$]daidzein

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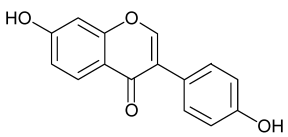
Abstract—The biological effects of the soy isoflavones have attracted considerable interest in recent years leading to numerous studies on dietary intake and epidemiology. Such studies require accurate and reproducible analytical methods. Herein we report the first synthesis of a multiply ^{13}C-labelled daidzein derivative, [3,4,8-$^{13}C_3$]daidzein, which has been employed as an internal standard in LC-MS and GC-MS analysis. The synthesis includes an improved three-step method for the synthesis of [2-^{13}C]resorcinol as one building block.

1. Introduction

The impact of phytoestrogens on human health is currently a subject of major interest.[1,2] In particular the soy isoflavones, including daidzein **1** and genistein **2**, have attracted considerable attention.[3,4] The soy isoflavones are present at significant levels in soya beans and soy products. They thus feature heavily in the diet particularly in Japan and Asia, where they have been associated with a low incidence of hormone dependent cancers.[5] These compounds have also been implicated in the prevention of cardiovascular disease,[6] lessening the symptoms of the menopause[7] and protection against osteoporosis.[8]

In order to understand the significance of the biological effects of phytoestrogens, analytical chemistry has had a key role to play. Accurate analysis has been important in attempts to establish the exposure of the population to the soy isoflavones through their diet and also in epidemiological studies to investigate the associations between isoflavone exposure and disease.



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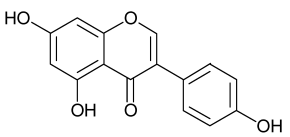

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A number of analytical methods have been used to quantify the low levels of phytoestrogens found in food and biological fluids. HPLC has been employed with UV detection, but this suffers from poor selectivity and low sensitivity.[9] Time-resolved fluoroimmunoassay (TR-FIA) methods have been developed for both daidzein and genistein.[10] These are undoubtedly rapid methods with high sensitivity but can suffer from a lack of specificity.[11] However, by far the most widely used technique has been mass spectrometry due to its inherent sensitivity and selectivity. GC-MS has been used extensively for the quantification of isoflavones in urine, even though it requires complex purification procedures and derivatisation of the analytes.[12–14] More recently LC-MS procedures have become more popular as these can be carried out without derivatisation and often with little or no purification of the sample prior to analysis, allowing the use of smaller samples.[15–18]

An important aspect of any quantitative analytical procedure is the nature of the internal standard. The optimum internal standard for LC-MS and GC-MS is a pure, stable, isotopically labelled analogue of the analyte, which must have a large enough mass difference to nullify the effect of natural abundance heavy isotopes in the analyte. This mass difference will depend on the molecular weight of the analyte. For isoflavone type structures a minimum of three extra mass units is required. Initially a number of deuterated standards were used for the analysis of isoflavones.[18] These were prepared using acid, or base, catalysed exchange methods to incorporate the deuterium atoms into the phenol rings.[19–21] The problem with these deuterated isoflavones is that they are always a mixture of species with varying numbers of deuteriums incorporated into the isoflavone, which is an inevitable consequence of the exchange procedures employed. This produces a range of molecular ions and complicates the analysis procedure unnecessarily. More significantly it has been shown that under the analysis conditions back exchange occurs and the deuteriums are slowly replaced by hydrogen.[22,23] This results in a reduction of the amount of internal standard present and, also, an apparent increase in the amount of the phytoestrogen being analysed.

Keywords: Daidzein; Isoflavones; Phytoestrogens; ^{13}C-labelling.
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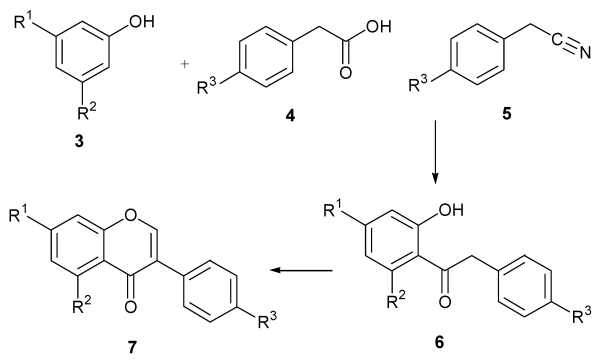


Scheme 1.

Due to the inherent problems with deuterated internal standards the aim of this work was to synthesise daidzein with three ^{13}C atoms incorporated into the carbon framework of the molecule for use as an internal standard. Using commercially available starting materials with 99% incorporation of ^{13}C, it should be possible to obtain the final product with 99% ^{13}C at the corresponding position in the phytoestrogen molecule. Thus with three ^{13}C atoms one major signal three mass units bigger than the unlabelled compound will be observed. This is in contrast to the variable levels of deuterium incorporation obtained through exchange procedures, which give an envelope of signals. Also the ^{13}C standards will be chemically stable and the ^{13}C atoms will not exchange back out from the molecule during the analysis procedures. We had previously prepared both daidzein and genistein labelled with a single ^{13}C-atom at the 4-position[24,25] and these compounds have been employed in metabolic studies on menopausal women.[26] This previous synthetic strategy was used as a suitable starting point.

2. Results and discussion

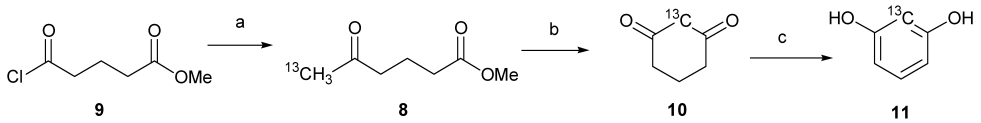
The general synthetic route towards the isoflavonoid phytoestrogens (Scheme 1) involves the condensation of an appropriate phenol **3** with either a substituted phenylacetic acid[24,25,27] **4** or a benzyl nitrile **5**.[24,25,28–30] This gives a deoxybenzoin **6** which then undergoes formylation and finally cyclisation to give the isoflavonoid **7**.[24,25,30,31] The synthesis presents various opportunities for the incorporation of ^{13}C atoms. However, the obvious strategy of putting put one ^{13}C atom into the 2-position in the formylation step at the end of the synthesis had already been shown to be unsuitable.[24] It was thus decided to incorporate one ^{13}C atom into the resorcinol and two into the side chain of the phenylacetic acid.

A previous literature procedure was discovered for the synthesis of [2-^{13}C]resorcinol[32] involving the synthesis of methyl 5-oxo-[6-^{13}C]hexanoate **8**, cyclisation via an intramolecular Claisen condensation and finally aromatisation to give the desired product. Firstly, methyl 4-(chloroformyl)butyrate **9** was converted into a mixed anhydride by reaction with 2-methoxybenzoic acid[33] and then reacted with [^{13}C]methyl magnesium iodide to give **8** in a low 30% yield. This step was thus modified and the methyl 4-(chloroformyl)butyrate **9** was reacted directly with a cuprate derived from [^{13}C]methyl iodide, giving the product in a much better 57% yield (Scheme 2).

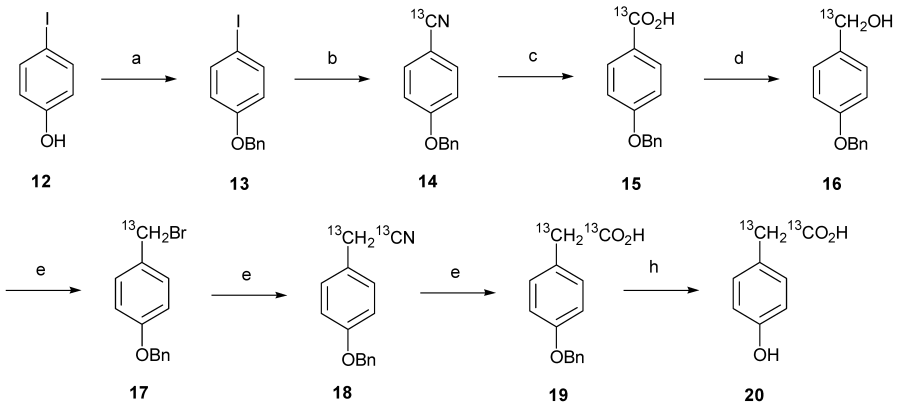
The cyclisation to [2-^{13}C]cyclohexane-1,3-dione **10** was previously[32] carried out in DMF with sodium methoxide in 61% yield, but we obtained a better 86% yield by using carefully dried potassium ^{t}butoxide in THF. Dehydrogenation was achieved using a palladium on charcoal catalyst in refluxing xylene at 137–140 °C giving [2-^{13}C]resorcinol **11** in 49% yield. A lower temperature was employed for this reaction compared to the 190 °C in refluxing triglyme previously reported.[32] The yields were comparable for the two procedures, but the lower temperature produced a cleaner more easily purified product. The spectral data for the [2-^{13}C]resorcinol were identical to the literature data.[32] The ^{13}C atom was clearly observed as an enhanced signal at 103.8 ppm in the ^{13}C NMR spectrum. This represents a much improved synthesis of [2-^{13}C]resorcinol in 24% yield over the three steps.

It was then necessary to incorporate two ^{13}C-atoms into the phenylacetic acid fragment and it was envisaged that these could both be derived from ^{13}C-labelled potassium cyanide, a very cheap source of ^{13}C atoms. In our previous work potassium [^{13}C]cyanide was reacted with a suitably protected benzyl bromide to give a single labelled precursor.[25] Therefore incorporation of two ^{13}C atoms required a ^{13}C-labelled benzyl bromide, which could be synthesised via an aromatic cyanation using another mole of potassium [^{13}C]cyanide and a suitably substituted aryl halide, followed by functional group transformations.

4-Iodophenol **12** was used as the starting material and the hydroxyl group was first protected as the benzyl ether **13** (Scheme 3). A large number of procedures are available for aromatic cyanation[34] but our choice was restricted by the requirement for ^{13}C-labelled cyanide, of which only the sodium and potassium salts are commercially available. Thus all the copper(I) cyanide methods were ruled out and instead a procedure employing potassium cyanide and a palladium(II) acetate catalyst in DMF under basic conditions (calcium hydroxide) was identified.[35] Only a limited range of aryl iodides had been examined under these conditions but we found that 4-benzyloxy-1-iodobenzene **13** reacted smoothly with unlabelled potassium cyanide to give the desired product. The reaction conditions were then optimised and using K^{13}CN the purified ^{13}C-labelled nitrile was obtained in 70% yield. The ^{13}C atom was clearly identified by the enhanced signal in the ^{13}C NMR spectrum at 119.6 ppm. The nitrile **14** was then hydrolysed under



Scheme 2. (a) $H_3^{13}CI$, Li, CuI, Et_2O, (57%); (b) KO^{t}Bu, THF then aq. HCl (86%); (c) 10% Pd/C, xylene, reflux (49%).



Scheme 3. (a) BnBr, K_2CO_3, acetone (90%); (b) $K^{13}CN$, $Pd(OAc)_2$, $Ca(OH)_2$, DMF (70%); (c) 2 N NaOH, MeOH (83%); (d) $LiAlH_4$, THF (93%); (e) PBr_3, Et_2O (93%); (f) $K^{13}CN$, 18-crown-6, MeCN (80%); (g) 2 M aq. NaOH, reflux (98%); (h) H_2, 10% Pd/C, EtOAc (96%).

alkaline conditions, sodium hydroxide in methanol, to give the acid **15** in 83% yield and reduced to the alcohol **16** in 93% yield using lithium aluminium hydride in THF.

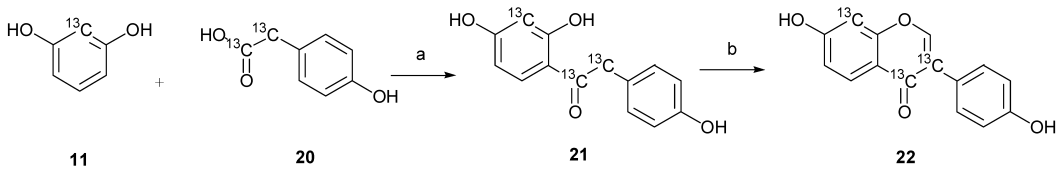
A number of procedures were examined for the conversion of the alcohol to the bromide **17** including trimethylsilyl bromide and carbon tetrabromide with triphenylphosphine. However, the best yield was obtained using phosphorus tribromide. The bromide was found to be reasonably unstable due to the 4-substituent and so was used without purification in the next step.

Reaction of the benzyl bromide **17** with a second mole of $K^{13}CN$ in acetonitrile, using 18-crown-6 to aid solubility, effected nucleophilic displacement to give the benzyl nitrile with **18** two ^{13}C-atoms, observed at 118.6 and 23.2 ppm in the ^{13}C NMR spectrum. Alkaline hydrolysis to the carboxylic acid, **19** was followed by hydrogenation to remove the benzyl ether and provide the precursor **20** for the synthesis of [3,4,8-$^{13}C_3$]daidzein (Scheme 3).

With the two ^{13}C-labelled building blocks prepared, the [3,4,8-$^{13}C_3$]daidzein was synthesised using adaptations of our previous methods (Scheme 4).[25] Condensation of the [2-^{13}C]resorcinol **11** and the phenylacetic acid **20** was carried out in neat boron trifluoride etherate, giving the deoxybenzoin intermediate **21** in 55% yield. The formylation/cyclisation step was achieved using dimethylformamide dimethylacetal in THF. In our previous work this had been carried out at reflux in 60% yield.[24,25] However, the step was carefully optimised by varying the temperature, time of reaction and solvent (THF, diethyl ether and DMF) and it was found that the yield increased as the reaction temperature was decreased, giving an optimum yield of 80% after stirring at room temperature in THF for 3 h. The crude product from this reaction was found to be much cleaner than from reactions at higher temperature and readily purified by simple crystallisation, whereas HPLC was required to clean up the products from the higher temperature reactions. This represents the best reported yield for this procedure in the synthesis of daidzein and we have found it to be more reproducible than many of the published methods.[25,30,31]

The [3,4,8-$^{13}C_3$]daidzein **22** was found to be identical to daidzein in all respects except for the expected increase in mass and NMR spectroscopic data. The three ^{13}C atoms were observed at 178.8 ppm, for the carbonyl at C-4, 126.0 ppm for C-3 and 103.5 ppm for C-8, with the expected coupling between C-3 and C-4 (J=54 Hz). The effect of the ^{13}C incorporation was also evident on the ^1H NMR spectrum, for example 8-H exhibited a 162 Hz coupling with the attached ^{13}C atom. The purity of the compound was confirmed by reverse phase HPLC (Kingsorb 3μ C18 Column (150×4.6 mm)) giving a retention time of 8 min 40 s with a mobile phase of acetonitrile:water (1:1) and a 0.3 mL min^{-1} flow rate. This gave a single peak at the identical retention time to unlabelled daidzein. Furthermore, the UV spectrum was measured giving a λ_{max} (EtOH) of 262 nm and ε= 23,894 $dm^3\ mol^{-1}\ cm^{-1}$, compared with literature value[36] of ε=24,739 $dm^3\ mol^{-1}\ cm^{-1}$, implying 96% purity within experimental error.

The compound has since been used as an internal standard in both GC-MS[37] and LC-MS[22,38] using isotope dilution methods. To be used over an extended dynamic range the isotopically labelled internal standard must have no significant mass overlap with the unlabelled analyte. This is obviously complicated by the natural abundance distribution of carbon, hydrogen and oxygen. When isotope distributions are taken into consideration the predicted mass intensity pattern for daidzein examined by negative electrospray ionisation is 253 (100%):254 (18%):255(3%). For quantitative analysis selective ion monitoring (SIM) is used at 253 for optimal instrumental sensitivity, so the



Scheme 4. (a) $BF_3{\cdot}Et_2O$ (55%); (b) $DMF{\cdot}(OMe)_2$, DMF (80%).

daidzein internal standard must not give any significant signal at this mass. When the isotopic purity of the [3,4,8-$^{13}C_3$]daidzein was examined by LC-MS, the major signal was observed, as expected, at 256 along with a signal of 4% relative intensity at 255 for daidzein molecules having only two ^{13}C atoms.[22] However, there were no discernible ions due to molecules with zero or one ^{13}C per molecule, thus comfortably meeting the criteria for use as an internal standard for isotope dilution mass spectrometry. Recent isoflavone analysis by GC-MS[37] demonstrated improved linearity over a range of concentrations due to the use of the ^{13}C-labelled internal standards, along with lower limits of detection. Quality control was also improved with lower intra- and interassay coefficients of variation (CV) for isoflavones where a ^{13}C-labelled standard was available.

3. Experimental

3.1. General

NMR spectra were recorded on a Varian Gemini 2000 (^1H 300 MHz, ^{13}C 75.45 MHz) or a Bruker Avance 300 (^1H 300 MHz, ^{13}C 75.45 MHz) spectrometer chemical shifts (δ) in ppm are given relative to Me_4Si, coupling constants (J) in Hz. Elemental analyses were carried out in the departmental microanalytical laboratory. IR spectra were recorded on a Perkin–Elmer series 1420 FT IR spectrophotometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs and recorded in cm^{-1}. EI and CI mass spectra were recorded on a VG Autospec. Electrospray mass spectra recorded on Micromass LC-T UV spectra were recorded on a Kontron Uvikon 930 spectrometer. Melting points were recorded on an electrothermal melting point apparatus and are uncorrected. Analytical TLC was carried out on Merck 5785 Kieselgel $60F_{254}$ fluorescent plates. Flash chromatography was performed according to the procedure of Still[39] using silica gel of 35–70 μ particle size. DMF was distilled from magnesium sulfate. Diethyl ether and tetrahydrofuran were distilled from sodium metal and benzophenone.

3.1.1. Methyl 5-oxo-[6-^{13}C]hexanoate (8).[32] Under the protection of a nitrogen atmosphere, lithium pieces (489 mg, 70.4 mmol) were added to dry diethyl ether (70 mL). ^{13}C-Methyl iodide (1.8 mL, 4.104 g, 28.91 mmol) was then introduced and the reaction mixture stirred at room temperature for 1 h, then cooled to 0 °C and copper iodide (3.352 g, 17.6 mmol) was added. After 0.5 h stirring at 0 °C, the resulting lithium dimethylcuprate was cooled to −20 °C. Precooled methyl 4-chloroformyl butyrate (2 mL, 2.386 g, 14.5 mmol) was then added dropwise with vigorous stirring. The reaction mixture was maintained at −20 °C for 1 h, then room temperature overnight. The reaction was quenched with saturated ammonium chloride (45 mL) and filtered to remove the copper residue. The filtrate was extracted with diethyl ether (3×20 mL), washed with brine (20 mL) and dried ($MgSO_4$). Further purification was carried out by column chromatography, eluting with petroleum ether (40–60 °C)/ethyl acetate (1:1) to give a colourless oil (1.19 g, 57%); ν_{max} (nujol)/cm^{-1} 1725 (C=O); δ_H (300 MHz, $CDCl_3$) 3.64 (3H, s, $-OCH_3$), 2.48 (2H, t, J=7.2 Hz, CH_2-3), 2.31 (2H, t, J=7.2 Hz, CH_2-4), 2.11 (3H, d, J=127 Hz, CH_2-6), 1.86 (2H, quin, J=7.2 Hz, CH_2-2); δ_C (75.45 MHz, $CDCl_3$) 202 (d, $J_{5,6}$=45 Hz, *C*-5), 174.4 (*C*-1), 52.0 (OCH_3), 42.9 (d, $J_{4,6}$=22 Hz, *C*-4) 33.4 (*C*-2), 30.2 (enhanced, *C*-6), 26.4 (*C*-6); *m/z* (ES^+) 168 [$(M+Na)^+$, 100%]; (EI) 145 (M^+, 15%), 114 (40, $[M-OCH_3]^+$), 86 (30, $[M-COOCH_3]^+$), 44 (100, CH_3CO^+).

3.1.2. [2-^{13}C]Cyclohexane-1,3-dione (10). To the solution of methyl 5-oxo-[6-^{13}C]hexanoate **8** (758 mg, 5.22 mmol) in dry THF (100 mL), was added potassium ^{t}butoxide (2.345 g, 20.9 mmol). The mixture was heated under reflux for 6 h, then the THF was removed at reduced pressure. The residue was dissolved in water (20 mL), acidified to pH 1 with concentrated hydrochloric acid, extracted with ethyl acetate (6×20 mL) and dried ($MgSO_4$). After removal of the solvent at reduced pressure, the desired product was obtained as a pale yellow solid (510 mg, 86%) mp 102–103 °C (lit.[32] 103–104 °C); ν_{max} (nujol)/cm^{-1} 2480, 1627, 1600, 1180; δ_H (300 MHz, $CDCl_3$) 3.36 (2H, d, J=130 Hz, CH_2-2), 2.55 (4H, t, J=6.7 Hz, CH_2-4 and 6), 2.0–1.8 (2H, m, CH_2-5); δ_C (75.45 MHz, $CDCl_3$) 58.8 (enhanced, *C*-1); *m/z* (EI) 113 (M^+, 50%), 85 (50, $[M-CO]^+$).

3.1.3. [2-^{13}C]Resorcinol (11). To a flask containing [2-^{13}C]cyclohexane-1,3-dione **10** (500 mg, 4.42 mmol) and xylene (75 mL), palladium on carbon (10%, 2.5 g) was added. The mixture was heated under reflux for 3 h, then the palladium catalyst was filtered off through celite. The reaction mixture was extracted with aqueous sodium hydroxide (20%, 4×20 mL). The combined aqueous layers were cooled to 0 °C, acidified to pH 2 with concentrated hydrochloric acid, and extracted with diethyl ether (3×25 mL). After removal of the solvent at reduced pressure, red oil was obtained. Further purification was carried out by column chromatography on silica, eluting with diethyl ether/petroleum ether (40–60 °C) (2:1) to give the desired product as a white solid (238 mg, 49%) mp 105–108 °C (lit.[32] 108–109 °C); ν_{max} (nujol)/cm^{-1} 3240 (OH), 1615, 966; δ_H (300 MHz, acetone-d_6) 8.04 (2H, s, $-OH$), 6.84 (1H, dt, J=8.1, 1.4 Hz, *H*-5), 6.21 (1H, dt, $J_{C,H}$=156, $J_{2,4}=J_{2,6}$=2.3 Hz, *H*-2), 6.26–6.1 (2H, m, *H*-4 and 6); δ_C (75.45 MHz, acetone-d_6) 103.8 (enhanced, *C*-2); *m/z* (CI) 112.0480 (MH^+, $^{12}C_5{}^{13}CH_6O_2$ requires 112.0484).

3.1.4. 4-Benzyloxyiodobenzene (13). To a solution of 4-iodophenol (10 g, 45.5 mmol) in acetone (250 mL) were added potassium carbonate (30.273 g, 227.3 mmol) and benzyl bromide (4.5 mL, 6.471 g, 37.9 mmol). The mixture was heated under reflux for 6 h, then cooled and filtered to remove the solid. The solvent was evaporated at reduced pressure and the crude product dissolved in diethyl ether (50 mL), washed with water (3×20 mL) and dried ($MgSO_4$). Further purification was carried out by column chromatography, eluting with ethyl acetate/petroleum ether (40–60 °C) (1:10) to give the product as white crystals (11.1 g, 94%) mp 60–62 °C (lit.[40] 62–63 °C) (found: C, 50.51; H, 3.58, $C_{13}H_{11}IO$ requires C, 50.35; H, 3.58%); ν_{max} (nujol)/cm^{-1} 1600, 1119, 972; δ_H (300 MHz, $CDCl_3$) 7.57 (2H, d, $J_{2,3}=J_{5,6}$=8.9 Hz, *H*-2, 6), 7.54–7.32 (5H, m, *H*-2′ to H-6′), 6.76 (2H, d, $J_{2,3}=J_{5,6}$=8.9 Hz, *H*-3, 5), 5.04 (2H, s, OCH_2); δ_C (75.45 MHz, $CDCl_3$) 159.0 (*C*-4), 138.6 (*C*-2 and 6), 136.9 (*C*-1′), 129.0 (*C*-3′ and 5′), 128.5 (*C*-4′), 127.8

(*C*-2′ and 6′), 117.7 (*C*-3 and 5), 83.4 (*C*-1), 70.4 (*C*-7); *m/z* (CI) 311 (MH^+, 35%), 310 (23, M^+), 184 (97, $[M-I]^+$), 91 (58, $C_7H_7^+$).

3.1.5. 4-Benzyloxybenzo[^{13}C]nitrile (14). To a solution of 4-benzyloxyiodobenzene **13** (20.04 g, 64.62 mmol) in dry DMF (250 mL) were added calcium hydroxide (2.33 g, 31.5 mmol), potassium [^{13}C]-cyanide (4.30 g, 65 mmol) and palladium acetate (2.25 g, 10.0 mmol). Under a nitrogen atmosphere, the mixture was heated at reflux for 4 h, then the DMF was removed under reduced pressure. The crude product was extracted with diethyl ether (3×50 mL). The combined organic extracts were then washed with water (3×50 mL) and dried ($MgSO_4$). Further purification was carried out by column chromatography, eluted with ethyl acetate/petroleum ether (40–60 °C) (3:10). After removal of the solvent, a white solid was obtained (9.52 g, 70%) mp 96–99 °C; (found: C, 80.31; H, 5.05; N, 6.99. $^{12}C_{13}{}^{13}CH_{11}NO$ requires C, 80.45; H, 5.27; N, 6.66%); ν_{max} (KBr)/cm^{-1} 2169 (CN); δ_H (300 MHz, $CDCl_3$) 7.60 (2H, dd, $J_{2,3}$=$J_{5,6}$=9.0 Hz, $J_{C,H}$=5.0 Hz, *H*-2 and 6), 7.43–7.34 (5H, m, *H*-2′ and 6′), 7.03 (2H, d, $J_{2,3}$=$J_{5,6}$=9.0 Hz, *H*-3 and 5), 5.12 (2H, s, OCH_2); δ_C (75.45 MHz, $CDCl_3$) 162.3 (*C*-4), 136.1 (*C*-1′), 134.0 (*C*-2 and 6), 129.2 (*C*-3′ and 5′), 128.8 (*C*-4′), 127.9 (*C*-2′ and 6′), 119.6 (enhanced ^{13}CN), 116.0 (*C*-3 and 5), 104.6 (d, $J_{C,C}$=83 Hz, *C*-1), 70.7 (*C*-7); *m/z* (CI) 211 (MH^+, 100%), 91 (10, $C_7H_7^+$).

3.1.6. 4-Benzyloxy[*carboxy*-^{13}C]benzoic acid (15). 4-Benzyloxybenzo[^{13}C]nitrile **14** (8.53 g, 40.6 mmol) was mixed with sodium hydroxide (2 N, 500 mL) and methanol (50 mL). The reaction was followed by TLC (silica, ethyl acetate/methanol (50:1)) and heated under reflux until the starting material had disappeared. The resulting mixture was cooled and adjusted to pH 1 with concentrated hydrochloric acid. The solid that precipitated was collected, then heated to reflux with sodium hydroxide (2 N, 500 mL) for another 8 h. The resulting mixture was then cooled and adjusted to pH 1 with concentrated hydrochloric acid again, extracted with diethyl ether/methanol (6:1). The organic layers were combined, washed with brine (2×50 mL), dried ($MgSO_4$) and the solvent removed at reduced pressure to give the product as white crystals (7.74 g, 83%) mp 192–196 °C; (found: C, 73.58; H, 5.08. $^{12}C_{13}{}^{13}CH_{12}O_3$ requires C, 73.78; H, 5.28%); ν_{max} (nujol)/cm^{-1} 1648 (CO_2H), 1601, 972; δ_H (300 MHz, CDCl) 7.94 (2H, dd, $J_{2,3}$=$J_{5,6}$=9.0 Hz, $J_{C,H}$=3.9 Hz, *H*-2 and 6), 7.38–7.24 (5H, m, *H*-2′ and 6′), 6.92 (2H, d, $J_{2,3}$=$J_{5,6}$=9.0 Hz, *H*-3 and 5), 5.04 (2H, s, OCH_2); δ_C (75.45 MHz, $CDCl_3$) 168.9 (enhanced, ^{13}COOH), 162.7 (*C*-4), 136.7 (*C*-1′), 132.2 (*C*-2 and 6), 129.0 (*C*-3′ and 5′), 128.5 (*C*-4′), 127.8 (*C*-2′ and 6′), 123.7 (d, $J_{C,C}$=74 Hz, *C*-1), 114.7 (*C*-3 and 5), 70.4 (*C*-7); *m/z* (EI) 229 (M^+, 10%), 91 (100, $C_7H_7^+$).

3.1.7. 4-Benzyloxy[*methylene*-^{13}C]benzyl alcohol (16). Under the protection of a nitrogen atmosphere, 4-benzyloxy[*carboxy*-^{13}C]benzoic acid **15** (3.50 g, 15.27 mmol) in dry THF (50 mL) was added dropwise to a suspension of lithium aluminium hydride (2.277 g, 60 mmol) in dry THF (60 mL). The mixture was stirred at room temperature overnight, then sulfuric acid (10%, 150 mL) was added carefully to quench the reaction. The resulting mixture was extracted with diethyl ether (4×50 mL), washed with water (3×30 mL) and dried ($MgSO_4$). After removal of the solvent at reduced pressure the desired product was obtained as a pale white solid (3.0 g, 93%); mp 94–96 °C (lit.[41] 94–96 °C); ν_{max} (nujol)/cm^{-1} 3424 (OH), 1601, 972; δ_H (300 MHz, $CDCl_3$) 7.38–7.18 (7H, m, *H*-2′ to 6′, *H*-2 and 6), 6.89 (2H, d, $J_{2',3'}$=$J_{5',6'}$=8.5 Hz, *H*-3 and 5), 5.00 (2H, s, OCH_2), 4.54 (2H, d, $J_{C,H}$=143 Hz, $^{13}CH_2O$); δ_C (75.45 MHz, $CDCl_3$) 158.8 (*C*-4), 137.3 (*C*-1′), 133.7 (d, *J*=48 Hz, *C*-1), 129.1 (*C*-2 and 6), 129.0 (*C*-3′ and 5′), 128.4 (*C*-4′), 127.8 (*C*-2′ and 6′), 115.3 (*C*-3 and 5), 70.4 (C-7), 65.5 (enhanced, $^{13}CH_2O$); *m/z* (EI) 215 (M^+, 25%), 91 (100, $C_7H_7^+$).

3.1.8. 4-Benzyloxy[*methylene*-^{13}C]benzyl bromide (17). To a solution of 4-benzyloxy[*methylene*-^{13}C]benzyl alcohol **16** (2.50 g, 11.61 mmol) in dry diethyl ether (50 mL) was added phosphorus tribromide (3.3 mL, 34.83 mmol). The reaction mixture was stirred at room temperature overnight. The pale yellow solution formed was then poured into ice water (200 mL), extracted with diethyl ether (5×30 mL) and dried ($MgSO_4$). After removal of the solvent at reduced pressure a white solid was obtained (3.02 g, 93%) and this product was employed in next step immediately without further purification. δ_H (300 MHz, $CDCl_3$) 7.37–7.23 (7H, m, *H*-2′ to 6′, *H*-2 and 6), 6.86 (2H, d, $J_{2',3'}$=$J_{5',6'}$=8.6 Hz, *H*-3 and 5), 4.99 (2H, s, OCH_2), 4.43 (2H, d, $J_{C,H}$=153 Hz, $^{13}CH_2Br$); *m/z* (EI) 277/279 (M^+, 5%), 198 (50, $[M-Br]^+$), 91 (100, $C_7H_7^+$).

3.1.9. 4′-Benzyloxy[1,2-$^{13}C_2$]phenylacetonitrile (18). 4′-Benzyloxy[*methylene*-^{13}C]benzyl bromide **17** (2.80 g, 10.06 mmol) was dissolved in acetonitrile (120 mL) then 18-crown-6 (2.66 g, 10.06 mmol) and potassium ^{13}C-cyanide (666 mg, 10.06 mmol) were added. The mixture was heated under reflux for 4 h, then cooled, and the solvent was removed to give a pale white residue, which was then extracted with diethyl ether (3×30 mL). The combined ether layers were washed with water (2×30 mL), dried ($MgSO_4$) and the solvent removed at reduced pressure. Purification by column chromatography on silica, eluting with petroleum ether (40–60 °C)/ethyl acetate (10:3 to 2:1) gave the desired product as a white solid (1.82 g, 80%); mp 66–69 °C; (found: C, 80.46; H, 5.82; N, 6.15. $^{12}C_{13}{}^{13}C_2H_{13}NO$ requires C, 80.86; H, 5.82; N, 6.22%); ν_{max} (nujol)/cm^{-1} 2192 (CN); δ_H(300 MHz, $CDCl_3$) 7.46–7.21 (H, m, *H*-2′ to 6′, *H*-2 and 6), 6.98 (2H, d, $J_{2',3'}$=$J_{5',6'}$=8.7 Hz, *H*-3 and 5), 5.08 (2H, s, OCH_2), 3.69 (2H, dd, $J_{C,H}$=136, 10.6 Hz, $^{13}CH_2{}^{13}CN$); δ_C (75.45 MHz, $CDCl_3$) 158.9 (*C*-4), 137.0 (*C*-1′), 129.5 (*C*-2 and 6), 129.0 (*C*-3′ and 5′), 128.5 (*C*-4′), 127.8 (*C*-2′ and 6′), 122.4 (d, $J_{2,1'}$=42 Hz, *C*-1), 118.6 (enhanced d, $J_{1,2}$=58 Hz, ^{13}CN), 115.9 (*C*-3 and 5), 70.5 (*C*-7), 23.2 (enhanced d, $J_{1,2}$=58 Hz, $^{13}CH_2$); *m/z* (EI) 225 (M^+, 10%), 91 (100, $C_7H_7^+$).

3.1.10. 4′-Benzyloxy[1,2-$^{13}C_2$]phenylacetic acid (19). 4′-Benzyloxy[1,2-$^{13}C_2$]phenylacetonitrile **18** (1.666 g, 7.40 mmol) was dissolved in aqueous sodium hydroxide (2 N, 90 mL) and heated to reflux for 6 h, then cooled and adjusted pH to 1 with concentrated hydrochloric acid. The precipitate formed was collected by filtration, washed with plenty of water and then diethyl ether. After drying under vacuum, the desired product was obtained as a white solid (1.77 g, 98%) mp 120–122 °C; (lit.[42] 120–122 °C) ν_{max}

(nujol)/cm^{-1} 1654 (CO_2H), 1600, 970; δ_H (300 MHz, $CDCl_3$) 7.37–7.21 (5H, m, *H*-2′ and 6′), 7.12 (2H, m, *H*-2, 6), 6.86 (2H, d, $J_{2',3'}=J_{5',6'}=8.7$ Hz, *H*-3 and 5), 4.97 (2H, s, OCH_2), 3.49 (2H, dd, $J_{C,H}=129$, 7.7 Hz, $^{13}CH_2$); δ_C (75.45 MHz, $CDCl_3$) 177.2 (enhanced d, $J_{1,2}=56$ Hz, ^{13}COOH), 158.4 (*C*-4), 137.3 (*C*-1′), 130.8 (*C*-2 and 6), 129.0 (*C*-3′ and 5′), 128.4 (*C*-4′), 127.9 (*C*-2′ and 6′), 126.2 (d, $J=48$ Hz, *C*-1), 115.3 (C3, 5), 70.4 (*C*-7), 40.5 (enhanced d, $J_{1,2}=56$ Hz, $^{13}CH_2$); *m*/*z* (EI) 244 (M^+, 15%), 91 (100, $C_7H_7^+$).

3.1.11. 4′-Hydroxy[1,2-$^{13}C_2$]phenylacetic acid (20). 4′-Benzyloxy[1,2-$^{13}C_2$]phenylacetic acid **19** (300 mg, 1.23 mmol) was dissolved in ethyl acetate (12 mL). After flushing with nitrogen, palladium on carbon (10%, 100 mg) was added. The mixture was stirred under a hydrogen atmosphere at room temperature overnight. The mixture was then filtered through celite, and the solvent removed at reduced pressure to give the product as a pale white solid (181 mg, 96%) mp 149–151 °C (lit.[42] 149–152 °C); (found: C, 63.29; H, 5.15. $^{12}C_6{}^{13}C_2H_8O_3$ requires C, 63.63; H, 5.23%); ν_{max} (nujol)/cm^{-1} 3395 (OH), 1654 (CO_2H); δ_H (300 MHz, $CDCl_3$, ppm) 7.03 (2H, dd, $J_{2',3'}=J_{5',6'}=8.4$ Hz, $J_{C,H}=4.2$ Hz, *H*-2′ and 6′), 6.70 (2H, d, $J_{2',3'}=J_{5',6'}=8.4$ Hz, *H*-3 and 5), 3.42 (2H, dd, $J_{C,H}=129$, 7.6 Hz, $^{13}CH_2$); δ_C (75.45 MHz, $CDCl_3$) 175.0 (enhanced, d, $J_{1,2}=55$ Hz, ^{13}COOH), 156.1 (*C*-4′), 130.5 (*C*-2′ and 6′), 125.5 (d, $J_{2,1'}=42$ Hz, *C*-1′), 115.6 (*C*-3′ and 5′), 40.5 (enhanced d, $J_{1,2}=55$ Hz, $^{13}CH_2$); *m*/*z* (ES^+) 177 ($[M+Na]^+$, 100%); *m*/*z* (ES^-) 153 [$(M-H)^-$, 65%].

3.1.12. [1,2,3′-$^{13}C_3$]-1-(2′,4′-Dihydroxyphenyl)-2-(4″-hydroxyphenyl)ethanone (21). To [2-^{13}C]resorcinol **11** (100 mg, 0.9 mmol) and 4′-hydroxy[1,2-$^{13}C_2$]phenylacetic acid **20** (139 mg, 0.9 mmol) was added boron trifluoride diethyl etherate (5 mL) under a nitrogen atmosphere. The mixture was heated at 70–80 °C for 3 h, then cooled, and poured into saturated sodium acetate (50 mL) and basified with saturated sodium hydrogen carbonate (40 mL). The mixture was then extracted with diethyl ether (4×20 mL), the organic layers combined and concentrated at reduced pressure to yield a pink gum. This crude product was purified by column chromatography on silica, eluting with dichloromethane/ethyl acetate (4:1) to give the desired product as a white solid (114 mg, 55%) mp 186–188 °C (lit.[30] 188–190 °C); ν_{max} (nujol)/cm^{-1} 3395 (OH), 1610 (C=O); δ_H (300 MHz, CD_3OD) 7.74 (1H, ddd, $J_{5',6'}=8.9$ Hz, $J_{C,H}=4.0$, $J_{3',6'}=1.2$ Hz, *H*-6′), 7.00 (2H, dd, $J_{2'',3''}=J_{5'',6''}=8.2$ Hz, $J_{2,2''}=J_{2,6''}=4.1$ Hz, *H*-2″ and 6″), 6.62 (2H, d, $J_{2'',3''}=J_{5'',6''}=8.2$ Hz, *H*-3″ and 5″); 6.14 (1H, dd, $J_{C,H}=160$ Hz, $J_{3',6'}=1.2$, *H*-3′), 6.18–6.32 (1H, m, *H*-5′), 4.01 (2H, dd, $J_{C,H}=128$, 6.0 Hz, CH_2-2); δ_C (75.45 MHz, CD_3OD) 203.2 (enhanced, d, $J_{1,2}=44$ Hz, *C*-1), 102.5 (enhanced, *C*-3′), 43.5 (enhanced, d, $J_{1,2}=44$ Hz, *C*-2); *m*/*z* (ES^-) 246.0764 ([$(M-H)^-$], $^{12}C_{11}{}^{13}C_3H_{12}O_4$ ES^- requires 246.0760).

3.1.13. [3,4,8-$^{13}C_3$]Daidzein (22). Under the protection of a nitrogen atmosphere, the deoxybenzoin **21** (80 mg, 0.35 mmol) was dissolved in dry DMF (3 mL). Then DMF dimethyl acetal (0.9 mL, 0.807 g, 6.77 mmol) was introduced dropwise. The mixture was stirred at room temperature for 3 h, and then poured into aqueous hydrochloric acid (1 M, 70 mL) and stirred for a further 2 h. The suspension formed was left at room temperature overnight. The precipitate was then collected by filtration, washed with water (3×5 mL), then diethyl ether (3×2 mL) and dried under vacuum. Further recrystallisation of the product from methanol gave the pure product as white solid (72 mg, 80%) mp 221 °C (lit.[31] 212–214 °C); λ_{max} (EtOH)/nm 262 (ε/23894 dm^3 mol^{-1} cm^{-1}, lit.[36] 24,739); ν_{max} (nujol)/cm^{-1} 3360 (OH), 1735 (C=O); δ_H (300 MHz, d^6-acetone) 9.6 (1H, s, 7-O*H*), 8.4 (1H, s, 4′-O*H*), 8.06 (1H, ddd, $J_{5,6}=8.8$ Hz, $J_{4,5}=3.8$ Hz ($^{13}C-^1H$ coupling), $J_{5,8}=1.3$ Hz, *H*-5), 8.14 (1H, t, $J_{2,3}=J_{2,4}=6.4$ Hz ($^{13}C-^1H$ couplings), *H*-2), 7.48 (2H, dd, $J_{2',3'}=J_{5',6'}=8.6$ Hz, $J_{3,2'}=J_{3,6'}=3.5$ Hz ($^{13}C-^1H$ coupling), 2′ and *H*-6′), 7.0 (1H, m, *H*-6), 6.9 (1H, dt, $J=162$ Hz ($^{13}C-^1H$ coupling), $J_{6,8}=1.9$ Hz, *H*-8), 6.89 (2H, d, $J_{2',3'}=J_{5',6'}=8.6$ Hz, *H*-3′ and 5′); δ_C (75.45 MHz, d^6-acetone) 178.8 (d, enhanced, $J_{3,4}=54$ Hz, *C*-4), 126.0 (d, enhanced, $J_{3,4}=54$ Hz, *C*-3), 103.5 (enhanced, *C*-8); *m*/*z* (CI) 258.0750 (MH^+, $^{12}C_{12}{}^{13}C_3H_{11}O_4$ requires 258.0758).

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