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A gram scale synthesis of a multi-^{13}C-labelled anthocyanin, [6,8,10,3′,5′-$^{13}C_5$]cyanidin-3-glucoside, for use in oral tracer studies in humans†

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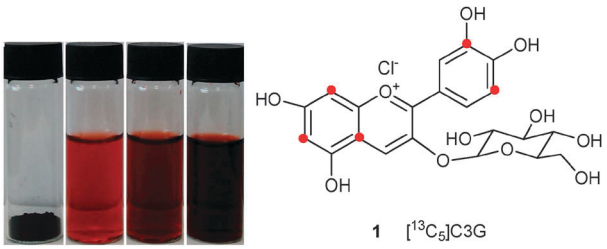
The major dietary anthocyanin, cyanidin-3-glucoside, was prepared on a 4 g scale from three units of diethyl [2-^{13}C]malonate and one unit of [1,3-$^{13}C_2$]acetone, such that five isotope locations were distributed throughout the molecule to provide a penta-$^{13}C_5$-labelled anthocyanin, [6,8,10,3′,5′-$^{13}C_5$]cyanidin-3-glucoside chloride, for use in human stable-isotope tracer studies.

Anthocyanins are naturally occurring polyphenols belonging to the flavonoid family of natural products. These pigments are pyrylium salts which are responsible for the rich colours from red, purple to blue displayed in various flowers, seeds, leaves, fruits and vegetables. Epidemiological evidence suggests that those who consume the highest proportions of anthocyanins in the diet are at the lowest risk of cardiovascular diseases such as hypertension and stroke.[1–3] However, due to their low stability at physiological pH and high propensity to bind with other molecules, their absorption, distribution, metabolism and elimination (ADME) in humans has yet to be fully established. The complete characterisation of the ADME of anthocyanins requires the use of multi-isotopically labelled anthocyanins in human feeding studies to identify the metabolites of cyanidin-3-glucoside (C3G) degradation products *in vivo*.

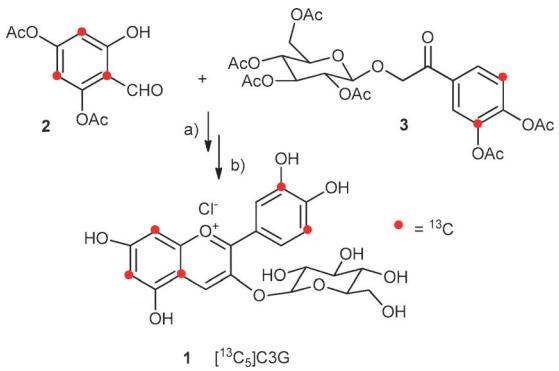
Isotopically labelled anthocyanins have been prepared previously in plant cell culture or catalysed by enzymes using ^{14}C and ^{3}H labels,[4–6] however these radioactive isotopes are not suitable for use in modern human feeding trials. Regio-selective ^{13}C-labelling studies have also been reported in plants from [4-^{13}C]enrichment.[7] C3G **1** is the most commonly occurring anthocyanin, found in 90% of all fruits (Fig. 1). Herein, we report a unique synthesis with five ^{13}C-isotopes located in both the aryl moieties of C3G. The resultant [6,8,10,3′,5′-$^{13}C_5$]-cyanidin-3-glucosyl chloride [[$^{13}C_5$]C3G] is targeted for *in vivo* pharmacokinetic and metabolism studies in humans, and thus several grams of C3G **1** were required such that aerosol (CO_2), urine, blood and faeces samples could be collected post bolus for analysis *via* LC-MS/MS and isotope ratio mass spectroscopy (IRMS).



1 [$^{13}C_5$]C3G

Fig. 1 Anthocyanin **1** is a deep red solid. The photo shows the solid and solutions of increasing concentration in dil. aq. HCl.

Due to the inherent instability of anthocyanins/anthocyanidins in neutral and basic conditions, only a few syntheses have been reported to date.[8–14] Among them, the aldol condensation between a phenolic aldehyde and an aryl ketone, pioneered by Robert Robinson in the early 1900s, remains efficient and has subsequently been further developed.[15] We adopted this key disconnection for the synthesis of [6,8,10,3′,5′-$^{13}C_5$]**1** as it allowed the incorporation of several ^{13}C atoms into both the building blocks, the phenolic aldehyde **2** and the glycosylated aryl ketone **3**, as illustrated in Scheme 1. Retrosynthetic analysis of each of the required precursors **2** and **3** suggested a synthetic route starting from commercially available diethyl [2-^{13}C]malonate and [1,3-$^{13}C_2$]acetone respectively.



1 [$^{13}C_5$]C3G

Scheme 1 Robinson's final step in anthocyanin synthesis. *Reagents and conditions*: (a) anhydrous HCl, EtOAc, −20 to 4 °C; (b) MeOH, H_2O, KOH, then 2 N HCl, 40–50%.

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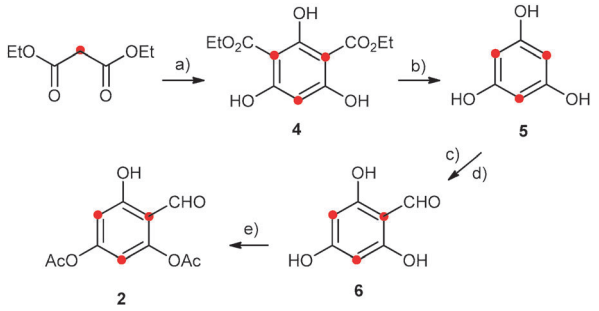


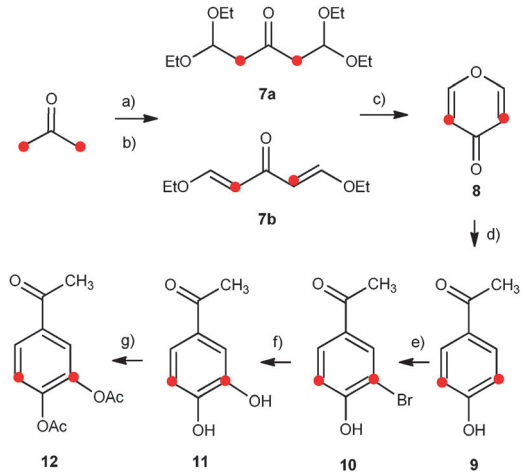
Scheme 2 *Reagents and conditions*: (a) EtONa, EtOH, 135–138 °C, 23–27%; (b) 60% aq. KOH, 130 °C, 96%; (c) oxalyl chloride, DMF, acetonitrile, −17 °C to RT; (d) H_2O, 70 °C, 72–81%; (e) $(CH_3CO)_2O$ (2 eq.), DMAP (0.2 eq.), EtOAc, reflux, 39–40%.

The synthesis of **2** exploited a route previously described for the synthesis of [2,4,6-$^{14}C_3$]phloroglucinol (Scheme 2).[16] This approach incorporates three ^{13}C atoms in a regiospecific manner.

The conversion of diethyl [2-^{13}C]malonate to diethyl 2,4,6-trihydroxy-[1,3,5-$^{13}C_3$]isophthalate **4** proved to be the most problematic step in the synthesis of **2**. The process was optimized with freshly dried, natural abundance substrate, on a scale of up to 8 g of diethyl malonate to give **4** in a yield of 40–42%, with a recovery of 20% of starting material. However, reaction with diethyl [2-^{13}C]malonate purchased to order, and carefully pre-dried, gave a yield of only 23–27% of [1,3,5-$^{13}C_3$]**4** under the same conditions. Due to the need to prepare multiple grams of **1**, this first step was carried out on ~30–40 g of diethyl [2-^{13}C]malonate, giving an average yield of 25% of [1,3,5-$^{13}C_3$]**4**. The starting material (*ca.* 22%) was recovered at a yield of only 10–12% for the second cycle. The hydrolysis of **4** and *in situ* decarboxylation to **5** was more efficient and was carried out on ~6 g batches of [1,3,5-$^{13}C_3$]**4**. A Vilsmeier–Haack reaction was used for formylation to generate **6**. A modification[17] using oxalyl chloride in place of $POCl_3$ improved the conversion and led to greater recovery of **6** (72% at 6 g scale of **5** and 79–81% at 3 g of **5**). The reaction of the Vilsmeier iminium cation with **5** was extremely viscous and due to the difficulty of stirring reactions on a larger scale (>10 g) led to di-formylation side products. Thus reactions were kept between 3–6 grams of **5** to ensure more uniform mixing.

For an efficient coupling reaction, 2,4,6-trihydroxybenzaldehyde **6** was protected as diacetate **2**.[15] This reaction was problematic as a result of competing C-aryl acylation side products, and provided a diacetate [1,3,5-$^{13}C_3$]**6** in a yield of ~40% after column chromatography and recrystallisation.

The construction of the "Eastern" aryl ring is illustrated in Scheme 3 and utilised the method of Hobuss and co-workers,[18] which was further developed in our laboratory,[19] to achieve a condensation between [1,3-$^{13}C_2$]acetone and triethyl orthoformate giving 4*H*-[3,5-$^{13}C_2$]pyran-4-one **8**. Accordingly, treatment of triethyl orthoformate (2 equiv.) with $BF_3{\cdot}OEt_2$, followed by the addition of [1,3-$^{13}C_2$]acetone (1 equiv.) and *N*,*N*-diisopropylethylamine gave a mixture of intermediates **7a**:**7b** (57:43). Simply heating this mixture in EtOH/HCl resulted in a cyclisation to 4*H*-[3,5-$^{13}C_2$]pyran-4-one **8**, which was an efficient reaction providing quantitative recovery of **8** without purification, or an 80% recovery after recrystallisation.



Scheme 3 *Regents and conditions*: (a) $HC(OEt)_3$ (2 eq.), $BF_3{\cdot}OEt$ 40 °C; (b) $^{i}PrNEt_2$ (6 eq.), −78 °C; quant.; (c) EtOH, aqueous HCl, 80–100%; (d) $CH_3COCH_2COCH_3$ (2.53 eq.), $^{t}BuOK$ (1.86 eq.), $^{t}BuOH$, then aq. HCl, 47–53%; (e) Br_2 (1.2 eq.), DCM, 70–76%; (f) aq. NaOH, $CuSO_4{\cdot}5H_2O$ (catalytic), 88%; (g) $(CH_3CO)_2O$, DMAP (catalytic), EtOAc, 75%. Overall yield of step (e–g) 50%.

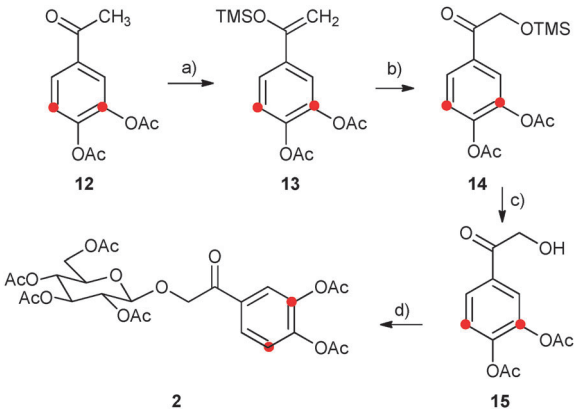
Treatment of **8** with an excess of acetylacetone and $^{t}BuOK$ in refluxing *tert*-butanol gave 4-hydroxy-[3,5-$^{13}C_2$]acetophenone **9** after acidic workup.[20]

Several bromination conditions were explored for the conversion of **9** to **10**, including *N*-methylpyrrolidin-2-one hydrotribromide (MPHT)/H_2O_2,[21] Br_2/Selectfluor,[22] and HBr/H_2O_2;[23] however, these gave complex mixtures. After significant optimisation, bromination with elemental bromine in DCM at 0 °C proved to be the most straightforward method. A slight excess of Br_2 and a relatively short reaction time (40 min) gave the monobrominated product **10** in ~76% yield with minor amounts of unreacted starting material (~15%) and ~10% dibrominated product.

Hydrolysis of **10** using aqueous sodium hydroxide and activated copper sulfate (as a catalyst) under reflux led to 3,4-dihydroxy-[3,5-$^{13}C_2$]acetophenone **11**,[19] which was subsequently converted into diacetate **12** with acetic anhydride in the presence of DMAP.

Silyl enol ether **13**, generated from acetophenone [3,5-$^{13}C_2$]**12**, was treated with *m*CPBA, and after *in situ* hydrolysis gave α-hydroxy-3,4-diacetoxy-[3,5-$^{13}C_2$]acetophenone **15** as illustrated in Scheme 4.[15,24]

Glycosylation of **15** required avoiding the use of heavy metals such as mercury and silver[13,15,25] and therefore a $BF_3{\cdot}OEt_2$ catalysed coupling of α-hydroxyacetophenone **15**, using 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate was employed.[26–28] This resulted in the glycosylated aromatic ketone **3** in a modest yield of 40–50%. Finally, the condensation and cyclisation of **2** and **3** were accomplished to generate [$^{13}C_5$]C3G **1** using anhydrous HCl/EtOAc, followed immediately by hydrolysis in MeOH and aq. KOH (Scheme 1).[13,15] Purification of this rather unstable and water soluble flavylium salt proved to be a significant challenge, and was carried out on Amberlite XAD-7 resin, which was further purified using cellulose microcrystalline and carbon-18 preparative column chromatography, followed by recrystallisation from EtOH



Scheme 4 *Reagents and conditions*: (a) TMSCl, Et_3N, DMF, 70 °C; (b) *m*CPBA, DCM, −20 °C to RT; (c) MeOH, yield for three steps 44%; (d) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate, $BF_3{\cdot}OEt_2$, 4 Å MS, −20 to 10 °C; 40–50%.

and acetonitrile (0.1% HCl). This provided a satisfactory yield of 40–50% of the product as a dark red powder.

In summary, a total of 4.30 grams of [$^{13}C_5$]C3G **1** was obtained, with a purity of >99.9% (by HPLC). This carbon-13 enriched material as formulated for human consumption will be utilised to study the ADME of anthocyanins. Ultimately any metabolite identified will provide future targets for exploring the mechanistic actions of anthocyanins in cellular models of disease.

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