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A new short synthesis of coumestrol and its application for the synthesis of [6,6a,11a-$^{13}C_3$]coumestrol

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Abstract—A convenient and simple two-step method for the synthesis of coumestrol has been established, which involves a base catalysed condensation of phenyl acetate with benzoyl chloride, followed by demethylation and subsequent tandem intramolecular cyclisation. This method was then employed for the efficient synthesis of multiply ^{13}C-labelled coumestrol.

1. Introduction

Coumestans represent a fully oxidised version of the flavonoid pterocarpans and share the same systematic numbering.[1] A number of coumestans were isolated from *Pueraria* and *Glycyrrhia*.[2] The most common and the most representative, coumestrol **1** (3,9-dihydroxy-6-benzofuro[3,2-*c*][1]benzopyran-6-one), which is related to the coumarins, was isolated from the roots of *Pueraria lubata* and *mirifica*,[3] roots of soybeans,[4] the whole plants of *Tephrosia purpurea*,[5] *Phaseolus coccineus*[6] and *lunatus*.[7] It is found mainly in red clover sprouts, mature alfalfa (lucerne) and alfalfa sprouts, soybean sprouts, and kudzu leaf. However, only small amounts are present in the human diet.[8] Low-coumestrol varieties of alfalfa and red clover are bred for forage and for commercial extraction of other phytoestrogens. Coumestrol **1** is an estrogen agonist that has been shown to be effective in reducing bone loss in model systems.[9] It has a higher binding affinity for the estrogen receptor than genistein,[10] consistent with the receptor binding model that appears to depend upon the phenolic group in the 4′-position of genistein and in the 12′-position of coumestrol. Its useful biological activities have made it an attractive synthetic target. In order to better understand, quantify and deduce the importance of these biological effects there is a need for the development of stable isotopically labelled coumestrol derivatives. Recently multiply ^{13}C-labelled derivatives of isoflavones, a related class of phytoestrogens which includes daidzein and genistein, have been employed as internal standards for LC–MS[11,12] and GC–MS[13] analysis of these compounds in plants and biological fluids resulting in considerable improvements in both sensitivity and reproducibility. Thus, a similarly ^{13}C-labelled version of coumestrol is a key synthetic target and may also have application in the deduction of the metabolic pathway for coumestrol **1** in mammals.

Three methods for the synthesis of coumestrol **1** have been previously described. The first method involved a condensation of methyl-(2-hydroxy-4-methoxyphenyl) glyoxylate and 2,4-dimethoxybenzyl alcohol, followed by photocyclisation, cyclisation and demethylation.[14] The second synthesis was based on a Pd-catalysed coupling reaction of aryl iodides and phenylacetylene, followed by deprotection and $PdCl_2$ catalysed intramolecular carbonylative cyclisation under an atmosphere of CO.[15] In the third procedure, coumestrol was constructed by an annulation onto the corresponding coumarin, forming the furan ring in the last step.[16–18] However, these methods all proved to be poor yielding, and are not suitable for the synthesis of coumestrol labelled with three ^{13}C atoms as is required for an internal standard for LC–MS and GC–MS analysis.[11–13]

In search of a route for preparation of coumestrol containing three ^{13}C-labelled atoms, a new, facile, short and easy procedure has been established. This has then been employed for the synthesis of coumestrol labelled with three ^{13}C atoms located in positions C-6, C-6a and C-11a.

2. Results and discussion

A retrosynthetic analysis illustrates that coumestrol **1** can be constructed by condensation of a phenyl acetate **2** and a benzoyl chloride **3** to give methyl 2,3-bis(2,4-dimethoxyphenyl)-3-oxopropanoate **4**. Demethylation and subsequent

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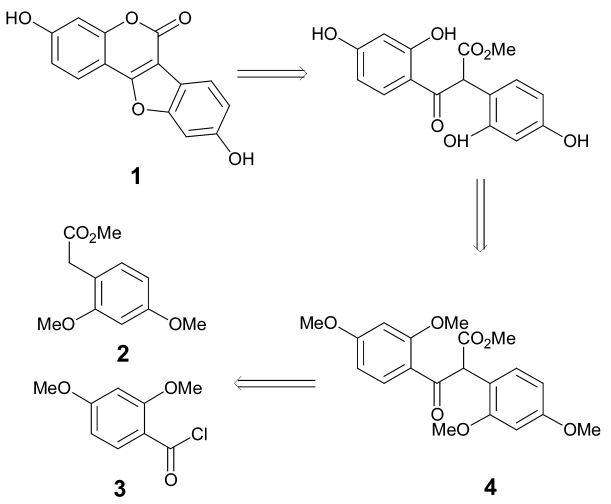
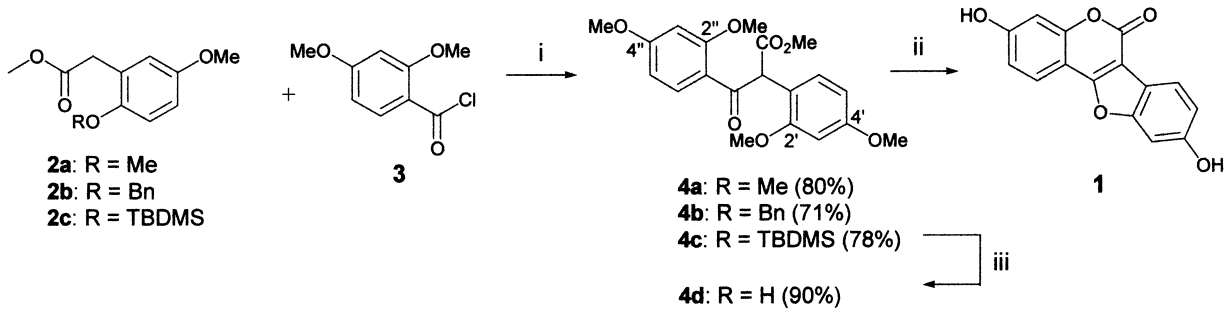


Scheme 1. Retro-synthesis of coumestrol.

C18 Column (150×4.6 mm)) giving a retention time of 8.25 min with a mobile phase of acetonitrile/water (1:1) and a 0.5 mL min^{-1} flow rate.

Treatment of **4b** under the same conditions gave a mixture of compounds, which was shown by ^1H NMR spectroscopy to be a result of only partial removal of the benzyl group. Two steps were required to obtain coumestrol **1** from the silyl protected precursor **4c**. The TBDMS group was first removed with TBAF to give **4d** in 90% yield, which on standard treatment with BBr_3, as for **4a**, gave **1** in almost quantitative yield (Scheme 2).

It was thus clear that use of the two fully methoxylated precursors **2** and **4a** gave the most high-yielding synthesis of coumestrol, superior to any of the previous literature methods. Therefore this procedure was then adapted for the synthesis of [6,6a,11a-$^{13}C_3$]coumestrol **5** starting from



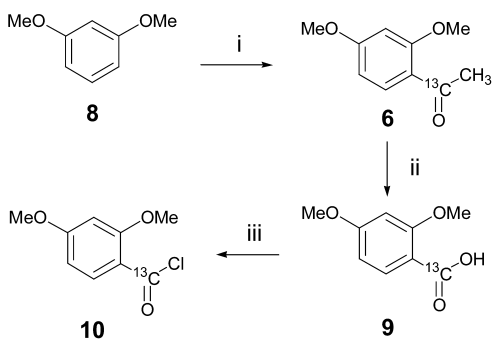
Scheme 2. (i) *n*-BuLi, *i*-Pr_2NH, THF, −78 °C for 2 h then 0 °C for 3 h; (ii) TBAF, THF, 15 min; (iii) 10 equiv. of 1 M BBr_3 in CH_2Cl_2, rt, 72 h (100% from **4a**).

intramolecular cyclisation would then afford coumestrol **1** (Scheme 1). Both the starting materials are amenable to C-labelling as the side chains could be built up using readily available C-labelled precursors, such as [1,2-$^{13}C_2$]acetyl chloride, [1-$^{13}C_1$]acetyl chloride or K^{13}CN.

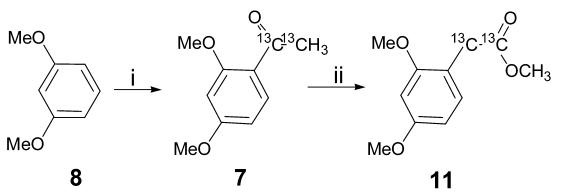
The synthesis of **1** is depicted in Scheme 2, starting from commercially available 2,4-dimethoxybenzoyl chloride **3** and a variety of protected methyl 2-hydroxy-4-methoxyphenyl acetates **2a**–**c**. The conditions for the C-acylation reaction were optimised using three different methyl 2-*O*-substituted 4-methoxyphenylacetates, namely the 2-methoxy **2a**, 2-benzyloxy **2b**, and 2-*tert*-butyldimethylsilyloxy **2c** derivatives, under a range of conditions using dry diethyl ether and dry THF as solvent (Scheme 2). The best results were obtained by reaction of the methyl acetates **2a**–**c** with a slight excess of **3** in the presence of LDA in dry THF at −78 °C and then warming to 0 °C to afford the methyl 2-methoxy-, benzyloxy- and methyl *tert*-butyldimethylsilyloxy 3-oxopropanoates **4a**–**c** in 71–80% yield.

When *O*-demethylation of **4a** was carried out using an excess of BBr_3 in CH_2Cl_2 at room temperature for 72 h a spontaneous tandem intramolecular cyclisation took place as envisaged to afford coumestrol **1** in almost quantitative yield. The product gave identical spectral data to that in the literature. The melting point (360–365 °C (dec)) was a poor indicator of purity due to decomposition of the sample and the wide variation in literature data, encompassing values from 290–293 °C[5] to 385 °C.[16] However, the compound was shown to be pure by reverse phase HPLC (Kingsorb 3μ

2′,4′-dimethoxy[1-$^{13}C_1$]acetophenone **6** and 2′,4′-dimethoxy[1,2-$^{13}C_2$]acetophenone **7**. The two ^{13}C-labelled acetophenones can be readily synthesised from 1,3-dimethoxybenzene **8** through acetylation with commercially available [1-$^{13}C_1$]acetyl chloride or [1,2-$^{13}C_2$]acetyl chloride (Schemes 3 and 4). Firstly, **8** was acetylated with [1-$^{13}C_1$]acetyl chloride using aluminium trichloride in nitroethane to give **6** in 88% yield. Oxidative cleavage of the acetophenone **6** with O_2 using a catalytic amount of $Co(NO_3)_3$ and $Mn(NO_3)_3$ in glacial acetic acid at 110 °C, afforded the 2,4-dimethoxybenzoic[*carboxy*-^{13}C]acid **9** in 75% yield, which on treatment with an excess of oxalyl chloride in CH_2Cl_2 at room temperature resulted in the formation of 2,4-dimethoxy[*carboxy*-^{13}C]benzoyl chloride **10** in 92% yield as indicated by ^1H NMR spectroscopy



Scheme 3. (i) $AlCl_3$, [1-$^{13}C_1$]AcCl, nitroethane, 45 °C, 30 min (88%); (ii) O_2, 0.04 equiv. of $Mn(NO_3)_3$, 0.04 equiv. of $Co(NO_3)_3$, AcOH, 110 °C, 24 h (75%); (iii) 2 equiv. of CO_2Cl_2, a drop of DMF, CH_2Cl_2, rt, 24 h.

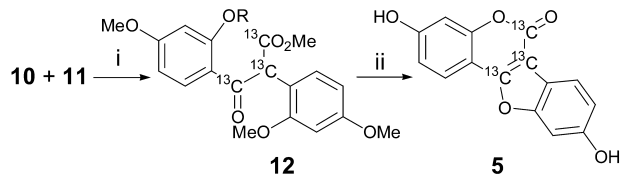


Scheme 4. (i) $AlCl_3$, [1,2-^{13}C]AcCl, nitroethane, 45 °C, 30 min (89%); (ii) TTN, 70% $HClO_4$, MeOH, rt, 2 h (80%).

(Scheme 3). The crude **10** was used in the condensation reaction without further purification.

The other fragment for the condensation reaction, methyl 2′,4′-dimethoxy[1,2-$^{13}C_2$]phenylacetate **11**, was synthesised in 80% yield via oxidative rearrangement of 2′,4′-dimethoxy[1,2-$^{13}C_2$]acetophenone **7** using thallium(III) nitrate (TTN) in MeOH and in presence of 70% $HClO_4$ as reported previously for the unlabelled analogue (Scheme 4).[19]

Condensation between 2,4-dimethoxy[*carboxy*-^{13}C]benzoyl chloride **10** and methyl 2′,4′-dimethoxy[1,2-$^{13}C_2$]-phenylacetate **11**, employing LDA for enolate generation, afforded methyl 2,3-bis(2,4-dimethoxyphenyl)-3-[1,2,3-$^{13}C_3$]oxopropanoate **12** in 83% yield (Scheme 5). Demethylation and subsequent intramolecular cyclisation of **12** using BBr_3 in CH_2Cl_2 resulted in [6,6a,11a-$^{13}C_3$]-coumestrol **5** in almost quantitative yield. The presence of the three ^{13}C-atoms was confirmed by mass spectrometry and by the enhanced signals for C-6,6a and 11a in the ^{13}C NMR spectrum, observed at 157.5, 102.0 and 159.4 ppm, respectively.



Scheme 5. (i) *n*-BuLi, *i*-Pr_2NH, THF, −78 °C for 2 h then 0 °C for 3 h (83%); (ii) 10 equiv. of 1 M BBr_3 in CH_2Cl_2, rt, 72 h (82%).

In conclusion, we have established a simple, two-step, high yielding method for the synthesis of coumestrol **1** from commercially available starting materials. This synthetic route is readily adaptable for the synthesis of different analogues as it is flexible with regard to substitution on both the phenyl acetate and benzoyl chloride. The method has been employed for the synthesis of multiply ^{13}C-labelled coumestrol for use as an internal standard in LC–MS and GC–MS analysis.

3. Experimental

3.1. General

Melting points were determined in open capillary tubes with an electrothermal apparatus and are uncorrected. THF was freshly distilled from sodium/benzophenone. For ^{1}H NMR (300 MHz) spectra the residual peak of $CHCl_3$ (7.26 ppm) and CH_3SOCH_3 (2.59 ppm) were used as internal reference, while for ^{13}C NMR (75 MHz) spectra the central peak of $CDCl_3$ (77.0 ppm) and that of CD_3SOCD_3 (39.95 ppm) were used as reference. Chemical shifts are given in δ and J values in Hz. Peak assignments were performed for the new compounds with the aid of the 2D COSY, GHSQCTOCSY and GHMBC spectra. Mass spectra were recorded at 70 eV. HRMS were recorded on a Finnigan VG AutoSpec instrument. The *O*-substituted methyl 2-hydroxy-4-methoxyphenyl acetates **2a**,[19] **2b**[20] and **2c**[20] were synthesized according to literature procedures.

3.1.1. Methyl 2,3-bis(2,4-dimethoxyphenyl)-3-oxopropanoate (4a). Under an Ar atmosphere, a solution of the acetate **2a** (1.00 g, 4.76 mmol) in dry THF (5 mL) was slowly added at −78 °C to a solution of LDA in THF (20 mL), prepared from diisopropylamine (0.53 g, 0.734 mL, 5.24 mmol) and *n*-BuLi (2.5 M in hexane, 0.21 mL, 5.24 mmol) at 0 °C. The light yellow solution was stirred for 30 min at −78 °C, and then transferred via cannula to a solution of 2,4-dimethoxybenzoyl chloride **3** (1.146 g, 5.71 mmol) in THF (5 mL) at −78 °C. The solution was stirred for 2 h at −78 °C, and then was allowed to warm to 0 °C. After 2 h stirring, the solution was poured into 2% aqueous HCl (20 mL), and extracted with ethyl acetate (3×50 mL). The combined extracts were washed with water (50 mL), dried over $MgSO_4$, and solvent was removed at reduced pressure. The resulting orange viscous oil was subjected to flash chromatography on silica (CH_2Cl_2/EtOAc 97:3) to give title compound **4a** (1.43 g, 80%) as a light yellow solid: mp 107–108 °C; ν_{max} (nujol)/cm^{-1} 1743 (CO_2Me), 1654 (C=O), 1597; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.73 (s, 3H, OCH_3), 3.78 (s, 9H, OCH_3), 3.83 (s, 3H, OCH_3), 5.95 (s, 1H, H-2), 6.37 (d, J=2.4 Hz, 1H, H-3″), 6.43 (dd, J=8.7, 2.4 Hz, 1H, H-5′), 6.43 (d, J=2.4 Hz, 1H, H-3′), 6.52 (dd, J=8.7, 2.4 Hz, 1H, H-5″), 7.04 (d, J=8.7 Hz, 1H, H-6′), 7.91 (d, J=8.7 Hz, 1H, H-6″); ^{13}C NMR ($CDCl_3$, 75 MHz): 52.2 (CO_2CH_3), 55.2 (OCH_3), 55.3 (OCH_3), 55.5 (2×OCH_3), 57.2 (C-2), 98.1 (C-3″), 98.6 (C-3′), 104.2 (C-5′), 105.5 (C-5″), 115.7 (C-1′), 119.7 (C-1″), 130.3 (C-6′), 133.59 (C-6″), 157.8 (C-2′), 160.3 (C-4′), 160.6 (C-4″), 164.8 (C-2″), 170.8 (C-1), 193.0 (C-3); m/z (EI) 374.1361 (M^+, requires 374.1365), 374 (5) and 165 (100); Anal. calcd for $C_{20}H_{22}O_7$: C, 64.16; H, 5.92. Found: C, 63.99; H, 5.75.

3.1.2. Methyl 2-(2-benzyloxy-4-methoxyphenyl)-3-(2,4-dimethoxyphenyl)-3-oxopropanoate (4b). Reaction of methyl 2-benzyloxy-4-methoxyphenyl acetate **2b** (0.28 g, 0.97 mmol) sequentially with LDA (1.07 mmol) and **3** (0.214 g, 1.07 mmol), as described for the preparation of **4a**, afforded the title compound **4b** (0.31 g, 71%) as a colorless waxy solid: mp 59–60 °C; ν_{max} (nujol)/cm^{-1} 1734 (CO_2Me), 1664 (C=O), 1604, 834, 737; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.69 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 5.03 (s, 2H, $OCH_2C_6H_5$), 6.08 (s, 1H, C-2), 6.35 (d, J=2.4 Hz, 1H, H-3″), 6.47 (dd, J=8.7, 2.4 Hz, 1H, H-5′), 6.49 (dd, J=8.7, 2.4 Hz, 1H, H-5″), 6.52 (d, J=2.4 Hz, 1H, H-3′), 7.10 (d, J=8.7 Hz, 1H, H-6′), 7.28–7.34 (m, 5H, C_6H_5), 7.87 (d, J=8.7, 2.4 Hz, 1H, H-6″); ^{13}C NMR ($CDCl_3$, 75 MHz): 52.2 (CO_2CH_3), 55.2 (OCH_3), 55.3 (OCH_3), 55.5 (OCH_3), 57.2 (C-2), 70.1 $OCH_2C_6H_5$), 98.2 (C-3″), 99.8 (C-3′), 104.6 (C-5′), 105.5 (C-5″), 116.2 (C-1′), 119.9 (C-1″), 126.9,

127.7, 128.4, 130.3 (C-6′), 133.6 (C-6″), 136.8, 156.9 (C-2′), 160.2 (C-4′), 160.6 (C-4″), 164.7 (C-2″), 170.8 (C-1), 193.1 (C-3); *m/z* (CI$^+$) 451 (M$^+$, 100%), 361 (10), 165 (16); Anal. calcd for $C_{26}H_{26}O_7$: C, 69.32; H, 5.82. Found: C, 68.87; H, 6.13.

3.1.3. Methyl 2-(2-*t*-butyldimethylsilyloxy-4-methoxyphenyl)-3-(2,4-dimethoxyphenyl)-3-oxopropanoate (4c). Reaction of methyl 2-*t*-butyldimethylsilyloxy-4-methoxyphenyl acetate **2c** (0.30 g, 0.968 mmol) sequentially with LDA (1.07 mmol) and **3** (0.214 g, 1.07 mmol), as described for the preparation of **4a**, afforded the title compound **4c** (0.37 g, 78%) as a light yellow wax: ν_{max} (nujol)/cm^{-1} 1736 (CO_2Me), 1670 (C═O), 1600; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.20 (s, 6H, 2×SiCH_3), 0.94 (s, 9H, Si-Bu-*t*), 3.71 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 5.94 (s, 1H, H-2), 6.37 (d, *J*=2.4 Hz, 1H, H-3″), 6.39 (d, *J*=2.4 Hz, 1H, H-3′), 6.47 (dd, *J*=8.7, 2.4 Hz, 1H, H-5′), 6.52 (dd, *J*=8.7, 2.4 Hz, 1H, H-5″), 7.06 (d, *J*=8.7 Hz, 1H, H-6′), 7.93 (d, *J*=8.7 Hz, 1H, H-6″); ^{13}C NMR ($CDCl_3$, 75 MHz): δ −4.5 (Si*C*H$_3$), −4.0 (Si*C*H$_3$), 18.1 (Si*C*(CH$_3$)$_3$), 25.6 (SiC(*C*H$_3$)$_3$), 52.1 (CO_2*C*H$_3$), 55.16 (OCH_3), 55.21 (OCH_3), 55.5 (OCH_3), 57.4 (C-2), 98.1 (C-3″), 105.2 (C-3′), 105.5 (C-5″), 105.6 (C-5′), 117.9 (C-1′), 119.7 (C-1″), 130.5 (C-6′), 133.7 (C-6″), 154.1 (C-2′), 159.7 (C-4′), 160.7 (C-4″), 164.9 (C-2″), 170.8 (C-1), 192.7 (C-3); *m/z* (EI) 475.2165 (M$^+$, $C_{25}H_{35}O_7Si$ requires 475.2152), 475 (2), 385 (6) and 165 (100).

3.1.4. Methyl 2-(2-hydroxy-4-methoxyphenyl)-3-(2,4-dimethoxyphenyl)-3-oxopropanoate (4d). Under a N_2-atmosphere, a solution of TBAF (1 M in THF, 3.76 mL, 3.75 mmol) was added to a solution of **4c** (0.80 g, 1.69 mmol) in THF (7 mL) at room temperature. After 15 min, the green solution was poured into water (20 mL), and extracted with ethyl acetate (3×30 mL). The combined extracts were dried over $MgSO_4$, and the solvent was evaporated. The residue was subjected to silica gel chromatography (CH_2Cl_2/EtOAc 95:5) to give the title compound **4d** (0.55 g, 90%) as a white solid: mp 55–56 °C; ν_{max} (nujol)/cm^{-1} 3375 (OH), 1735 (CO_2Me), 1654 (C═O), 1598; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.75 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 5.73 (s, 1H, C-2), 6.42 (d, *J*=2.4 Hz, 1H, H-3″), 6.42 (dd, *J*=8.7, 2.4 Hz, 1H, H-5′), 6.52 (d, *J*=2.4 Hz, 1H, H-3′), 6.54 (dd, *J*=8.7, 2.4 Hz, 1H, H-5″), 7.00 (d, *J*=8.7 Hz, 1H, H-6′), 7.83 (d, *J*=8.7 Hz, 1H, H-6″), 8.29 (s, 1H, 2′-OH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 52.8 (COO*C*H$_3$), 55.2 (OCH_3), 55.5 (OCH_3), 55.6 (OCH_3), 61.1 (C-2), 98.3 (C-3″), 103.8 (C-3′), 105.7 (C-5″), 106.8 (C-5′), 113.6 (C-1′), 119.5 (C-1″), 132.7 (C-6′), 133.9 (C-6″), 156.9 (C-2′), 160.6 (C-4″), 161.0 (C-4′), 165.6 (C-2″), 170.7 (C-1), 196.7 (C-3); *m/z* (EI) 360.120822 (M$^+$, $C_{19}H_{20}O_7$ requires 360.120903), 360 (3%), 342 (9) and 165 (100).

3.1.5. Coumestrol (1). An excess of 1 M BBr_3/CH_2Cl_2 (13.34 mL, 13.34 mmol) was added with stirring to a solution of **4a** (0.5 g, 1.34 mmol) in CH_2Cl_2 (5 mL) at room temperature under Ar. The mixture was stirred for 72 h, then water was added and CH_2Cl_2 was evaporated at reduced pressure. The mixture was refluxed for 3 h. After cooling to room temperature the yellow precipitate was filtered, and the filtrate was extracted with EtOAc (3×30 mL), dried over $MgSO_4$. The combined brown solid was purified by silica gel chromatography (CH_2Cl_2/EtOAc 8:2) to give title compound **1** (0.29 g, 82%) as a light yellow solid: mp 360–365 °C (dec); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.99 (d, *J*=2.1 Hz, 1H, H-4), 7.02 (dd, *J*=8.7, 2.1 Hz, 1H, H-2), 7.04 (d, *J*=8.7, 2.1 Hz, 1H, H-8), 7.25 (d, *J*=2.1 Hz, 1H, H-10), 7.78 (d, *J*=8.7 Hz, 1H, H-7), 7.93 (d, *J*=8.7, 2.4 Hz, 1H, H-1), 10.11 (br s, 1H, 9-OH), 10.77 (br s, 1H, 3-OH); ^{13}C NMR ($CDCl_3$, 75 MHz): 98.6 (C-10), 102.0 (C-6a), 103.0 (C-4), 104.1 (C-1a), 113.7 (C-2), 113.9 (C-10), 114.5 (C-7a), 120.6 (C-7), 122.6 (C-1), 154.6 (C-4a), 155.9 (C-9), 156.9 (C-10a), 157.5 (C-6), 159.4 (C-11a), 161.1 (C-3); Anal. calcd for $C_{15}H_8O_5$: C, 67.17; H, 3.01. Found: C, 66.83; H, 2.78.

3.1.6. 2′,4′-Dimethoxy[1,2-$^{13}C_2$]acetophenone (7). Finely powdered anhydrous $AlCl_3$ (1.68 g, 12.6 mmol) was added to a well-stirred solution of 1,3-dimethoxybenzene **8** (2 g, 14.47 mmol) in freshly distilled nitroethane (10 mL) under a N_2-atmosphere. Then [1,2-$^{13}C_2$]acetyl chloride (1 g, 0.905 mL, 12.58 mmol) was slowly added, and the resulting red solution was stirred at 45 °C for 30 min. The mixture was allowed to cool to room temperature, poured into ice water (100 mL), and extracted with diethyl ether (3×50 mL). The combined organic layers were washed with brine (50 mL) and dried over $MgSO_4$. The solvent was removed at reduced pressure, and the orange oily residue was purified by column chromatography (silica, CH_2Cl_2/hexane, 4:1) to afford the title compound **7** (2.03 g, 89%) as a white solid: mp 40–41 °C (Lit[21] mp 39–41 °C); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.56 (dd, *J*=128.1, 6.3 Hz, 3H, H-2), 3.84 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.44 (dd, *J*=2.4, 1.5 Hz, 1H, H-3′), 6.51 (dd, *J*=8.7, 2.4 Hz, 1H, H-5′), 7.82 (dd, *J*=8.7, 4.2 Hz, 1H, H-6′); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 31.8 (d, *J*=168.7 Hz, C-2), 55.4 (OCH_3), 55.5 (OCH_3), 98.2 (d, *J*=10.8 Hz, C-3′), 105.0 (d, *J*=15.3 Hz, C-5′), 120.5 (d, *J*=132.9 Hz, C-1′), 131.8 (s, C-6′), 161.0 (d, *J*=8.7 Hz, C-2′), 164.5 (C-4′), 197.7 (d, *J*=168.7 Hz, C-1); *m/z* (EI) 182.0852 (M$^+$, $C_8{}^{13}C_2H_{12}O_3$ requires 182.0853), 182 (32%), 166 (100), 151 (6) and 122 (7).

3.1.7. 2′,4′-Dimethoxy[1-$^{13}C_1$]acetophenone (6). The title compound was prepared according to the procedure described for **7**, using [1-$^{13}C_1$]acetyl chloride to give the product as white solid (2.1 g, 88%): 40–41 °C (Lit[21] mp 39–41 °C); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.56 (d, *J*=6.3 Hz, 3H, H-2), 3.84 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.44 (dd, *J*=2.4, 1.5 Hz, 1H, H-3′), 6.51 (dd, *J*=8.7, 2.4 Hz, 1H, H-5′), 7.82 (dd, *J*=8.7, 3.9 Hz, 1H, H-6′); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 31.8 (d, *J*=169.5 Hz, C-1), 55.4 (OCH_3), 55.5 (OCH_3), 98.2 (C-3′), 105.0 (d, *J*=13.8 Hz, C-5′), 120.5 (d, *J*=132.9 Hz, C-1′), 132.6 (C-6′), 161.0 (d, *J*=8.7 Hz, C-2′), 164.5 (C-4′), 197.7 (C-1); *m/z* (EI) 181.0818 (M$^+$, $C_9{}^{13}C_1H_{12}O_3$ requires 181.0819), 181 (29%), 166 (100), 151 (8) and 122 (6).

3.1.8. 2,4-Dimethoxy[*carboxy*-^{13}C]benzoic acid (9). A solution of the acetophenone **6** (1.0 g, 5.52 mmol), $Mn(NO_3)_3$ (0.064 g, 0.22 mmol), and $Co(NO_3)_3$ (0.064 g, 0.22 mmol) glacial acetic acid (10 mL) was stirred at 110 °C for 24 h under an O_2-atmosphere. The solvent was evaporated at reduced pressure, and the residue was

dissolved in 2 N $NaHCO_3$ (15 mL) at 60 °C, which was extracted with ethyl acetate (3×30 mL). The aqueous solution was acidified with concentrated H_2SO_4, and extracted with ethyl acetate (3×30 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. The resulting yellow solid was subjected to silica gel chromatography (CH_2Cl_2/EtOAc 97:3) to furnish the title compound **9** (0.76 g, 75%) as a white solid: 105–106 °C (Lit[22] mp 106 °C); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.87 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 6.53 (dd, J=2.4, 1.8 Hz, 1H, H-3′), 6.64 (dd, J=9.0, 2.4 Hz, 1H, H-5′), 8.12 (dd, J=9.0, 2.4 Hz, 1H, H-6′), 10.45 (br s, 1H, COOH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 55.7 (OCH_3), 56.6 (OCH_3), 98.6 (d, J=12.9 Hz, C-3), 106.5 (d, J=17.1 Hz, C-5), 111.3 (d, J=302.1 Hz, C-1), 135.5 (d, J=9.0 Hz, C-6), 159.5 (d, J=8.7 Hz, C-2), 165.1 (C-4), 165.2 (s, COOH); *m/z* (EI) 183.0617 (M^+, $C_8{}^{13}C_1H_{10}O_4$ requires 183.0612), 183 (98%), 166 (100), 154 (26), 136 (58) and 122 (5).

3.1.9. 2,4-Dimethoxy[*carboxy*-^{13}C]benzoyl chloride (10). Oxalyl chloride (1.26 g, 0.86 mL, 9.93 mmol) was added to a solution of the carboxylic acid **9** (0.90 g, 4.92 mmol) in dry CH_2Cl_2 (20 mL) containing a drop of dry DMF under an Ar atmosphere. The solution was stirred at room temperature under light exclusion for 24 h. The solvent was then evaporated at reduced pressure and the yellow orange solid was washed with dry hexane (2×30 mL). Drying in vacuo gave the title compound **10** (0.98 g, 99%) as light yellow solid, which was pure enough (purity ≈90%, as indicated by ^{1}H NMR spectroscopy) to be used in the next step. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.90 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.46 (t, J=2.4 Hz, 1H, H-3), 6.55 (dd, J=9.0, 2.4 Hz, 1H, H-5), 8.16 (dd, J=9.0, 6.6 Hz, 1H, H-6).

3.1.10. Methyl 2′,4′-dimethoxy[1,2-$^{13}C_2$]phenyl acetate (11). A solution of the acetophenone **7** (1.0 g, 5.49 mmol) in methanol (10 mL) was dropwise added to a well stirred solution of TTN (2.68 g, 6.04 mmol) in methanol (10 mL) containing perchloric acid (5 mL, 70% w/w) under Ar. The reaction mixture was stirred at room temperature for 2 h, and the resulted white precipitate thallium(I) nitrate was removed by filtration and the filtrate was carefully poured into 2 N aq. $NaHCO_3$ (100 mL). The aqueous solution was extracted with CH_2Cl_2 (3×50 mL), washed with brine (2×50 mL) and dried ($MgSO_4$). The solvent was removed at reduced pressure, and the orange oily residue was purified by silica gel chromatography (CH_2Cl_2/hexane 4:1) to afford the title compound **11** (0.93 g, 80%) as a light yellow solid: 48–49 °C (Lit[20] mp 50 °C); ν_{max} (nujol)/cm^{-1} 1698 (CO_2Me), 1617, 1590; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.56 (dd, J=129.9, 8.1 Hz, 2H, H-2), 3.68 (d, J=3.9 Hz, 3H, OCH_3), 3.795 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 6.45 (dd, J=9.0, 2.4 Hz, 1H, H-5′), 6.50 (d, J=2.4 Hz, 1H, H-3′), 7.08 (dd, J=9.0, 2.4 Hz, 1H, H-6′); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 35.0 (d, J=231.5 Hz, C-2), 51.50 (d, J=13.8 Hz, CO_2CH_3), 55.4 (OCH_3), 55.5 (OCH_3), 98.7 (d, J=9.0 Hz, C-3′), 104.1 (d, J=13.2 Hz, C-5′), 115.4 (dd, J=187.6, 11.7 Hz, C-1′), 131.1 (t, J=9.0 Hz, C-6′), 158.9 (d, J=8.7 Hz, C-2′), 160.2 (C-4′), 172.6 (d, J=231.5 Hz, C-1); *m/z* (EI) 212.0960 (M^+, $C_9{}^{13}C_2H_{14}O_4$ requires 212.0959), 212 (34%), 152 (100) and 122 (26).

3.1.11. Methyl 2,3-bis(2,4-dimethoxyphenyl)-3-[1,2,3-$^{13}C_3$]oxopropanoate (12). Condensation of benzoyl chloride **10** (0.50 g, 2.49 mmol) and ^{13}C-labelled acetate **11** (0.44 g, 2.07 mmol) as described for the preparation of **4a**, afforded the title compound **12** (0.65 g, 83%) as a light yellow solid: mp 106–107 °C; ν_{max} (nujol)/cm^{-1} 1700 (CO_2Me), 1623 (C=O), 1591; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.73 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 3.77 (s, 6H, OCH_3), 3.82 (s, 3H, OCH_3), 5.95 (ddd, J=133.2, 8.7, 6.3 Hz, 1H, H-2), 6.37 (q, J=2.4 Hz, 1H, H-3″), 6.43 (dd, J=9.0, 2.4 Hz, 1H, H-5′), 6.43 (d, J=2.4 Hz, 1H, H-3′), 6.52 (dd, J=8.7, 2.4 Hz, 1H, H-5″), 7.04 (dd, J=8.7, 4.2 Hz, 1H, H-6), 7.91 (dd, J=8.7, 4.2 Hz, 1H, H-6′); ^{13}C NMR ($CDCl_3$, 75 MHz): 52.2 (OCH_3), 55.2 (OCH_3), 55.3 (OCH_3), 55.5 (2×OCH_3), 57.2 (dd, J=235.6, 162.5 Hz, H-2), 98.1 (d, J=11.7 Hz, C-3″), 98.6 (d, J=8.7 Hz, C-3′), 104.2 (d, J=13.8 Hz, C-5′), 105.5 (d, J=13.8 Hz, C-5″), 115.7 (C-1′), 119.7 (C-1″), 130.3 (C-6′), 133.6 (C-6″), 157. 8 (C-2′), 160.3 (C-4′), 160.6 (C-4″), 164.8 (C-2″), 170.8 (dd, J=235.5, 8.7 Hz, C-1), 193.0 (dd, J=162.5, 8.7 Hz, C-3); *m/z* (EI) 377.147475 (M^+, $C_{17}{}^{13}C_3H_{22}O_7$ requires 377.146618), 377 (4%), 166 (100); Anal. calcd for $C_{17}{}^{13}C_3H_{20}O_7$: C, 63.47; H, 5.66. Found: C, 63.65; H, 5.89.

3.1.12. [6,6a,11a-$^{13}C_3$]Coumestrol (5). The title compound was prepared according to the procedure described for **1**, using **12** to give the product as white solid (0.283 g, 80%): mp 360–365 °C (dec); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.99–7.06 (m, 3H, H-2,4,8), 7.25 (d, J=2.1 Hz, 1H, H-10), 7.78 (d, J=8.7, 2.7 Hz, 1H, H-7), 7.93 (d, J=8.7, 4.2 Hz, 1H, H-1), 10.11 (br s, 1H, 9-OH), 10.77 (br s, 1H, 3-OH); ^{13}C NMR ($CDCl_3$, 75 MHz): 98.6 (C-10), 102.0 (dd, J=348, 251.7 Hz, C-6a), 103.0 (C-4), 104.1 (C-1a), 113.7 (C-2), 113.9 (C-10), 114.5 (C-7a), 120.6 (C-7), 122.6 (C-1), 154.6 (C-4a), 155.9 (C-9), 156.9 (C-10a), 157.5 (dd, J=348, 22.8 Hz, C-6), 159.4 (dd, J=252, 22.8 Hz, C-6), 161.1 (C-3); *m/z* (EI) 271.047053 (M^+, $C_{12}{}^{13}C_3H_8O_5$ requires 271.047238), 132 (44) and 117 (9).

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