Occurrence of phenylpyruvic acid in woody plants: biosynthetic significance and synthesis of an enolic glucoside derivative

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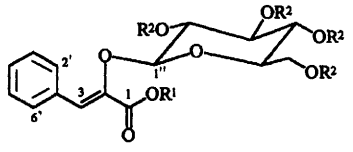
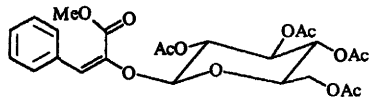
The leaves and stems of *Aspalathus linearis*, a member of the Fabaceae, contains (*Z*)-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid 1, an enolic glucoside of phenylpyruvic acid, representing the first unequivocal evidence for the latter's presence in woody plants. The synthesis of a derivative 2 of the natural product, and of related regiomeric and geometrical isomers 3, 4 and 5, and the biosynthetic significance in relation to the shikimic acid pathway are discussed.

Introduction

Phenylpyruvic acid (PPA) features as an intermediate in the shikimic acid pathway for the biosynthesis of the crucial aromatic amino acids L-phenylalanine and L-tyrosine in plants and bacteria.[1,2] PPA occurs abundantly in the urine of patients suffering from the congenital biochemical disorder phenylketonuria,[3] and is effectively produced by several microorganisms,[4,5] certain marine sponges[6] and presumably also in tobacco plants.[7] Unequivocal proof for its presence in woody plants, where it presumably plays a key role in the biosynthesis of the flavonoids and a variety of other secondary metabolites,[1] has, however, not yet been documented. Continued investigation[8] of the physiologically significant compounds in *Aspalathus linearis*, a member of the Fabaceae, which is used for the manufacture of Rooibos Tea, an important health beverage,[9] has revealed the presence of an enolic β-D-glucopyranoside of PPA,[10] hence lending credence to the latter's role in the shikimic acid pathway in woody plants. Its structure elucidation, biosynthetic significance and synthesis, and the syntheses of some geometrical and regioisomers are discussed.



1 $R^1 = R^2 = H$
2 R^1 = Me, R^2 = Ac



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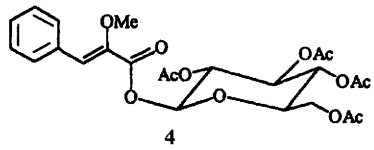

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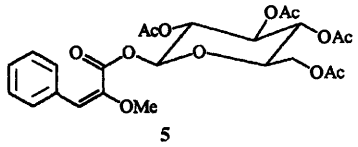

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Results and discussion

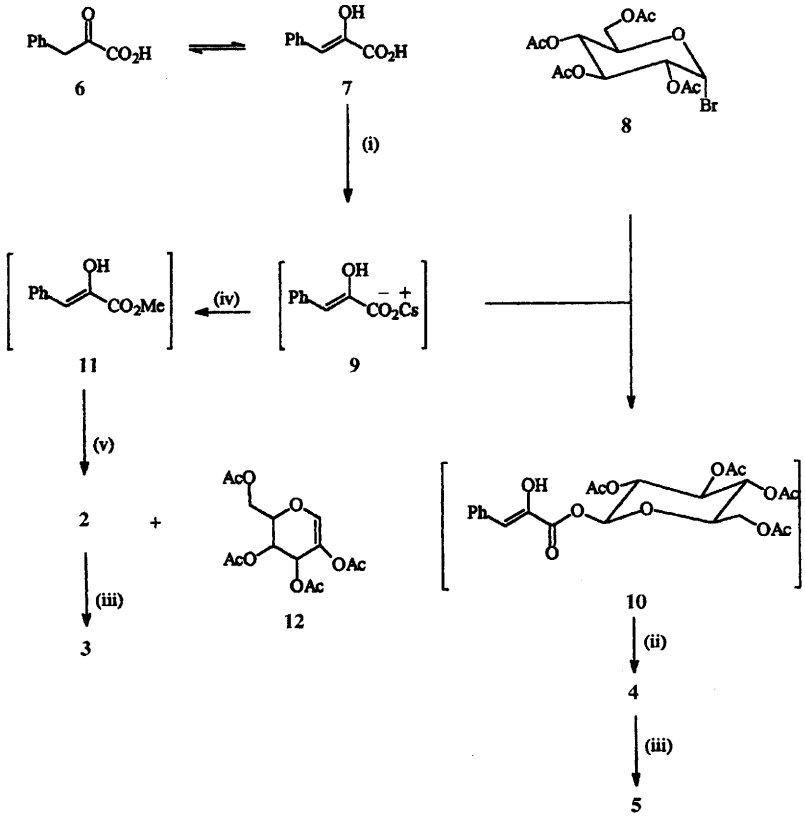
In addition to the phenolic compounds described previously,[8] the ethanol solubles of the aqueous extract of *A. linearis*, kindly supplied by 'Rooibos Tea Natural Products Ltd.', Clanwilliam contains (*Z*)-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid **1** which was identified as the methyl ester acetate derivative **2**. Its ^{1}H NMR spectrum at 300 MHz in $(CD_3)_2CO$–D_2O indicated an unsubstituted phenyl ring, a vinylic proton (δ 7.03, s), an *O*-methyl (δ 3.83) and four *O*-acetyl (δ 2.01, 1.98, 1.96 and 1.90) resonances, and the characteristic seven-spin system of a β-D-glucopyranosyl moiety ($^3J_{1'',2''}$ = 8.0, $^3J_{2'',3''} = {}^3J_{3'',4''}$ = 9.5 and $^3J_{4'',5''}$ = 10.0 Hz) substituted at one of its oxygen functionalities. The methoxy protons exhibited a nuclear Overhauser enhancement (NOE) association with the vinylic hydrogen (1%), the hydrogen of the anomeric carbon (δ_H 5.56, 0.6%) and two of the *O*-acetyl groups (δ 2.01, 0.3%; δ 1.90, 0.2%), while the vinylic hydrogen showed an NOE association with H-2′ and -6′ (δ 7.86, 5%). A COLOC experiment at 500 MHz correlated the methoxy protons with a carbonyl carbon (δ_C 164.51), the proton (δ 5.56) at the anomeric carbon (δ_C 99.69) with a vinylic carbon (δ_C 141.39) attached to oxygen, and the remaining carbon (δ_C 126.09) of the double bond with H-2′ and -6′ of the phenyl ring. Collectively these data are suggestive of structure **2** for the derivative of the novel natural product, (*Z*)-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid **1**.

In order to confirm the aforementioned tentative structure and especially the absolute D-configuration of the glycosidic unit, derivative **2** and its *E*-geometrical isomer **3**, and the closely related regiomeric (*Z*)-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) 2-methoxy-3-phenylpropenoate **4** and its *E*-isomer **5**, were synthesized with PPA **6**[11] and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8**[12] as starting materials (Scheme 1). The ubiquitous natural occurrence of glycosidic esters[13] incidentally prompted us to first synthesize the glucopyranosyl ester **4** while awaiting the results of the 500 MHz NMR investigation. The physical data emanating from such a synthetic endeavour should contribute towards the structure elucidation of related phenylpyruvic analogues which will, no doubt, eventually be encountered in natural sources.

The synthetic protocol had to reconcile several crucial features, *i.e.* effecting *O- vs. C*-glucosylation of the enolic functionality and substitution *vs.* elimination at C-1 of the glucosyl moiety with preferential formation of the β-anomer. Synthesis of the glucopyranosyl ester **4** was effected under mild conditions by utilizing the caesium effect.[14,15] PPA **6**, existing entirely in the enolic form **7** in solution, was thus activated by trans-

Table 1 ^{1}H NMR peaks (δ_H) of PPA **6**, its derivatives **2** and **4**, and their goemetrical isomers **3** and **5** at 300 MHz (23 °C). Splitting patterns and *J*-values (Hz) are given in parentheses

Proton	**6**, $(CD_3)_2CO$	**2**, $(CD_3)_2CO$–D_2O	**3**, $(CD_3)_2CO$–D_2O	**4**, $(CD_3)_2CO$–D_2O	**5**, $(CD_3)_2CO$–D_2O
3	6.53 (s)	7.03 (s)	6.76 (s)	7.03 (s)	6.25 (s)
2′/6′	7.82 (m)	7.86 (m)	7.31–7.19 (m)	7.80 (m)	7.32–7.20 (m)
3′/5′	7.36 (m)	7.36 (m)		7.41 (m)	
4′	7.25 (m)				
1″		5.56 (d, 8.0)	5.24 (d, 8.0)	6.03 (d, 8.0)	6.00 (d, 8.1)
2″		5.21 (dd, 8.0, 9.5)	5.09 (dd, 8.0, 9.8)	5.20 (dd, 8.0, 9.5)	5.01 (dd, 8.1, 10.0)
3″		5.37 (t, 9.5)	5.33 (dd, 9.8, 9.6)	5.46 (t, 9.5)	5.38 (dd, 10.0, 9.5)
4″		5.08 (dd, 9.5, 10.0)	5.06 (t, 9.6)	5.16 (dd, 9.5, 10.5)	5.08 (dd, 9.5, 10.0)
5″		3.96 (m)	4.13 (m)	4.21 (m)	4.16 (m)
6″		3.99 (dd, 2.5, 12.5)	4.15 (dd, 2.5, 12.5)	4.11 (dd, 2.2, 12.6)	4.11 (dd, 2.5, 12.0)
		4.12 (dd, 5.5, 12.5)	4.25 (dd, 5.8, 12.5)	4.31 (dd, 4.8, 12.6)	4.28 (dd, 4.5, 12.0)
OMe		3.83 (s)	3.63 (s)	3.77 (s)	3.75 (s)
OAc		2.01, 1.98, 1.96, 1.90 (each s)	1.97 (×2), 1.93, 1.86 (each s)	2.01, 2.00 (×2), 1.98 (each s)	2.01, 2.00, 1.93, 1.91 (each s)
CO_2H	8.02 (br s)				



Scheme 1 Synthesis of the PPA derivatives **2** and **4**, and their geometrical isomers **3** and **5**. *Reagents and conditions:* (i) Cs_2CO_3–dry MeOH; (ii) CH_2N_2–MeOH; (iii) *hν* (350 nm), MeOH; (iv) MeI–dry DMF; (v) NaH, followed by compound **8** in dry DMF at 0 °C ⟶ 20 °C.

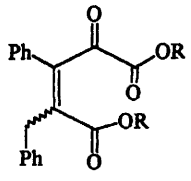
formation into the carboxylate **9** using caesium carbonate in anhydrous methanol at room temperature. Evaporation afforded the caesium salt **9**, which was dissolved in dry dimethylformamide (DMF) and added to 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** in dry DMF under vigorous stirring.[15] The resultant ester **10** was not isolated but was directly transformed into (*Z*)-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) 2-methoxy-3-phenylpropenoate **4** by methylation with diazomethane.

Comparison of the ^{1}H NMR data (Table 1) of the glucopyranosyl ester **4** with those of the natural product derivative **2** indicated identical chemical shifts for the vinylic protons (δ 7.03) but conspicuous shift differences especially for the glucosidic protons. The *Z*-glucopyranosyl ester **4** was thus photolytically[16] transformed into (*E*)-(2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl) 2-methoxy-3-phenylpropenoate **5** by irradiation at 350 nm in methanol. The ^{1}H NMR data (Table 1) again differed substantially from those of the natural product derivative **2** with the vinylic proton (δ 6.25) significantly shielded ($\Delta\delta$ 0.78 ppm) compared with the chemical shifts of the same proton in compounds **2** and **4** (see also below).

The synthesis of the natural product derivative **2** was less straightforward and was complicated by the instability of methyl phenylpyruvate, the tendency of PPA to give aldol-type products **13** under mild conditions† (see Experimental section), and the susceptibility of tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** to base-induced dehydrobromination and thus formation of the glucal derivative **12**. The caesium carboxylate **9** (*vide supra*) was eventually dissolved in anhydrous DMF and methylated with methyl iodide under vigorous stirring. Owing

† This contrasts with reports that methyl phenylpyruvate is readily available *via* methylation of PPA with MeOH in ethylene dichloride.[17]

to its rapid and complete decomposition during work-up, the DMF solution of methyl phenylpyruvate **11** was immediately chilled to 0 °C and added to a suspension of sodium hydride in DMF at 0 °C, again under vigorous stirring. This mixture was slowly added to a solution of tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** in dry DMF at 0 °C and the temperature was allowed to rise to 20 °C. Work-up and purification afforded the 2,3,4,6-tetra-*O*-acetyl-D-glucal (**12**, 27%) and the methyl 3-phenyl-2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)propenoate (**2**, 21%) with ^{1}H NMR data (Table 1) identical with those of the same derivative of the natural product. Unequivocal confirmation for the *Z*-configuration of the double bond was obtained by photolytic conversion of the natural product derivative **2** in low yield into the *E*-geometrical isomer **3**, which again showed shielding ($\Delta\delta$ 0.27) of its vinylic proton (δ 6.76) compared with the chemical shift (δ 7.03) of the same proton in the *Z*-isomer **2**.



13 R = H
14 R = Me

The conspicuous preference for the existence of the *Z* enolic form **7** of PPA, and for the exclusive formation of the *Z* geometrical isomers **2** and **3** is at present being investigated by semi-empirical calculations, details of which will be discussed elsewhere.

Methyl (*Z*)-3-phenyl-2-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)propenoate **2** not only represents the first glucoside of PPA, but also provides the first unambiguous evidence for the occurrence of this secondary metabolite in woody plants where it may serve as the precursor to α-hydroxychalcones[18] and hence to C-3 oxygenated flavonoids, and to a range of other key natural products.[1] Formation of the enolic glucoside presumably stabilizes PPA which is then 'stored' in this state and released into the biogenetic pool when required. Since biosynthetic processes are often compartmentalized the PPA glucoside may plausibly represent the form that permits intercompartmental transport. The results described here shed new light on the shikimic acid pathway and should provide some new impetus to biosynthetic research in this area. In addition, PPA may contribute to the positive dermatological effects[19,20] of the health beverage, Rooibos Tea.

Experimental

^{1}H NMR spectra were recorded on Bruker AM-300 and AMXR-500 spectrometers for solutions as indicated with Me_4Si as internal standard. *J*-Values are given in Hz. Mass spectra were obtained with a Kratos MS-80 instrument. TLC was performed on pre-coated Merck plastic sheets (silica gel 60 PF_{254}, 0.25 mm) and the plates were sprayed with H_2SO_4–HCHO (40:1, v/v) after development. Preparative plates (PLC), 20 × 20 cm, Kieselgel PF_{254} (1.0 mm) were air-dried and used without prior activation. Column chromatography was on Sephadex LH-20, Fractogel TSK HW 40(S) or Cellulose in various columns, solvent systems and flow rates (to be specified in each instance). Column chromatography under medium pressure (MPLC) was performed on a Büchi system (18 bar pressure; 1 bar = 10^5 Pa). Methylations were performed with an excess of diazomethane in MeOH–diethyl ether over a period of 48 h at −15 °C, while acetylations were in acetic anhydride–pyridine at ambient temperature. Evaporations were done under reduced pressure at ambient temperatures in a rotary evaporator, and freeze drying of aqueous solutions was carried out on a Virtis 12 SL freezemobile.

Extraction and fractionation

The commercial form of *A. linearis* (600 g) was boiled in water (3.0 dm^3) for 1 h and left at room temperature for 18 h. After decantation and filtration the mixture was freeze-dried to give a reddish brown solid (49 g). A portion (43.6 g) of this material was extracted with chloroform (3 dm^3) in a Soxhlet apparatus for 6.5 h to remove chlorophyll. This process was repeated several times, the chloroform-insolubles were air-dried and a part (85 g) was stirred for 20 min in EtOH (2 dm^3) at 60 °C. Filtration and evaporation of the filtrate afforded a reddish brown solid (4.3 g). Combination of these solids from several repetitions of this procedure eventually gave 45.7 g of extract, which was subjected to column chromatography on Sephadex LH-20 [90.5 × 6 cm column; 1.9 cm^3 min^{-1} flow rate; 17 cm^3 fractions; gradient solvent system, EtOH (6.77 dm^3), 10% MeOH–EtOH (1.55 dm^3) and 50% acetone–MeOH (5.47 dm^3)] to give fractions AF_1 (tubes 1–38, 1.83 g), AF_2 (39–62, 4.28 g), AF_3 (63–86, 7.4 g), AF_4 (87–119, 4.49 g), AF_5 (120–218, 6.29 g), AF_6 (219–414, 8.37 g) and AF_7 (415–532, 2.91 g). Fraction AF_3 was suspended in EtOH (5 cm^3), and the mixture was stirred for 5 min at room temperature and filtered. The filtrate was evaporated to dryness and the residual material (6.21 g) was subjected to column chromatography on Fractogel TSK HW 40(S) [50.5 × 6 cm column; 2.5 cm^3 min^{-1} flow rate; 13 cm^3 fractions; gradient solvent system, EtOH (4.51 dm^3), 50% acetone–water (2 dm^3)] to give fractions AF_3F_1 (tubes 1–44, 93 mg) AF_3F_2 (45–58, 1.81 g), AF_3F_3 (59–68, 333 mg), AF_3F_4 (69–76, 84 mg), AF_3F_5 (77–193, 1.125 g), AF_3F_6 (194–347, 804 mg) and AF_3F_7 (348–501, 1.96 g). Fraction AF_3F_6 was subjected to MPLC [Lichroprep RP-18, 5–20 μm; 45 × 2 cm column; 2.5 cm^3 min^{-1} flow rate; 16.5 cm^3 fractions; gradient solvent system, 20% MeOH–water (5.94 dm^3), 40% MeOH–water (1.47 dm^3), 60% MeOH–water (2.19 dm^3) and 80% MeOH–water (2.76 dm^3)] to give four fractions $AF_3F_6F_1$ (tubes 1–80, 268 mg), $AF_3F_6F_2$ (81–426, 10 mg), $AF_3F_6F_3$ (427–430, 15 mg) and $AF_3F_6F_4$ (431–750, 49 mg).

Fraction $AF_3F_6F_1$ was methylated and the mixture was separated by PLC in benzene–acetone–methanol (4:5:1, v/v) to give a band at R_F 0.40 (7.4 mg). This was acetylated and purified by preparative TLC (PLC) in hexane–benzene–acetone–methanol (40:40:15:5, v/v) to give (*Z*)-*methyl 3-phenyl-2-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyloxy)propenoate* **2** as an amorphous yellow solid (R_F 0.57, 4.4 mg) (Found: M$^+$, 508.1579. $C_{24}H_{28}O_{12}$ requires M, 508.1581); δ_H (Table 1); *m/z* 331 (55%), 271 (15), 229 (3), 211 (10), 179 (21) and 169 (100). The results from the investigation of the remaining fractions indicated above will be described elsewhere.

Syntheses of the regiomeric PPA glucosides 2 and 4, and their geometrical isomers 3 and 5

PPA **6** was synthesized from acetylglycine and benzaldehyde *via* 4-benzylidene-2-methyloxazol-5-one and α-acetamidocinnamic acid according to standard literature procedures.[11] 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** was similarly available[12] *via* treatment of a mixture of the α- and β-anomer of 1,2,3,4,6-penta-*O*-acetylglucose with HBr in acetic acid.

(*Z*)-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 2-methoxy-3-phenylpropenoate 4. Caesium carbonate (29 mg, 0.09 mmol) was added to a stirred solution of PPA **6** (28 mg, 0.17 mmol) in MeOH (2.2 cm^3). After 30 min the solvent was evaporated off under reduced pressure, the caesium carboxylate **9** was dissolved in freshly distilled anhydrous DMF (1.7 cm^3), and a solution of the glucosyl bromide **8** (69 mg, 0.17 mmol) in dry DMF (1 cm^3) was added to the vigorously stirred mixture at room temperature. The precipitated CsBr was filtered off after 30 min, and the DMF was removed under vacuum at 60 °C to give

a yellow residue (85 mg). This was methylated with diazomethane and the mixture was separated by PLC in hexane–benzene–acetone–methanol (40:40:15:5, v/v) to give the *title compound* **4** as a light yellow syrup (R_F 0.60; 18.2 mg, 22%) (Found: M^+, 508.1581. $C_{24}H_{28}O_{12}$ requires M, 508.1581); δ_H (Table 1); *m/z* 331 (55%), 271 (15), 229 (2.0), 211 (9.0) and 169 (100).

(*E*)-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl) 2-methoxy-3-phenylpropenoate 5. The *Z*-isomer **4** (18 mg) was dissolved in MeOH (80 cm^3), and the solution was purged with N_2 for 30 min, and subsequently irradiated for 15 h at 350 nm in a Rayonet photochemical reactor. Evaporation of the mixture followed by PLC *in hexane*–benzene–acetone–methanol (40:40:15:5, v/v) afforded starting material **4** (R_F 0.60; 9.6 mg recovery) and the *title compound* **5** as an oil (R_F 0.54, 2.7 mg) (Found: M^+, 508.1578. $C_{24}H_{28}O_{12}$ requires M, 508.1581); δ_H (Table 1); *m/z* 331 (40%), 271 (12), 229 (3), 211 (8), 179 (17) and 169 (100).

(*Z*)-Methyl 3-phenyl-2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)propenoate 2. PPA **6** (110 mg, 0.67 mmol) and caesium carbonate (110.8 mg, 0.34 mmol) were stirred under N_2 in dry MeOH (5 cm^3) for 30 min at room temperature. The methanol was evaporated off in a stream of N_2, the caesium carboxylate **9** was dissolved in anhydrous DMF (5 cm^3), and methyl iodide (0.04 cm^3, 0.68 mmol) was added to the vigorously stirred mixture. After 30 min the mixture was chilled to 0 °C and transferred under anhydrous conditions and nitrogen atmosphere, over a period of 30 min, to a vigorously stirred suspension of sodium hydride (18 mg, 0.75 mmol) in DMF (5 cm^3) at 0 °C. This mixture was stirred for a further 1 h at 0 °C and was added during 30 min to a vigorously stirred solution of the glucosyl bromide **8** (290 mg, 0.73 mmol) in dry DMF (2 cm^3) at 0 °C. The temperature was raised to 20 °C, stirring was continued for 16 h, and the reaction was quenched with chilled, saturated aq. NaCl (150 cm^3). Extraction with ethyl acetate–hexane (4:1, v/v; 3 × 150 cm^3), drying (Na_2SO_4), evaporation off of solvent, and subsequent PLC in hexane–benzene–acetone–methanol (50:40:5:5, v/v) afforded two bands, at R_F 0.44 and 0.35. The former band gave 2,3,4,6-tetra-*O*-acetyl-D-glycal **12** as an oil (66 mg, 27%); $\delta_H(CDCl_3)$ 6.60 (s, H-1), 5.52 (d, *J* 4.2, H-3), 5.19 (dd, *J* 4.2 and 5.5, H-4), 4.33 (m, H-5), 4.39 (dd, *J* 7.0 and 11.6, H-6), 4.18 (dd, *J* 3.0 and 11.6, H-6), 2.06 (3 × OAc) and 2.02 (OAc); *m/z* 331 (21%), 316 (1.0), 303 (54), 271 (45), 229 (69), 211 (40), 172 (2), 169 (100) and 157 (4). The R_F 0.35 band comprised the title compound **2** as an amorphous yellow solid (70 mg, 21%) (Found: M^+, 508.1578. $C_{24}H_{28}O_{12}$ requires M, 508.1581); δ_H (Table 1); $\delta_C([^2H_6]$ acetone; 500 MHz) CH_3CO_2 (170.55, 170.34, 170.09 and 169.94), CH_3CO_2 (20.64, 20.59, 20.53 and 20.42), C-1 (164.51), C-2 (141.39), C-1′ (133.80), C-2′/C-6′ (131.62), C-3′/C-4′/C-5′ (130.13 and 129.23), C-3 (126.09), C-1″ (99.69), C-3″ (73.22), C-2″/C-5″ (72.63 and 72.48), C-4″ (69.29), C-6″ (62.41) and OCH_3 (52.64); *m/z* 331 (55%), 271 (15), 229 (3), 211 (10), 179 (21) and 169 (100).

(*E*)-Methyl 3-phenyl-2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)propenoate 3. A solution of the *Z*-isomer **2** (20 mg) in MeOH was purged with N_2 for 30 min and subsequently irradiated under N_2 at 350 nm for 53 h. The solvent was evaporated off and the mixture was separated by PLC in hexane–benzene–ethyl acetate (5:4:1, v/v, × 7) to give the starting material **2** (R_F 0.59, 14 mg recovery) and the *title compound* **3** as an amorphous solid (R_F 0.60; 1.3 mg, 6.5%) (Found: M^+, 508.1582. $C_{24}H_{28}O_{12}$ requires M, 508.1581); δ_H (Table 1).

Aldol-type condensation of PPA. PPA **6** (200 mg) was dissolved in a mixture of dry MeOH (0.4 cm^3) and anhydrous ethylene dichloride (5 cm^3) and the solution was refluxed under argon for 18 h. The solvent was evaporated off and the mixture was resolved by PLC in hexane–benzene–acetone–methanol (40:40:15:5, v/v) to give a main band at R_F 0.72 (80 mg). This was methylated with diazomethane to give the dimethyl ester **14** of (*E*/*Z*)-2-benzyl-3-phenyl-4-oxopent-2-enedioic acid **13** as an amorphous solid (65 mg); $\delta_H[(CD_3)_2CO–D_2O]$ 7.60, 7.51, 7.16 and 6.87 (ArH), 3.59 (d, *J* 14.5, CH_2), 3.47 (d, *J* 14.5, CH_2) and 3.81 and 3.71 (each s, 2 × OMe).

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