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Note

A convenient gram-scale synthesis of uridine diphospho($^{13}C_6$)glucose

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Abstract—A simple gram-scale synthesis of uridine diphospho($^{13}C_6$)glucose is presented from D-($^{13}C_6$)glucose. The critical step uses a 1*H*-tetrazole-catalyzed coupling of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-1-phosphate and UMP-morpholidate. The uridine diphospho($^{13}C_6$)glucose was used in the structural identification of (1→3)-β-D-glucan from *Lolium multiflorum*.

Keywords: Sugar nucleotides; UDP-glucose; Morpholidate coupling; Isotopic labeling; Glucan

Sugar nucleotides are the primary building blocks from which most complex carbohydrates are built.[1] Despite the central importance of sugar nucleotides, there is still a need for simple practical routes for their preparation. Here we report a simple high-yielding route for the preparation of uridine diphospho($^{13}C_6$)glucose **6** from D-($^{13}C_6$)glucose on a gram scale, for use in ^{13}C NMR and mass spectrometric structural studies of glycans.[2–5] Unlike many of the methods used for the synthesis of sugar nucleotides that are described in the literature, this approach relies on the coupling of a protected glucose-1-phosphate derivative, thereby allowing purification of intermediates on silica gel.

Enzymatic methods for the synthesis of sugar nucleotides hold great promise for simple preparative approaches to sugar nucleotides.[6] However, despite the apparent simplicity of these approaches, there remain significant problems including the difficulty of acquiring many of the necessary enzymes, the problem of scale-up, and the difficulty of isolation of the products. For example, Ma and Stöckigt have reported a 450 mg enzymatic synthesis of UDP-(4-^{13}C)glucose from D-(4-^{13}C)glucose; however, this method required repeated column and preparative thin layer chromatography and thus is not amenable to scale-up.[7]

Synthetic methods, while superficially cumbersome, are in many cases more reliable for the preparation of sugar nucleotides. In general, there are two main approaches: condensation of a sugar and a nucleotide diphosphate,[8–11] or condensation of a sugar phosphate with an activated nucleotide monophosphate.[12,13] The latter is more widely used as issues of stereochemical control in the former can lead to anomeric mixtures.[9–11] Most commonly the condensation of an unprotected sugar phosphate with a nucleoside phosphomorpholidate under 1*H*-tetrazole catalysis is used.[14] Indeed, Fairweather and co-workers have reported the synthesis of UDP-($^{13}C_6$)glucose using this standard method.[5] However, this approach requires troublesome purification of the highly polar unprotected sugar nucleoside diphosphate from a complex reaction mixture. In a patent disclosure, Oehrlein and Baisch have reported the carbonyl diimidazole-promoted coupling of acetylated sugar phosphates with nucleoside monophosphates, followed by their deprotection using orange peel pectinesterase.[15] This method demonstrated the advantages of coupling protected sugar phosphates, but failed to exploit the advantageous physical properties of the acetylated product in the isolation step. Kosma and co-workers have

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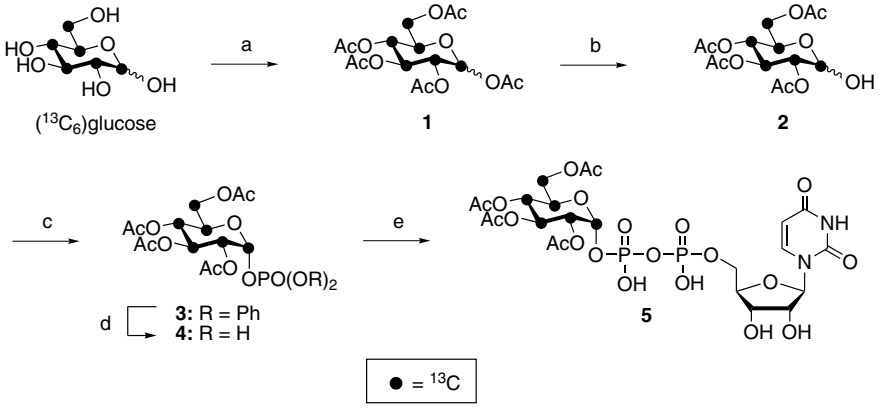
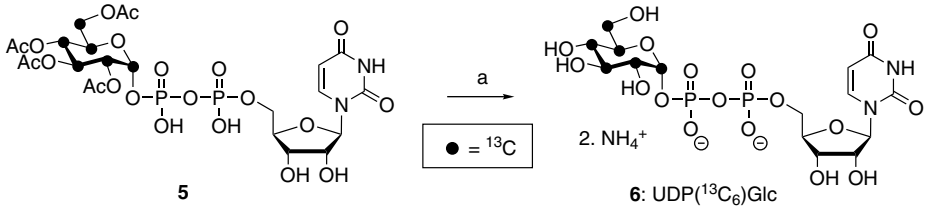
published a simple but significant extension to these methods that involves coupling of acetylated sugar phosphates with a nucleoside phosphomorpholidate,[16–18] where the acetylated sugar nucleotide was purified by flash chromatography prior to deacetylation. While very promising, this method has not been applied on a large scale and the utility of 1*H*-tetrazole as a catalyst for promoting the coupling of acetylated phosphosugars and morpholidates has not been widely investigated.[19] Here, we report on a simple practical route to UDP-($^{13}C_6$)glucose using 1*H*-tetrazole-catalyzed coupling of an acetylated sugar phosphate with a morpholidate as the key step, that proceeds in an excellent overall yield from D-($^{13}C_6$)glucose.

D-($^{13}C_6$)Glucose was acetylated using catalytic sulfuric acid in acetic anhydride to afford the pentaacetate **1**. The pentaacetate **1** was treated with hydrazine acetate to afford hemiacetal **2**. The hemiacetal was condensed with diphenylphosphoryl chloride according to the procedure of Sabesan and Neira to afford the anomeric phosphate **3**.[20] The diphenyl groups of **3** were cleaved by hydrogenolysis using platinum oxide under hydrogen and the resulting phosphoric acid **4** was coupled with uridine 5′-monophosphoromorpholidate in pyridine, according to the procedure of Kosma.[18] In our hands, this procedure was sluggish and even after several days significant starting material remained. Upon addition of 1*H*-tetrazole significant rate acceleration was noted and the reaction cleanly went to completion overnight. The coupled product was purified by flash chromatography to afford the tetraacetate **5** in excellent yield (84% over two steps) (Scheme 1). The overnight reaction time and excellent yield seen here stand in contrast to the 1*H*-tetrazole-catalyzed morpholidate couplings reported by the Schmidt group, which required 3 days to achieve low to moderate yields (13–30%) of UDP-*exo*-glycal derivatives.[19] Finally, deprotection of **5** was carried out by treatment of tetraacetate **6** with triethylammonium bicarbonate (TEAB) in methanol/water at −20 °C. The product was purified by Biogel chromatography to afford uridine diphospho($^{13}C_6$)glucose **6** as the ammonium salt (Scheme 2). Sugar nucleotide **6** was contaminated with trace amounts of uridine 5′-monophosphate (UMP) and ($^{13}C_6$)glucose 1,2-cyclic phosphate (≈5%), which arise spontaneously during the prolonged deprotection conditions. The presence of these impurities did not affect combustion analysis of **6**, nor its use in biological studies (see below). UMP has been found to act as a potent inhibitor of many glycosyltransferases and if its removal is necessary it is noted that UMP can be hydrolyzed by treatment with alkaline phosphatase.

The utility of UDP-($^{13}C_6$)glucose **6** for the study of polysaccharide synthesis and for structural analysis of the resultant product was illustrated by its use as substrate in reaction with a (1→3)-β-D-glucan synthase



Scheme 1. Reagents and conditions: (a) Ac_2O, H_2SO_4, rt, 96%; (b) NH_2NH_2·AcOH, DMF, 91%; (c) $(PhO)_2POCl$, DMAP, 83%; (d) H_2, PtO_2, EtOAc/CH_3CH_2OH (1:1); (e) uridine 5′-monophosphoromorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamide salt, 1*H*-tetrazole, pyridine, 84% over two steps.



Scheme 2. Reagents and conditions: (a) TEAB, CH_3OH, H_2O, −20 °C, 94%.

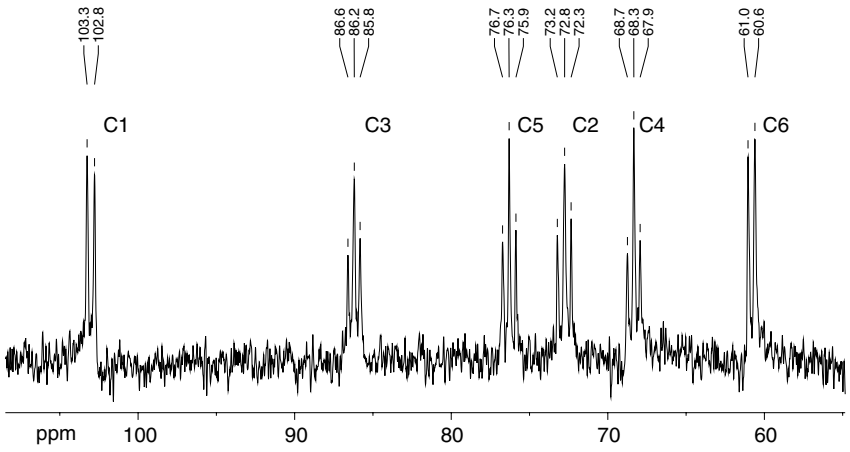


Figure 1. 100.59 MHz ^{13}C NMR spectrum of ≈25 μg (1→3)-β-D-glucan prepared from *Lolium multiflorum* by in vitro labeling with UDP-($^{13}C_6$)glucose (**6**).

preparation from suspension-cultured Italian ryegrass (*Lolium multiflorum*) cells.[21] The product (≈25 μg) was recovered using the procedure of Him et al.[22] The ^{13}C NMR spectrum of the product (Fig. 1) is identical to that from a similar ^{13}C-labeled (1→3)-β-D-glucan preparation from blackberry (*Rubus fruticulosus*),[5] and agrees with the characteristic shifts seen for a natural abundance ^{13}C NMR spectrum of a (1→3)-β-D-glucan.[23]

To conclude, a simple chemical synthesis of UDP-($^{13}C_6$)glucose **6** is described, with the critical step being a 1*H*-tetrazole-catalyzed coupling of the acetylated glucose-1-phosphate **1** and uridine 5′-monophosphoromorpholidate. Starting with 2 g of D-($^{13}C_6$)glucose over 1 g of labeled UDP-($^{13}C_6$)glucose **6** was synthesized in an overall yield of 50%. This route is scaleable and should be readily applied to even larger scale syntheses, or to the synthesis of other isotopically labeled derivatives.

1. Experimental

1.1. General methods

Petroleum spirits refer to a mixed fraction boiling at 40–60 °C. Thin layer chromatography (TLC) was performed with Merck Silica Gel 60 F_{254}, using mixtures of petroleum spirits–EtOAc unless otherwise stated. Detection was effected by either charring in a mixture of 5% sulfuric acid–CH_3OH and/or by visualization in UV light. NMR spectra were obtained on Varian Inova 400 MHz instruments (Melbourne, Australia). Flash chromatography was performed according to the method of Still et al. with Merck Silica Gel 60, using adjusted mixtures of EtOAc–petroleum spirits unless otherwise stated.[24] CH_2Cl_2 and pyridine were dried over CaH_2. Solvents were evaporated under reduced pressure using a rotary evaporator. High resolution mass spectra were performed by Sally Duck at the Chemistry Department, Monash University. D-($^{13}C_6$)Glucose and hydrazine acetate were obtained from Sigma–Aldrich. Elemental analysis was performed by C.M.A.S. (Belmont, Victoria).

1.2. Diphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)glucopyranosyl phosphate (3)

1.2.1. 1,2,3,4,6-Penta-*O*-acetyl-D-($^{13}C_6$)glucopyranose (1). A suspension of D-($^{13}C_6$)glucose (2.00 g, 10.7 mmol) in Ac_2O (11 mL) was treated with H_2SO_4 (100 μL) at 0 °C and the mixture was stirred at rt for 2 h. Water (40 mL) was then added followed by EtOAc (50 mL). The organic phase was separated and washed with water (3 × 50 mL) and satd aq $NaHCO_3$ (3 × 50 mL), dried ($MgSO_4$), and concentrated in vacuo to give 1,2,3,4,6-penta-*O*-acetyl-D-($^{13}C_6$)glucopyranose (**1**) as a clear oil (4.07 g, 96%, β:α ratio = 4:5). ^1H NMR (400 MHz, $CDCl_3$): δ 2.01 (s, 3H, $CH_3\beta$), 2.02 (s, 3H, $CH_3\alpha$), 2.03 (s, 3H, $CH_3\beta$), 2.04 (s, 3H, $CH_3\alpha$), 2.05 (s, 3H, $CH_3\alpha$), 2.08 (s, 3H, $CH_3\beta$), 2.09 (s, 3H, $CH_3\alpha$), 2.12 (s, 3H, $CH_3\beta$), 2.18 (s, 3H, $CH_3\alpha$), 2.22 (s, 3H, $CH_3\beta$), 3.62–5.74 (m, 6H, H-2α,β, 3α,β, 4α,β, 5α,β, 6aα,β, 6bα,β), 5.71 (dd, $J_{C,H} = 165$ Hz, $J_{1,2} = 9.2$ Hz, 1H, H-1β), 6.33 (dm, $J_{C,H} = 175$ Hz, 1H, H-1α).

1.2.2. 2,3,4,6-Tetra-*O*-acetyl-D-($^{13}C_6$)glucopyranose (2). A solution of 1,2,3,4,6-penta-*O*-acetyl-D-($^{13}C_6$)glucopyranose (**1**) (4.05 g, 10.2 mmol) in DMF (10 mL) was treated with hydrazine acetate (1.13 g, 12.3 mmol) and stirred under nitrogen for 2 h. A second portion of hydrazine acetate (470 mg, 5.11 mmol) was added and stirred for a further 2 h. EtOAc (50 mL) was then added followed by water (50 mL) and the organic layer was washed with water (2 × 50 mL), dried ($MgSO_4$), and concentrated in vacuo to give 2,3,4,6-tetra-*O*-acetyl-D-($^{13}C_6$)glucopyranose (**2**) as a clear oil (3.28 g, 91%). ^1H

NMR (400 MHz, $CDCl_3$): δ 2.02–2.10 (m, 12H, $4 \times CH_3$), 3.57–5.80 (m, 7H, H-1,2,3,4,5,6a,6b).

1.2.3. Diphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)glucopyranosyl phosphate (3). DMAP (2.20 g, 18.0 mmol) was added to a solution of 2,3,4,6-tetra-*O*-acetyl-D-($^{13}C_6$)glucopyranose (**2**) (2.66 g, 7.51 mmol) in CH_2Cl_2 (53 mL) and stirred at rt for 15 min. The reaction mixture was cooled to −10 °C, then diphenyl chlorophosphate (3.75 mL, 18.1 mmol) was added dropwise. After stirring for 1 h, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed successively with ice cold water (2×100 mL), ice cold 0.5 M HCl (2×100 mL), and satd aq $NaHCO_3$ (2×100 mL). The organic fraction was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography (40–50% EtOAc in petroleum spirits) to give diphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)glucopyranosyl phosphate (**3**) as a pale yellow oil (2.70 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 2.00 (s, 6H, $CH_3 \times 2$), 3.84 (dd, $J_{C,H} = 150$ Hz, $J_{6a,6b} = 12.8$ Hz, 1H, H-6b), 4.03 (dm, $J_{C,H} = 148$ Hz, 1H, H-5), 4.15 (ddd, $J_{C,H} = 147$ Hz, $J_{6a,6b} = 12.8$ Hz, $J_{5,6a} = 3.6$ Hz, 1H, H-6a), 5.00 (dm, $J_{C,H} = 146$ Hz, 1H, H-2), 4.88–5.72 (m, 2H, H-3,4), 6.04 (dm, $J_{C,H} = 180$ Hz, 1H, H-1); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 20.21, 20.51, 20.58 (×2) (4C, CH_3), 60.93 (d, $J_{C,C} = 44.2$ Hz, 1C, C-6), 68.87–70.09 (m, 4C, C-2,3,4,5), 94.95 (d, $J_{C,C} = 44.2$ Hz, 1C, C-1), 120.00–150.24 (4C, Ph), 169.32, 169.76, 169.95, 170.52 (4C, C=O); ^{31}P NMR (161.8 MHz, $CDCl_3$): δ −14.38 to −14.22 (m, 1P, P-1); ESIMS *m/z* calcd for $^{13}C_6C_{20}H_{29}O_{13}P$ $[M+Na]^+$: 609.1445. Found 609.1445.

1.3. Uridine 5′-diphospho-2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)glucopyranose (5)

A solution of diphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)-glucopyranosyl phosphate (3) (2.01 g, 3.42 mmol) in EtOAc/EtOH (1:1, 60 mL) containing PtO_2 (486 mg) was shaken under H_2 in a Parr apparatus at 380 kPa for 18 h. The reaction mixture was filtered through Celite, and the filtrate treated with Et_3N (0.95 mL, 6.84 mmol), and then concentrated in vacuo to yield crude bis(triethylammonium) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**4**). Without purification, this crude material was co-evaporated with dry pyridine (3×20 mL). UMP-morpholidate (3.38 g, 4.91 mmol) was co-evaporated with pyridine (3×20 mL), then transferred via a cannula into the reaction flask. The combined reagents were co-evaporated with pyridine (2×20 mL) then 1*H*-tetrazole (772 mg, 11.0 mmol) was added and the reaction mixture stirred at rt for 24 h. The reaction mixture was concentrated in vacuo and the resulting material was purified by flash chromatography (7:2:1 then 5:2:1, EtOAc/CH_3OH/H_2O). The combined fractions were pooled, concentrated in vacuo and purified a second time by flash chromatography (10–25% H_2O in acetonitrile) to give uridine 5′-diphospho-2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)glucopyranose (**5**) (2.14 g, 84%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 1.88 (s, 3H, CH_3), 1.91 (s, 3H, CH_3), 1.94 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 3.75–5.53 (m, 7H, H-1,2,3,4,5,6a,6b), 3.99–4.19 (m, 4H, H-2′,3′,4′,5′), 5.80 (d, $J_{1',2'} = 4.0$ Hz, 1H, H-1′), 5.83 (d, $J_{5'',6''} = 8.0$ Hz, 1H, H-5″), 7.84 (d, $J_{5'',6''} = 8.0$ Hz, 1H, H-6″); ^{13}C NMR (100.5 MHz, D_2O): δ 20.24, 20.29, 20.30, 20.40 (4C, CH_3), 61.51–62.01 (m, 1C, C-6), 65.13 (d, $J_{C5',P} = 5.3$ Hz, C-5′), 67.55–68.95 (m, 2C, H-4,5), 69.50–71.80 (m, 3C, C-2,3,3′), 74.18 (C-2′), 83.10 (d, $J_{C4',P} = 9.1$ Hz, C-4′), 88.82 (C-1′), 92.20–92.69 (m, 1C, C-1), 102.84 (C-5″), 141.97 (C-6″), 151.88 (C-2″), 166.31 (C-4″), 172.97 (×2), 173.25, 173.83 (4C, C=O); ^{31}P NMR (161.8 MHz, D_2O): δ −11.74, −9.47 ($2 \times$ br s, P-1,2); ESIMS *m/z* calcd for $^{13}C_6C_{17}H_{32}N_2O_{21}P_2$ $[M-H]^-$: 739.1099. Found 739.1114. $[M+Na-2H]^-$: 761.0929. Found 761.0926.

1.4. Bis(ammonium) uridine 5′-diphospho-α-D-($^{13}C_6$)-glucose (6)

Uridine 5′-diphospho-2,3,4,6-tetra-*O*-acetyl-α-D-($^{13}C_6$)-glucopyranose (**5**) (1.65 g, 2.23 mmol) was suspended in a mixture of 0.1 M TEAB (60 mL) and CH_3OH (45 mL) and chilled to −40 °C. Et_3N (750 μL, 10.2 mmol) was then added and the reaction mixture was stored at −20 °C for 4 days with occasional mixing. A mixture of 0.1 M TEAB (30 mL) and CH_3OH (22.5 mL) then Et_3N (375 μL, 5.1 mmol) were added and the reaction mixture was stored at −20 °C for a further 4 days. The reaction mixture was then concentrated in vacuo, keeping the temperature below 30 °C. The resulting material was passed through size exclusion resin (Bio-Gel P-2, 3.5 cm × 45 cm) eluted with 250 mM NH_4HCO_3. The fractions containing the product were pooled and lyophilized. The product was passed through cation exchange resin (Dowex 50W X8-400, ammonium form, 2×5 cm). Fractions containing the product were pooled and lyophilized to give bis(ammonium) uridine 5′-diphospho-α-D-($^{13}C_6$)glucose (**6**) as a white solid (1.28 g, 94%). ^{1}H NMR (400 MHz, D_2O): δ 3.05–4.15 (m, 9H, H-2,3,3′,4,4′,5,5′,6a,6b), 4.19 (d, $J_{1',2'} = 3.6$ Hz, 1H, H-2′), 5.43 (dm, $J_{C,H} = 173$ Hz, 1H, H-1), 5.74–5.80 (m, 2H, H-1′,5″), 7.77 (d, $J_{5'',6''} = 8.4$ Hz, 1H, H-6″); ^{13}C NMR (100.5 MHz, D_2O): δ 60.99 (d, $J_{C,C} = 42.7$ Hz, 1C, C-6), 65.63 (d, $J_{C5',P} = 5.3$ Hz, 1C, C-5′), 70.02 (ddd, $J_{C3,C4} = 37.4$ Hz, $J_{C4,C5} = 37.4$ Hz, $J_{C2,C4} = 3.0$ Hz, 1C, C-4), 70.34 (s, 1C, C-3′), 71.79–72.74 (m, 1C, C-2), 74.97 (m, 3C, C-2′,3,5), 83.93 (d, $J_{C4',P} = 9.1$ Hz, 1C, C-4′), 89.08 (1C, C-1′), 96.29 (dm, $J_{C1,C2} = 44.2$ Hz, 1C, C-1), 103.37 (C-5″), 142.33 (C-6″), 152.56 (C-2″), 166.98 (C-4″); ^{31}P

NMR (161.8 MHz, D_2O): −10.91 (ddd, $J_{P1,P2} = 19.8$ Hz, $J_{C1,P2}$ 6.9 Hz, $J_{C2,P2} = 6.1$ Hz, 1P, P-1), −9.28 (d, $J_{P1,P2} = 19.8$ Hz, 1P, P-2); LRMS (ESI^-, m/z): $[M-H]^-$ 571.2, $[M-2H]^{2-}$ 285.2. ESIMS m/z calcd for $^{13}C_6C_9H_{24}N_2O_{17}P_2$ $[M-H]^-$: 571.0673. Found 571.0665. $[M+Na-2H]^-$ 593.0492. Found 593.0494. Anal. Calcd for $^{13}C_6C_9H_{30}N_4O_{17}P_2$: C, 30.70; H, 4.99; N, 9.24. Found: C, 30.67; H, 5.02; N, 9.31.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.02.033.

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