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Note

Synthesis and esterase-catalysed de-esterification of radiolabelled methyl 2,6-di-*O*-pivaloyl-α-D-glucopyranoside

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Carboxylesterases hydrolyse a wide range of carboxylic esters and are important in the transformation of many toxic pesticides, insecticides, drugs, and endogeneous compounds. They are found in many tissues (*e.g.*, liver and kidneys) and in serum[1]. They have been used in organic synthesis. Hog-liver carboxylesterase can hydrolyse[2–4] asymmetric chiral and prochiral esters and has been used in stereospecific transesterifications producing optically active esters and alcohols from racemates[5]. Numerous liver carboxylase preparations have been described[1,6], but there is much less data on serum esterases[7–9].

Wheat-germ lipase[10,11] has been used for the deacylation of sugar derivatives. Acetyl groups tend to migrate, which can complicate the identification of the products of hydrolysis. Thymidine 5-pivaloate is a prodrug which is de-esterified by plasma esterases and is now used to maintain high levels of thymidine for longer periods, thereby preventing or reversing the toxic effects of methotrexate therapy without any loss of its antitumour activity[12].

We now report a one-pot synthesis of ^{14}C-labelled methyl *O*-pivaloyl-α-D-glucopyranosides and the susceptibility of methyl 2,6-di-*O*-pivaloyl-α-D-glucopyranoside to esterases from rabbit sera. The pivaloyl group is a versatile protecting-group[13,14] which is relatively stable towards migration. This work is part of a project on prodrugs containing esterified sugar moieties. In order to simulate physiological conditions more closely, unpurified esterases were used.

In a one-pot synthesis, convenient for radiolabelled compounds, D-[U-^{14}C]glucose was treated with methanolic hydrogen chloride to give methyl α-D-[U-^{14}C]glucopyranoside (**1**, 95% based on t.l.c. data). The crude product was treated with pivaloyl chloride (656 nmol)–pyridine, to give a mixture of the 2- (**2**), 6- (**3**), and 2,6-di-*O*-pivaloyl (**4**) derivatives, which were isolated by column chromatography and identified by comparison with their unlabelled analogues. Unlabelled **2** and **4**

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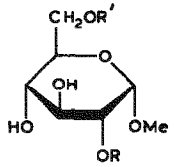
have been described[14], but **3** was unknown hitherto. Unlabelled **3** was prepared, and its structure was determined on the basis of analytical and ^{1}H-n.m.r. data for **3** and its triacetate. The position of the pivaloyl group was assigned by comparison of the chemical shift of the *tert*-butyl singlet with those of a series of methyl *O*-pivaloyl-α-D-glucopyranosides[13].



1 R = R' = H
2 R = ^{t}BuCO, R' = H
3 R = H, R' = ^{t}BuCO
4 R = R' = ^{t}BuCO

The enzymic de-esterification of the 2,6-dipivaloate **4** (see Experimental) proceeded via the 2- (**2**) and 6- (**3**) esters and ultimately gave **1**. The results of a series of experiments are given in Table I. Thus, with 1 and 2mM concentrations of **4** (*cf.* K_m 1.14mM), **2** and **3** would be detected after 15 min, and after 40 min with 4 and 5mM concentrations of **4**. With 1mM **4**, **2** and **3**, but not **1**, were present after 25 min; after 1 h, 38% of **4** had reacted but only 3.8% of **1** was detected. After 24 h, virtually all of **4** had reacted and 52% of **1** had been formed. Thus, the conversion of **4** into **2** and **3** is more rapid than the conversion of **2** and **3** into **1**. The ratio of **2** and **3** remained essentially the same (~6:1) regardless of reaction times or concentrations of incubation mixtures, and reflected the preferential cleavage of the 6-ester group in **4** and the similar susceptibilities of **2** and **3** to the enzyme. Pig-liver esterase has been reported[1–4] to hydrolyse various esters stereoselectively, and prochiral diesters gave chiral monoesters with large enantiomeric excess. The action of rabbit-serum esterase on **4** is stereoselective, with the 6-ester group being hydrolysed much more readily than the 2-ester group.

On the basis of kinetic studies, the values for K_m (1.14 ±0.31 mmol.dm^{-3}) and V_{max} (1.08 ±0.097 nmol.mg^{-1}.min^{-1}) were determined for substrate disappearance.

EXPERIMENTAL

General. — Column chromatography was performed on silica gel (Kemika, 0.063–0.2 mm), and t.l.c. on Silica Gel 60 (Merck), using *A*, acetonitrile–water (5:1); *B*, benzene–ethyl acetate (1:1); and *C*, ethyl acetate–benzene–ethanol–chloroform (100:20:1:1). ^{1}H-N.m.r. spectra (internal Me_4Si) were recorded with a JEOL FX Q FT spectrometer. Radioactivity was measured by using a Beckman

TABLE I

ENZYMIC DEACYLATION OF **4**: EFFECT OF TIME OF REACTION AND INITIAL CONCENTRATION OF SUBSTRATE

Initial substrate conc. (mM)	*Composition of products*[a]														
	15 min			*25 min*			*40 min*			*1 h*			*24 h*		
	1[b]	**(2+3)**[c]	**4**[d]	**1**[b]	**(2+3)**[c]	**4**[d]	**1**[b]	**(2+3)**[c]	**4**[d]	**1**[b]	**(2+3)**[c]	**4**[d]	**1**[b]	**(2+3)**[c]	**4**[d]
1	—	17.3	79.1	—	27.6	69.6	—	N.d.		3.8	54.3	38.3	52.5	43.0	2.8
2	—	10.2	87.7	—	15.9	81.7		N.d.		1.6	41.1	54.1	48.1	43.4	6.8
4	—	—	—		N.d.[e]		—	14.8	80.1	—	26.2	69.8	30.7	67.5	—
5	—	—	—		N.d.		—	13.4	81.3	—	22.7	69.4	26.3	72.7	—

[a]Analysis of products based on radioactivity, and given as a percentage of the total radioactivity scraped off the t.l.c. plate. [b]Methyl α-D-[U-^{14}C]glucopyranoside. [c]Mixture of 2- and 6-pivaloates. [d]Methyl 2,6-di-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside. [e]Not determined.

LS-100C liquid scintillation counter and a scintillation cocktail of toluene (720 mL), Triton X-100 (280 mL), 2,5-diphenyl-1,3-oxazole (5 g), and *p*-bis[2-(5-phenyloxazolyl)]benzene (50 mg).

Kinetic calculations were done on an HP 9845B computer, and values for K_m and V_{max} were obtained by using the Michaelis–Menten equation.

D-[U-^{14}C]Glucose (aqueous solution containing 3% of ethanol; 98% radiochemically pure; specific activity, 10.2 GBq/nmol) was obtained from Amersham International, and the solvent was removed under reduced pressure prior to use.

*Methyl α-*D*-[U-^{14}C]glucopyranoside* (**1**) *and its pivaloylation* — A solution of D-[U-^{14}C]glucose (7.4 MBq) and D-glucose (50 mg) in dry methanol (2 mL) was boiled under reflux for 14 h in the presence of catalytic quantities of hydrogen chloride (0.3 mL of 3.3M HCl in methanol). The solvent was removed under reduced pressure and methanol was distilled thrice from the residue in order to remove traces of HCl. T.l.c. (solvent *A*) indicated that the residue consisted mainly of **1** (95% based on radioactivity).

A solution of crude **1** in dry pyridine (1.5 mL) was treated with pivaloyl chloride (0.08 mL, 656 nmol) at room temperature for 15 min. Ethanol was then added, the solvents were evaporated under reduced pressure, and a solution of the residue in ethanol was partially concentrated. This procedure was repeated 5 times in order to remove traces of pyridine. The solution of the residual mixture in ethanol (1.4 mL) was applied to a column (1 × 60 cm) of silica gel and eluted with solvent *B*, to give chromatographically homogeneous methyl 2,6-di-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**4**; 36 mg, 35.8% based on glucose); R_F 0.5 (solvent *B*) and 0.8 (solvent *C*); specific activity, 28.0 MBq/mmol.

Further elution with solvent *C* gave a mixture of **2** and **3** (37.8 mg, 48.0%). Re-chromatography of the mixture (solvent *C*) produced methyl 2-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**2**; 6 mg, 7.8%), R_F 0.3 (solvent *C*); specific activity, 29.7 MBq/mmol; and methyl 6-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**3**; 14.8 mg, 19.2%), R_F 0.2 (solvent *C*); specific activity, 30.0 MBq/mmol.

Products **2**–**4** were identified by comparison (t.l.c. and ^{1}H-n.m.r. spectra) with authentic unlabelled compounds.

*Methyl 6-*O*-pivaloyl-α-*D*-glucopyranoside* (**3**). — A solution of methyl α-D-glucopyranoside (**1**, 54 mg) in dry pyridine (1.5 mL) was treated with pivaloyl chloride (0.08 mL) at room temperature for 15 min, and then concentrated. Column chromatography (solvent *B*, then solvent *C*) of the product mixture gave the 2,6-ester[13] (**4**; 50 mg, 49.7%), the 2-ester[13] (**2**; 7 mg, 9%), and the 6-ester (**3**; 20 mg, 25.9%), isolated as a glass, R_F 0.2 (solvent *C*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.23 (s, 9 H, PivO-6), 3.41 (s, 3 H, MeO).

Conventional acetylation of **3** with acetic anhydride–pyridine[15] gave, after column chromatography (solvent *B*), methyl 2,3,4-tri-*O*-acetyl-6-*O*-pivaloyl-α-D-glucopyranoside, isolated as a glass (19 mg, 85.2%). ^{1}H-N.m.r. data ($CDCl_3$): δ

1.23 (s, 9 H, PivO-6), 2.01, 2.03, 2.08 (3 s, each 3 H, 3 AcO), and 3.41 (s, 3 H, MeO).

Anal. Calc. for $C_{18}H_{28}O_{10}$: C, 53.46; H, 7.00. Found: C, 53.44; H, 6.72.

Enzymic deacylations of methyl 2,6-di-O-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**4**). — The incubation mixture contained 75 μL of 0.1M phosphate-buffered saline (PBS, pH 7.2), 50 μL of rabbit serum, and 10 μL of methyl sulfoxide containing various concentrations of **4**. The resulting mixtures (135 μL) were 0.5, 1, 2, 4, and 5mM with respect to **4**. Incubations were allowed to proceed variously for 15, 25, 40 min, 1 h, and 24 h (Table I), depending on the concentration of **4**.

In the kinetic studies, the incubations were allowed to proceed for 15, 20, and 25 min for 1 and 2mM **4**, and for 40 min and 1 h for 4 and 5mM **4** (Table I). Incubations containing 0.5mM **4** were allowed to proceed for 5 (87.8% of **4** then remained, and 10.6% of **2** and **3** had been formed), 10 (82.5% of **4**, 16.1% of **2** and **3**), and 15 min (77.5% of **4**, 21.3% of **2** and **3**). The time intervals were chosen to allow plots of time *vs.* disappearance of the substrate to be linear.

In another series of experiments, incubation mixtures (135 μL, containing mM **4**) containing 10, 20, 50, and 60 μL of methyl sulfoxide, with the volumes of PBS appropriately decreased, were incubated for 15 min. No significant difference could be observed in the ratios of the substrate and reaction products.

Each incubation was performed at 37° and stopped by the addition of 0.6 mL of ethanol. Serum proteins were then removed by centrifugation (1500*g*, 15 min, Tehtnica LC-320 bench centrifuge). Each supernatant solution was concentrated under reduced pressure and an aliquot (20 μL) of a solution of the residue in 100 μL of aqueous 50% ethanol was subjected to t.l.c. (solvent *C*) with unlabelled **1–4** as references; R_F values: **1**, 0; **2**, 0.3; **3**, 0.2; **4**, 0.8. By using solvent *A* (R_F values: **1**, 0.44; **2** and **3**, 0.67; and **4**, 0.9), it was found that radioactivity was associated only with the expected products.

The reference compounds were detected by charring with sulfuric acid. The absorbent containing each product (**1–4**) of enzymic action was scraped off and the radioactivity was measured. Because the R_F values of **2** and **3** were similar, the combined radioactivity was determined. The absorbent containing each product was suspended in a mixture of 2 mL of aqueous 75% ethanol and 2 mL of scintillation cocktail, and the radioactivity was measured and expressed as a percentage of the total radioactivity (Table I). The recovery of the radioactivity was 97–99%.

Each enzymic deacylation experiment was performed at least twice and the results were 95% reproducible.

T.l.c. and determinations of radioactivity were repeated 2–3 times, the reproducibility being 96–98%.

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