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Binding properties and esterase activity of monoclonal antibodies elicited against sucrose 6-heptylphosphonate

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Abstract

Various sugar phosphonates were prepared by a Mitsunobu condensation between phosphonic diacids and properly protected carbohydrates. 6′-*O*-*p*-Aminophenylsucrose 6-heptylphosphonate was coupled to Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH) and the KLH conjugate was used for generation of monoclonal antibodies. Binding properties of these antibodies were screened by competitive enzyme-linked immunosorbent assay (ELISA) using the BSA conjugate. A monoclonal antibody with good binding properties showed a regioselective esterase activity toward 6-octanoylsucrose compared with 6′-octanoylsucrose.

Keywords: Sugar phosphonate; Sucrose; Monoclonal antibodies; Abzyme

1. Introduction

Since the first examples of catalytic antibodies reported by the groups of Lerner[1] and Schultz,[2] a remarkable number of papers reported that antibodies with good binding specificity toward an haptenic transition state analogue could provide powerful tools for regio- and stereoselective organic synthesis.[3–6] Among these reports, some have dealt with carbohydrate chemistry, most of the time to obtain antibodies with glycosidase activity[7–11] while only one group has reported an antibody which hydrolysed regio- and stereoselectively acylated carbohydrates.[12,13] Since the very beginning of the discovery of abzymes, it was shown that monoclonal antibodies generated against phosphonates, which are transition state analogues for the hydrolysis of esters, can also catalyse transesterification reactions.[14–20] Following this concept, we focused on the generation of catalytic antibodies designed for the preparation of monoesters of sucrose **1** (Scheme 1). Sucrose esters can be used as surfactants in the food, detergent and cosmetic industries.[21] Usually, these fatty acid esters are prepared as mixtures, either by esterification of sucrose with acyl chlorides or acyl chloroformates, or by transesterification between sucrose and fatty esters.[22–26] Pure and clearly defined esters in a primary position have been obtained using tributylstannyloxide.[27] Selective acylation of unprotected sucrose has also been accomplished with 3-acylthiazolidine-2-thiones introduced by Plusquellec and coworkers.[28–30] Alternatively, these esters can also be prepared through bio-

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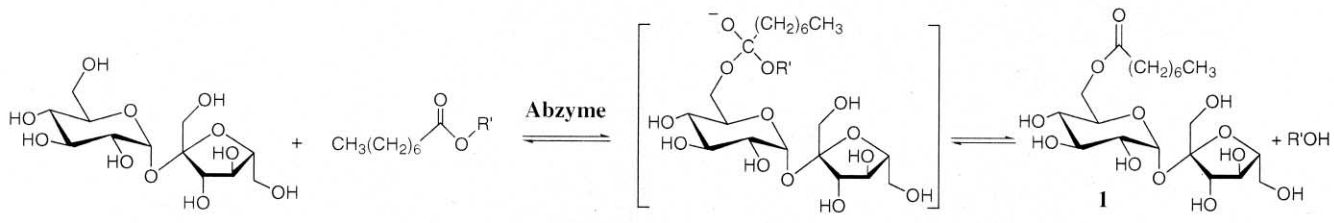
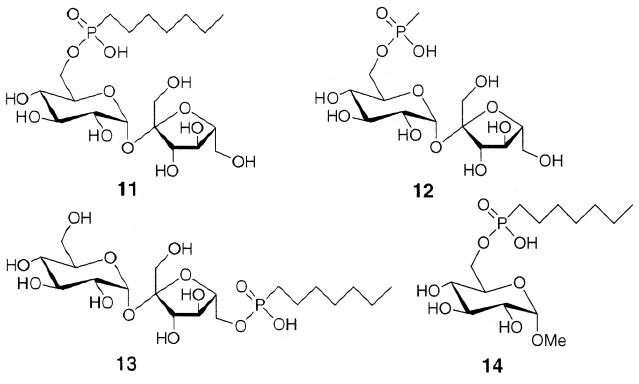


Scheme 1. Conversion of sucrose to 6-octanoylsucrose proceeding through a tetrahedral intermediate.

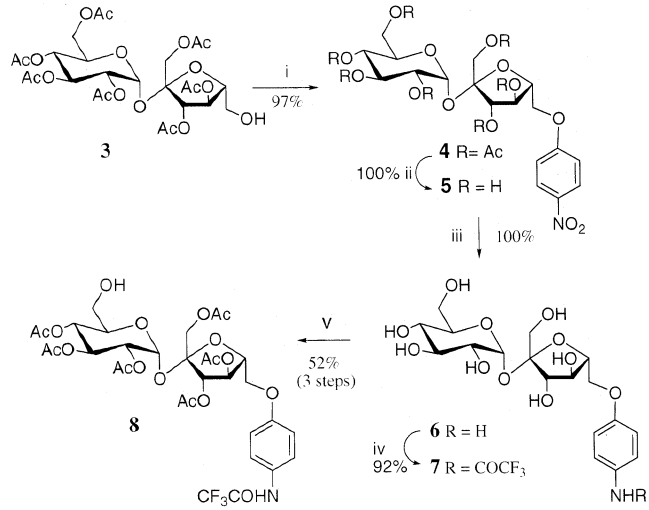
chemical methods. However, lipase-catalysed esterifications of sucrose in aqueous buffers gave rise to complex mixtures of mono-, di-, and polyacylsucrose.[31] In contrast, monoesters at the 1′ position have been obtained in anhydrous DMF[32,33] or in pyridine[34] with a protease which catalysed the transesterification between sucrose and activated esters while the use of a lipase in *tert*-butyl alcohol[35] led to the formation of a mixture of 6 and 6′ monoesters. Therefore, selective reactions that could provide esters at the 6 position would be valuable.

We decided to prepare compound **2** as hapten to generate monoclonal antibodies. The *p*-aminophenyl group installed at the 6′ position was designed to couple the hapten to a carrier protein to obtain an immune response in immunised mice. In order to evaluate the recognition properties of the monoclonal antibodies, other sugar phosphonates (**11-14**) were also synthesised and used as inhibitors in competitive enzyme-linked immunosorbent assay (ELISA). We hereby report these results as well as the catalytic properties of one monoclonal antibody toward the hydrolysis of several sucro esters.

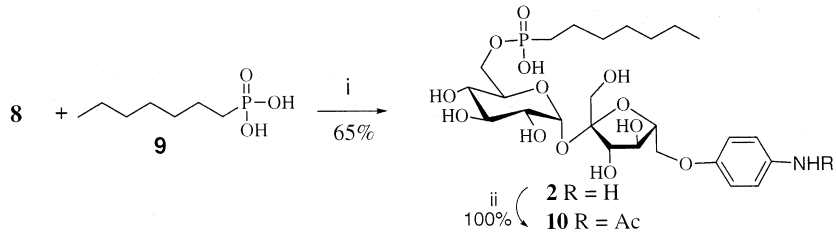


2. Results and discussion

Synthesis of sugar phosphonates.—Hapten **2** was prepared by coupling heptylphosphonic acid with compound **8** which was obtained in seven steps from 2,3,4,6,1′,3′,4′-hepta-*O*-acetylsucrose (**3**)[36,37] according to Scheme 2. The *p*-nitrophenyl group was introduced by a Mitsunobu reaction using triphenylphosphine and diisopropyl azodicarboxylate (DIAD) in 97% yield. After Zemplén deacetylation and hydrogenation in the presence of 10% palladium-on-carbon catalyst in a 9:1 *t*-BuOH–water mixture, **6** was obtained in quantitative yield. Then, treatment with thioethyl trifluoroacetate in methanol afforded **7** in 92% yield. Compound **8** was then prepared in 52% overall yield from **7** through a three-step procedure without purification of the intermediates. First, the OH-6 of polyol **7** was regioselectively protected by treatment with dimethoxytrityl chloride (DMTrCl) in pyri-



Scheme 2. (i) *p*-Nitrophenol, PPh_3, DIAD, THF; (ii) MeONa, MeOH; (iii) H_2, Pd/C, *t*-BuOH–H_2O; (iv) CF_3COSEt, MeOH; (v) (1) DMTrCl, pyridine, (2) Ac_2O, pyridine (3) TsOH, CH_2Cl_2–MeOH.



Scheme 3. (i) (1) PPh_3, DEAD, pyridine (2) LiOH aq 1 M, CH_3CN then Dowex H^+; (ii) Ac_2O, MeOH, Et_3N.

dine, then peracetylation in the same pot with acetic anhydride gave the fully protected derivative in which selective hydrolysis of the methoxytrityl ether (*p*-TsOH in 2:1 CH_2Cl_2–MeOH) gave, after flash chromatography, pure compound **8**.

Phosphonic acid monoesters are usually prepared by selective cleavage of symmetrical or non-symmetrical phosphonate diesters.[38] Another strategy is based on the direct monoesterification of phosphonic acid with alcohol in the presence of a condensing reagent such as 1,3-dicyclohexylcarbodiimide[39] or trichloroacetonitrile.[40] However these methods require a large excess of alcohol. Alternatively, the synthesis of phosphonate esters has been reported from phosphonic monoacids and alcohols using Mitsunobu coupling conditions.[41,42] This strategy has been applied to achieve phosphonylation and phosphorylation of a purine nucleoside[43] and yields were improved by performing the reaction in anhydrous pyridine. This solvent effect was explained by the enhanced nucleophilic reactivity of the phosphate and the phosphonate anions in the presence of pyridine.[44] Accordingly, we found that the Mitsunobu reaction can provide monophosphonates when conducted in pyridine under alcohol limiting conditions. Hence, compound **8** was reacted with heptylphosphonic acid (3 equiv), triphenylphosphine and diethyl azodicarboxylate (DEAD) in pyridine at 120 °C for 4 h (Scheme 3). As the resulting phosphonate was difficult to purify at this stage, it was deprotected directly with LiOH added to the crude mixture. Pure **2** was obtained, after C-18 flash chromatography, in 65% yield from **8**. Then, *N*-acetylation with acetic anhydride in MeOH in the presence of triethylamine afforded **10** in quantitative yield.

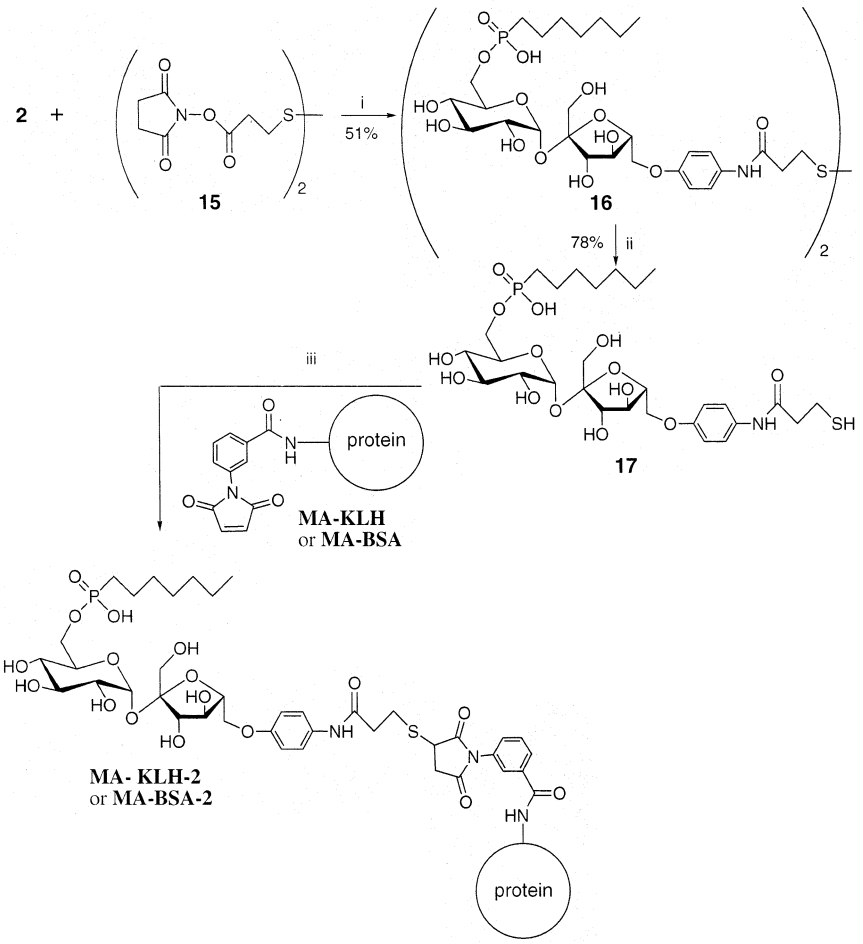
Following the same strategy, phosphonates **11** and **12** were prepared by reacting 2,3,1′,3′,4′,6′-hexa-*O*-acetylsucrose[45] with heptyl- or methyl-phosphonic acid respectively. After treatment with LiOH, **11** was obtained in 68% yield after C-18 flash chromatography, while **12** was isolated as its triethylammonium salt in 66% yield after DEAE-Sephadex A25 ion-exchange chromatography. The regioselectivity of the phosphonylation was confirmed by ^1H NMR. The two H-6 protons of **11** and **12** appeared as two ddd with 6.5 and 7.5 Hz proton-phosphorous coupling constants. The signals were simplified upon phosphorous irradiation. 2,3,4,6,1′,3′,4′-Hepta-*O*-acetylsucrose[40,41] and methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranoside[46] were similarly reacted with heptylphosphonic acid. Deacetylation and purification by C-18 flash chromatography afforded **13** and **14** in 67 and 70% yields, respectively.

Hapten coupling to carrier proteins. Production of monoclonal antibodies.—To produce antibodies, animals must be immunised with an hapten–protein conjugate. Two strategies were tested to carry out the conjugation. In the first one, maleimide-activated proteins[47] were used. Coupling was performed by a three-step procedure. At first, compound **2** was reacted with disuccinimido dithiobispropionate **15** in DMF (Scheme 4). The disulfide **16** was then reduced with *n*-tributylphosphine in MeOH–water to give the thioderivative **17**, which was coupled to the maleimide-activated carrier proteins MA-KLH and MA-BSA to give the conjugates MA-KLH-**2** and MA-BSA-**2**, respectively. Quantification of the hapten–carrier ratio (see Section 4) afforded ratios in the range 4:1.

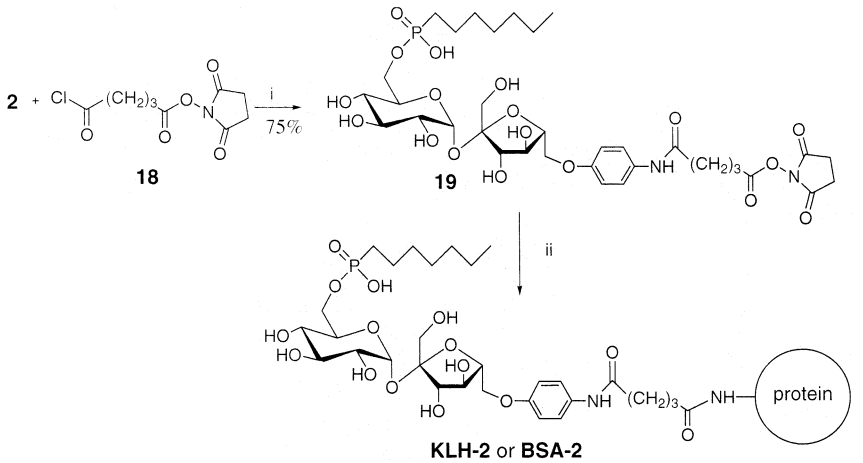
In the second strategy, the conjugation was carried out using the heterobifunctionnal glutaryl reagent **18**,[48] to afford KLH-**2** and BSA-

2 (Scheme 5). This method proved to be more efficient since the hapten-to-protein ratios were higher (16:1).

Then, keyhole limpet hemocyanin conjugate KLH-**2** was used to immunise mice (Balb/c). A standard protocol for cell fusion was adopted and the resulting hybridoma cells were screened by an ELISA technique for binding to the hapten-BSA conjugate BSA-**2**. Cloning by limiting dilution gave seven mono-



Scheme 4. (i) DMF; (ii) *n*-Bu_3P, MeOH–water; (iii) phosphate buffer pH 7.3.



Scheme 5. (i) DMF, Et_3N; (ii) pyrophosphate buffer pH 8, BSA of KLH.

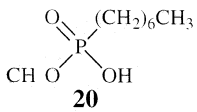
Table 1
IC_{50} in μM for phosphonates-antibodies binding, determined by competitive ELISA

Antibody	Phosphonates					
	10	**11**	**12**	**13**	**14**	**20**
10A10	1	1	>500	>500	50	>500
7E8	14	5	>500	>500	240	>500
XA6	nd	3	>500	9.5	7	70
1D11	nd	5	>500	>500	87	>500
8C8	nd	10	>500	>500	140	>500
3G10	nd	15	>500	>500	24	100
7E11	15	15	>500	>500	60	200

nd: not determined.

clonal antibodies specific for hapten **2** (the *N*-acetylated hapten **10** was used as inhibitor). They were purified by chromatography on protein A-coupled Sepharose 4B.

Binding properties of monoclonal antibodies.—In order to evaluate the relative contribution of the transition-state structural elements to binding, the affinity for the various phosphonates **10**–**14** as well as for the methyl ester **20**[49] was determined by competitive inhibition enzyme-linked immunoassay and was expressed as IC_{50} in μM (Table 1).



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All the antibodies revealed a binding affinity for the sucrose-6-heptylphosphonate (**11**) showing that the *p*-acetamidophenyl group did not contribute to a great extent to the recognition. Only XA6 bound to the regioisomer **13**. Phosphonate **12** without the hydrophobic chain did not appear to bind up to 500 μM, whereas binding of **20** without sugar is weak or non existent; these results suggested that both the fatty acid chain and the sugar are important for the recognition process. Furthermore, the binding properties of the glucoside derivative **14** revealed a relative importance of the fructose unit in the recognition.

Esterase activity of antibodies.—All the antibodies were initially tested for their esterase properties toward 6-octanoylsucrose (**1**).[26,30] The reaction was monitored by an enzymatic determination of sucrose.[50] The described protocol was adapted in order to carry out the measurement in 96-well polyvinyl assay plate (ELISA plate, see Section 4). An aliquot of the reacting mixture was treated with β-fructosidase (from yeast) at pH 4.6. The sucrose obtained in the abzymatic reaction gave fructose and glucose while the remaining octanoylsucrose gave fructose and 6-octanoylglucose. Hexokinase (HK) was then added at pH 7.6 to catalyse the phosphorylation of glucose and fructose by adenosine-5′-triphosphate (ATP). The glucose 6-phosphate obtained was oxidised in gluconate-6-phosphate with nicotinamide-adenine-dinucleotide-phosphate (NADP) in the presence of glucose-6-phosphate dehydrogenase (G6P-DH) with the formation of an equimolar amount, relative to sucrose, of reduced nicotinamide-adenine-dinucleotide-phosphate (NADPH). The amount of NADPH was determined by its absorbance at 340 nm. Only the antibody XA6 showed hydrolytic activity. In the initial screening, the hydrolysis was tested at pH 8 (Bicine buffer), 37 °C with 10% Me_2SO with an initial concentration of the sucro-ester of 0.1 mM and an antibody concentration of 4 μM. Parallel experiments were carried out in the presence of phosphonate **11** (10^{-2} mM). Antibody XA6 proved to accelerate the reaction with respect to the uncatalysed hydrolysis (in the absence of antibody). Furthermore, the acceleration was completely suppressed in the presence of compound **11**, demonstrating that catalysis took place in the combining site of the antibody. For this antibody, experiments were repeated in the same conditions (concentration and temperature) but at different pH values, ranging from 4.5 to 9. The optimal pH was found to be around 7. This antibody was then further studied at pH 7.35. The antibody-catalysed hydrolysis rates of 6-octanoylsucrose in the presence of 9 μM antibody were measured as a function of substrate concentration under the conditions used in the aforementioned screening. The ester concentrations were varied from 0.11 to 1.1 mM and the observed rates were corrected for the uncatalysed rate of hydrolysis. Antibody XA6

was found to obey Michaelis–Menten kinetics for the hydrolysis of 6-octanoylsucrose. The corrected rates were analysed by a Lineweaver–Burk plot to determine the Michaelis–Menten parameters. The first-order rate constant k_{cat} was 6.5 h^{-1} and Michaelis constant K_M was 0.25 mM. The background hydrolysis rate constant (k_{uncat}) was 2.4×10^{-3} h^{-1}. The hydrolytic properties of this antibody were then examined toward 6′-octanoylsucrose[26,28] as well as toward methyl 6-octanoyl-α-D-glucopyranoside.[51] In this case, the abzymatic reaction was also monitored by an enzymatic method. In the protocol described above, β-fructosidase was replaced with α-glucosidase (maltase recombinant from *Saccharomyces cerevisiae*). The esters' concentrations were varied from 0.1 to 1 mM and the observed rates were corrected for the uncatalysed rate of hydrolysis. The Michaelis–Menten parameters obtained by a Lineweaver–Burk plot are shown in Table 2 as well as the kinetic parameters determined for 6-octanoylsucrose as substrate.

XA6 showed a significant rate acceleration for the sucrose esters hydrolysis with values of k_{cat}/k_{uncat} of 2.6×10^3 and 1.2×10^3 whereas the acceleration with methyl-6-octanoyl-α-D-glucopyranoside was only 529. Thus, abzyme XA6 can distinguish regioisomers as shown by the specific constants (k_{cat}/K_M) 4.5-fold higher for 6-octanoylsucrose with respect to 6′-octanoylsucrose. This is mainly due to a variation of k_{cat} since K_M is similar for the two esters. It is also interesting to note that the rate enhancements and the specific constants showed great differences for sucrose derivatives and methyl 6-octanoyl-α-D-glucopyranoside (Table 2).

3. Conclusions

We developed a straightforward access to sugar phosphonates from phosphonic acids using a Mitsunobu reaction. This allowed us to prepare a hapten for the generation of monoclonal antibodies toward sucrose-6-heptylphosphonate and other structurally related phosphonates to elicit binding properties of the antibodies. Seven monoclonal antibodies specific for hapten **2** were obtained. Competitive inhibition ELISA revealed that both the fatty acid chain and the sugar are important for the recognition process. Among the seven antibodies, one proved to be catalytic for the hydrolysis of 6 and 6′-octanoylsucrose with a regioselectivity for the ester at C-6. This abzyme has now to be tested for ester synthesis in thermodynamic (reverse of hydrolysis) or kinetic (transesterification) conditions.

4. Experimental

General methods.—All moisture-sensitive reactions were performed under Ar atmosphere using oven-dried glassware. All solvents were dried over standard drying agents and freshly distilled prior to use. Flash-column chromatography was performed on Silica Gel 60A C.C. (6–35 μm, SDS) or on Lichroprep RP-18 (25–40 μm, E. Merck). Reactions were monitored by TLC on Silica Gel (E. Merck) 60 F_{254} with detection by charring with H_2SO_4. Melting points were determined with a capillary apparatus and are uncorrected. Optical rotations were measured at 26 ± 2 °C. NMR spectra were recorded at rt with Bruker AC 200, AC 250 or AM 400 spectrometers. 85% H_3PO_4 was used as external standard for ^{31}P NMR. Mass spectra were

Table 2
Michaelis–Menten parameters for XA6 toward various substrates (pH 7.35; 37 °C; 10% Me_2SO)

	6-Octanoylsucrose	6′-Octanoylsucrose	Methyl-6-octanoyl-α-D-glucopyranoside
k_{cat} (h^{-1})	6.50	1.70	0.9
K_M (mM)	0.25	0.30	1.0
k_{uncat} (h^{-1})	$2.4\ 10^{-3}$	$1.4\ 10^{-3}$	$1.7\ 10^{-3}$
k_{cat}/K_M (h^{-1}/mM^{-1})	26	5.7	0.9
k_{cat}/k_{uncat}	$2.6\ 10^3$	$1.2\ 10^3$	529

recorded on a MAT 95S instrument. Elemental analyses were performed at the CNRS Microanalytical Laboratory (Gif-sur-Yvette, France).

1′,2,3,3′,4,4′,6-Hepta-O-*acetyl-6′*-O-p-*nitrophenylsucrose* (**4**).—To a solution of 2,3,4,6,1′,3′,4′-hepta-*O*-acetylsucrose (10.0 g, 15.7 mmol), *p*-nitrophenol (2.6 g, 1.2 equiv) and triphenylphosphine (5.6 g, 1.5 equiv) in toluene (150 mL) was added diisopropylazodicarboxylate (4.5 mL, 1.5 equiv) over a 5 min period. After 12 h the mixture was concentrated and the residue was purified by flash chromatography (1:2 petroleum ether–EtOAc) to afford **4** (11.5 g, 97%); mp 95 °C (from EtOAc–petroleum ether); $[\alpha]_D$ 61° (*c* 1.02, $CHCl_3$); IR (KBr): *v* 2963, 1751, 1594, 1517, 1371, 1344, 1224, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz): δ 8.19–8.27 (m, 2 H, Ar), 7.01–7.11 (m, 2 H, Ar), 5.71 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.55 (d, 1 H, $J_{3',4'}$ 5.5 Hz, H-3′), 5.48 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 9.6 Hz, H-3), 5.47 (dd, 1 H, $J_{4',5'}$ 5.5 Hz, H-4′), 5.05 (t, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 4.85 (dd, 1 H, H-2), 4.06–4.46 (m, 8 H, 2 H-1′, H-5, H-5′, 2 H-6, 2 H-6′), 2.18, 2.14, 2.13, 2.10, 2.08, 2.03 and 1.94 (7 s, 21 H, 7 $COCH_3$); ^{13}C NMR ($CDCl_3$, 62.9 MHz): δ 169.7–170.9 (7 $COCH_3$), 163.0 (C–NO_2), 141.9 (Ar*C*O), 125.9 and 114.7 (4 ArC), 104.3 (C-2′), 90.2 (C-1), 79.4 (C-5′), 75.7 (C-3′), 75.2 (C-4′), 70.2 (C-2), 69.2 (C-5), 68.8 (C-6′), 68.4 and 68.1 (C-3 and C-4), 62.4 (C-1′), 61.6 (C-6), 20.8–21.1 (7 $COCH_3$). Anal. Calcd for $C_{32}H_{39}NO_{20}$: C, 50.73; H, 5.19; N, 1.85; O, 42.23. Found: C, 50.56; H, 5.48; N, 1.94; O, 42.11.

6′-O-p-*Nitrophenylsucrose* (**5**).—A solution of **4** (11.0 g, 14.5 mmol) in MeOH (150 mL) was made basic by the addition of NaOMe (80 mg, 1.4 mmol). After 2 h, the mixture was neutralized with Dowex-50 (H^+) resin, filtered, and concentrated to give **5** as a white solid (6.7 g, 100%); mp 115 °C; $[\alpha]_D$ 62° (*c* 0.4, MEOH); IR (KBr): *v* 3400, 2922, 1592, 1507, 1438, 1341, 1271, 1181, 1119, 1056 cm^{-1}; ^1H NMR (CD_3OD, 200 MHz): δ 8.00–8.10 (m, 2 H, Ar), 6.95–7.05 (m, 2 H, Ar), 5.21 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.47 (dd, 1 H, $J_{5',6'a}$ 7.0, $J_{6'a,6'b}$ 10.6 Hz, H-6′a), 4.1 (dd, 1 H, $J_{5',6'b}$ 1.5 Hz, H-6′b), 3.9–4.02 (m, 3 H, H-3′, H-4′, H-5′), 3.65–3.80 (m, 2 H, H-5, H-6a), 3.53 (dd, 1 H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5 Hz, H-3), 3.50 (d, 1 H, $J_{1'a,1'b}$ 11.0 Hz, H-1′a), 3.49 (dd, 1 H, $J_{5,6b}$ 6.5 $J_{6a,6b}$ 12.0 Hz, H-6b), 3.44 (d, 1 H, H-1′b), 3.24 (dd, 1 H, H-2), 3.10 (dd, 1 H, H-4); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 165.4 (C–NO_2), 142.7 (Ar*C*–O), 126.7 and 115.9 (4 ArC), 105.5 (C-2′), 93.4 (C-1), 81.3 (C-5′), 78.7 (C-3′), 76.4 (C-4′), 74.7 (C-3), 74.4 (C-2), 73.2 (C-5), 71.6 (C-4), 71.5 (C-6′), 63.6 (C-1′), 62.7 (C-6). Anal. Calcd for $C_{18}H_{25}NO_{13}\cdot 0.5\ H_2O$: C, 45.77; H, 5.55; N, 2.96. Found: C, 45.74; H, 5.63; N, 3.06.

6′-O-p-*Aminophenylsucrose* (**6**).—A vigorously stirred mixture of **5** (7.0 g, 15,1 mmol) and 10% Pd on activated carbon (1.2 g) in 9:1 *t*-BuOH–water (140 mL) was degassed under diminished pressure and saturated with hydrogen (by a H_2-filled balloon) three times. The suspension was stirred for an additional 3 h at rt under a slightly positive pressure of H_2, then filtered through a pad of Celite, and concentrated to afford **6** (6.5 g) as a white solid in quantitative yield; mp 111 °C; $[\alpha]_D$ 70° (*c* 0.4, MeOH); IR (KBr): *v* 3380, 2924, 1635, 1512, 1456, 1236, 1137, 1059 cm^{-1}; ^1H NMR (CD_3OD, 250 MHz): δ 6.5–6.7 (m, 4 H, Ar), 5.27 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.12 (dd, 1 H, $J_{5',6'a}$ 7.0, $J_{6'a,6'b}$ 12.0 Hz, H-6′a), 3.8–4.0 (m, 4 H, H-6′b, H-3′, H-5′, H-4′), 3.68 (ddd, 1 H, $J_{4,5}$ 9.6, $J_{5,6a}$ 2.0, $J_{5,6b}$ 5.0 Hz, H-5), 3.64 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, H-6a), 3.55 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3), 3.52 (dd, 1 H, H-6b), 3.47 (s, 2 H, 2 H-1′), 3.25 (dd, 1 H, H-2), 3.17 (dd 1 H, H-4); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 153.4 (C–NH_2), 141.8 (Ar*C*–O), 118.2 and 116.7 (4 ArC), 105.5 (C-2′), 93.4 (C-1), 81.5 (C-5′), 78.8 (C-3′), 76.5 (C-4′), 74.7 (C-3), 74.1 (C-2), 73.2 (C-5), 71.4 (C-4), 71.2 (C-6′), 63.7 (C-1′), 62.4 (C-6). Anal. Calcd for $C_{18}H_{27}NO_{11}\cdot 0.5H_2O$: C, 48.87; H, 6.38; N, 3.17. Found: C, 48.26; H, 6.39; N, 3.23.

6′ - O - p - Trifluoroacetamidophenylsucrose (**7**).—*S*-Ethyltrifluorothioacetate (9.5 mL, 74.5 mmol, 5 equiv) was added to a solution of **6** (6.2 g, 14.3 mmol) in MeOH (30 mL). The resulting solution was kept at rt for 5 h, then diluted with 60 mL of water and washed with 2×40 mL portions of hexane. The aqueous layer was concentrated to give **7** as a white solid. (7.0 g, 92%); mp 108 °C; $[\alpha]_D$ 55° (*c* 0.5, MeOH); IR (KBr): *v* 3378, 2930, 1710,

1513, 1205, 1158 cm^{-1}; ^1H NMR (CD_3OD, 250 MHz): δ 7.3–7.4 (m, 2 H, Ar), 6.8–6.9 (m, 2 H, Ar), 5.27 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.26 (dd, 1 H, $J_{5',6'a}$ 7.0, $J_{6'a,6'b}$ 10.6 Hz, H-6′a), 3.85–4.05 (m, 4 H, H-6′b, H-3′, H-5′, and H-4′), 3.70 (ddd, 1 H, $J_{4,5}$ 9.6, $J_{5,6a}$ 2.0, $J_{5,6b}$ 5.5 Hz, H-5), 3.66 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, H-6a), 3.55 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3), 3.51 (dd, 1 H, H-6b), 3.47 (s, 2 H, 2 H-1′), 3.25 (dd, 1 H, H-2), 3.17 (dd, 1 H, H-4); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 158.1 (*C*–NHCOCF$_3$), 130.6 (Ar*C*–O), 156.5 (q, $J_{C,F}$ 37.0 Hz, *C*OCF$_3$), 123.8 (2 ArC), 117.5 (q, $J_{C,F}$ 287.0 Hz, *C*F$_3$), 115.9 (2 ArC), 105.5 (C-2′), 93.4 (C-1), 81.5 (C-5′), 78.8 (C-3′), 76.5 (C-4′), 74.7 (C-3), 74.2 (C-2), 73.2 (C-5), 71.5 (C-4), 70.8 (C-6′), 63.7 (C-1′), 62.5 (C-6). Anal. Calcd for $C_{20}H_{26}F_3NO_{12}\cdot 2H_2O$: C, 42.48; H, 5.35; N, 2.48. Found: C, 42.74; H, 5.32; N, 2.19.

2,3,4,1′,3′,4′-Hexa-O-acetyl-6′-O-p-trifluoroacetamidophenylsucrose (**8**).—A solution of **7** (5.8 g, 10.9 mmol) and dimethoxytritylchloride (4.5 g, 13.2 mmol) in Py (25 mL) was kept at rt for 40 h. The mixture was then diluted with Py (15 mL) and Ac_2O (15 mL) was added. After 12 h, the mixture was concentrated to dryness. The residue was treated with a solution of *p*-TsOH (5 g, 29 mmol) in a 2:1 CH_2Cl_2–MeOH mixture (180 mL) at 0 °C under vigorous stirring. After an additional hour at 0 °C the mixture was neutralized with 5% aq $NaHCO_3$ and extracted with CH_2Cl_2 (250 mL). The organic layer was washed with water, dried ($MgSO_4$) and concentrated. The residual syrup was purified by flash chromatography (2:1 CH_2Cl_2–EtOAc) to afford **8** as a white solid (4.4 g, 52%); mp 86 °C (from EtOAc–cyclohexane); $[\alpha]_D$ 64° (*c* 0.9, $CHCl_3$); IR (KBr): *v* 3480, 3320, 2970, 1755, 1380, 1240, 1160, 1040 cm^{-1}; ^1H NMR (CD_3OD, 250 MHz): δ 7.90 (s, 1 H, N*H*), 7.46–7.55 (m, 2 H, Ar), 6.92–7.01 (m, 2 H, Ar), 5.79 (d, 1 H, $J_{1,2}$ 4.0 Hz, H-1), 5.55 (d, 1 H, $J_{3',4'}$ 5.5 Hz, H-3′), 5.52 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 10.0 Hz, H-3), 5.50 (dd, 1 H, $J_{4',5'}$ 5.5 Hz, H-4′), 4.97 (dd, 1 H, $J_{4,5}$ 10.0 Hz, H-4), 4.82 (dd, 1 H, H-2), 4.40 (ddd, 1 H, $J_{5',6'a}$ 4.0, $J_{5',6'b}$ 6.0 Hz, H-5′), 4.20–4.31 (m, 4 H, 2 H-6 and 2 H-1′), 4.13 (ddd, 1 H, $J_{5,6a}$ 2.0, $J_{5,6b}$ 4.5 Hz, H-5), 3.63 (ddd, 1 H, $J_{6a,OH}$ 8.0, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.50 (ddd, 1 H, $J_{6b,OH}$ 5.0 Hz, H-6b), 2.44 (dd, 1 H, OH), 2.13, 2.12, 2.07, 2.03, and 1.97 (6 s, 6 CO*C*H$_3$). ^{13}C NMR ($CDCl_3$, 62.9 MHz): δ 170.7–169.5 (6 *C*OCH$_3$), 156.0 (*C*-NH), 154.6 (q, $J_{C,F}$ 37.0 Hz, *C*OCF$_3$), 129.0 (Ar*C*–O), 124.3 (2 ArC), 115.7 (q, $J_{C,F}$ 288.0 Hz, *C*F$_3$), 112.8 (2 ArC), 103.9 (C-2′), 89.8 (C-1), 79.4 (C-5′), 75.7 (C-3′), 74.8 (C-4′), 70.6 (C-3), 70.3 (C-2), 69.2 (C-5), 68.5 (C-4), 67.7 (C-6′), 62.6 (C-1′), 60.8 (C-6), 20.5–20.8 (6 CO*C*H$_3$). Anal. Calcd for $C_{32}H_{38}F_3NO_{18}$: C, 49.17; H, 4.90; N, 1.79, Found: C, 49.01; H, 4.97; N, 1.62.

6′-O-p-Aminophenyl-6-heptylphosphonatosucrose (**2**).—To a stirred solution of **8** (1.0 g, 1.3 mmol), heptylphosphonic acid **9** (702 mg, 3.9 mmol), and triphenylphosphine (928 mg, 3.9 mmol), in dry Py (2 mL) at 120 °C, was added DEAD (640 μL, 3.9 mmol). Stirring was continued at 120 °C for 4 h, then the mixture was cooled to rt and concentrated. The residue was dissolved in MeCN (3 mL), then 1 M aq LiOH (3 mL) was added under vigorous stirring. After 1 h, the mixture was neutralised with Dowex-50 (H^+) resin, filtered and concentrated. The residue was triturated in a 1:1 MeOH–water mixture (20 mL) and filtered. The filtrate was concentrated and the residue was purified by C18 flash chromatography (step gradient from 9:1 water–MeOH to pure MeOH) to afford **2** (502 mg, 65%) as a colorless foam; $[\alpha]_D$ 56° (*c* 0.35, MeOH); IR (KBr): *v* 3374, 2928, 2361, 1635, 1512, 1456, 1252, 1143, 1059, 997 cm^{-1}. ^1H NMR (CD_3OD, 250 MHz): δ 7.0–7.1 (m, 2 H, Ar), 6.95-6.85 (m, 2 H, Ar), 5.28 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.34 (dd, 1 H, $J_{5',6'a}$ 8.0, $J_{6'a,6'b}$ 11.0 Hz, H-6′a), 3.7-4.1 (m, 7 H, H-6′b, H-5, H-6a, H-6b, H-3′, H-4′, H-5′), 3.53 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3), 3.44 (s, 2 H, 2 H-1′), 3.22 (dd, 1 H, H-2), 3.11 (dd, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 1.4–1.5 (m, 4 H, 2 C*H*$_2$), 1.00–1.15 (m, 8 H, 4 C*H*$_2$), 0.67–0.77 (m, 3 H, C*H*$_3$); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 155.4, 137.1, 120.1, and 116.9 (6 ArC), 105.2 (C-2′), 92.9 (C-1), 81.5 (C-5′), 78.3 (C-3′), 75.7 (C-4′), 74.6 (C-3), 73.6 (d, $J_{C,P}$ 6.9 Hz, C-5), 73.4 (C-2), 71.5 (C-4), 71.3 (C-6′), 64.7 (C-6), 54.8 (C-1′), 33.0 (P–(CH$_2$)$_3$–*C*H$_2$–), 32.3 (d, $J_{C,P}$ 16.6 Hz, P–(CH$_2$)$_2$–*C*H$_2$–), 30.2 (P–(CH$_2$)$_4$–*C*H$_2$–), 28.0 (d, $J_{C,P}$ 135 Hz, P–*C*H$_2$–), 24.7 (d, $J_{C,P}$ 3.7 Hz,

P–CH_2–CH_2–), 23.7 (P–$(CH_2)_5$–CH_2–), 14.5 (CH_3); ^{31}P NMR (CD_3OD, 100 MHz): δ 28.7; ESI–HRMS Calcd for $C_{25}H_{42}NO_{13}P$–H: 594.2315. Found 594.2312. Anal. Calcd for $C_{25}H_{42}NO_{13}P{\cdot}1.5\ H_2O$: C, 48.23; H, 7.29; N, 2.25. Found: C, 48.29; H, 7.03; N, 2.02.

6′-p-Acetamidophenyl-6-heptylphosphonato-sucrose (**10**).—To a solution of **2** (70 mg, 0.12 mmol) in MeOH (500 μL) was added Ac_2O (60 μL) and Et_3N (50 μL, 0.36 mmol). The solution was kept at rt for 2 h, then diluted with water (500 μL) and concentrated to afford quantitatively **10** (75 mg) as an amorphous solid; $[\alpha]_D$ 60° (*c* 0.5, MeOH); IR (KBr): *ν* 3417, 2927, 1657, 1512, 1460, 1409, 1243, 1143, 1060 cm^{-1}; ^{1}H NMR (CD_3OD, 250 MHz): δ 7.38–7.46 (m, 2 H, Ar), 6.90–6.98 (m, 2 H, Ar), 5.38 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.45 (dd, 1 H, $J_{5',6'a}$ 8.0, $J_{6'a,6'b}$ 11.0 Hz, H-6′a), 3.87–4.25 (m, 7 H, H-6′b, H-5, H-6a, H-6b, H-3′, H-4′, and H-5′), 3.71 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3), 3.61 (s, 2 H, 2 H-1′), 3.42 (dd, 1 H, H-2), 3.32 (dd, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 1.50–1.60 (m, 4 H, 2 CH_2), 1.20–1.32 (m, 8 H, 4 CH_2), 0.82–0.91 (m, 3 H, CH_3); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 171.2 (*C*O), 156.9 133.0, 122.8, and 115.8 (6 ArC), 105.2 (C-2′), 92.9 (C-1), 81.7 (C-5′), 78.3 (C-3′), 75.6 (C-4′), 74.6 (C-3), 73.7 (d, $J_{C,P}$ 6.9 Hz, C-5), 73.4 (C-2), 71.5 (C-4), 70.9 (C-6′), 65.0 (C-6), 64.0 (C-1′), 32.9 (P–$(CH_2)_3$–CH_2–), 32.4 (d, $J_{C,P}$ 16.6 Hz, P–$(CH_2)_2$–CH_2–), 30.2 (P–$(CH_2)_4$–CH_2–), 28.0 (d, $J_{C,P}$ 135 Hz, P–CH_2–), 24.8 (d, $J_{C,P}$ 3.7 Hz, P–CH_2–CH_2–), 23.7 (P–$(CH_2)_5$–CH_2–), 23.5 (COCH_3), 14.5 (CH_3); ^{31}P NMR (CD_3OD, 100 MHz): δ 28.7; ESI–HRMS calcd for $C_{27}H_{44}NO_{14}P$-H: 636.2421. Found 636.2422. Anal. Calcd for $C_{27}H_{44}NO_{14}P{\cdot}3\ H_2O$: C, 46.89; H, 7.29; N, 2.03. Found: C, 46.81; H, 6.85; N, 2.08.

6-Heptylphosphonatosucrose (**11**).—2,3,1′,3′,4′,6′-Hexa-*O*-acetylsucrose (400 mg, 0.67 mmol) was treated with heptylphosphonic acid (364 mg, 2.0 mmol), triphenylphosphine (480 mg, 2.0 mmol) and DEAD (330 μL, 2.0 mmol) in Py (1 mL) as described for the preparation of **2**. After LiOH treatment (see above) and C-18 flash chromatography (step gradient from 9:1 water–MeOH to pure MeOH), **11** (230 mg, 68%) was obtained as a white powder; $[\alpha]_D$ 38° (*c* 0.7, MeOH); IR (KBr): *ν* 3387, 2926, 2856, 1652, 1467, 1178, 1041 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.36 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.08 (ddd, 1 H, $J_{6a,6b}$ 11.5, $J_{6a,P}$ 6.5, $J_{5,6a}$ 2.0 Hz, H-6a), 4.06–4.09 (m, 2 H, H-3′ and H-4′), 4.02 (ddd, 1 H, $J_{6b,P}$ 7.5, $J_{5,6b}$ 4.5 Hz, H-6b), 3.93 (ddd, 1 H, $J_{4,5}$ 9.5 Hz, H-5), 3.74–3.83 (m, 3 H, H-5′, H-6′a, and H-6′b), 3.72 (dd, 1 H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5 Hz, H-3), 3.61 (d, 1 H, $J_{1'a,1'b}$ 12.0 Hz, H-1′a), 3.57 (d, 1 H, H-1′b), 3.43 (dd, 1 H, H-2), 3.40 (dd, 1 H, H-4), 1.52–1.65 (m, 4 H, $(CH_2)_2$), 1.27–1.40 (m, 8 H, $(CH_2)_4$), 0.87–0.93 (m, 3 H, CH_3); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 105.0 (C-2′), 93.2 (C-1), 83.5 (C-5′), 79.0 (C-3′), 75.8 (C-4′), 74.3 (C-3), 73.5 (d, $J_{C,P}$ 6.9 Hz, C-5), 73.2 (C-2), 71.1 (C-4), 64.3 (C-6), 64.1 (C-6′), 63.7 (C-1′), 33.0 (P–$(CH_2)_3$–CH_2–), 2.3 (d, $J_{C,P}$ 16.6 Hz, P–$(CH_2)_2$–CH_2–), 30.2 (P–$(CH_2)_4$–CH_2–), 28.0 (d, $J_{C,P}$ 135 Hz, P–CH_2–), 24.7 (d, $J_{C,P}$ 3.7 Hz, P–CH_2–CH_2–), 23.7 (P–$(CH_2)_5$–CH_2–), 14.5 (CH_3); ^{31}P NMR (CD_3OD, 100 MHz): δ 32.0; ESI–HRMS Calcd for $C_{19}H_{37}O_{13}P$–H: 503.1893. Found 503.1882. Anal. Calcd for $C_{19}H_{37}O_{13}P{\cdot}0.5\ H_2O$: C, 44.44; H, 7.46; Found: C, 43.13; H, 7.47.

Methylphosphonatosucrose triethylammonium salt (**12**).—2,3,1′,3′,4′,6′-Hexa-*O*-acetylsucrose (300 mg, 0.5 mmol) was treated with methylphosphonic acid (96 mg, 1.5 mmol), triphenylphosphine (360 mg, 1.5 mmol) and DEAD (250 μL, 1.5 mmol) in Py (800 μL) as described for the preparation of **2**. After LiOH treatment (see above) and DEAE-Sephadex A 25 (HCO_3^- form) chromatography (0.2 M bicarbonate triethylammonium), **12** was obtained as a white amorphous solid (172 mg, 66%); $[\alpha]_D$ 39° (*c* 0.69, MeOH); IR (KBr): *ν* 3414, 2937, 2738, 2678, 2491, 1652, 1476, 1171, 1036 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.36 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.09 (ddd, 1 H, $J_{6a,6b}$ 11.5, $J_{6a,P}$ 6.5, $J_{5,6a}$ 2.0 Hz, H-6a), 4.04–4.07 (m, 2 H, H-3′ and H-4′), 4.01 (ddd, 1 H, $J_{6b,P}$ 7.5, $J_{5,6b}$ 5.0 Hz, H-6b), 3.94 (ddd, 1 H, $J_{4,5}$,9.5 Hz, H-5), 3.74–3.84 (m, 3 H, H-6′a, H-6′b, and H-5′), 3.70 (dd, 1 H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5 Hz, H-3), 3.61 (d, 1 H, $J_{1'a,1'b}$ 11.5 Hz, H-1′a), 3.57 (d, 1 H, H-1′b), 3.41 (dd, 1 H, H-2), 3.36 (dd, 1 H, H-4), 3.19 (q, 6 H, *J* 7.0 Hz, $(CH_3CH_2)_3N$), 1.32 (t, 9 H, $(CH_3CH_2)_3N$), 1.23 (d, 3 H, $J_{H,P}$ 16.5 Hz,

CH_3); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 105.0 (C-2′), 93.1 (C-1), 83.7 (C-5′), 78.9 (C-3′), 75.8 (C-4′), 74.4 (C-3), 73.4 (d, $J_{C,P}$ 9.5 Hz, C-5), 73.3 (C-2), 71.2 (C-4), 64.5 (d, $J_{C,P}$ 5.0 Hz, C-6), 64.0 and 63.9 (C-1′ and C-6′), 47.5 (($CH_3CH_2)_3N$), 12.3 (d, $J_{C,P}$ 138 Hz, P–CH_3), 9.12 (($CH_3CH_2)_3N$). ^{31}P NMR (CD_3OD, 100 MHz): δ 26.4. ESI–HRMS Calcd for $C_{13}H_{25}O_{13}P$–H: 419.0954. Found 419.0949.

6′-Heptylphosphonatosucrose (**13**).—2,3,4,6,1′,3′,4′-Hepta-*O*-acetylsucrose (636 mg, 1 mmol) was treated with heptylphosphonic acid (540 mg, 3 mmol), triphenylphosphine (714 mg, 3 mmol) and DEAD (500 μL, 3 mmol) in Py (1.5 mL) as described for the preparation of **2**. After LiOH treatment (see above) and C-18 flash chromatography, **13** (337 mg, 67%) was obtained as a white powder; $[\alpha]_D$ 42° (*c* 0.5, MeOH); IR (KBr): *v* 3248, 2990, 2931, 1753, 1696, 1533, 1251, 1059 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz): δ 5.36 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.07–4.16 (m, 3 H, H-6a, H-6b, and H-6′a), 4.02 (ddd, 1 H, $J_{6'a,6'b}$ 11.5, $J_{6'b,P}$ 5.5, $J_{5,6'b}$ 4.0 Hz, H-6′b), 3.95 (ddd, 1 H, $J_{4,5}$ 10.5, $J_{5,6b}$ 5.5, $J_{5,6a}$ 2.5 Hz, H-5), 3.80–3.86 (m, 2 H, H-5′ and H-3′), 3.75 (dd, 1 H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.0 Hz, H-3), 3.70 (dd, 1 H, *J* 12.0, *J* 5.5 Hz, H-4′), 3.68 (d, 1 H, $J_{1'a,1'b}$ 12.0 Hz, H-1′a), 3.64 (d, 1 H, H-1′b), 3.41 (dd, 1 H, H-2), 3.26 (dd, 1 H, H-4); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 105.3 (C-2′), 93.5 (C-1), 82.3 (d, $J_{C,P}$ 7.1 Hz, C-5′), 78.8 (C-3), 76.3 (C-4′), 74.6 (C-3), 74.2 (C-5), 73.4 (C-2), 71.9 (C-4), 65.6 (d, $J_{C,P}$ 5.0 Hz, C-6′), 63.5 (C-1′), 62.6 (C-6), 32.9 ($P(CH_2)_3CH_2$), 32.4 (d, $J_{C,P}$ 17.1 Hz, $P(CH_2)_2CH_2$), 30.1 ($P(CH_2)_4CH_2$), 28.1 (d, $J_{C,P}$ 135.4 Hz, PCH_2), 24.7 (d, $J_{C,P}$ 4.7 Hz, PCH_2CH_2), 23.7 ($P(CH_2)_5CH_2$), 14.4 (CH_3); ^{31}P NMR (CD_3OD, 100 MHz): δ 28.5. ESI–HRMS Calcd for $C_{19}H_{37}O_{13}P$–H: 503.1893. Found 503.1889. Anal. Calcd for $C_{19}H_{37}O_{13}P{\cdot}0.5\ H_2O$: C, 44.44; H, 7.46; Found: C, 44.32; H, 7.62.

Methyl-6-heptylphosphonato-α-D-glucopyranoside (**14**).—Methyl-2,3,4-tri-*O*-acetyl-α-D-glucopyranoside (320 mg, 1 mmol) was treated with heptylphosphonic acid (540 mg, 3 mmol), triphenylphosphine (714 mg, 3 mmol) and DEAD (500 μL, 3 mmol) in Py (1.5 mL) as described for the preparation of **2**. After LiOH treatment (see above) and C-18 flash chromatography, **14** (249 mg, 70%) was obtained as a white amorphous solid. $[\alpha]_D$ 67° (*c* 0.53, MeOH); IR (KBr): *v* 3414, 2927, 2854, 1186, 1030 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz): δ 4.64 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 3.77 (ddd, 1 H, $J_{5,6}$ 4.5, $J_{6,P}$ 7.5, $J_{6,6'}$ 11.5 Hz, H-6), 3.70 (ddd, 1 H, $J_{5,6'}$ 2.0, $J_{6',P}$ 6.5 Hz, H-6′), 3.62 (dd, 1 H, $J_{2,3}$ 9.0, $J_{3,4}$ 9.0 Hz, H-3), 3.57 (ddd, 1 H, $J_{4,5}$ 9.5 Hz, H-5), 3.44 (dd, 1 H, $J_{3,4}$ 9.0 Hz, H-4), 3.40 (dd, 1 H, H-2), 3.39 (s, 3 H, OCH_3), 1.51–1.65 (m, 4 H, 2 CH_2), 1.26–1.41 (m, 8 H, $(CH_2)_4$), 0.09 (t, 3 H, *J* 7.0 Hz, CH_3); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 101.3 (C-1), 72.8 (d, $J_{C,P}$ 5.7 Hz, C-5), 74.7 (C-4), 73.6 (C-2), 71.1 (C-4), 64.5 (d, $J_{C,P}$ 5.2 Hz, C-6), 55.6 (OCH_3), 32.9 ($P(CH_2)_3CH_2$), 32.3 (d, $J_{C,P}$ 16.7 Hz, $P(CH_2)_2CH_2$), 30.1 ($P(CH_2)_4CH_2$), 28.1 (d, $J_{C,P}$ 136.4 Hz, PCH_2), 24.7 (d, $J_{C,P}$ 4.7 Hz, PCH_2CH_2), 23.7 ($P(CH_2)_5CH_2$), 14.4 (CH_3); ^{31}P NMR (CD_3OD, 100 MHz): δ 27.5; ESI–HRMS Calcd for $C_{14}H_{28}O_8P$ -H: 355.1521; Found 355.1519. Anal. Calcd for $C_{14}H_{29}O_8P$: C, 47.19, H, 8.20, Found: C, 47.41 H, 8.32.

General procedure for the coupling of **2** *to MA-proteins: Preparation of MA-KLH-***2** *and MA-BSA-***2** *conjugates.*—A solution of **2** (150 mg, 0.25 mmol) and 3,3′-dithiodipropionic acid bis(succinimido)ester (33 mg, 0.08 mmol) in DMF (450 μL) was heated at 60 °C for 8 h. The resulting solution was directly purified by LH-20 chromatography (85 × 2 cm, MeOH) to afford **16** (56 mg, 51%) as an amorphous solid. ^1H NMR (CD_3OD, 250 MHz) selected data: δ 7.15–7.35 (m, 2 H, Ar), 6.70–6.85 (m, 2 H, Ar), 5.22 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 2.89 (t, 2 H, *J* 7.0 Hz, CH_2–S), 2.62 (t, 2 H, CH_2–CH_2–S), 1.30–1.50 (m, 4 H, P–$(CH_2)_2$–), 1.03–1.20 (m, 8 H, P–$(CH_2)_2$–$(CH_2)_4$–), 0.71 (t, 3 H, *J* 5.5 Hz, P–$(CH_2)_6$–CH_3); ESI–MS: *m/z* 681.1 (M/2 − H), 1363.4 (M), 1385.4 (M − H + Na), 1386.3 (M + Na). To a solution of **16** (23 mg, 16.8 μmol) in a 9:1 MeOH–water mixture (1 mL) was added *n*-Bu_3P (10 μl, 33.6 μmol) at rt, and the mixture was stirred for 1 h then concentrated. Chromatography of the residue (C 18, 1:1 MeOH–water) gave **17** (18 mg, 78%) as a white amorphous solid. ^1H NMR (CD_3OD, 200 MHz) selected data: δ 7.35–7.20 (m, 2 H, Ar), 6.70–6.80 (m, 2 H, Ar), 5.24 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.28

(dd, 1 H, $J_{5',6'a}$ 7.5, $J_{6'a,6b}$ 11.5 Hz, H-6′a), 3.45 (s, 2 H, 2 H-1′), 3.26 (dd, 1 H, $J_{2,3}$ 9.5 Hz, H-2), 2.89 (t, 2 H, J 7.0 Hz, CH_2–SH), 2.61 (t, 2 H, CH_2–CH_2–SH), 1.31–1.50 (m, 4 H, P–(CH_2)$_2$), 1.10–1.25 (m, 8 H, (CH$_2$)$_2$–(CH_2)$_4$), 0.73 (t, 3 H, J 5.5 Hz, P–(CH$_2$)$_6$–CH_3). To a solution of maleimide–BSA or maleimide–KLH ($\sim$5 mg) in 0.5 mL of 10 mM phosphate buffer saline (PBS, pH 7.3) was added a solution of **17** (6 mg, 8.8 mmol) in PBS (1 mL) and the mixture was gently stirred overnight at 4 °C. Unreacted **12** was separated from the modified proteins by gel-filtration on Sephadex G25 in PBS. The concentration of MA-KLH-**2** and MA-BSA-**2** was determined by bicinchoninic acid assay;[52] 3 and 0.9 mg/mL were obtained, respectively, for the KLH and the BSA conjugates. The number of hapten molecules per BSA or KLH was estimated by amine titration and by measuring the absorbance at 271 nm (λ_{max} of **2**). 3.3 hapten/KLH (calcd for a molecular weight of 100,000) and 3.5 hapten/BSA were obtained.

General procedure for the coupling of **2** *to native proteins: preparation of BSA-***2** *and KLH-***2** *conjugates.*—To a solution of **2** (117 mg, 0.2 mmol) in a mixture of DMF (2 mL) and Et$_3$N (35 μL, 1.25 equiv) was added 5-[(2,5-dioxo-1-pyrrolidinyl)oxy]-5-oxopentanoyl chloride (62 mg, 1.2 equiv). The mixture was stirred for 90 min at rt, then concentrated. Flash chromatography of the residue (1:1 CH_2Cl_2–MeOH) gave **19** (122 mg, 75%) as an amorphous solid; ^{1}H NMR (CD_3OD, 250 MHz): δ 7.30–7.40 (m, 2 H, Ar), 6.80–6.90 (m, 2 H, Ar), 5.38 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.35 (dd, 1 H, $J_{5',6'a}$ 7.5, $J_{6'a,6'b}$ 11.5 Hz, H-6′a), 3.60–4.10 (m, 7 H, H-3′, H-4′, H-5′, H-6′b, H-5, and 2 H-6), 3.58 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3), 3.48 (s, 2 H, 2 H-1′), 3.30 (dd, 1 H, H-2), 2.70 (s, 4 H, CO(CH_2)$_2$CO), 2.60 (t, J 7.0 Hz, CH_2(CH$_2$)$_2$), 2.36 (t, J 7.0 Hz, (CH$_2$)$_2$CH_2), 1.95 (q, 1 H, J 7.0 Hz, CH$_2$CH_2CH$_2$), 1.30–1.50 (m, 4 H, (CH_2)$_2$), 1.05–1.30 (m, 8 H, (CH_2)$_4$), 0.70–0.85 (m, 3 H, CH_3). To a solution of BSA or KLH (10 mg) in 50 mM pyrophosphate buffer pH 8 (2 mL) was added a solution of **19** (60 mg, 74 μmol) in DMF (100 μL). The mixture was stirred for 2 h at rt then purified by gel-filtration on Sephadex G25 in PBS. The concentration (2.8 mg/mL for BSA-**2** and 1.3 mg/mL for KLH-**2**) and the number of haptens was determined as described above; 14 hapten/KLH and 11 hapten/BSA were obtained.

Antibody production.—Four Balb/c mice each received a subcutaneous injection of 100 μg of KLH-**2** (75 μL of the solution obtained as described above) emulsified in complete Freund's adjuvant (75 μL) on day 1. On day 7 and 14 mice received a new subcutaneous injection of KLH-**2** (75 μL) emulsified in incomplete Freund's adjuvant. On day 21, serum was taken from the mice and the titers determined by ELISA. On day 42 the mice with the highest titer receive a final boost intraperitoneally with 100 μg of KLH-**2** in 75 μL of PBS. Three days after the last boost, the spleens were taken from the mice and the cells (10^8) were fused with 2×10^7 X63/HGPRT$^-$ myeloma cells in the presence of PEG 4000. Cells were plated into eight 96-well cell culture plates, each well containing 100 μL of splenocyte feeders. After 9 days, the plates were screened by ELISA for binding to BSA-**2** conjugate and the cells that displayed positive response to binding test were subcloned and expanded to produce large quantities of monoclonal antibodies.

Purification of antibodies.—The tissue culture supernatant (150 mL) was spun at 1000*g* for 30 min. NaCl (26.3 g) and glycine (11.2 g) were added and the pH was adjusted to 8.9 with 0.5 M NaOH. The resulting solution was filtered on a 0.22 μm nylon membrane and loaded on a 5 mL HiTrap protein A-column (Pharmacia Biotech). The column was washed with 30 mL of binding buffer (Biorad) and the elution was realised with 100 mM glycine–HCl buffer pH 3. Each fraction was neutralised with 1 M tris buffer pH 8. The fractions containing the I*g*G were concentrated on a Centricon 30 (Amicon) and were stored at -20 °C in sterile PBS pH 7.35 at a concentration of $\sim$20 mg/mL. The concentration was determined by measurement of the absorbance at 280 nm. Generally 7–15 mg of antibodies were obtained.

Titration of antibodies in animal serum.—Each well of a 96-well polyvinyl assay plate (ELISA plate) was coated with 50 μL of the

conjugate BSA-**2** (30 μg/mL) in phosphate buffer salt (PBS 10 mM, pH 7.2 phosphate, 150 mM NaCl) and incubated at rt overnight. The plate was washed twice with PBS. A solution of BSA (3% in PBS, 300 μL) was added to the wells and the plate was incubated at 37 °C for 2 h. The plate was washed twice with PBS and serum was added at different dilutions (1/50 to 1/400,000). The plate was incubated for 2 h at 37 °C. Washing with PBS was repeated seven times and a solution of goat anti-mouse I*g*G-horseradish peroxidase in BSA 1.5% was added to each well. The plate was incubated for 1 h at 37 °C and washed seven times with PBS. To each well was added 50 μL of a solution of *o*-phenylenediamine (OPD, 0.5 mg/mL) in 0.1 M sodium citrate (pH 5.5) containing 0.1% hydrogen peroxide. The colour was allowed to develop for 10–20 min at rt in the dark and 100 μL of 2 N H_2SO_4 containing 0.5% Na_2SO_3 were added to stop the reaction. The absorbance at 490 nm was measured using a microtiter plate reader. The titer was defined as the serum dilution at which the absorbance became that of the background. Titers between $1/10^5$ and $1/4 \times 10^5$ were obtained.

Enzyme-linked immunosorbent assay.—Positive hybridoma clones producing I*g*G which specifically bind to the hapten were identified by ELISA. ELISA plates were prepared as described above (treatment with BSA-2, incubation, washing and saturation with 3% BSA). The hybridoma supernatant from the each wells of the culture plates was added to the ELISA plates and incubated for 2 h at 37 °C. The plates were washed with PBS and the above protocol was followed. Positive clones were identified by comparison with background.

Competitive enzyme-linked immunosorbent assay.—ELISA plates were prepared as described above (treatment with BSA-**2**, incubation, washing and saturation with 3% BSA). Dilutions of the appropriate inhibitor (**10**–**14** and **20**) were prepared from DMF stock solution in BSA 1% from concentration ranging from 10^{-3} to 1 mM. The proportion of DMF was adjusted to 2% (v/v) in each dilution. In each well of the ELISA plates were added 25 μL of these dilutions and 25 μL of an I*g*G dilution in BSA 1% (the I*g*G dilution was adapted in order to obtain a significant absorbance in the well without inhibitors). The ELISA protocol was then followed as already described. The IC_{50} were deduced from the analysis of the curves obtained by plotting the absorbance at 490 nm vs the inhibitors' concentrations.

Kinetics measurements.—Stock solutions of the esters were prepared in Me_2SO with varying concentrations from 5.3 to 1.1 mM. To 600 μL of phosphate buffer (pH 7.35) were added 80 μL of the above dilutions and 26 μL of a 0.13 mM antibody solution or 26 μL of buffer (uncatalysed reactions). The reactions were incubated in a controlled-temperature shaker at 37 °C and 140 rpm. Periodically, 30 μL aliquots were withdrawn and placed in an ELISA plate. A solution of β-fructosidase (20 μL, 72 u/mL in 0.32 M citrate pH 4.6) for the 6-octanoylsucrose hydrolysis kinetics, or 20 μL of a solution of α-glucosidase (60 u/mL in 0.32 M citrate pH 4.6) for the 6′-octanoylsucrose or the methyl-6-octanoyl-α-D-glucopyranoside hydrolysis, were added and the plate was incubated at 25 °C for 15 min. NADP (2.4 mg/mL, 90 μL) and ATP (5.7 mg/mL) solutions in triethanolamine buffer pH 7.6 were added. The plate was incubated 3 min at 25 °C and the absorbance A1 was read at 340 nm. A suspension of HK (5 μL, 300 u/mL) and G6P-DH (140 u/mL) were added and the plate was incubated for 15 min at 25 °C. The absorbance A2 was measured at 340 nM. The glucose concentrations (amount of hydrolysed ester) were deduced from the differences A2 − A1 by comparison with a standard curve obtained from dilutions of sucrose or methyl-α-D-glucopyranoside prepared in phosphate buffer containing 10% Me_2SO coated on the same plate.

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