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Carbohydrate Research 331 (2001) 119–127

CARBOHYDRATE RESEARCH

Synthesis of biotinylated bis(D-glucose) derivatives for glucose transporter photoaffinity labelling

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Received 20 September 2000; accepted 18 January 2001

Abstract

New diazirine based bis-glucose derivatives for tagging glucose transporters have been synthesised. These included two biotinylated compounds linked either by an aminocaproate or by a cleavable dithiol link. These compounds have been derivatised via a key skeleton compound that can be easily used for introduction of additional tags. Studies on the erythrocyte glucose transporter (GLUT1) and the insulin-stimulated adipose cell transporter (GLUT4) have revealed the biotinylated photoreactive bis-glucose compounds are effective labelling reagents. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bis-glucose; Biotin; Photolabels; Glucose transporters

1. Introduction

There are five major glucose transporters in mammalian tissues (GLUTs 1–5)[1] but new isoforms are being discovered.[2] These proteins catalyse one of the most important cell membrane transport events since glucose plays a central role in cellular homeostasis and metabolism.

The GLUT transporters have broad specificities for hexoses and, in addition to glucose, other monosaccarides such as galactose and mannose are transported. Previous studies have established that bulky groups are tolerated around the 4-OH position when the hexose occupies the exofacial binding sites of the transporters.[3] We have previously found that a bis-hexose structure has advantages for photolabelling of the exofacial surface of the transporters. The bis-structure allows a hydrophobic moiety to be inserted in the bridge in a form that produces a molecule, that overall, still has a hydrophilic character and is water-soluble. In addition, the bis-hexoses are bulky and consequently act as powerful inhibitors of glucose transport but are not themselves transported because of steric constraints imposed by the bulky substituents. Consequently as these probes are too hydrophilic to enter cells through the membrane lipid, they act specifically at the exofacial binding site. This bis-structure has previously been reported for bis-mannose[4] linked by a 2-propylamine bridge and bis-glucose derivatives linked by a secondary amine bridge.[5] The bis-mannose propyl-2-amine (BMPA) compounds includ-

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PII: S0008-6215(01)00025-8

ing a biotinylated derivative[6] have been particularly useful in tagging the glucose transporters and therefore we have examined here a bis-glucose compound with the versatile 2-propylamine bridge. The choice of photoreactive substituent for studying glucose transporters was also considered to be important. Bis-mannose derivatives in which aryl azide or benzophenone groups have been introduced have been previously described.[7,8] The disadvantages of the aryl azides is that nucleophilic side reactions reduce the specificity of the labelling reaction while the disadvantage of the benzophenone groups is that long irradiation times are needed to produce a high yield of cross-linked adduct. By contrast, diazirine groups have been found to generate highly reactive carbenes smoothly and specifically with short irradiation times. Furthermore, we have found that a carbene-introduced linkage is more stable than those introduced by the other two species.[9] We have consequently concluded that diazirine is the most useful photophor[10,11] and have synthesised new bis-glucose compounds that contain this photoreactive group. We have chosen to introduce biotin groups attached by either amino-hexanoate or cleavable dithiol links. The advantage of the latter is that it allows release, under mild reducing conditions at moderate temperatures, of the photolabelled protein from the avidin or streptavidin matrix used to isolate and purify the protein. We have found that the release of photolabelled transporters, tagged with the previously described biotin-amino-hexanoate linked bis-mannose photolabel Bio-LC-ATB-BMPA, from streptavidin agarose requires that the adduct be heated at 100 °C for 30 min in the presence of denaturing SDS.

In our consideration of the route to produce new bis-glucose photolabels we thought it useful to prepare a key intermediate that could subsequently be used to introduce new tags as required. In this paper we describe the synthesis of novel amino BOC protected diazirine-based photoreactive bis-glucose derivative as this key intermediate. Removal of a BOC protecting group leaves an amine group that can then be easily coupled to any probe moiety containing a carboxylate group via amide bond formation. To determine the utility of the approach we studied the interaction of the biotinylated bis-glucose photoaffinity probes with the human erythrocyte transporter, GLUT1 and the with the adipose cell transporter, GLUT4.

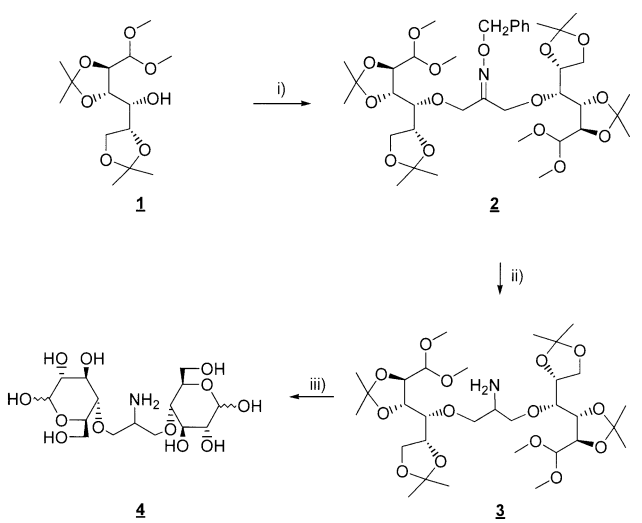

Fig. 1. (i) (a) NaH, DMF, room temperature; (b) 1,3-dichloro-2-propane benzyloxime, room temperature, 74%. (ii) H_2, Raney nickel, ethanol, H_2O, NH_4OH, room temperature, 94%. (iii) 1 M HCl, 100 °C, quant.

2. Results and discussion

Synthesis of bis-glucose photoreactive probes.—The route for synthesis of the bis-glucose compounds is shown in Fig. 1. 2,3,5,6-Di-*O*-isopropylidene-D-glucose dimethyl acetal (**1**)[12] was converted to the 4-O-linked dimer **2** with 1,3-dichloro-2-propanone *O*-benzyloxime. This crosslinking reagent has been previously used to synthesise bis-D-mannose-2-propylamine.[13] The reaction proceeds rapidly to a monochloro intermediate (which could be isolated and characterised) and then more slowly to the bis- compound. These conversions can be followed by thin layer chromatography (TLC). Following purification, **2** was hydrogenated using Raney nickel as catalyst to give the amine **3** in good yield. The protecting acetal groups were then removed by acid hydrolysis to generate the 1,3-bis(glucos-4-yloxy) 2-propylamine compound (**4**).

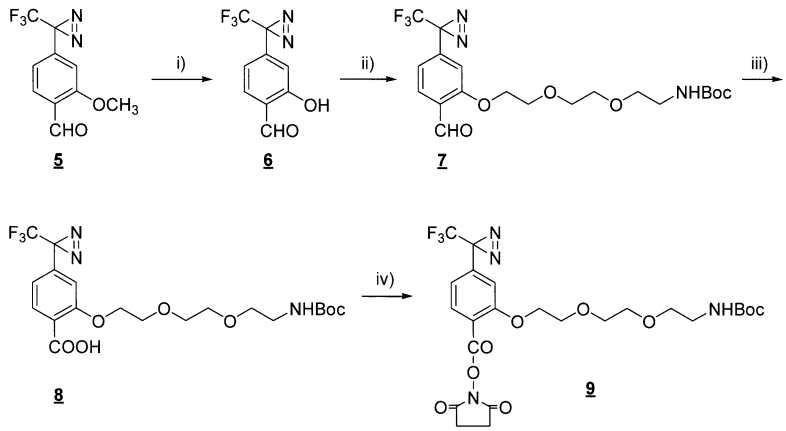

Fig. 2. (i) BBr_3, CH_2Cl_2, room temperature, 64%. (ii) 2-[2-(2-*tert*-Butoxycalbonylaminoethoxy)ethoxy]ethyl bromide, K_2CO_3, *n*-Bu_4NI, DMF, 60 °C, 91%. (iii) *n*-Bu_4NMnO_4, pyridine, room temperature, 90%. (iv) *N*-Hydroxysuccinimide, dicyclohexylcarbodiimide, CH_2Cl_2, CH_3CN, room temperature, 99%.

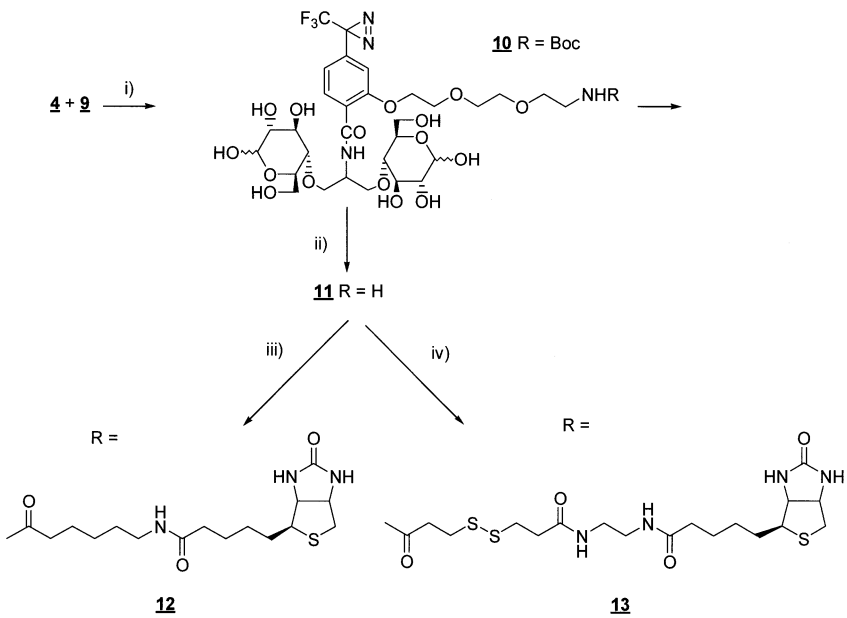

Fig. 3. (i) 0.1 M $NaHCO_3$, DMF, room temperature, 88%. (ii) 50% Trifluoroacetic acid, CH_2Cl_2, 0 °C, 86%. (iii) *N*-Biotinylamino caproic acid *N*-hydroxysuccinimide ester, 0.1 M $NaHCO_3$–DMF, room temperature, 37%. (iv) ([2-(Biotinyl)ethylamido]-3,3′-dithiodipropionic acid *N*-hydroxysuccinimide ester, 0.1 M $NaHCO_3$–DMF, room temperature, 40%.

We have previously reported the synthesis of a diazirinyl aryl carboxylate with a polyether spacer linked to biotin[14] that can be used to biotin tag proteins directly. However, we considered it more useful in the present study to synthesise from **4**, a photoreactive skeleton compound first and then subsequently couple this to biotin via ether aminocaproate or dithiol linkers (Figs. 2 and 3). Many more applications of the intermediate **11** are then possible as it can be further substituted with any additional tags as required for additional applications. For synthesis of the key skeleton compound **11**, we required the novel compound **9**, the production of which, itself required modifications of previously described syntheses (Fig. 2). We were able to achieve ether cleavage of **5**[15] with BBr_3 without protection of the aldehyde group of this diazirine compound. Phenol alkylation with the BOC protected ethylene glycol spacer[14] then afforded **7** in good yield. The aldehyde group of **7** was then oxidised to the carboxylic acid in **8** which was then converted to its *N*-hydroxy-succinimide ester derivative **9** (Fig. 2).

The condensation of compound **4** and **9** in nonaqueous conditions (triethylamine or morpholine in DMF) was slow and only produced low yields of **10** (less than 20%), possibly due to low solubility of the bis-glucose-2-propylamine compound in DMF We therefore used a water–DMF mixture for coupling. The bis-glucose-2-propylamine compound and the diazirine were dissolved in 0.1 M $NaHCO_3$ and DMF, respectively. The reaction proceeded smoothly with a moderate yield of the desired product.

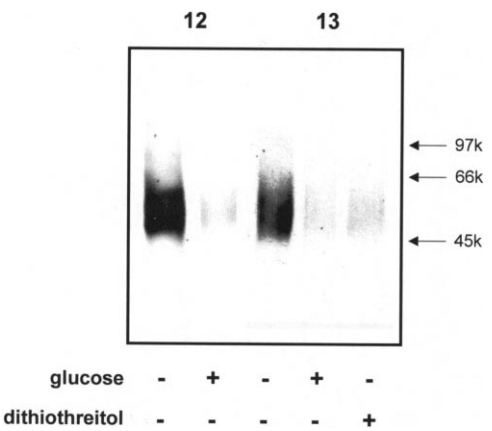

Fig. 4. Chemiluminescence detection of photoaffinity labelled human GLUT1. Compounds **12** and **13** were used to photolabel GLUT1 purified from human erythrocytes. Glucose (0.17 M) was added as a competitor for the binding site during the photolabelling reaction (lanes 2 and 4). The susceptibility of the dithiol bridge in GLUT1 photolabelled with compound **13** to reducing agent was examined by adding 0.5 M dithiothreitol at room temperature for 15 min after the photolabelling reaction. Photolabelled samples were resolved by SDS-PAGE on 10% gels run under reducing conditions (compound **12**) and non-reducing conditions (compound **13**) and then transferred to nitrocellulose for analysis of the biotin signal by using avidin-HRP and ECL detection.

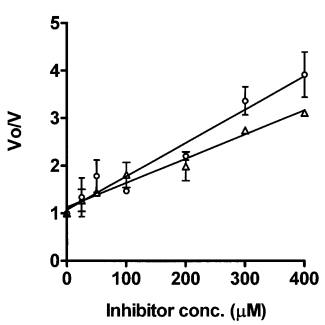

Fig. 5. Determination of half maximal inhibition constants for bis D-glucose ligands. The uptake of tracer 2-deoxy-[^{14}C]-D-glucose in insulin-stimulated rat adipose cells was determined at the indicated concentrations of compounds **12** (△) and **13** (○). The rate constants (mean and S.E.M. are shown) for uptake in the presence (V) and absence (V_0) of inhibitor (I) which were then used to calculate the K_i according to the equation $V_0/V = 1 + I/K_i$. Results shown are from three experiments.

The BOC protected skeleton was stable on storage and could be conveniently deprotected by TFA treatment for immediate coupling to an appropriate biotin-NHS ester as required. The biotinylated compounds studied here included compounds with aminocaproate and dithiol spacers, compounds **12** and **13**, respectively (Fig. 3).

Photoaffinity labelling of human GLUT1.—Erythrocytes provide a rich source of GLUT1, which is present as 3–5% of the membrane protein. GLUT1 was purified as previously described.[16] Purified GLUT1 was then mixed with stoichiometric amount of compound **12** or **13**, and the samples were then irradiated and the protein was resolved by SDS-PAGE and transferred to nitrocellulose as described in Section 3. Extravidin peroxidase conjugation revealed strong biotinylated bands at approximately 50 kDa for both compounds **12** and **13** and these signals were competitively reduced in the incubations with D-glucose (Fig. 4). Under the labelling conditions employed, the signal was therefore specific for the binding site of GLUT1. One of our aims was to test the feasibility of using compound **13** in experiments in which the biotin moiety is removed by a reducing agent that breaks the dithiol spacer arm. We therefore incubated the GLUT1 samples (phototagged with **13**) either in the absence or presence of dithiothreitol and resolved the labelled proteins under non-reducing conditions (Fig. 4). The biotin group was found to be cleaved under quite mild conditions and therefore, this compound is likely to be useful in future studies in which release of biotin-tagged protein or binding-site peptides under mild conditions is required.

Photolabelling of insulin-stimulated GLUT4 in adipocytes.—To determine the affinity of the interaction between the ligands **12** and **13** and GLUT4, each of the compounds was tested as a competitive inhibitor of 2-deoxy-D-glucose uptake into insulin-stimulated rat adipocytes (Fig. 5). K_i values or half-maximal inhibition constants for **12** and **13** were 195 and 142 μM, respectively. The K_i value for **12** was similar to, but slightly better than that of the equivalent bis-mannose compound Bio-

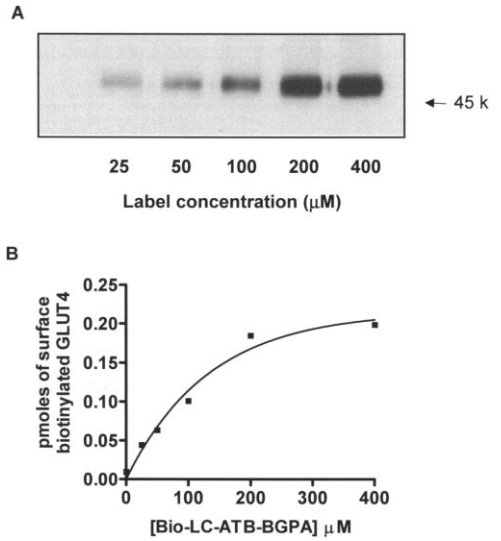

Fig. 6. Quantification of GLUT4 labelling. Rat adipose cells were UV irradiated in the presence of compound **12** at the indicated concentrations. Cell membrane protein was then isolated and solubilised. Streptavidin beads was then used to precipitate the biotin-tagged GLUT4, which was resolved by SDS-PAGE and detected using an anti-GLUT4 antibody, peroxidase coupled secondary antibody and ECL (A). The ECL signal was quantified by comparison with a GLUT4 standard and the extent to which the labelling became saturated with increasing ligand concentrations was determined (B).

LC-ATB-BMPA (K_i of 273 μM Ref. 6). We have also determined the most appropriate concentration of **12** to use in GLUT4-tagging experiments (Fig. 6). To do this we have photolabelled, washed and solubilised the adipose cell protein as described in Section 3 and then precipitated the biotin-tagged protein with streptavidin-agarose. A wash step then allowed removal of the non-biotinylated GLUT4. The precipitated material was next resolved by SDS-PAGE and an antibody to the C-terminal section of GLUT4 was used to detect the tagged protein (Fig. 6(A)). Only a single labelled band was obtained and the amount of GLUT4 was quantified by reference with a standard containing a known amount of this protein.[17] This quantification (Fig. 6(B)) revealed that maximal tagging with biotin occurred when 200–400 μM ligand was used, a value that is consistent with the affinity constant revealed in the transport inhibition experiments.

3. Experimental

General methods.—Silica Gel for column chromatography was Kieselgel 60 (E. Merck, 230–400 mesh). Structural characterisations of synthetic products were performed with a JEOL JNM GX-400 spectrometer (^{1}H NMR) and a Micromass Autospec (MS). Elemental analysis was carried out by the microanalysis service (Bath University). The purity of those compounds that were not available in sufficient quantity for elemental analysis were determined by a combination of diazirine UV detection, biotin detection and hexose detection techniques applied to the compounds on TLC plates. Furthermore, no extraneous lines were detected in the NMR spectra and UV spectra and extinction coefficients at 350 nM were consistent with those previously established compounds containing the diazirine moiety. (Extravidin-peroxidase conjugate was obtained from Sigma. Chemiluminescence detection reagent and Hyper-film ECL were obtained from Amersham. Nitrocellulose membrane (BioTrace NT) was obtained from GelmanScience.)

1,3-Bis(2,3, 5, 6-di-O-*isopropylidene*-D-*glucose dimethylacetal-4-yloxy)-2-propane*-O-*benzyloxime* (**2**).—2,3,5,6-Di-*O*-isopropylidene glucopyranosyl dimethyl acetal (**1**)[12] (2.36 g, 7.7 mmol) was dissolved in DMF (15 mL). Sodium hydride (60%, 0.34 g, 8.5 mmol) was added to this solution at rt. After bubbling had ceased, 1,3-dichloro-2-propanone *O*-benzyloxime[13] (0.89 g, 3.9 mmol) was added. The reaction mixture was stirred at rt for 2 h. Additional NaH (0.09 g, 2.2 mmol) was then added. The reaction mixture was stirred for a further 1 h, quenched by AcOH (0.3 mL) and partitioned between CH_2Cl_2 and water. The aqueous phase was further extracted into CH_2Cl_2 twice. The combined organic layer was washed with 1 M HCl, saturated $NaHCO_3$ and satd NaCl, and then dried over Na_2SO_4. After filtration, the product was concentrated. The residue was purified with silica column chromatography (4:1 *n*-hexane–EtOAc) to afford a colourless oil (2.21 g, 74%). ^{1}H NMR ($CDCl_3$): δ 7.28–7.38 (5 H, m, Ph), 5.07 (2 H, s, $NOCH_2Ph$), 4.58 (4 H, s, $OCH_2C{=}NO$), 4.35 (2 H, d, $J = 6.0$ Hz, H-1),

4.30 (2 H, ddd, $J = 5.0, 6.0, 6.0$ Hz, H-5), 4.17 (2 H, dd, $J = 6.0, 7.6$ Hz, H-2), 4.00–3.95 (6 H, m, H-3 and 6), 3.62 (2 H, dd, $J = 2.6, 5.0$ Hz, H-4), 3.40 (12 H, s, OCH_3), 1.37 (24 H, s, $C(CH_3)_2$); FABMS: m/z 772 ($[M + H]^+$); HR-FABMS: Calcd for $C_{38}H_{62}NO_{15}$ ($[M + H]^+$) 772.4119; Found 772.4110. Anal. Calcd for $C_{38}H_{61}NO_{15}$ C, 59.13; H, 7.97; N, 1.81. Found: C, 59.2; H, 7.82; N, 1.88.

1,3-Bis(2,3,5, 6-di-O-*isopropylidene*-D-*glucose dimethylacetal-4-yloxy)-2-propylamine* (**3**).—Compound **2** (2.02 g, 2.6 mmol) and Raney nickel (59%, 8 mL) were suspended in 16:8:1 water–EtOH–NH_3 (20 mL). The reaction mixture was vigorously stirred at rt under a hydrogen atmosphere for 19 h. The mixture was filtered through Celite and was then concentrated. The residue was partitioned between EtOAc and water. The organic phase was washed with saturated NaCl, dried over Na_2SO_4, filtered and then concentrated to afford a colourless oil (1.63 g, 94%). ^{1}H NMR ($CDCl_3$): δ 4.36 (2 H, d, $J = 6.2$ Hz, H-1), 4.23 (2 H, ddd, $J = 5.0, 6.0, 6.0$ Hz, H-5), 4.14 (2 H, dd, $J = 6.2, 7.4$ Hz, H-2), 4.03–3.98 (6 H, m, H-3 and 6), 3.67 (1 H, t, $J = 5.9$ Hz, $CHNH_2$), 3.61 (2 H, dd, $J = 2.4, 5.0$ Hz, H-4), 3.48 (4 H, d, $J = 5.9$ Hz, OCH_2), 3.40 (12 H, s), 1.37 (24 H, s), 4.36 (4 H, d, $J = 6.3$ Hz), 4.3–3.5 (14 H, m), 4.00 (4 H, d, $J = 6.3$ Hz), 3.45 (12 H, s, OCH_3), 3.20 (1 H, m), 1.76 (1 H, br), 1.42, 1.40, 1.34 (total 24 H, each s, $C(CH_3)_2$). FABMS: m/z 668 ($[M + H]^+$); HR-FABMS: Calcd for $C_{31}H_{58}N0_{14}$ ($[M + H]^+$) 668.3857; Found 668.3848. Anal. Calcd For $C_{31}H_{57}NO_{14}\cdot 0.5\ H_2O$: C, 55.02; H, 8.64; N, 2.07. Found: C, 55.2; H, 8.52; N, 2.09.

*1,3-Bis(*D-*glucopyranos-4-yloxy)-2-propylamine* (**4**).—Compound **3** (0.56 g, 0.83 mmol) was suspended in 1 M HCl (2 mL) and heated at 100 °C for 3 h. The reaction mixture was neutralised with Amberlite IRA-93 (OH form) and the resin was removed by filtration. The filtrate was concentrated to afford light brown oil (0.39 g, quant.). ^{1}H NMR (CD_3OD): δ 5.05 (1 H, d, $J = 3.6$ Hz, 1-α, 4.40 (1 H, d, $J = 7.6$ Hz, 1-β), 3.4–4.0 (17 H, m). FABMS m/z 416 ($[M + H]^+$). HRFABMS Calcd for $C_{15}H_{30}NO_{12}$ ($[M + H]^+$): 416.1768. Found: 416.1745.

2-Hydroxy-4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzaldehyde (**6**).—2-Methoxy-4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzaldehyde (**5**)[15] (3.396 g, 13.9 mmol) was dissolved in CH_2Cl_2 at −20 °C. BBr_3 (1.4 mL, 14.8 mmol) was added in portions. The reaction mixture was stirred at 0 °C for 1 h and water was added to quench the reaction. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, filtered and then concentrated. The residue was purified with silica column chromatography (5:1 *n*-hexane–CH_2Cl_2) to afford a pale yellow oil (2.048 g, 64%). ^{1}H NMR ($CDCl_3$): δ 9.90 (1 H, s, CHO), 7.59 (1 H, d, $J = 7.9$ Hz, Ph), 6.07 (1 H, d, $J = 7.9$ Hz, Ph), 6.75 (1 H, s, Ph). EIMS: m/z 230 ($[M]^+$). HRMS Calcd for $C_9H_5F_3N_2O_2$ ($[M]^+$): 230.0303; Found: 230.0291.

*2-[2-[2-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzaldehyde* (**7**).—Compound **6** (2.001 g, 8.69 mmol), 2-[2-(2-*tert*-butoxycalbonylaminoethoxy)ethoxy]ethyl bromide,[14] K_2CO_3 (1.999 g, 14.4 mmol) and nBu$_4$NI (0.403 g, 1.08 mmol) were suspended in DMF (109 mL). The suspension was heated at 60 °C for 12 h, concentrated and partitioned between EtOAc and water. The organic layer was washed with 1 M HCl, satd $NaHCO_3$ and satd NaCl, and then dried over $MgSO_4$. After filtration, the product was concentrated and subjected to silica column chromatography (1:1 *n*-hexane–EtOAc) to afford a pale yellow oil (3.658 g, 91%). ^{1}H NMR ($CDCl_3$): δ 10.48 (1 H, s, CHO), 7.85 (1 H, d, $J = 8.2$ Hz, Ph), 6.86 (1 H, d, $J = 8.2$ Hz, Ph), 6.74 (1 H, s, Ph), 4.27 (2 H, t, $J = 4.7$ Hz), 3.92 (2 H, t, $J = 4.7$ Hz), 3.71 (2 H, m), 3.64 (2 H, m), 3.54 (2 H, m), 3.32 (2 H, m), 1.43 (9 H, s, $C(CH_3)_3$). FABMS m/z 462 ($[M + H]^+$). HRFABMS Calcd for $C_{20}H_{27}F_3N_3O_6$ ($[M + H]^+$): 462.1582; Found: 462.1844.

*2-[2-[2-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3yl] benzoic acid* (**8**).—Compound **7** (2.135 g, 4.63 mmol) and tetrabutylammonium permanganate (2.485 g, 6.87 mmol) were dissolved in Py (45 mL) at rt and stirred for 2 h. The reaction mixture was diluted with toluene and washed with 1 M Na_2SO_4, 1 M HCl and satd NaCl.

The organic layer was dried over $MgSO_4$, filtered and concentrated to afford a yellow oil (1.988 g, 90%). ^{1}H NMR ($CDCl_3$): δ 8.16 (1 H, d, $J = 8.2$ Hz, Ph), 6.94 (1 H, d, $J = 8.2$ Hz, Ph), 6.78 (1 H, s, Ph), 4.37 (2 H, m), 3.92 (2 H, m), 3.72 (2 H, m), 3.65 (2 H, m), 3.55 (2 H, m), 3.32 (2 H, m), 1.42 (9 H, s, s, $C(CH_3)_3$). FABMS: m/z 478 ($[M + H)^+$). HRFABMS Calcd for $C_{20}H_{27}F_3N_3O_7$ ($[M + H]^+$): 478.1801; Found: 478.1799.

*2-[2-[2-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl] benzoic acid* N*-hydroxysuccinimide ester* (**9**).—*N*-hydroxysuccinimide (0.490 g, 4.26 mmol) and compound **8** (1.837 g, 3.85 mmol) were dissolved in 5:1 CH_2Cl_2–CH_3CN (12 mL) at rt. Dicyclohexylcarbodiimide (0.877 g, 4.25 mmol) in CH_2Cl_2 was added. The reaction mixture was stirred at rt for 3 h, filtered, and then concentrated. The residue was partitioned between EtOAc and water. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, filtered and concentrated to afford a yellow oil (2.186 g, 99%). ^{1}H NMR ($CDCl_3$): δ 8.03 (1 H, d, $J = 8.2$ Hz, Ph), 6.87 (1 H, d, $J = 8.2$ Hz, Ph), 6.76 (1 H, s, Ph), 4.24 (2 H, m), 3.91 (2 H, m), 3.72 (2 H, m), 3.61 (2 H, m), 3.53 (2 H, m), 3.31 (2 H, m), 2.90 (s, 4 H, CH_2CH_2), 1.43 (9 H, s, $C(CH_3)_3$). FABMS: m/z 575 ($[M + H]^+$); HRFABMS Calcd for $C_{24}H_{30}F_3N_4O_9$ ($[M + H]^+$): 575.1965; Found: 575.1973. Anal. Calcd For $C_{24}H_{29}F_3N_4O_9 \cdot 0.5\ H_2O$: C, 49.40; H, 5.18; N, 9.60. Found: C, 49.55; H, 5.35; N, 9.35.

N*-[2-[2-[2-(2-*tert*-Butoxycarbonylamino-ethoxy)ethoxy] ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl]-1,3-bis(*D*-glucopyranose-4-yloxy)-2-propylamine* (**10**).—Compound **9** (0.162 g, 0.28 mmol) was dissolved in DMF (2.5 mL) and compound **4** (0.162 g, 0.17 mmol) was added in 3 mL of 0.1 M $NaHCO_3$. The reaction mixture was stirred at rt for 12 h. The product was then applied to a column of Amberlite IRN-150L (2 mL, neutral form) and was washed through the column with water. The eluate was concentrated and the product was purified with silica column chromatography (13:5:1 $CHCl_3$–CH_3OH–water) to afford a colourless oil (0.131 g, 88%). ^{1}H NMR (D_2O): δ 7.88 (1 H, d, $J = 8.2$ Hz, Ph), 7.05 (1 H, d, $J = 8.2$ Hz, Ph), 6.89 (1 H, s, Ph), 5.23 (1 H, d, $J = 3.6$ Hz, 1-α), 4.44 (1 H, d, $J = 7.6$ Hz, 1-β), 4.40–3.20 (29 H, m), 1.37 (9 H, s, $C(CH_3)_3$). FABMS: m/z 875 ($[M + H]^+$). HRFABMS Calcd for $C_{35}H_{54}F_3N_4O_{18}$ ($[M + H]^+$): 875.3385; Found 875.3370.

N*-[2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-benzoyl]1,3-bis(*D*-glucopyranos-4-yloxy)-2-propylamine* (**11**).—Trifluoroacetic acid (1 mL) was added to compound **10** (0.181 g, 0.21 mmol) in CH_2Cl_2 (1 mL) at 0 °C and the mixture was stirred for 0.5 h. The reaction mixture was concentrated and then redissolved in water (2 mL). The solution was passed through a 3 mL column of Amberlite IRA-93 (OH form) and the product was washed through with distilled water. The eluate was concentrated to afford colourless oil (0.138 g, 86%). FABMS m/z 775 ($[M + H]^+$).

N*-[2-[2-[2-(2-(*N*-biotinylcaproylamino)-ethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl]1,2-bis(glucopyranosyl-4-yloxy)-2-propylamine* (**12**).—*N*-biotinyl caproic acid succinimide ester (0.301 g, 0.66 mmol) was dissolved in DMF (2 mL). To this solution compound **11** (0.464 g, 0.60 mmol) in 0.1 M $NaHCO_3$ (2 mL) was added at rt and the reaction mixture was stirred for 12 h. The reaction mixture was then concentrated. The residue was dissolved in water and applied to a column of Amberlite IRN-150L (2 mL, neutral form) and the product was washed through the column with distilled water. The eluate was then concentrated to afford pale-yellow oil. The eluate was concentrated and the product was purified with silica column chromatography (13:5:1 $CHCl_3$–CH_3OH–water) to afford a colourless oil (0.271 g, yield 37%). ^{1}H NMR (CD_3OD): δ 7.95 (1 H, d, $J = 8.2$ Hz, Ph), 7.02 (1 H, d, $J = 8.2$ Hz, Ph), 6.85 (1 H, s, Ph), 5.10 (1 H, d, $J = 3.6$ Hz, 1-α), 4.58 (1 H, d, $J = 7.6$ Hz, 1-β), 4.40–2.80 (37 H, m), 1.80–1.20 (10 H, m). FABMS: m/z 1114 ($[M + H]^+$). HR-FABMS m/z ($[M + H]^+$) Calcd for $C_{46}H_{71}F_3N_7O_{19}S$: 1114.4478; Found 1114.4530.

N*-[2-[2-[2-(2-(2-Biotinyl)ethylamido]-3,3′-dithiodipropionylamino)ethoxy)ethoxy]-ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-*

*3-yl]benzoyl]1,3-bis(*D*-glucopyranos-4-yloxy)-2 - propylamine* (**13**).—[2 - (Biotinyl)ethylamido]-3,3′-dithiodipropionic acid *N*-hydroxysuccinimide ester (0.105 g, 0.18 mmol) was dissolved in DMF (1 mL). To this solution, compound **11** (0.163 g, 0.21 mmol) in 0.1 M $NaHCO_3$ (0.5 mL) was added at rt and the reaction mixture was stirred for 12 h. The reaction mixture was then concentrated. The residue was dissolved in water and applied to a column of Amberlite IRN-150L (2 mL, neutral form) and the product was washed through the column with distilled water. The eluate was then concentrated to afford pale yellow oil. The eluate was concentrated and the product was purified with silica column chromatography (50:25:1 $CHCl_3$–CH_3OH–water) to afford a colourless oil (0.089 g, yield 40%). ^1H NMR (CD_3OD): δ 7.42 (1 H, d, $J = 8.2$ Hz, Ph), 7.10 (1 H, d, $J = 8.2$ Hz, Ph), 6.90 (1 H, s, Ph), 5.23 (1 H, d, $J = 3.6$ Hz, 1-α), 4.60 (1 H, d, $J = 7.6$ Hz, 1-β), 4.50–4.20 (4 H, m), 4.00–3.20 (28 H, m), 3.0–2.50 (14 H, m), 2.25 (2 H, m), 1.80–1.40 (6 H, m). FABMS: m/z 1235 ($[M + H]^+$). HRFABMS m/z ($[M + H]^+$) Calcd for $C_{48}H_{74}F_3N_8O_{20}S_3$: 1235.4117; Found 1235.4134.

Photoaffinity labelling of GLUT1.—Human GLUT1 was prepared as described by Baldwin and Lienhard.[16] GLUT1 (10 μg) and compound **12** or **13** (0.25 nmol) were suspended in 0.1 M NaCl, 1 mM EDTA, 50 mM sodium phosphate buffer pH 7.4 (23 μL) at rt for 10 mm. Where indicated in the figure legends, D-glucose was included for competitive inhibition. The mixture was irradiated three times for 30 s with 30 s intervals between successive irradiations at 0 °C in a Rayonet photochemical reactor using 300 nm lamps. The solution was then centrifuged at 400,000*g* at 4 °C for 30 mm. The pellet was resuspended in (20 μL) of 1 mM EDTA, 50 mM Tris pH 7.4. A sample (0.5 μg) was then subjected to electrophoresis on a 10% acrylamide gel and transferred to nitrocellulose. The nitrocellulose was blocked for 1 h in 10% skimmed milk suspended with 0.1% Tween 20, Tris-buffered saline pH 7.4 (T-TBS), washed twice with T-TBS for 5 min and immersed in Extravidin peroxidase (a 1:4000 dilution of the manufacturers solution) with 1% skimmed milk in T-TBS for 40 mm. After washing 6 times with T-TBS for 5 min, the membrane was immersed in chemiluminescence reagents for 1 min and exposed to film for 0.5 min.

Photoaffinity labelling of GLUT4.—Rat adipocytes isolation, K_i measurements, and UV irradiation for labelling experiments were carried out as previously described. After labelling, cell samples were homogenised in HES buffer (255 mM sucrose, 1 mM EDTA, 20 mM HEPES, 1 μg/mL antipain, aprotinin, pepstatin, leupeptin and 100 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), pH 7.2). Homogenates were washed once with HES buffer and subjected to centrifugation (554,000*g* for 30 min at 4 °C) to obtain a total membrane fraction. This pellet was solubilised in PBS pH 7.2, with 2% of Thesit ($C_{12}E_9$) and protease inhibitors (antipain, aprotinin, pepstatin, and leupeptin each at a concentration of 1 μg/mL and 100 μM AEBSF). The samples were solubilised for 50 min at 4 °C with rotation and were then subjected to centrifugation (20,000*g* for 20 min at 4 °C). Biotinylated proteins in the supernatants were either immuno-precipitated using a GLUT4 antiserum as previously described[6] or were precipitated with streptavidin beads (Pierce, Rockford, IL). Following GLUT4 immuno-precipitation complexes were released into electophoresis sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol) at rt. The steptavidin precipitates were washed four times with PBS buffer con taining 1% Thesit with protease inhibitors, four times with PBS containing 0.1% Thesit plus protease inhibitors and once in PBS. Electrophoresis sample buffer was added to each pellet. The sample was then heated to 95 °C for 30 min. The samples were subjected to centrifugation (2,300*g* for 1 min) and the supernatants were removed. The pellets were washed with additional electrophoresis sample buffer, heated to 95 °C for 30 min and resubjected to centrifugation. Mercaptoethanol was added (10% final concentration) to the above samples in the electrophoresis sample buffer and these were then subjected to SDS-PAGE (10% gel). Proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in TBS-0.1%

Tween (TBS-T) and washed six times with TBS-T. Membranes were then incubated with affinity purified anti-GLUT4 C-terminal antibody[18] in TBS-T containing 1% BSA (2 h at rt), followed by washing (6 times in TBST) and detection using secondary antibody linked to horseradish peroxidase. GLUT4 protein was visualised with enhanced chemiluminescence (ECL). Pmoles of labelled GLUT4 were determined by comparison with a GLUT4 standard protein as described.[17]

Acknowledgements

This work was supported by the Medical Research Council (UK), the Wellcome Trust and Diabetes (UK).

References

1. Gould, G. W.; Holman, G. D. *Biochem. J.* **1993**, *295*, 329–341.
2. Ibberson, M.; Uldry, M.; Thorens, B. *J. Biol. Chem.* **2000**, *275*, 4607–4612.
3. Barnett, J. E. G.; Holman, G. D.; Munday, K. A. *Biochem. J.* **1973**, *135*, 539–541.
4. Holman, G. D.; Midgley, P. J. W. *Carbohydr. Res.* **1985**, *135*, 337–341.
5. Abbadi, M.; Holman, G. D.; Morin, C.; Rees, W. D.; Yang, J. *Tetrahedron Lett.* **1999**, *40*, 5861–5864.
6. Koumanov, F.; Yang, J.; Jones, A. E.; Hatanaka, Y.; Holman, G. D. *Biochem. J.* **1998**, *330*, 1209–1215.
7. Holman, G. D.; Parkar, B. A.; Midgley, P. J. W. *Biochim. Biophys. Acta* **1986**, *855*, 115–126.
8. Holman, G. D.; Karim, A. R.; Karim, B. *Biochim. Biophys. Acta* **1988**, *946*, 75–84.
9. Yoshida, E.; Nakayama, H.; Hatanaka, Y.; Kanaoka, Y. *Chem. Pharm. Bull.* **1990**, *38*, 982–987.
10. Clark, A. E.; Holman, G. D. *Biochem. J.* **1990**, *269*, 615–622.
11. Gillingham, A. K.; Koumanov, F.; Hashimoto, M.; Holman, G. D. In *Membrane Transport. A Practical Approach*; Baldwin, S. A., Ed.; Oxford University Press: London, 2000; pp. 193–207.
12. Stevens, J. D. *Carbohydr. Res.* **1975**, *45*, 143–150.
13. Sher, P. M.; Kronenthal, D. R. *J. Label. Compd. Radiopharm.* **1997**, *39*, 21–27.
14. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *Bioorg. Med. Chem.* **1994**, *2*, 1367–1373.
15. Hashimoto, M.; Kanaoka, Y.; Hatanaka, Y. *Heterocycles* **1997**, *46*, 119–122.
16. Baldwin, S. A.; Lienhard, G. E. *Methods Enzymol.* **1989**, *174*, 39–50.
17. Ryder, J. W.; Yang, J.; Galuska, D.; Rincón, J.; Björnholm, M.; Krook, A.; Lund, S.; Pedersen, O.; Wallberg-Henriksson, H.; Zierath, J. R.; Holman, G. D. *Diabetes* **2000**, *49*, 647–654.
18. Holman, G. D.; Kozka, I. J.; Clark, A. E.; Flower, C. J.; Saltis, J.; Habberfield, A. D.; Simpson, I. A.; Cushman, S. W. *J. Biol. Chem.* **1990**, *265*, 18172–18179.