Cell-Surface Recognition of Biotinylated Membrane Proteins Requires Very Long Spacer Arms: An Example from Glucose-Transporter Probes

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Glucose transporters (GLUTs) can be photoaffinity labelled by (diazirinetrifluoroethyl)benzoyl-substituted glucose derivatives and the adduct can be recognised, after detergent solubilisation of membranes, by using streptavidin-based detection systems. However, in intact cells recognition of photolabelled GLUTs by avidin and anti-biotin antibodies only occurs if the bridge between the photoreactive and the biotin moieties has a minimum of 60 – 70 spacer atoms. We show that a suitably long bridge can be synthesised with a combination of polyethylene glycol and tartarate groups and that introduction of these spacers generates hydrophilic products that can be cleaved with periodate. Introduction of the very long spacers does not appreciably reduce the affinity of interaction of the probes with the transport system.

KEYWORDS:

bridging ligands · carbohydrates · membranes · photoaffinity labelling

Introduction

Membrane proteins are often only partially exposed to the external solution surrounding cells and have binding sites that are buried within their central regions, which lie deep within the lipid bilayer. This inaccessibility presents major problems in attempts to tag these proteins by biotin in a form where they can be recognised by avidin or anti-biotin antibodies. The problem is compounded because the biotin binding site in avidin, which can be used for detection, is buried 9 Å below the surface.[1] Detection will therefore require the close approach of two large macromolecules that both have deep binding pockets. This problem particularly applies to membrane glucose transporters (GLUTs) which expose short exofacial loops at the outer surface of the cell[2] which are difficult to label with conventional biochemical approaches. In addition, mutagenesis studies on these proteins indicate that the substrate binding pocket lies deep between the transmembrane segments.[3, 4]

The GLUT4 protein is of particular importance as a pharmacological target as it is present only in insulin-responsive tissues.[5] In the basal state, GLUT4 is sequestered into an intracellular reservoir membrane compartment. The exposure of GLUT4 at the cell surface is regulated by insulin signalling, which initiates a signalling cascade that ultimately results in the stimulation of the exocytosis of GLUT4-containing vesicles and the fusion of the vesicles with the plasma membrane.[6–8] This process is defective in Type II diabetes[9–11] and, therefore, methods for rapid monitoring of the extent of the GLUT4 translocation between membrane compartments are needed.

We have developed a series of photoaffinity probes based on a dihexose structure, in which hexose moieties (D-mannose or D-glucose) are linked through their 4-OH positions to a 2-aminopropyl spacer, to probe the cell-surface exposure of GLUT4. Comparisons of aryl azide,[12] benzophenone[13] and aryl diazirinetrifluoroethane[14] photoaffinity labelling derivatives of hexoses have suggested that the latter are most suitable as they are highly efficient cross-linkers with good specificity. A (diazirinetrifluoroethyl)benzoyl moiety[15–17] has been attached through an amide link to the central amine group. More recently a biotinylated version of this compound has been developed in which a linker to biotin is attached through the hydroxy group of the 2-hydroxy-4-(1-diazirine-2,2,2-trifluoroethyl)benzoyl moiety.[18, 19] This compound has been used to assess the cell-surface exposure of GLUT4 in the human muscle of control and Type II diabetic patients and marked impairments in the insulin-stimulated translocation of GLUT4 were evident using this technique.[11]

New applications that were envisaged for the biotinylated photoaffinity probes for GLUT4 (including high-throughput screening of pharmacological reagents) required recognition of the biotin moiety by avidin or anti-biotin antibodies in intact cells. We were initially surprised to find that the first generation of biotinylated hexose affinity probes referred to above (which were used in detergent-solubilised extracts of cells) were not suitable for assays in intact cells. We therefore developed a range

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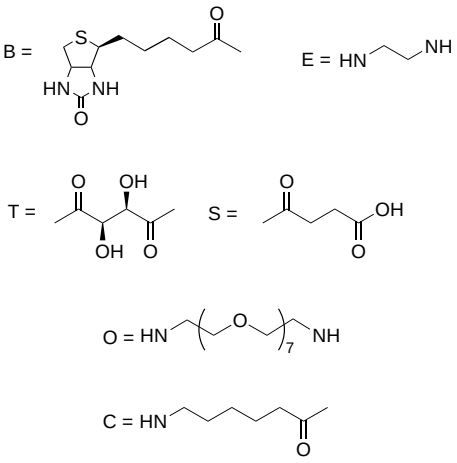
of new compounds with additional spacers between the biotin and the labelling site on the GLUT4 protein. In the course of developing new spacers to allow more exposure of biotin to detection systems, it also seemed desirable to develop a cleavable link in the spacer as this would allow easy release of the tagged protein from the macromolecule detection system.

Results and Discussion

Synthesis of biotinylated spacers

The synthesis of a relatively short spacer, compound **1**, containing biotin was readily accomplished by the sequential solution assembly of biotin with diethylamine, then tartaric acid, and followed by another reaction with diethylamine (Scheme 1). Succinate (from succinic anhydride) was used as a terminal acid

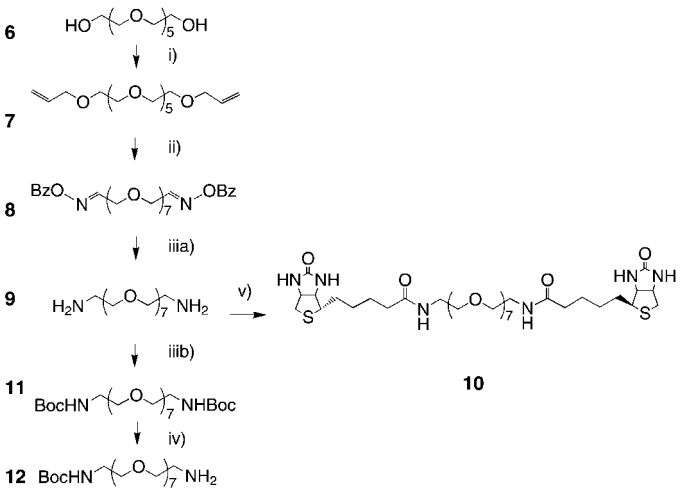


1 B-E-T-E-S
2 B-E-T-E-T-E-S
3 B-O-T-E
4 B-O-T-O-S
5 B-C-O

Scheme 1. *Assembly of biotinylated and tartarate-linked spacer arms. Components were assembled by conventional amide coupling methods with either solid-phase supports (for **1**, **2**) or through solution chemistry employing* N*-hydroxysuccinimide esters (**3** – **5**). An amide bond between the substituents is indicated by a* –.

for convenient coupling to any ligand containing an amino group. Conventional methods for amide bond formation, which involve dicyclohexylcarbodiimide and *N*-hydroxysuccinimide, were initially used. However, solid-phase methods[20] were found to be more convenient for assembly of both E-T-E-S and E-T-E-T-E-S. Release from the support was followed by reaction of the terminal amino group with biotin *N*-hydroxysuccinimide. In this way, compounds **1** and **2** (Scheme 1) were generated in good overall yield (50 – 60% from the carbamate-linked ethylene diamine on Wang resin[20]).

The synthesis of the long spacers **3** – **5** involved generation of a diamino compound **9** from hexaethylene glycol (**6**, Scheme 2). Allylation of **6** using allylbromide and sodium hydride gave **7**. The allyl compound was then oxidised to a diol and cleaved to



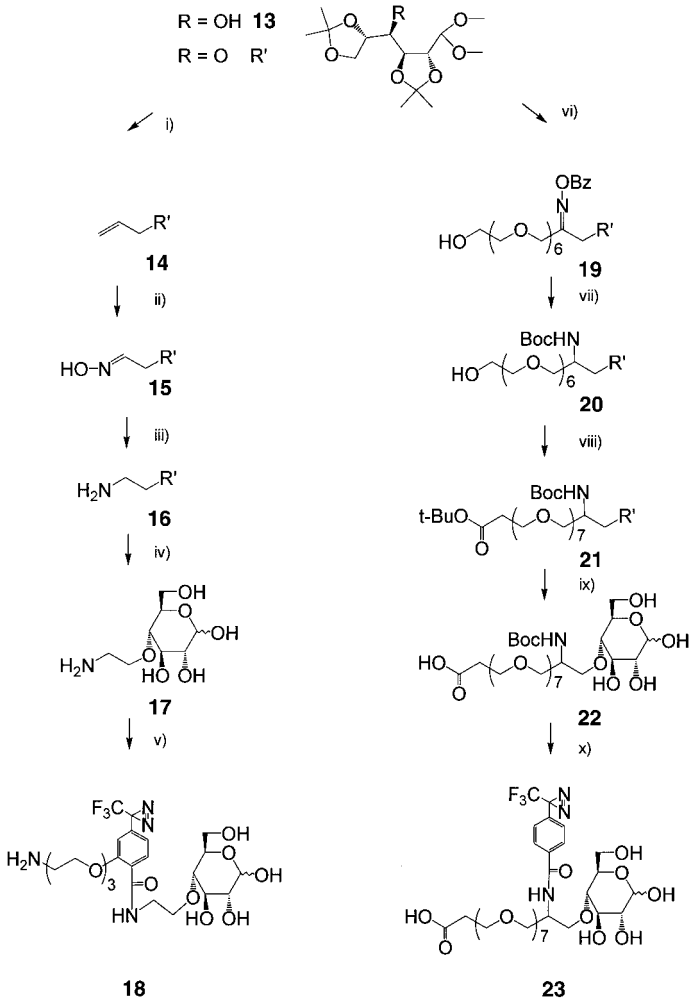
Scheme 2. *Synthesis of a 25-atom spacer from hexaethylene glycol. i) Allylbromide, NaH, DMF, 0 °C, 90%; ii) a. OsO_4, periodate, diethyl ether/water, 0 °C; b. benzylhydroxylamine hydrochloride, pyridine/EtOH, RT, 68%; iii) a. $LiAlH_4$, THF, 0 °C; b. $(Boc)_2$, THF, 0 °C, 55%; iv) TFA/EtOAc, extraction with water then $CHCl_3$, 58%; v) biotin* N*-hydroxysuccinimide, DMF, RT, 70%. Boc* = tert*-butoxycarbonyl, TFA = trifluoracetic acid.*

the aldehyde using catalytic quantities of osmium tetroxide in the presence of sodium periodate. Treatment of the crude aldehyde with benzylhydroxylamine hydrochloride gave the protected oxime **8**, which was purified by silica gel chromatography. Reduction of the oxime with lithium aluminium hydride gave the diamino compound **9**. Attempts to use **9** in solid-phase assembly were unsuccessful. Furthermore, it was found that **9** could not be mono-biotinylated even with very low stoichiometric amounts of biotin *N*-hydroxysuccinimide. The bis-biotin compound **10** was always produced. The bis-biotin compound was most efficiently produced in good yield with a 2:1 ratio of biotin *N*-hydroxysuccinimide to **9**. In order to obtain a mono-biotinylated derivative we therefore synthesised the mono-Boc compound **12**. This was most efficiently accomplished by conversion of **9** into the di-Boc compound **11**. Compound **11** was then partially hydrolysed by brief treatment with trifluoroacetic acid. The mono-Boc compound **12** could be separated from the di-Boc compound **11** using water extraction from an ethyl acetate solution of the hydrolysate. The mono-Boc derivative was then further purified from the bicarbonate-neutralised water layer by extraction into chloroform.

Once obtained, **12** was found to be a very useful and versatile compound for producing the desired mono-biotin and subsequently tartarate-linked derivatives **3** and **4** and the caproate-linked compound **5**. The free amino groups in **3** and **5** were considered suitable for coupling to any ligand (or protein) containing a free carboxylic acid group. Similarly, the succinylated compounds **1**, **2** and **4** were considered useful compounds for coupling to any ligand (or protein) with a free amino group.

Synthesis of hexose photolabels

The 4-OH position has been found to most useful for derivatisation with photoreactive groups as there is good tolerance of bulky groups at this position.[21] We have therefore synthesised a series of compounds starting from the glucose acetal compound **13** (Scheme 3).[22] Allylation of **13** with allyl bromide and sodium hydride gave **14** in quantitative yield. Subsequent oxidation with osmium tetroxide and cleavage with periodate gave an ethanal derivative which was converted into the oxime **15**. Reduction of **15** with lithium aluminium hydride then produced the protected 4-aminoethyl derivative of glucose, **16**. Acid hydrolysis then gave **17**, which was crystallised as the hydrochloride salt. An alternative stategy was adopted to produce a 4-OH linked glucose containing a terminal carboxylic acid group. Compound **13** was first derivatised with 1,3-dichloro-2-benzyloxime (1.5 equivalents) and sodium hydride and then cross-linked by the addition of 2 equivalents of hexaethylene glycol to give **19**. Reduction of the benzyloxime using Raney Nickel as the catalyst gave an amine, which was subsequently protected by conversion into the Boc derivative **20**. Compound **20** was then treated with *tert*-butyl acrylate in the presence of sodium as catalyst to give **21**. Acid deprotection was followed by amino group reprotection to produced **22**. Following purification, **22** could be conveniently deprotected to give the corresponding amine compound. The amine group was considered useful for coupling to aryl diazirinetrifluoroethyl groups while the carboxylic acid group was considered useful for coupling to the biotinylated linkers.



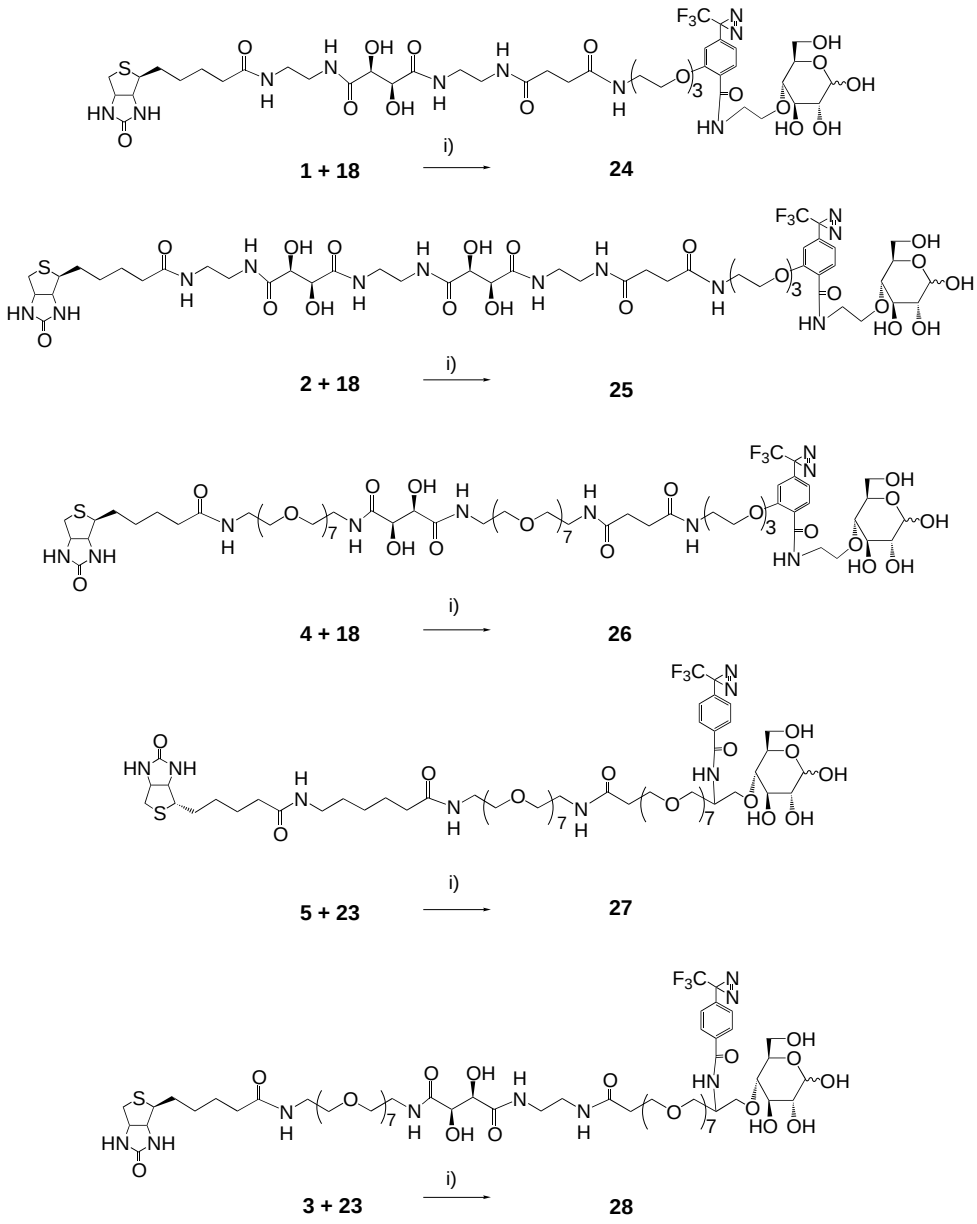
Scheme 3. *Synthesis of 4-OH-substituted* D*-glucoses. i) Allylbromide, NaH, DMF, 0 °C, 88 %; ii) a. OsO_4, periodate, diethyl ether/water, 0 °C, 75 %; b. NH_2OH, HCl, pyridine/ethanol, 83 %; iii) $LiAlH_4$, THF, 0 °C, 67 %; iv) 1 M HCl, 100 °C, 90 min, quantitative; v) a. 2-[2-(2-(2-*tert*-butoxycarbonylaminoethoxy)ethoxy)ethoxy]-4-[1-diazirine-2,2,2-trifluoroethyl] benzoic acid* N*-hydroxysuccinimide ester, DMF, $(Et)_3N$, RT, 50 – 70 %; b. TFA/water, 0 °C; vi) NaH, 1,3-dichloro-2-propane benzyl oxime, DMF, then NaH, hexaethylene glycol, 43 %; vii) H_2, Raney nickel, ethanol, water, then $(Boc)_2$, THF, RT, 73 %; viii)* tert*-butylacrylate, Na, THF, RT, 64 %; ix) a. 1 M HCl, 100 °C, 90 min; b. $(Boc)_2$, THF, RT, 49 %; x) a. TFA, water, 0 °C; b 4-(1-diazirine-2,2,2-trifluoroethyl)benzoic acid* N*-hydroxysuccinimide ester, DMF, $(Et)_3N$, RT, 50 – 70 %.*

Compounds **17** and **22** were converted into photoreactive diazirinyl compounds **18** and **23** with 2-[2-(2-(2-*tert*-butoxycarbonylaminoethoxy)ethoxy)ethoxy]-4-[1-diazirine-2,2,2-trifluoroethyl] benzoic acid *N*-hydroxysuccinimide ester[18, 19] and 4-(1-diazirine-2,2,2-trifluoroethyl) benzoic acid *N*-hydroxysuccinimide ester[23], respectively, with Boc deprotection steps as required. Finally the photoreactive hexose products were amide linked to the biotinylated linkers to give the final products (**24** – **28**, Scheme 4) for biological evaluation.

Biological studies

Each of the compounds was tested as a competitive inhibitor of 2-deoxy-D-glucose uptake into insulin-stimulated rat adipocytes to determine whether the long spacer arms altered the affinity of the interaction between the ligands and GLUT4. The extents of inhibition are indicated by the K_i or half-maximal inhibition values (Figure 1 and Table 1). For comparison, the K_i values for the previously used compounds ATB-BMPA and Bio-LC-ATB-BMPA are listed. It can be seen that introduction of a biotin group, or indeed the very long spacer arms, does not markedly alter the affinity of interaction (Figure 1 and Table 1). The slightly higher K_i values for the glucose derivatives **24** – **28** are in part due to the monohexose structure. We have previously shown that a dihexose structure increases the affinity by about twofold relative to a monohexose compound.[24] The advantage of the dihexose structure lies mainly in the increased hydrophilicity, in contrast to the monohexose compounds in which the hydrophobicity of the aryl diazirinetrifluoroethyl moiety is not so well balanced. The increased hydrophilicity produces compounds that are readily soluble in aqueous buffers and which do not permeate cell membrane lipids. Using the linkers described here, the requirement for a dihexose structure is reduced as the presence of the hydrophilic tartarate groups and amide linkages balances the hydrophobicity of the aryl diazirinetrifluoroethyl group.

After labelling of intact rat adipocytes with compound **24**, we treated the cells with 5 mm periodate for 15 min. This was sufficient to cleave the link to the biotin. Subsequently, biotinylated cells were solubilised in the nonionic detergent Thesit ($C_{12}E_9$) and subjected to a precipitation protocol using streptavidin agarose. In this procedure the non-biotinylated and



Scheme 4. *Assembly of the biotinylated diazirinetrifluorobenzoyl glucose compounds **24** – **28** by coupling of the indicated spacers and photoreactive* D*-glucose compounds. i) DMF, 0.1* M *$NaHCO_3$, RT, 30 – 50 %.*

periodate-cleaved GLUT4 cannot bind and was removed in the washing steps. The bound protein was then eluted and resolved by SDS-PAGE. The GLUT4 was detected using a GLUT4 C-terminal peptide antibody (Figure 2)

The data in Figure 2 suggest that the link to biotin in **24** was accessible to a small molecule, such as periodate, added to intact cells. However, when we assessed the extent to which biotin attached to GLUT4 could combine with avidin in the intact cell situation, we were surprised to find a problem associated with accessibility to this large macromolecule (avidin tetramers have a molecular mass of 60 kDa). This difference in accessibility to periodate and avidin was assumed to be related to a steric constraint imposed by the juxtaposition of two large proteins. One method chosen for assessing the extent of avidin interaction involved precipitation of GLUT4 from a Thesit-solubilised cell lysate. This was followed by resolution of the precipitated protein by SDS-PAGE and detection of the biotin by a streptavidin-HRP polymer, Amdex (Figure 3 a). No significant reduction of the biotin signal from **24** occurred following the avidin treatment in intact cells, suggesting that no complex was formed. Also shown in Figure 3a are comparisons of these data with the shorter compounds Bio-LC-ATB-BMPA and the longer compounds **25**, **26** and **27**. Only the biotin signals from the very long compounds **26** and **27** were reduced following the avidin treatment. The spacer atoms between the photoreactive diazirine and the carbonyl group of the biotin number 21 for Bio-LC-ATB-BMPA and 30, 38, 72 and 63 for compounds **24**, **25**, **26** and **27**, respectively.

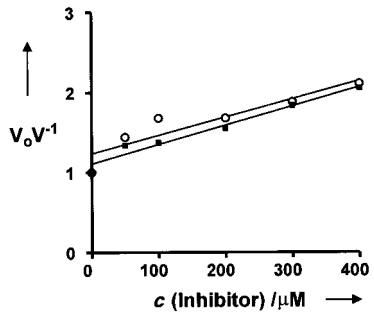


Figure 1. *Determination of affinity constants for biotinylated glucose analogues with varying spacer-arm length. Half maximal inhibition constants (K_i values) for analogue inhibition of glucose-transport activity were determined in insulin-stimulated rat adipocytes. Compound **24** (○) with a 30-atom spacer is compared with compound **26** which has a 72-atom spacer (■).*

We also studied the cell-surface avidin interaction by carrying out streptavidin precipitation of the biotinylated protein, followed by detection with the GLUT4 antibody. No cell-surface avidin inhibition of the GLUT4 signal was detected for compounds **24** or **25** when this alternative detection method was employed (results not shown). Figure 3 b is an example of this type of experiment using the long linker compounds **27** and **28**,

Table 1. *Length of spacer arms for compounds **24** – **28** and several reference compounds, and half maximal inhibition values (K_i) obtained from experiments with these compounds.*

Compound	Spacer length[a]	K_i [μM][b]
ATB-BMPA[c]	none	247
Bio-LC-ATB-BMPA[d]	21	273
24	30	438
25	38	477
26	72	409
27	63	488
28	64	464

[a] The spacer length was taken as the number of atoms between the carboxylate of biotin and the carbon of the diazirine group. [b] The K_i values were from single experiments for compounds **24** and **25** and from the mean of two experiments for compounds **26** – **28**, each with transport rates from at least five concentrations of inhibitor determined in triplicate. [c] See ref. [14]. [d] See ref. [19].

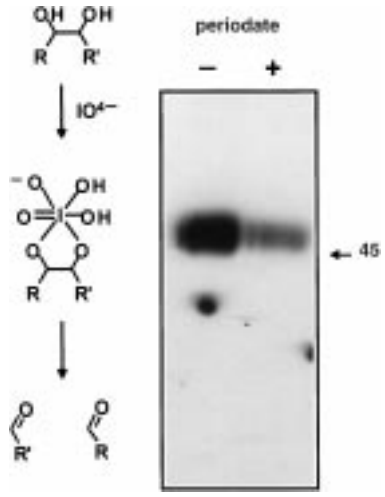


Figure 2. *Periodate cleavage of biotinylated GLUT4 in rat adipose cells. Rat adipose cells were labelled using **24** and the labelled membrane proteins were streptavidin agarose precipitated and resolved by SDS-PAGE, transferred to nitrocellulose and blotted for GLUT4. Treatment with 5 mM periodate for 15 min in intact adipose cells cleaved the central C–C bond in the tartarate group of the spacer to generate two aldehyde groups (left panel, R′ is the biotin moiety and R is the photolabel-GLUT4 adduct). This treatment reduced the GLUT4 signal (right panel).*

which both showed an avidin interaction in the intact-cell situation. Comparable avidin-inhibition results were obtained using either detection method for compound **27** (Figure 3).

We have also studied the interaction of biotinylated GLUT1 from human erythrocytes with an anti-biotin antibody in the presence and absence of competing avidin. The rationale was to establish that the accessibilty problem also existed when a different macromolecule was allowed to interact with a biotinylated glucose transporter in intact cells. The extent of interaction of biotinylated GLUT1 with the FITC-labelled anti-biotin antibody was monitored by confocal microscopy. No signal was detected when GLUT1 was biotinylated with Bio-LC-ATB-BMPA or compounds **24** and **25**. However, when using compounds **27** (with the 63 spacer atoms) we obtained a very sharp punctate signal

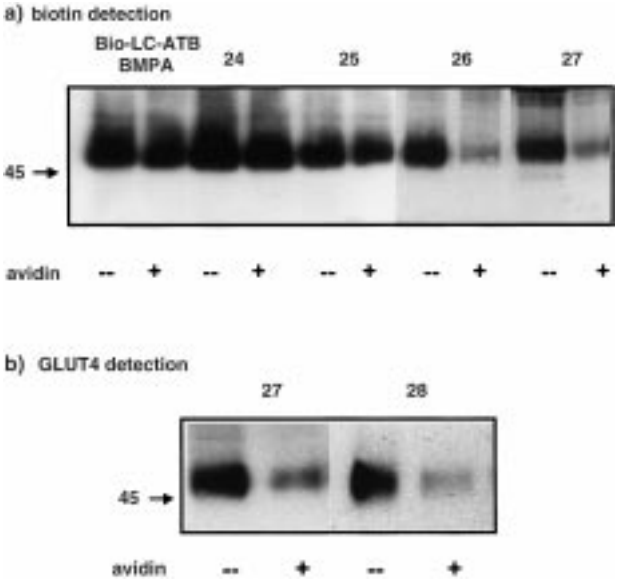


Figure 3. *Avidin interaction with biotinylated GLUT4 requires a very long linker in the photolabelling reagent. In (a) rat adipocyte GLUT4 was photolabelled by Bio-LC-ATB-BMPA and compounds **24** – **27** and immunoprecipitated with a GLUT4 antiserum conjugated to protein-A sepharose. The biotin signal was detected by streptavidin – horse radish peroxidase (HRP) polymer (Amdex). Only the signals from the longer spacer compounds **26** and **27** were reduced by addition of avidin to intact cells. In (b) rat adipocyte GLUT4 was labelled by **27** and **28**, streptavidin agarose precipitated and detected using a GLUT4 antibody. The signals from these long spacer-arm compounds were reduced by addition of avidin to intact cells.*

(Figure 4a) that was completely displaced by competing avidin (not shown) or by competing glucose during the labelling step (Figure 4b).

In conclusion, new linker reagents have been generated and have been shown to have utility in overcoming the problem of detecting a biotin-tagged membrane protein using macromolecules such as avidin and anti-biotin antibodies in the intact cell situation. The reagents contain periodate-cleavable tartarate groups. This feature increases the utility of the compounds as the tartarate groups provide increased hydrophilicity and a means of releasing the tagged protein from the detection system. The incorporation of the long linkers described into suitable ligand probes should facilitate the high-throughput analysis required for drug screening. With the increased accessibility to detection systems, intact cells could be used in multiple-array systems.

Experimental Section

Chemical synthesis: Generally, chemical products were purified by silica gel chromatography using mixtures of ethyl acetate and methanol, or (for the hydrophilic final products) ethyl acetate, methanol and water with 1 – 5% ammonium hydroxide. Protected sugars and the polyethylene glycol derivatives were analysed by ^{1}H NMR spectroscopy (400 MHz, JEOL JNM GX-400) and FAB mass spectrometry (VG Autospec). Deprotected final derivatives were analysed by FAB mass spectrometry. As examples of the synthesis methods employed, we describe below the synthesis of the key

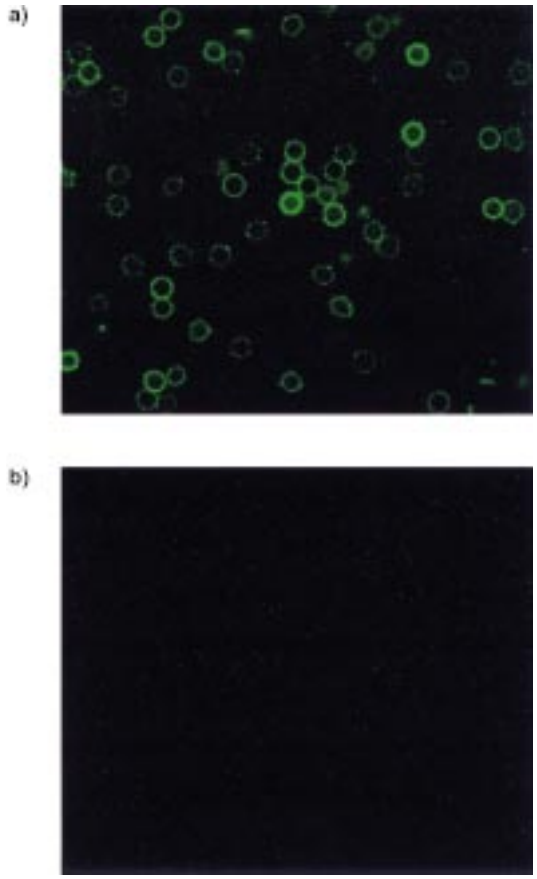


Figure 4. *Confocal images of biotinylated GLUT1 in human erythrocytes. Compound* ***27*** *(0.5 mM) was used to biotinylate GLUT1 either in the absence (a) or presence (b) of 0.5 M glucose. Following labelling, the cells were washed to remove excess reagents, conjugated with fluoroscein-isothiocyanate-labelled anti-biotin antibody and then examined by confocal microscopy with a* $\times$ *64 lens and 488 nm excitation of the fluorescein-tagged transporter. The z-plane focal image illustrates cell-surface labelling.*

spacer intermediate **12** and the key glucose carboxylic acid intermediate **22**. The routes to the aminoethyl glucose compound **17** employed similar methods to those described for **22** (ether formation, oxime reduction and acetal hydrolysis). All other compounds were produced by amide coupling in solution or on solid phase supports.

Preparation of the mono-Boc polyoxyethylene spacer compound 12: NaH (60% with oil, 8.4 g, 210 mmoles) was added to hexaethyleneglycol (**6**; 20 g, 71 mmoles) in DMF (100 mL) at 0–4 °C. After 30 min, allylbromide ($d = 1.4$, 20 mL, 231 mmoles) was added and the reaction was stirred at 0–4 °C for 3 h. MeOH (5 mL) was then added to quench the reaction, and the solution was partitioned between water and CH_2Cl_2. The water layer was further extracted twice with CH_2Cl_2 and the combined CH_2Cl_2 layers were dried over Na_2SO_4. Evaporation under reduced pressure afforded **7** as colourless oil (23 g, 64 mmoles, 90%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 5.92$ (2H, m, ^3J(H,H) = 18.8, 10.5, 5.5, 1.2 Hz, CH_2CH), 5.27 (2H, d, ^3J(H,H) = 18.8 Hz, CH_2CH), 5.18 (2H, d, ^3J(H,H) = 10.5 Hz, CH_2CH), 4.03 (4H, d $\times$ 2, ^3J(H,H) = 5.5, 1.2 Hz, OCH_2), 3.68–3.57 (24H, m, OCH_2CH_2O); FAB-MS: *m/z* 363 $[M+H]^+$, 385 $[M+Na]^+$.

Compound **7** (21.7 g, 60 mmoles) in diethyl ether (100 mL) was stirred vigorously with water (100 mL). OsO_4 (2.5% solution in 2-methyl-2-propanol, 8.75 mL) and $NaIO_4$ (50 g, 234 mmoles) were then added. The solution was stirred for 4 h at room temperature. MeOH (100 mL) was added and the precipitated salts were removed by filtration through celite. The solution was concentrated and EtOH (300 mL) and pyridine (150 mL) were added, followed by benzyl hydroxylamine hydrochloride (22 g, 138 mmoles). After 4 h at room temperature, the solution was concentrated and partitioned between water and CH_2Cl_2. The water layer was further extracted twice with CH_2Cl_2 and the combined CH_2Cl_2 layers were dried over Na_2SO_4, concentrated and applied to a silica gel column equilibrated in EtOAc:light petroleum ether (1:1). The product was eluted with EtOAc:2% MeOH. This was concentrated to afford **8** as a colourless oil (23.5 g, 41 mmoles, 68%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 7.51$ (1H, t, ^3J(H,H) = 5.9 Hz, NCH), 7.38–7.28 (10H, m, Ph), 6.90 (1H, t, ^3J(H,H) = 3.5 Hz, NCH), 5.09 and 5.08 (each 2H, s, OCH_2Ph), 4.34 (2H, d, ^3J(H,H) = 3.5 Hz, OCH_2), 4.12 (2H, d, ^3J(H,H) = 5.9 Hz, OCH_2), 3.66–3.58 (24H, m, OCH_2CH_2O); FAB-MS: *m/z* 577 $[M+H]^+$, 599 $[M+Na]^+$.

$LiAlH_4$ (1 M solution in THF, 100 mmoles) was added to **8** (21.8 g, 38 mmoles) in THF (600 mL) at 0–4 °C. After 4 h, 10 M NaOH (5 mL) and enough water to decompose the excess $LiAlH_4$ were added slowly at 0–4 °C. The precipitated salts were removed by filtration through Celite and di-*tert*-butyl-dicarbonate (1 M solution in THF, 80 mmoles) was added. After 1 h, the solution was concentrated and partitioned between EtOAc and water. The water layer was further extracted three times with EtOAc. The combined organic layer was washed with saturated NaCl solution and dried over Na_2SO_4. The solution was filtered, concentrated and applied to a silica gel column equilibrated in EtOAc. The product was eluted with EtOAc:2–10% MeOH. Concentration then afforded **11** as a colourless oil (11.9 g, 21 mmoles, 55%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 3.68–3.60$ (24H, m, OCH_2CH_2O), 3.54 (4H, t, ^3J(H,H) = 5.1 Hz, OCH_2), 3.31 (4H, t, ^3J(H,H) = 5.1 Hz, CH_2NCO), 1.44 (18H, s, $C(CH_3)_3$); FAB-MS: *m/z* 569 $[M+H]^+$, 591 $[M+Na]^+$.

Compound **11** (0.28 g, 0.5 mmol) was dissolved in EtOAc (3 mL), and TFA (1 mL) was added at 0–4 °C. The reaction mixture was heated at 40 °C for 30 min and then partitioned between EtOAc and water. The EtOAc layer was extracted three times with water. The EtOAc layer contained **11**, which was reutilised. The combined aqueous phase was neutralised with saturated $NaHCO_3$ and extracted five times with $CHCl_3$. The combined $CHCl_3$ layers were dried over Na_2SO_4, filtered and concentrated to afford **12** as a colourless oil. (0.14 g, 0.29 mmoles, 58%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 3.66–3.57$ (24H, m, OCH_2CH_2O), 3.54 (4H, t, ^3J(H,H) = 4.7 Hz, OCH_2), 3.32 (2H, t, ^3J(H,H) = 4.7 Hz, CH_2NCO), 2.93 (2H, br.t, CH_2N), 1.44 (9H, s, $C(CH_3)_3$); FAB-MS: *m/z* 469 ($[M+H]^+$).

Preparation of the glucose carboxylic acid compound 22: Compound **13** (1.84 g, 6 mmoles) was dissolved in DMF (15 mL). NaH (60% with oil, 360 mg, 9 mmoles) was added and then, after 15 min at 0–4 °C, 1,3-dichloro-2-propanone-*O*-benzyloxime[25] (2.08 g, 9 mmoles) was added with rapid mixing. The reaction was then stirred for a further 4 h at room temperature. Monitoring the progress of the reaction by thin layer chromatography in hexane: EtOAc (4:2) revealed formation of the monochloro intermediate and consumption of **13** (R_F of the intermediate 0.6, R_F of **13** 0.25). Compound **6** (3.4 g, 12 mmoles) in DMF (15 mL) and NaH (60% with oil, 360 mg, 9 mmoles) were then added and the reaction was left overnight at room temperature. AcOH (2 mL) was added slowly followed by enough water to quench the reaction. The product was partitioned between CH_2Cl_2 and water and the water layer was further extracted twice with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated and the product was applied to a silica gel column equilibrated with EtOAc:light petroleum ether (1:1). Impurities and residual DMF were eluted with EtOAc and the product was eluted with EtOAc:1% MeOH. The product was

concentrated to give **19** as a colourless oil (2.15 g, 2.88 mmoles, 48%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 7.38 - 7.28$ (5H, m, Ph), 5.09 and 5.07 (2H, s × 2, CH_2Ph), 4.46 – 4.14 (4H, m, OCH_2), 4.35 (1H, d × 2, $^3J(H,H) = 6.4$ Hz, H-1), 4.22 (1H, m, H-5), 4.11 (1H, m, H-2), 4.04 – 3.94 (3H, m, H-3 and H-6), 4.30 – 4.15 (4H, m, OCH_2), 4.08 – 3.94 (4H, m, OCH_2), 3.65 – 3.56 (25H, m, OCH_2CH_2O and H-4), 3.42 – 3.39 (6H, m, OCH_3), 1.42 – 1.33 (12H, m, $C(CH_3)_2$); FAB-MS: *m/z* 770 $[M+Na]^+$.

Compound **19** (2.6 g, 3.48 mmoles) in EtOH (10 mL) and Raney Nickel (50% suspension in water, 10 mL) were stirred rapidly under hydrogen for 2 h. The solution was filtered and concentrated and then dissolved in THF (20 mL). Saturated $NaHCO_3$ solution was added to bring the pH value to 8.0 and then this solution was rapidly stirred with di-*tert*-butyl-dicarbonate (1 M solution in THF, 6 mmoles). After 30 min at room temperature, the solution was partitioned between CH_2Cl_2 and water. The water layer was further extracted twice with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated and then applied to silica gel column eqilibrated in EtOAc. The product was eluted using EtOAc:3 – 6% MeOH and concentrated to give **20** as a colourless oil (1.9 g, 2.54 mmoles, 73%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 4.37$ (1H, d × 2, $^3J(H,H) = 6.4$ Hz, H-1), 4.22 (1H, m, H-5), 4.11 (1H, m, H-2), 4.04 – 3.93 (3H, m, H-3 and H-6), 3.90 – 3.65 (3H, m, CHN and OCH_2), 3.65 – 3.56 (27H, m, OCH_2, OCH_2CH_2O and H-4), 3.48 – 3.38 (6H, m, OCH_3), 1.44 – 1.34 (21H, m, $C(CH_3)_2$ and $C(CH_3)_3$); FAB-MS: *m/z* 744 $[M+H]^+$, 766 $[M+Na]^+$.

Compound **20** (1.75 g, 2.36 mmoles) in THF (20 mL) was stirred at room temperature with Na (11 mg, 0.5 mmoles). After 30 min, *tert*-butylacrylate ($d = 0.875$, 500 μL, 3.4 mmoles) was added and the reaction was stirred for 3 h. AcOH (30 μL) was then added to neutralise the solution. The solution was concentrated and applied to a silica gel column equilibrated in EtOAc. The product was eluted with EtOAc:0.2 – 1% MeOH to give **21** as a colourless oil (1.31 g, 1.51 mmoles, 64%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 4.37$ (1H, d × 2, $^3J(H,H) = 6.4$ Hz, H-1), 4.22 (1H, m, H-5), 4.11 (1H, m, H-2), 4.03 – 3.94 (3H, m, H-3 and H-6), 3.90 – 3.69 (3H, m, CHN and OCH_2), 3.69 – 3.57 (29H, m, OCH_2, OCH_2CH_2O and H-4), 3.48 – 3.37 (6H, m, OCH_3), 2.50 (2H, t, CH_2COO, $^3J(H,H) = 6.6$ Hz), 1.44 – 1.32 (total 30H, m, $C(CH_3)_2$ and $C(CH_3)_3$); FAB-MS: *m/z* 894 $[M+Na]^+$.

Compound **21** (1.27 g, 1.46 mmoles) in 1 M HCl (6 mL) was heated at 100 °C for 90 min. The solution was cooled and neutralised with NaOH (10 M solution, 6 mmoles). Thin layer chromatography analysis (MeCN:EtOH:AcOH:water; 9:1:1:1) revealed a single product (R_F 0.3). The amine was converted into its *N*-Boc-protected derivative for purification and analysis. The solution of amine was concentrated to ca. 1 mL and the pH value was further adjusted to 8.5 by careful addition of 10 M NaOH. Di-*tert*-butyl-dicarbonate (1 M solution in THF, 3 mmoles) was added and the solution was vigourously stirred at room temperature for 16 h. The solution was concentrated and dissolved in EtOAc:MeOH (1:1). Precipitated salts were removed by filtration and, after further concentration, the solution was applied to a silica gel column equilibrated in EtOAc:MeOH (1:1). The product was then eluted with EtOAc:MeOH:water (12:6:1) to give **22** as a slightly yellow oil (0.49 g, 0.71 mmoles, 49%). ^{1}H NMR (400 MHz, D_3O, TMS): $\delta = 5.25$ and 4.60 (together 1H, d and d, $^3J(H,H) = 2.9$, 7.8 Hz, H1-α and H1-β respectively), 4.20 – 3.15 (37H, m, all other protons), 2.48 (2H, t, $^3J(H,H) = 6.6$ Hz, CH_2COO), 1.52 – 1.40 (9H, m, $C(CH_3)_3$); FAB-MS: *m/z* 712 $[M+Na]^+$.

Preparation of cells, and assays of glucose transport activity: Human erythrocytes[26] or rat adipose cells[14] were prepared as previously described. Rat adipocytes were stimulated with 20 nM insulin and incubated with the indicated inhibitors at concentrations of up to 800 μM, before addition of the transported substrate, 2-deoxy-D-{^{3}H}-glucose, at a trace concentration (50 μM) that was well below its K_m value. The rate constant for uptake in the presence (V) and absence (V_o) of inhibitor (I) were then used to calculate the K_i value according to the equation $V_o/V = 1 + I/K_i$.[14]

Photoaffinity labelling: Human erythrocytes[26] or rat adipose cells[14] were maintained at 18 °C in the presence of 300 – 500 μM photoreactive probe for 1 min either in the presence or absence of the competing ligands, as described in the figure legends. The erythrocyte and adipocyte samples were then irradiated for 1 min in a Rayonet photochemical reactor using 300 nm bulbs. Excess reagent was removed by extensive washing in physiological buffers[19] and then, where indicated in the figure legends, 0.3 μM avidin was added to block the cell-surface tagged transporters. Excess avidin was then removed by washing the cells.

Detection of biotinylated GLUT4 by blotting: Cell samples were homogenised in HEPES/EDTA/sucrose (HES) buffer (255 mM sucrose, 1 mM ethylenediaminetetraacetate (EDTA), 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 1 μg mL^{-1} antipain, aprotinin, pepstatin and 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 (554000 g for 30 min at 4 °C) to obtain a total membrane fraction. This pellet was solubilized in phosphate buffer saline (PBS; pH 7.2) with 2% of Thesit ($C_{12}E_9$) and the protease inhibitors (antipain, aprotinin, pepstatin and leupeptin, each at a concentration of 1 μg mL^{-1}) and 100 μM AEBSF. The samples were solubilized for 50 min at 4 °C with rotation and were then subjected to centrifugation (20000 g for 20 min at 4 °C). Biotinylated proteins in the supernatants were either immunoprecipitated using a GLUT4 antiserum as previously described[19] or were precipitated with streptavidin beads (Pierce, Rockford, IL). Following GLUT4 immunoprecipitation, complexes were released into electophoresis-sample buffer (62.5 mM tri(hydroxymethyl)aminomethane (Tris; pH 6.8), 2% SDS, 10% glycerol) at room temperature. The steptavidin precipitates were washed four times with PBS buffer containing 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 (2300 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 electrophoresis-sample buffer and these samples were then subjected to SDS-PAGE (10% gel). Proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline – 0.1% Tween (TBS-T) and washed six times with TBS-T. Membranes were either incubated with streptavidin-HRP (Amdex, Amersham) or with affinity-purified anti-GLUT4 C-terminal antibody[14] in TBS-T containing 1% bovine serum albumin (BSA; 2 h at room temperature), followed by washing (six times in TBS-T) and detection using secondary antibody linked to horseradish peroxidase. GLUT4 protein was visualized with enhanced chemiluminescence (ECL).

Detection of biotinylated GLUT1 by fluorescence microscopy: Human erythrocytes were photolabelled as described above and then washed five times in PBS with 3% goat serum and 1% BSA to remove excess ligand. The erythrocytes were then mixed with a 1:25 dilution of the manufacturers (Vector Lab) solution of fluorescein isothiocyanate labelled anti-biotin antibody at 4° C for 2 h and then washed six times in PBS with 3% goat serum and 1% BSA. Confocal

images were obtained using a Zeiss LSM510 system with a × 64 lens and 488 nm excitation. *z*-plane focal sections were obtained to demonstrate cell-surface labelling.

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