

Glycosylated cell-penetrating peptides and their conjugates to a proapoptotic peptide: preparation by click chemistry and cell viability studies

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Abstract Cell-penetrating peptides (CPPs), which are usually short basic peptides, are able to cross cell membranes and convey bioactive cargoes inside cells. CPPs have been widely used to deliver inside cells peptides, proteins, and oligonucleotides; however, their entry mechanisms still remain controversial. A major problem concerning CPPs remains their lack of selectivity to target a specific type of cell and/or an intracellular component. We have previously shown that myristoylation of one of these CPPs affected the intracellular distribution of the cargo. We report here on the synthesis of glycosylated analogs of the cell-penetrating peptide (R6/W3): Ac-RRWRRR-NH₂. One, two, or three galactose(s), with or without a spacer, were introduced into the sequence of this nonapeptide *via* a triazole link, the Huisgen reaction being achieved on a solid support. Four of these glycosylated CPPs were coupled *via* a disulfide bridge to the proapoptotic KLAK peptide,

(KLAKLAKKLAKLAK), which alone does not enter into cells. The effect on cell viability and the uptake efficiency of different glycosylated conjugates were studied on CHO cells and were compared to those of the nonglycosylated conjugates: (R6/W3)S-S-KLAK and penetratinS-S-KLAK. We show that glycosylation significantly increases the cell viability of CHO cells compared to the nonglycosylated conjugates and concomitantly decreases the internalization of the KLAK cargo. These results suggest that glycosylation of CPP may be a key point in targeting specific cells.

Keywords Cell-penetrating peptide · Glycosylation · Click chemistry · Triazole · Apoptose

Introduction

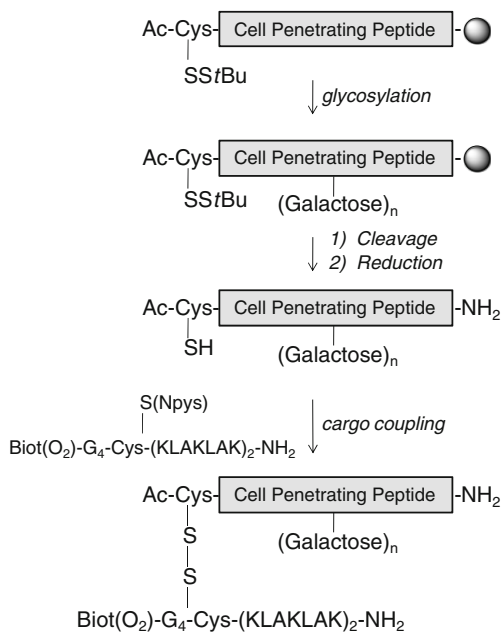
The possibility for a highly basic peptide to enter into cells was viewed as an artifact for a long time, as this was in total contradiction with the established dogma on the impermeability of the plasma membrane to cationic species [1, 2]. Today, plethora of basic peptides which correspond to segments of natural proteins, synthetic peptides, or platforms have been described and are classified as cell-penetrating peptides (CPPs) [3–6]. It has also been demonstrated that CPPs have the capacity to shuttle bioactive cargoes inside eukaryotic cells. The final localization in the cell of the CPP/cargo conjugates or complexes is undoubtedly associated with the mechanism of entry; however, the mechanism is still an ongoing matter of debate [7–17]. The capacity for CPPs to deliver a bioactive molecule inside of cells has opened avenues for intracellular pharmacology, and recently, significant advances have been accomplished for siRNA delivery and also for specific

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Scheme 1 Schematic view depicting the strategy for the syntheses of CPP conjugates with the proapoptotic KLAFLAK peptide. The CPP, without or with a Pra residue at the desired position, are assembled on a solid support by Fmoc strategy, the N-terminal residue being Ac-CysS-S*t*Bu. The galactose(s) moiety is introduced on the peptidyl-resin *via* click chemistry. Cleavage is performed on the solid support and followed by purification. The cysteine side chain is deprotected to conjugate the KLAFLAK cargo, which contains an electrophilic cysteine (Cys-S*N*pys, *N*pys 3-nitropyridinesulfonyl). The CPP cargo conjugate is finally purified by HPLC

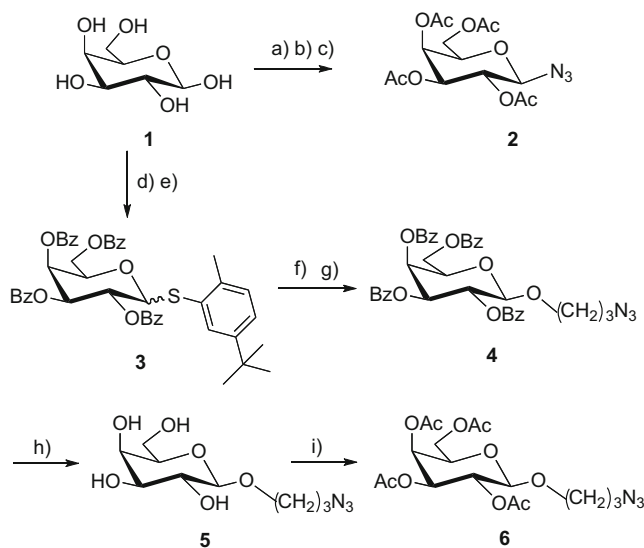
cellular targeting with homing peptides to name a few examples [16, 17].

Our pioneering work on penetratin along with Alain Prochiantz's team was started 15 years ago [7]. We have synthesized minimalist analogs of CPPs, and we have studied the effect of CPP myristoylation on the delivery of a peptide cargo [18]. This work has now led us to explore the influence of glycosylation on the cellular uptake, taking as a starting point our lead (R6/W3) CPP sequence [19–21]. Glycosylation of bioactive compounds has been used mainly for increasing either the hydrophilicity, the enzymatic stability of the compound, and/or its delivery into the brain [22–25]. In the context of CPPs, we have hypothesized that glycosylation may regulate and or affect the internalization of the corresponding glycosylated analogs of (R6/W3) [26–30]. According to the strategy depicted in Scheme 1, we have synthesized glycosylated analogs of (R6/W3), in which galactose unit(s) linked *via* click chemistry replace(s) one, two, or the three tryptophans. We have subsequently analyzed the viability of CHO cells treated by conjugates with the proapoptotic peptide KLAFLAK, [31–33] and have visualized their localizations in these cells by fluorescence.

Results and discussion

Syntheses of azido galactose analogs 2, 5, and 6

Two series of azido-functionalized galactose derivatives have been prepared: compound **2** with the azido group directly attached on the anomeric position of the tetra-acetylated galactose and analogs with a $(O-CH_2)_3$ spacer on the anomeric position, with either *OBz* **4**, free OH **5**, or *OAc* **6**. The syntheses of these analogs are summarized in Scheme 2. The analog 1-azido-2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranoside **2** was prepared from galactose **1**, as previously described [22]. The (2-methyl-5-tert-butylphenyl)-2,3,4,6-tetra-*O*-benzoyl-1-thio- β -D-galactopyranoside **3** was prepared, according to a strategy we developed recently in the mannose series, which turned out to be very convenient to modify the anomeric position [34]. Compound **3** was obtained with a good yield and was further reacted with 3-bromopropan-1-ol catalyzed by *N*-bromosuccinimide in the presence of triflic acid. This reaction was followed by a substitution to afford 3-azidopropyl-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranoside **4**. The benzoates were subsequently removed to yield 3-azidopropyl- β -D-galactopyranoside **5**, which was then acetylated leading to 3-azidopropyl-2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranoside **6**. It is worth mentioning that compound **6** may also be obtained from galactose **1**, as previously described [35, 36].



Scheme 2 Reagents and conditions a) Ac_2O , dry pyridine, 99%; b) HBr/CH_3COOH , $CHCl_3$, 2 h, RT, 64%; c) NaN_3 , acetone, 60 °C, 94%; d) $BzCl$, dry pyridine, 0 °C, Argon; e) 2-Methyl-5-tert-butylthiophenol, $BF_3 \cdot Et_2O$, dry CH_2Cl_2 , Argon, 74%; f) 3-bromopropan-1-ol, NBS, $TfOH$, 4 Å molecular sieves, dry CH_2Cl_2 , Argon, 72%; g) NaN_3 , dry DMF, 60 °C, 81%; h) Na, dry MeOH, 82%; i) Ac_2O , dry pyridine, 85%

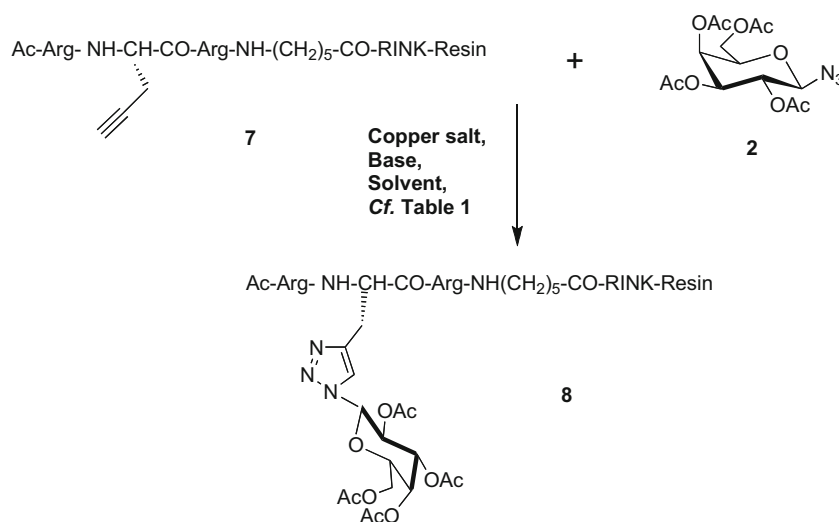
Click reaction on a solid support, general conditions with Ac-Arg-Pra-Arg-Ahx-Rink-amide resin

All of the glycopeptides synthesized are analogs of the CPP (R6/W3). One, two, or the three tryptophans of the (R6/W3) have been substituted by a protected or unprotected galactose-containing residue. Galactose moieties were covalently linked by a triazole linkage *via* the Huisgen reaction [37, 38]. Using the model tripeptide **7**, Ac-Arg-Pra-Arg-Ahx-CONH₂ (Pra standing for propargyl amino acid, Fmoc strategy), we first searched for the optimal conditions to couple the azidosugar to the propargyl residue on to the solid support. The glycosylated peptide Ac-Arg-[Pra(Gal(OAc))-Arg-Ahx-CONH₂ **8** was obtained after cleavage from the resin with standard conditions (TFA/H₂O/TIS), Scheme 3 (refer to [Supplementary files](#)). It is worth noting that these conditions are not detrimental to the carbohydrate and the triazole link [39, 40]. The reproducibility and sensitivity of IR analysis (alkyne signal at 2,200 cm⁻¹) was not sufficient to monitor the reaction by taking aliquots of the resin [41, 42]. Azidorhodamine analog **9** (refer to [Supplementary files](#)), prepared from rhodamine B, exhibited fluorescence quenching (approximately ten times) when covalently linked *via* a triazole to the solid support, as previously reported [43]. This analog, therefore, cannot be used for the quantification of remaining Pra residues on the support. It should be noted that no quenching was observed when azidorhodamine analog **9** was dissolved in the same solvent in the presence of a Rink amide resin. The different glycosylated peptidyl-resin

samples were cleaved from the solid support and analyzed by RP-HPLC. Triazole formation was found to be complete within 5 h with 2 eq. of sugar **2**. Four eq. of Cu(OAc)₂ or 2 eq. of CuI were efficient in the presence of 2 eq. of ascorbic acid and 5 eq. of diisopropylamine, in DMF containing 30% of either pyridine, lutidine, or piperidine. Five hours of coupling reaction time was estimated to be the best compromise for the equivalents of azido-sugar which were required for the reaction (refer to [Supplementary files](#)). Only the β-anomer was detected in solution as demonstrated by NMR analysis (data not shown).

Syntheses of the glycopeptide analogs of the cell-penetrating peptide (R6/W3)

Using these optimal conditions, all of the glycopeptides listed in Table 1 were synthesized, as described for the model glycopeptide **8**, starting from the propargyl peptides **12**, **13**, or **14**. Glycopeptides **15** and **16** contain one galactose unit, with acetylated or free OH, respectively, while **17** and **18** have two and three acetylated galactose units, respectively. Glycopeptide **19** possesses three galactose units, all OH being free. Glycopeptides **20–22** all have a single galactose unit with a propyl spacer between the sugar and the triazole, with either acetylated (**20**), benzoylated (**21**), or free OH (**22**). After cleavage from the Rink amide resin, all of the peptides were purified by RP-HPLC (for yields and characterization by MALDI–TOF mass spectrometry, refer to [Supplementary files](#)). It should be noted that the *t*Bu-protecting group of the cysteine residue



Scheme 3 Optimization of the coupling reaction on to a solid support with a model peptidyl-resin. The model tripeptide, Ac-Arg-Pra-Arg-aminohexanoyl-resin, assembled on a Rink-amide resin, was used to optimize the conditions for coupling the azido galactose **2**. The various parameters (copper salt, base, solvent, and coupling time) were analyzed; see Table 1 in [Supplementary materials](#). The evolution

of the coupling reaction (ratio of starting peptide vs peptide **8**) was monitored after the cleavage from the resin and HPLC analysis. Triazole formation was complete within 5 h with 2 eq. of sugar **2**. Four eq. of Cu(OAc)₂ or 2 eq. of CuI were both efficient in the presence of 2 eq. of ascorbic acid and 5 eq. of diisopropylethylamine, in DMF containing 30% of pyridine, lutidine, or piperidine

Table 1 Peptides sequences and abbreviations

Peptides and primary sequences	Abbreviations
10 Ac-C(SS <i>t</i> Bu)-RRWRRWRR-NH ₂	(R6/W3)S-S <i>t</i> Bu
11 Ac-C-RRWRRWRR-NH ₂	(R6/W3)-SH
12 Ac-C(SS <i>t</i> Bu)-RRW-Pra-RRWRR-NH ₂	(R6/W2)-Pra-S-S <i>t</i> Bu
13 Ac-C(SS <i>t</i> Bu)-RRW-Pra-RR-Pra-RR-NH ₂	(R6/W1)-Pra ₂ -S-S <i>t</i> Bu
14 Ac-C(SS <i>t</i> Bu)-RR-Pra-Pra-RR-Pra-RR-NH ₂	(R6/W0)-Pra ₃ -S-S <i>t</i> Bu
15 Ac-C(SS <i>t</i> Bu)-RRW-[Pra-Gal(OAc)]-RRWRR-NH ₂ (from click reaction of 12 with 2)	(R6/W2)-[Pra-Gal(OAc)]S-S <i>t</i> Bu
16 Ac-C-RRW-[Pra(Gal-OH)]-RRWRR-NH ₂ (from de- <i>O</i> -acylation/reduction of 15)	(R6/W2)-[Pra-Gal(OH)]-SH
17 Ac-C(SS <i>t</i> Bu)-RRW-[Pra-Gal(OAc)]-RR-[Pra-Gal(OAc)]-RR-NH ₂ ^a (from click reaction of 13 with 2)	(R6/W1)-[Pra-Gal(OAc)] ₂ S-S <i>t</i> Bu
18 Ac-C(SS <i>t</i> Bu)-RR-[Pra-Gal(OAc)] ₂ -RR-[Pra-Gal(OAc)]-RR-NH ₂ (from click reaction of 14 with 2)	(R6/W0)-[Pra-Gal(OAc)] ₃ S-S <i>t</i> Bu
19 Ac-C-RR-[Pra(Gal-OH)] ₂ -RR-[Pra-Gal(OH)]-RR-NH ₂ (from de- <i>O</i> -acylation/reduction of 18)	(R6/W0)-[Pra-Gal(OH)] ₃ -SH
20 Ac-C(SS <i>t</i> Bu)-RRW-[Pra-Gal(CH ₂) ₃ (OAc)]-RRWRR-NH ₂ (from click reaction of 12 with 5)	(R6/W2)- [Pra-Gal(CH ₂) ₃ (OAc)]S-S <i>t</i> Bu
21 Ac-C(SS <i>t</i> Bu)-RRW-[Pra-Gal(CH ₂) ₃ (OBz)]-RRWRR-NH ₂ (from click reaction of 12 with 4)	(R6/W2)-[Pra-Gal(CH ₂) ₃ (OBz)]S-S <i>t</i> Bu
22 Ac-C(SS <i>t</i> Bu)-RRW-[Pra-Gal(CH ₂) ₃ (OH)]-RRWRR-NH ₂ ^a (from click reaction of 12 with 6)	(R6/W2)-[Pra-Gal(CH ₂) ₃ (OH)]S-S <i>t</i> Bu
23 Ac-C-RRW-[Pra-Gal(CH ₂) ₃ (OH)]-RRWRR-NH ₂ (from reduction of 22)	(R6/W2)-[Pra-Gal(CH ₂) ₃ (OH)]-SH
24 Ac-C-RRW-[Pra-Gal(CH ₂) ₃ (OAc)]-RRWRR-NH ₂ (from reduction of 20)	(R6/W2)-[Pra-Gal(CH ₂) ₃ (OAc)]-SH

Pra propargylglycine, *Pra-Gal(OH)* β-D-galactopyranoside triazole, *Pra-Gal(OAc)* 2,3,4,6-tetra-acetyl-Pra-Gal(OH), *Pra-Gal(CH₂)₃(OH)* 3-(β-D-galactopyranosyloxy)propyl triazole, *Pra-Gal(CH₂)₃(OAc)* 2,3,4,6-tetra-*O*-acetyl-Pra-Gal(CH₂)₃(OH), *Pra-Gal(CH₂)₃(OBz)* 2,3,4,6-tetra-*O*-benzyl-Pra-Gal(CH₂)₃(OH)

^a Fig. 1

does not interfere with the Huisgen reaction, which required the presence of ascorbic acid to reduce Cu²⁺ to Cu⁺ species. Galactose unit(s) in **15** and **18** was(were) de-*O*-acetylated using ammonia in methanol to give **16** and **19**, respectively. During this deacetylation step, the S-S*t*Bu bond was partially cleaved, leading to a mixture of S-S*t*Bu peptides and the free thiol analogs, which were isolated and identified by MALDI-TOF mass spectrometry. Reduction by DTT was required for complete removal of S*t*Bu. The SH glycopeptides were then used in the coupling reaction with the cargo (see below).

Syntheses of conjugates with the proapoptotic peptide KLAKE

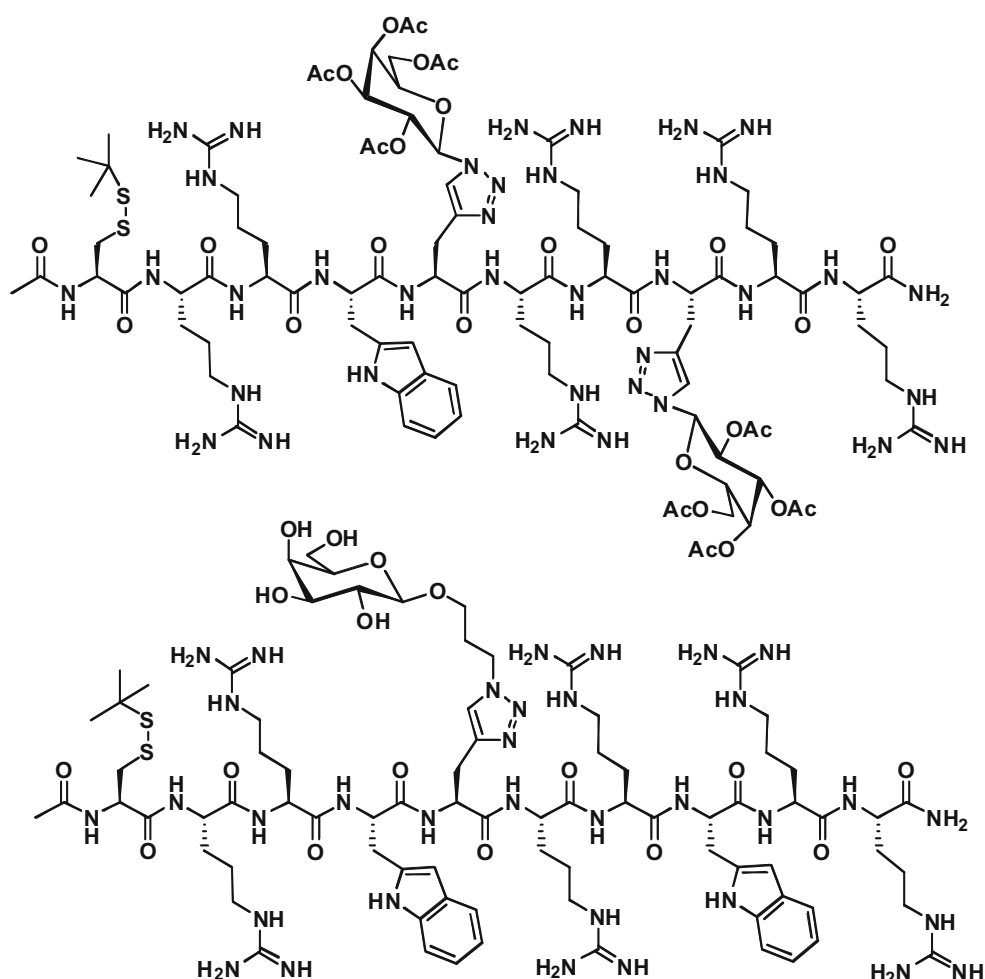
The proapoptotic peptide KLAKE (KLAKEKLAKE) does not enter into cells and does not affect cell viability, but KLAKE induces cell death when delivered to the mitochondria by a cell-penetrating peptide [31–33]. Four of the glycopeptides, **16**, **19**, **23**, and **24**, were conjugated through a labile disulfide linker to a biotinylated analog of KLAKE: Biotin(O₂)-(Gly)₄-C(Npys)-KLAKEKLAKE-NH₂, **25**. The KLAKE peptide was modified in the N-terminal region for further characterization of its cellular delivery either by fluorescence (biotin revealed with

fluorescent streptavidin) and/or by mass spectrometry (¹H-Gly or ²H-Gly) [46]. The activated protecting group of cysteine (Npys) allows for a rapid and efficient coupling to a thiol-containing peptide [18, 20, 44, 45]. Conjugates with (R6/W3) and penetratin (Ac-CRQIKIWFQNRRMKWKK-NH₂) were also synthesized as references, molecules **26** and **32**, respectively. The structures and characterizations of the different conjugates **26–29** are reported in Table 2 and in Supplementary files. It is interesting to note that in contrast to previous studies [45], the deprotection of the S-S*t*Bu glycopeptides was never complete with TCEP as indicated by HPLC even with higher amounts of TCEP and prolonged reaction times. However, reduction was quantitative using 20 equivalents of DTT in Tris-HCl buffer.

Cell viability studies

In this study, we have focused our analysis on the activity of the various KLAKE conjugates, inferring that cell death should be related to the uptake efficiency of the conjugates. The CCK8 cell counting kit was used to quantify the extent of dead cells at the end of 2 h incubation with CHO-K1 cells. The viability of the cells incubated with the glycosylated conjugates were compared to those of the KLAKE conjugates with penetratin, **32**, and (R6/W3), **26**,

Fig. 1 Two peptides from Table 2 are illustrated as examples. *Top* Ac-Cys(SS*t*Bu)-RRW-[Pra-Gal(OAc)]-RR-[Pra-Gal(OAc)]-RR-NH₂, abbreviated in the text as (R6/W1)-[Pra-Gal(OAc)]₂S-S*t*Bu, **17**, and *bottom* Ac-Cys(SS*t*Bu)-RRW-[Pra-Gal(CH₂)₃(OH)]-RRRR-NH₂, abbreviated in the text as (R6/W2)-[Pra-Gal(CH₂)₃(OH)]S-S*t*Bu, **22**



and of the free cargo Biotin(O₂)-(Gly)₄-Cys(Acm)-KLA-KLAKKLAKLAK-NH₂ **31**. The thiol function of this KLA analog was masked with an acetamidomethyl group. We have recently demonstrated that the activated SH protecting group may contribute to the internalization of peptides by cell surface thiol/disulfide exchange [45].

Penetratin, (R6/W3), and the glycosylated CPPs (at 50 μM; data not shown) and the KLA cargo **31** did not decrease the number of viable cells even at 50 μM, the highest concentration which was used. It has been previously demonstrated with other KLA analogs or with other cell lines that KLA peptides are unable to cross the bilayer of eukaryotic cells unless they are coupled to a CPP [31] or a scaffold [32], which shuttle KLA inside cells. The extent of cell death induced by all of the CPP-KLA conjugates tested herein is reported in Fig. 2. The conjugates penetratin-S-S-KLA, **32**, and (R6/W3)S-S-KLA, **26**, induced cell death of CHO-K1 cells, the latter being significantly more potent. The free CPPs penetratin and (R6/W3) were found previously to be internalized in CHO cells with the same efficiency when quantified by

mass spectrometry and penetratin was slightly more efficient to deliver a peptide inhibitor of protein kinase C [18, 45]. All of the glycoconjugates, **27–30** were found to be less active than the nonglycosylated reference conjugate (R6/W3)S-S-KLA **26**. (R6/W2)-[Pra-Gal(OH)]S-S-KLA **27** which was already significantly active at 20 μM. This glycoconjugate was more potent in inducing cell death than the three other glycosylated conjugates. The introduction of a propyl spacer between the CPP and the galactose unit decreased the conjugate activity. The glycoconjugate (R6/W2)-[Pra-Gal(CH₂)₃(OH)]S-S-KLA **28**

Table 2 Glycopeptides conjugated to the KLA peptides

Number—peptides
26 —(R6/W3)S-S-KLA
27 —(R6/W2)-[Pra-Gal(OH)]S-S-KLA
28 —(R6/W2)-[Pra-Gal(CH ₂) ₃ (OH)]S-S-KLA
29 —(R6/W2)-[Pra-Gal(CH ₂) ₃ (OAc)]S-S-KLA
30 —(R6/W0)-[Pra-Gal(OH)] ₃ S-S-KLA

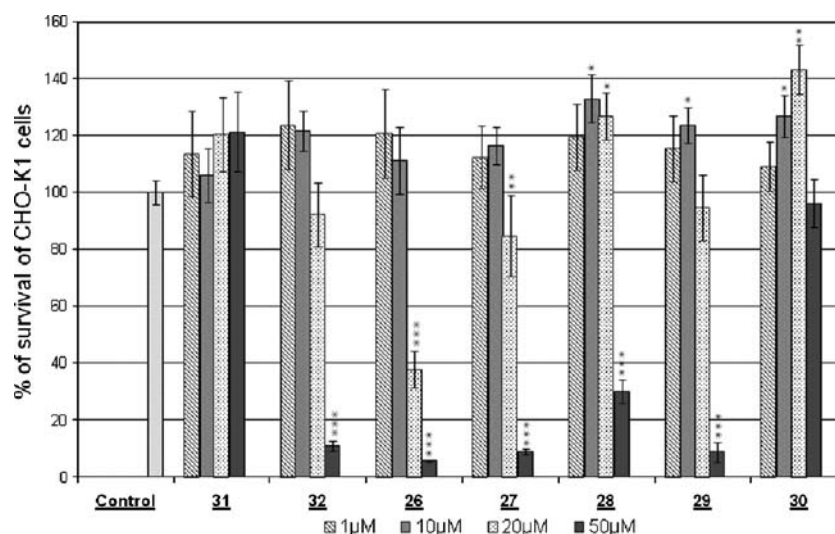


Fig. 2 Cell viability experiments were expressed as a percentage of living CHO-K1 cells. Adherent CHO cells (10,000 cells per well) were incubated for 2 h at 37 °C with various concentrations of CPP-KLAK conjugates, from left to right: 1, 10, 20, and 50 μM of conjugate (compounds **31**, Biotin(O₂)-(Gly)₄-Cys(Acm)-KLAKLAK-KLAKLAK-NH₂; **32**, penetratinS-S-KLAK; **26**, (R6/W3)S-S-KLAK; **27**, (R6/W2)-[Pra-Gal(OH)]S-S-KLAK; **28**, (R6/W2)-[Pra-Gal(CH₂)₃(OH)]S-S-KLAK; **29**, (R6/W2)-[Pra-Gal(CH₂)₃(OAc)]S-S-KLAK; and **30**, (R6/W0)-[Pra-Gal(OH)]₃S-S-KLAK). After incuba-

tion, the supernatant was removed; a solution of CCK8 cell counting kit was added, and the absorbance was measured at 450 nm after 2 h. Results are expressed as the average of at least three independent experiments performed in triplicate; differences (Student's test) being * significant, ** very significant, and *** extremely significant from control value (100%). Only the free KLAK peptide and the conjugate with three galactose units **30** (R6/W0)-[Pra-Gal(OH)]₃S-S-KLAK did not affect the cell viability, even at 50 μM

was found to induce cell death only at the higher concentration used (50 μM). The difference in the efficacies of conjugates **27** and **28** is significant at 20 μM ($P=0.04$) and extremely significant at 50 μM ($P=0.0004$). Interestingly, acetylation of galactose is associated with a decrease in cell viability, as shown with analog **29**, (R6/W2)-[Pra-Gal(CH₂)₃(OAc)]S-S-KLAK. The difference between the efficiencies of conjugates **28** and **29** is very significant at 50 μM ($P=0.008$). It is interesting to note that the conjugate with the three galactoses, (R6/W0)-[Pra-Gal(OH)]₃S-S-KLAK, **30**, does not affect cell viability even at 50 μM.

Fluorescence labeling of CHO-K1 cells incubated with KLAK, **31**, or KLAK conjugates, **26**, **27**, **30**

The delivery and intracellular localization of the KLAK cargo into cells were studied by fluorescence. The alternative for such studies is to use either fluorescent peptides and live cells or biotinylated peptides and fixed cells. The biotinylated peptide is revealed by fluorescent streptavidin. Both of these strategies require modifications of the peptide and have inherent drawbacks. On the one hand, it has been suggested that CPPs tend to diffuse during cell fixation [47, 48]. On the other hand, functionalization with large hydrophobic fluoroprobes may affect the peptide uptake mechanism and its final localization, more than biotin, a smaller size tag (data to be published, S. Aubry

et al.). A procedure with fixed cells was set up to discriminate the internalized peptide from the membrane-bound peptide [49]. This procedure minimized the artifact due to an eventual diffusion of the peptide, as the membrane-bound cargo was first quenched with unlabeled avidin to visualize only the internalized species. The cells were subsequently fixed with paraformaldehyde and permeabilized. The intracellular biotinylated cargo was revealed with fluorescent Alexa-488-streptavidin. It is worth mentioning that the fluorescent streptavidin will label the conjugated with KLAK as well as the free KLAK peptide that is released after reduction with glutathione in the cytosol. The free KLAK cargo **31** and glycoconjugates (R6/W3)S-S-KLAK **26**, (R6/W2)-[Pra-Gal(OH)]S-S-KLAK **27** and (R6/W0)-[Pra-Gal(OH)]₃S-S-KLAK **30** were analyzed with this procedure. These compounds were incubated at a concentration (10 or 20 μM; Fig. 3) where their respective activity killed only part of the CHO cells. No fluorescence was detected when CHO-K1 cells were incubated with the free KLAK cargo **31**, showing that KLAK was not internalized alone and corroborating its lack of effect on cell viability. The fluorescent labelings observed with the conjugates **26**, **27**, and **30** have also corroborated their activities on cell viability. Conjugate **30** did not label any cell and did not lead to cell death (Fig. 2).

Rothbard *et al.* have proposed (Arg)_n (with n as being up to 16 residues) as a powerful CPP, showing that the short analogs ($n=6-8$) were still potent [50, 51]. In a subsequent

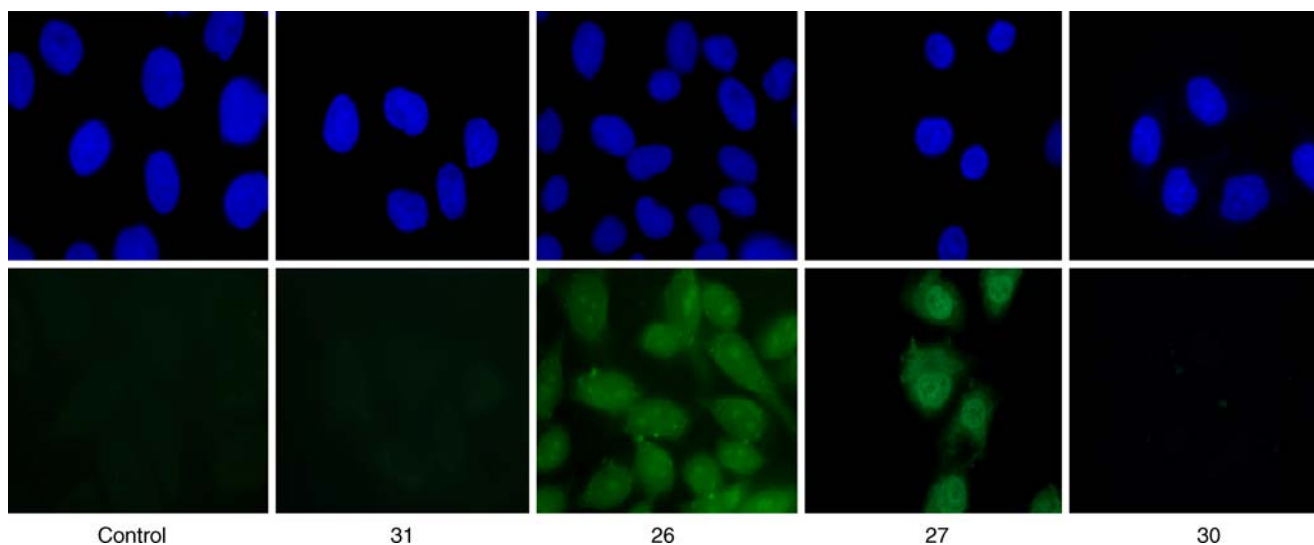


Fig. 3 The biotinylated internalized cargo was visualized by confocal microscopy. The CPP-KLAK conjugates were incubated for 2 h at a single concentration: 10 μ M (conjugate **26**) or 20 μ M (KLAK, conjugates **27** and **30**). No peptide was incubated in the control experiment. After incubation, the membrane-bound cargo was quenched with avidin. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton \times 100. The intracellular cargo was

then visualized with fluorescent streptavidin (streptavidin-Alexa488, in *green*). Nuclei were stained with DAPI (in *blue*). These experiments were duplicated which lead to comparable images in all cases. Cargo delivery inside cells was observed only when using (R6/W3) and (R6/W2)-[Pra-Gal(OH)] carriers. No intracellular KLAK was detected with the carrier bearing three galactose units

study, they modified the decapeptide (Arg)₁₀ by systematically replacing with other amino acids the three arginines which project on the same side of a 3₁₀ helical structure (*i.e.*, the second, fifth, and eighth positions). Only the introduction of either an aspartic acid or a glutamic acid (seven arginines and three acidic residues in the sequence) induced a drastic decrease in the internalization efficiency of the corresponding peptides. In contrast, decamers with seven arginines and three serines or threonines (residues with hydroxylated side chains) were as potent as (Arg)₁₀ [52]. The data produced herein show that the viability of the cells incubated with KLAK conjugates is closely related to conjugate internalization. The conjugate with three galactoses did not affect cell viability, and no internalized KLAK peptide was observed by fluorescence with this glycoconjugate. It is worth mentioning that this conjugate should not be metabolized faster than its corresponding unglycosylated conjugate, as it has been proposed that glycosylation generally increases peptide stability [53, 54]. Thus, the presence of carbohydrate(s) decrease the capacity of the glycosylated CPP cargo conjugate to enter cells, in contrast to serine or threonine residues. This conclusion applies even though (Arg)₆ is still efficiently taken up into cells [50, 51], and the glycosylated CPPs reported here have six arginines in their sequences.

Noticeably, CPPs have been designed either starting from carbohydrate synthons [55] or with both carbohydrates on one part of a construct and basic side-chains on the other part [56]. All of these glycosylated analogs were

efficiently taken up into cells [55, 56]. The glycosylated CPP designed herein with three carbohydrate moieties is not internalized. Thus, depending on the “place/spatial organization” of the carbohydrates and basic chains on the final synthon, the uptake efficiency is or is not affected. It is difficult to correlate the uptake efficiency of the glycosylated conjugate described in this study to a significant variation in the hydrophilicity/hydrophobicity balance of the starting CPP (R6/W3). If the HPLC retention time of the glycosylated conjugates is taken into consideration as an overall index of the relative hydrophilicity, monogalactosylation shows a slight increase in hydrophilicity, whereas trigalactosylation led to a slight increase in hydrophobicity, when compared to the (R6/W3) conjugate. One might be tempted to propose that the glycoconjugates with galactoses (especially the one with three galactoses) may bind to some extracellular component competing/impairing the interactions of guanidiniums with either heparan sulfates or lipids, which are necessary for cellular uptake independent of the entry mechanism. This extracellular binding does not lead to a rapid intracellular delivery during the 2 h of incubation time. The absence of intracellular labeling also proves that the glycoconjugate was not subjected to significant disulfide exchange with thiolated proteins present on the cell membrane. As recently shown by Aubry *et al.*, these thiol functions are involved in the extracellular transfer of some disulfide-bridged peptides. [45].

From this study, one must conclude that the introduction of galactose(s) *per se* in the sequence of a CPP affects the

intracellular delivery. We have shown that glycosylation with three galactoses of a cationic peptide (with six arginines) yielded peptides unable to enter into cells. The exact reason for this decreased efficiency remains to be unveiled at the molecular level. This apparent “negative result” (inactive CPPs) must in fact be considered as an opportunity to regulate the internalization of bioactive molecules and provides an insight into addressing CPPs, which still remains a crucial problem. We can theorize that glycosylation *via* an immolative linker between a CPP and a carbohydrate may yield a more specific addressing to cells. In this framework, a specific linker between the peptide and the carbohydrate residues, which is designed to be cleaved in the environment of tumor cells, must allow a selective internalization inside of these cells for the CPP and the shuttled drug.

Experimental section

1-Azido-2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranoside, **2**

Prepared in two steps from galactose **1**, as previously described [22].

(2-Methyl-5-tert-butylphenyl) 2,3,4,6-tetra-*O*-benzoyl-1-thio- β -D-galactopyranoside, **3**

To a solution of 1,2,3,4,6-penta-*O*-benzoyl-D-galactopyranose (4.0 g, 5.71 mmol) prepared from galactose **1** [34] in anhydrous dichloromethane (10 mL) were added, under argon, 2-methyl-5-tert-butylthiophenol (1.35 mL, 6.85 mmol, 1.2 eq.) and BF₃.Et₂O (3.62 mL, 28.54 mmol, 5 eq.). An orange solution is obtained in a few minutes after the addition of BF₃.Et₂O. The reaction was monitored by TLC (cyclohexane-ethyl acetate 7:3). After 16 h, the reaction was complete. The reaction mixture was cooled to 0 °C then cautiously neutralized with a saturated NaHCO₃ solution. The product was extracted with CH₂Cl₂; the combined organic layers were dried (MgSO₄), filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (cyclohexane/EtOAc, 9:1 then 8:2) to afford **3** (3.22 g, 74%) as a white foam containing two anomers (α , β 25:75). α anomer: Rf=0.48 (cyclohexane/EtOAc, 8:2). $[\alpha]_D^{25}$ 151.7 (*c* 1.0, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ : 8.12–8.03 (m, 4H, HAr ortho), 7.94–7.91 (m, 2H, HAr ortho), 7.85–7.82 (m, 2H, HAr ortho), 7.63–7.32 (m, 13H, HAr), 7.16 (dd, 1H, HAr, *J*=1.9 Hz, *J'*=8.0 Hz), 7.06 (d, 1H, HAr, *J*=8.0 Hz), 6.25 (d, 1H, H1, *J*_{H1–H2}=4.9 Hz), 6.17–6.16 (m, 1H, H4), 6.06–5.94 (m, 2H, H3+H2), 5.22–5.17 (m, 1H, H5), 4.59 (dd, 1H, H6a, *J*_{H6a–H5}=6.5 Hz, *J*_{H6a–H6b}=11.4 Hz), 4.44 (dd, 1H, H6b, *J*_{H6b–H5}=6.5 Hz), 2.32 (s, 3H, CH₃), 1.21 (s,

9H, tBu); ¹³C NMR (62.5 MHz, CDCl₃) δ : 165.7, 165.5, 165.4 (4 C=O), 149.8–124.9 (30 CAR), 86.5 (C1), 69.1, 68.9 (C4+C3+C2), 68.1 (C5), 62.2 (C6), 34.3 (C(CH₃)₃), 31.1 (C(CH₃)₃), 20.2 (CH₃); MS ESI⁺-HRMS (m/z) calculated for C₄₅H₄₂NaO₁₉S: 781.24417, found 781.24353. β anomer: Rf=0.44 (cyclohexane/EtOAc, 8:2). $[\alpha]_D^{25}$ 101.0 (*c* 1.0, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ : 8.02–7.97 (m, 6H, HAr ortho), 7.78 (dd, 2H, HAr ortho, *J*_{Hortho–Hméta}=7.1 Hz, *J*_{Hortho–Hpara}=1.4 Hz), 7.63–7.36 (m, 12H, HAr), 7.30–7.21 (m, 2H, HAr), 7.14 (d, 1H, HAr, *J*=7.9 Hz), 6.02 (d, 1H, H4, *J*_{H4–H3}=3.1 Hz), 5.86 (t, 1H, H2, *J*_{H2–H1}=*J*_{H2–H3}=9.9 Hz), 5.61 (dd, 1H, H3), 4.98 (d, 1H, H1), 4.65 (dd, 1H, H5, *J*_{H5–H6a}=5.9 Hz, *J*_{H5–H6b}=10.8 Hz), 4.44–4.30 (m, 2H), 2.33 (s, 3H, CH₃), 1.23 (s, 9H, tBu); ¹³C NMR (62.5 MHz, CDCl₃) δ : 166.3, 165.8, 165.5 (4 C=O), 149.8–126.2 (30 CAR), 88.1 (C1), 75.1 (C5), 73.2 (C3), 68.7, 68.6 (C4+C2), 62.5 (C6), 34.5 (C(CH₃)₃), 31.4 (C(CH₃)₃), 20.9 (CH₃); MS ESI⁺-HRMS (m/z) [MNa]⁺ calculated for C₄₅H₄₂NaO₁₉S: 781.24417, found 781.24352.

3-Azidopropyl-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranoside, **4**

3-Azidopropyl-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranoside, **4** was prepared in two steps from compound **3** *via* 3-bromopropyl-2,3,4,6-tetra-*O*-benzoyl- β -D-galactopyranoside. To a solution of (2-methyl-5-tert-butylphenyl)-2,3,4,6-tetra-*O*-benzoyl-1-thio- β -D-galactopyranoside **3** (3.14 g, 4.13 mmol) in dry dichloromethane (10 mL), under argon, were added 4 Å molecular sieve (3.10 g), 3-bromopropanol (540 μ L, 6.20 mmol, 1.5 eq.), *N*-bromosuccinimide (1.47 g, 8.26 mmol, 2 eq.), and trifluoromethanesulfonic acid (44 μ L, 0.41 mmol, 0.1 eq.). The reaction mixture was stirred under an argon atmosphere for 15 min at room temperature and was then filtered over celite. The filtrate was washed first with a saturated solution of Na₂S₂O₃ (10 mL) and then with a saturated solution of NaHCO₃ (10 mL). The product was extracted with CH₂Cl₂ (3 \times 15 mL). The combined organic layers were dried (MgSO₄), filtered, and concentrated *in vacuo* to give a yellow syrup, which was purified by flash column chromatography (cyclohexane/EtOAc, 9:1 then \rightarrow 8:2) to yield 2.14 g (72% yield) of product as a white foam. Rf=0.28 (cyclohexane/EtOAc, 8:2); $[\alpha]_D^{25}$ 87.8 (*c* 1.0, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ : 8.01 (d, 2H, *J*=7.5 Hz), 7.94 (d, 2H, *J*=7.5 Hz), 7.89 (d, 2H, *J*=7.6 Hz), 7.71 (d, 2H, *J*=7.6 Hz), 7.55–7.12 (m, 12H, HAr), 5.93 (d, 1H, *J*_{H4–H3}=3.1 Hz, H4) 5.72 (dd, 1H, *J*_{H2–H3}=10.3 Hz, *J*_{H2–H1}=7.9 Hz, H2), 5.55 (dd, 1H), 4.78 (d, 1H, H1), 4.62 (dd, 1H, *J*_{H6a–H6b}=10.7 Hz, *J*_{H6a–H5}=6.1 Hz, H6a), 4.38–4.25 (m, 2H, H6b+H5), 4.04–3.98 (m, 1H, OCHaHb), 3.71–3.66 (m, 1H, OCHaHb), 3.30–3.24 (m, 2H, CH₂Br),

2.08–1.92 (m, 2H, OCH₂CH₂CH₂Br); ¹³C NMR (62.5 MHz, CDCl₃) δ: 166.1, 165.6, 165.4 (C=O), 133.6–128.3 (24 CAr), 102.0 (C1), 71.7 (C3), 71.4 (C5), 69.8 (C2), 68.2 (C4), 67.7 (OCH₂CH₂CH₂Br), 62.1 (C6), 32.4 (OCH₂CH₂CH₂Br), 30.1 (OCH₂CH₂CH₂Br). MS FAB⁺-HRMS (m/z) [MNa]⁺ calculated for C₃₇H₃₃O₁₀BrNa: 739.1155, found 739.1182.

To a solution of 3-bromopropyl-2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside (2.04 g, 2.85 mmol) in dry DMF (30 mL), under argon, sodium azide (1.11 g, 17.10 mmol, 6 eq.) was added. The mixture was stirred at 60 °C for 3 h, and the solvent was evaporated. The residue was diluted in CH₂Cl₂ (20 mL), washed with water (3x15mL), dried (MgSO₄), filtered, and evaporated in vacuo. The crude material was purified by column chromatography on silica gel (cyclohexane/EtOAc 8:2) to give 1.57 g of **4** (81% yield), isolated as a white foam. Rf=0.23 (cyclohexane/EtOAc, 8:2); [α]_D²⁵ 89.0 (*c* 1.0, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ: 8.01–7.68 (m, 8H, HAR ortho), 7.59–7.13 (m, 12H, HAR), 5.93 (d, 1H, *J*_{H4–H3}=3.1 Hz, H4), 5.72 (dd, 1H, *J*_{H2–H3}=10.4 Hz, *J*_{H2–H1}=7.9 Hz, H2), 5.54 (dd, 1H), 4.75 (d, 1H, H1), 4.62 (dd, 1H, *J*_{H6a–H6b}=10.9 Hz, *J*_{H6a–H5}=6.3 Hz), 4.38–4.26 (m, 2H, H6b+H5), 4.01–3.97 (m, 1H, OCHaHb), 3.67–3.52 (m, 1H, OCHaHb), 3.21–3.16 (m, 2H, CH₂N₃), 1.77–1.73 (m, 2H, OCH₂CH₂CH₂N₃); ¹³C NMR (62.5 MHz, CDCl₃) δ: 166.1, 165.6, 165.3 (C=O), 133.6–128.3 (24 CAr), 101.8 (C1), 71.7 (C3), 71.4 (C5), 69.8 (C2), 68.1 (C4), 66.8 (OCH₂CH₂CH₂N₃), 62.0 (C6), 47.9 (OCH₂CH₂CH₂N₃), 29.0 (OCH₂CH₂CH₂N₃); MS FAB⁺-HRMS (m/z) [MNa]⁺ calculated for C₃₇H₃₃O₁₀N₃Na: 702.2064, found 702.2059.

3-Azidopropyl-β-D-galactopyranoside, **5**

To a suspension of 3-azidopropyl-2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranoside **4** (1.0 g, 1.47 mmol) in anhydrous methanol (15 mL), under argon, was added sodium (23 mg, 1.0 mmol). The suspension slowly disappeared; the reaction was complete after 15-h stirring (TLC: cyclohexane/EtOAc, 7:3). The clear solution was neutralized by adding a cation exchange resin (IR 120-H⁺ form) previously treated by successive washings with H₂O (3 × 20 mL) and MeOH (3x20mL). After filtration, the filtrate was concentrated to give an oil, which was purified by column chromatography on silica gel (EtOAc/MeOH 9:1) to give 315.6 mg of **5** (82% yield), isolated as a pale yellow syrup. Rf=0.2 (EtOAc/MeOH, 9:1); [α]_D²⁵ -5.3 (*c* 1.0, DMSO); ¹H NMR (250 MHz, MeOD) δ: 4.24 (d, 1H, *J*_{H1–H2}=6.8 Hz, H1), 4.04–3.95 (m, 1H, OCHaHb), 3.86 (d, 1H, *J*_{H3–H2}=2.2 Hz, H3), 3.77 (d, 1H, *J*=2.0 Hz, H6b), 3.75 (s, 1H, H6a), 3.71–3.62 (m, 1H, OCHaHb), 3.58–3.45 (m, 5H, CH₂N₃+H2+H4+H5), 1.94–1.84 (m, 2H, OCH₂CH₂CH₂N₃); ¹³C NMR (62.5 MHz, MeOD) δ:

105.1 (C1), 76.6, 74.9, 72.5, 70.3 (C3), 67.6 (OCH₂CH₂CH₂N₃), 62.5 (C6), 49.4 (OCH₂CH₂CH₂N₃), 30.3 (OCH₂CH₂CH₂N₃), Lit. [57]; MS FAB⁺-HRMS (m/z) [MNa]⁺ calculated for C₉H₁₇O₆N₃Na: 286.10096, found 286.10078.

3-Azidopropyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside, **6**

To a solution of 3-azidopropyl-β-D-galactopyranoside **5** (157.8 mg, 0.60 mmol) in anhydrous pyridine (2.5 mL) was added acetic anhydride (395 μL, 4.20 mmol, 7 eq.) under argon. The reaction mixture was stirred overnight at room temperature. Pyridine was then evaporated, and the residue was diluted in dichloromethane, washed with diluted H₂SO₄ (1 M; 2 × 5 mL) and then with a saturated solution of NaHCO₃. The combined organic layers were dried (MgSO₄), filtered, and concentrated *in vacuo*. The crude material was purified by flash column chromatography (cyclohexane/EtOAc, 7:3) to give 220.6 mg of **6** (85% yield), isolated as an oil. Rf=0.25 (cyclohexane/EtOAc, 7:3); [α]_D²⁵ 0.28 (*c* 1.0, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ: 5.33 (d, 1H, *J*_{H4–H3}=3.1 Hz, H4), 5.13 (dd, 1H, *J*_{H2–H3}=10.4 Hz, *J*_{H2–H1}=7.9 Hz, H2), 4.96 (dd, 1H, H3), 4.42 (d, 1H, H1), 4.13–4.03 (m, 2H, H6a+H6b), 3.95–3.87 (m, 2H, H5+OCHaHb), 3.59–3.50 (m, 1H, OCHaHb), 3.32 (t, 2H, *J*=6.3 Hz, CH₂N₃), 2.09 (s, 3H, CH₃ Ac), 2.01 (s, 3H, CH₃ Ac), 1.99 (s, 3H, CH₃ Ac), 1.92 (s, 3H, CH₃ Ac), 1.86–1.73 (m, 2H, OCH₂CH₂CH₂N₃); ¹³C NMR (62.5 MHz, CDCl₃) δ: 101.2 (C1), 70.8 (C3), 70.6 (C5), 68.7 (C2), 66.9 (C4), 66.4 (OCH₂CH₂CH₂N₃), 61.2 (C6), 47.8 (OCH₂CH₂CH₂N₃), 29.6 (OCH₂CH₂CH₂N₃), 20.6 (4CH₃ Ac), Lit. [35]; MS FAB⁺-HRMS (m/z) [MNa]⁺ calculated for C₁₇H₂₅N₃NaO₁₀: 454.14322, found 454.14252.

Azido-propyl-rhodamine, **9**

To a CH₂Cl₂ solution of rhodamine B (75.0 mg, 0.157 mmol) was added EDC.HCl (90.0 mg, 0.471 mmol, 3 eq.) followed by *N*-hydroxysuccinimide (54.2 mg, 0.471 mmol, 3 eq.) [58]. The reaction mixture was stirred for 0.5 h at room temperature, before dropwise addition of 3-azido-1-propanol [58] (79.0 mg, 0.783 mmol, 5 eq.), and 4-dimethylaminopyridine (1.95 mg, 0.016 mmol, 0.1 eq.) in CH₂Cl₂. The reaction was stirred at room temperature overnight, after dilution in CH₂Cl₂ the solution was washed with H₂O (3×). The organic layer was dried (MgSO₄), filtered, and concentrated under vacuum. The crude product was purified by flash column chromatography (CH₂Cl₂/MeOH: 9/1) to give 58.0 mg of rhodamine-N₃ **9** as an oil (66%). ¹H NMR (250 MHz, CDCl₃) δ: 8.25 (m, 1H, HAR), 7.79 (m, 2H, HAR), 7.31 (s, 1H, HAR), 7.05 (m, 2H, HAR),

6.85 (m, 2H, HAr), 6.75 (m, 2H, HAr), 4.10 (t, 2H, $J=6.0$ Hz, CH₂), 3.65 (q, 8H, $J=8.0$ Hz, CH₂CH₃), 3.20 (t, 2H, $J=6.1$ Hz, OCH₂CH₂CH₂N₃), 1.69 (t, 2H, $J=6.0$ Hz, CH₂), 1.47 (t, 12H, $J=8.0$ Hz, CH₂CH₃); ¹³C NMR (62.5 MHz, CDCl₃) δ: 166.0 (C=O), 158.9–155.4 (6 CAr), 133.3–129.5 (8 CAr), 114.2 (2 CAr), 113.3 (CAr), 96.2 (CAr), 62.4 (OCH₂CH₂CH₂N₃), 47.8 (OCH₂CH₂CH₂N₃), 46.0 (2*N(CH₂CH₃)₂), 27.7 (OCH₂CH₂CH₂N₃), 12.5 (2*N(CH₂CH₃)₂). MS MALDI-TOF (m/z) [M]⁺ calculated for C₃₁H₃₆N₅O₃: 526.28, found 526.28.

Peptides syntheses

MBHA resin LL (100–200 mesh)-HCl, (4-methylbenzhydrylamine-hydrochloride polystyrene resin, 0.59 mmol/g), Rink amide MBHA resin (100–200 mesh) (4-(2'-4'-diméthoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA polystyrene resin, 0.64 mmol/g), *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-Cys(*t*BuS)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH, Boc-Ala-OH, Boc-Leu-OH, and Boc-Lys(2-CI-Z)-OH were purchased from Novabiochem. Boc-Gly-OH was purchased from Senn Chemicals, Boc-Cys(Npys)-OH from Bachem, and Fmoc-Pra-OH from IRIS Biotech. Other reagents for amino acid couplings were obtained from Applied Biosystems. Biotin(O₂) was prepared from biotin as previously described in the literature [59]. Peptides were characterized by MALDI-TOF MS (DE-Pro, PerSeptive Biosystems) in positive ion reflector mode using the matrix CHCA. The m/z of the protonated molecule (first isotope) are given as experimental (calculated) and theoretical (found).

Model peptide: Ac-Arg-Pra-Arg-Ahx-NH₂, 7 The tripeptide was synthesized manually by solid-phase methodology on a Rink amide resin (0.64 mmol/g) by Fmoc strategy. Fmoc protection was removed with a solution of piperidine in NMP (20%, 3×5 min). Stepwise coupling reactions were performed with Fmoc-protected amino acids, HBTU, and DIPEA (3/2.85/3 eq.) at RT. After removal of the last Fmoc protecting group, the peptide was *N*-acetylated with a solution of acetic anhydride in NMP (20%, 45 min). The resin was washed with NMP, CH₂Cl₂, MeOH and dried under vacuum. For analysis, approximately 5 mg of the resin were treated with TFA/H₂O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), precipitated in diethyl ether, lyophilized and purified by RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 8 min (0–95% acetonitrile over 30 min). The peptide was characterized by MALDI-TOF MS (m/z) [MH]⁺ calculated 580.37, found 580.38.

Click chemistry with the model tripeptide Ac-Arg-Pra-Arg-Ahx-Rink-amide resin

To the dry resin-bound Ac-R-Pra-R-Ahx-Rink (Ahx: aminohexanoic acid) in a fritted syringe were added Cu(OAc)₂ or CuI, ascorbic acid (in a 1:2 ratio with Cu(OAc)₂ and a 1:1 ratio with CuI salt) and a solution containing 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl azide **2** (0.06 M), DIEA in a solution of 30% lutidine, pyridine or piperidine in DMF, (Refer to [supplementary materials](#) for the equivalents of Cu salt, and DIEA used). The solution was sonicated for 5 min and shaken at RT. The resin was then washed with DMF, DMF/pyridine (6/5) containing ascorbic acid (0.02 g/mL), CH₂Cl₂ and MeOH. The resin was dried and then cleaved by TFA/H₂O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), precipitated in diethyl ether, lyophilized and purified by RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 10 min (0–95% acetonitrile over 30 min). Conversion of the reaction was determined by RP-HPLC. The final glycopeptide **8** was characterized by MALDI-TOF MS (m/z) [MH]⁺ calculated 953.48, found 953.48.

Coupling of azido-propyl rhodamine to Ac-Arg-Pra-Arg-Ahx-Rink-amide resin

To the dry resin-bound model peptide in a fritted syringe were added Cu(OAc)₂ (40 eq.), ascorbic acid (40 eq.) and a solution containing Rhodamine-N₃ **9** (20 eq.), DIEA (50 eq.) in a solution of 30% pyridine in DMF. The solution was sonicated for 5 min and shaken at RT. The resin was then washed with DMF, DMF/Pyridine (6/5) containing ascorbic acid (0.02 g/mL), CH₂Cl₂ and MeOH. The resin was dried and then cleaved by treatment with TFA/H₂O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), precipitated in diethyl ether, lyophilized and purified by RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). The peptide was characterized by MALDI-TOF MS (m/z) [M]⁺ calculated 1,105.64, found 1,105.64.

(R6/W3)-S-StBu, 10 The peptide was synthesized manually by solid-phase methodology on a Rink amide resin (0.64 mmol/g) by Fmoc strategy in a fritted syringe. Fmoc protection was removed with a solution of piperidine in NMP (20%, 3×5 min). Stepwise coupling reactions were performed with Fmoc-protected amino acids, HBTU and DIPEA (3/2.85/3 eq.) at RT. After removal of the last Fmoc protecting group, the peptide was *N*-acetylated with a solution of acetic anhydride in NMP (20%, 45 min). The resin was washed with NMP, CH₂Cl₂, and MeOH and dried under vacuum. The resin was then treated with TFA/H₂O/TIS: 95/2.5/2.5

(15 mL/g resin, 2 h, RT), precipitated in diethyl ether, lyophilized, and purified by RP-HPLC on a preparative C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) to yield the product as a white powder after lyophilisation (14% yield). The purity of the peptide was determined by analytical RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 17 min (15–55% acetonitrile over 30 min). The peptide was characterized by MALDI–TOF MS (m/z) $[MH]^+$ calculated 1,744.93, found 1,744.94.

Syntheses of Pra-containing R/W peptides

The peptides were synthesized manually by solid-phase methodology on a Rink amide resin (0.64 mmol/g) by Fmoc strategy in a fritted syringe, as described for the model tripeptide Ac-R-Pra-R-Ahx-NH₂. For analysis, approximately 5 mg of the resin were treated with TFA/H₂O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), precipitated in diethyl ether, lyophilized, and purified by RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA; 0–95% acetonitrile over 30 min). Pure peptides were characterized by MALDI–TOF MS.

(*R6/W2*)-Pra-*S*-StBu, **12** RP-HPLC retention time is 12.5 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[M,H]^+$ calculated is 1,654.89, and found 1,654.82.

(*R6/W1*)-Pra₂-*S*-StBu, **13** RP-HPLC retention time is 12.9 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 1,563.85, found 1,563.56; $[M2H]^{2+}$ calculated is 782.43, found 782.26.

(*R6/W0*)-Pra₃-*S*-StBu, **14** RP-HPLC retention time is 9.9 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 1,472.81, found 1,472.80.

Syntheses of the glycosylated peptides

In a fritted syringe were added, to the corresponding dried Pra containing-peptidyl-resin, Cu(OAc)₂ (4 eq.), ascorbic acid (2 eq.), and a solution of DMF/pyridine (7/3) containing the azido-sugar (two equivalents relative to Pra aminoacid, 0.06 M) and DIPEA (5 eq.). The solution was sonicated for 5 min and shaken for 15 h at RT. The resin was then washed with DMF, DMF/Pyridine (6/5) containing ascorbic acid (0.02 g/mL), CH₂Cl₂, and MeOH. The resin was dried under vacuum and then cleaved by TFA/H₂O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT). Analytical RP-HPLC showed

complete conversion of the peptide into the glycopeptide. The crude glycopeptide was precipitated in diethyl ether, lyophilized, and purified by RP-HPLC on a preparative C8 column using a linear gradient of acetonitrile (0.1% TFA) in an aqueous solution (0.1% TFA). Peptide purity was analyzed by analytical RP-HPLC with a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) and characterized by MALDI–TOF MS.

(*R6/W2*)-[Pra-Gal(OAc)]-*S*-StBu, **15** RP-HPLC retention time is 14.5 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 2,028.00, found 2,027.96. Overall yield (peptide synthesis, click chemistry, and purification) is 3.0% with a purity >95%.

(*R6/W1*)-[Pra-Gal(OAc)]₂-*S*-StBu, **17** RP-HPLC retention time is 18 min (10–60% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 2,310.07, found 2,309.83; $[M2H]^{2+}$ calculated is 1,155.54, found 1,155.88. Overall yield (peptide synthesis, click chemistry, and purification) is 4.5% with a purity >95%.

(*R6/W0*)-[Pra-Gal(OAc)]₃-*S*-StBu, **18** RP-HPLC retention time is 18.2 min (10–60% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 2,592.14, found 2,591.87; $[M2H]^{2+}$ calculated is 1,296.57, found 1,296.90. Overall yield (peptide synthesis, click chemistry, and purification) is 7.5% with a purity >95%.

(*R6/W2*)-[Pra-Gal(CH₂)₃(OAc)]-*S*-StBu, **20** RP-HPLC retention time is 14.5 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated 2,086.04, found 2,085.92. Overall yield (peptide synthesis, click chemistry, and purification) is 4.8% with a purity >95%.

(*R6/W2*)-[Pra-Gal(CH₂)₃(OBz)]-*S*-StBu, **21** RP-HPLC retention time is 24.0 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 2,334.11, found 2,334.38. Overall yield (peptide synthesis, click chemistry, and purification) is 2.3% with a purity >95%.

(*R6/W2*)-[Pra-Gal(CH₂)₃(OH)]-*S*-StBu, **22** RP-HPLC retention time is 9.7 min (15–55% acetonitrile over 30 min). MALDI–TOF MS (m/z) $[MH]^+$ calculated is 1,918.00, found 1,917.59. $[M2H]^{2+}$ calculated is 959.51, found 959.79. Overall yield (peptide synthesis, click chemistry, and purification) is 3.4% with a purity >95%.

Reduction of the *t*-buthio disulfide

To the disulfide peptide dissolved in a degassed solution of Tris–HCl 50 mM (~2 mM), DTT (20 eq.) was added, and

the reaction was stirred at RT for 2 h. Crude thiol peptides were then purified by RP-HPLC on a preparative C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Peptide purity was analyzed by analytical RP-HPLC on a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) and characterized by MALDI-TOF MS.

(R6/W3)-[Pra-Gal(OH)]₃-SH, **11** RP-HPLC retention time is 11.7 min (15–55% acetonitrile over 30 min). MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated is 1,657.90, found 1,658.00. Yield is 87% with a purity >95%.

(R6/W2)-[Pra-Gal(CH₂)₃(OH)]-SH, **23** RP-HPLC retention time is 7.0 min (15–55% acetonitrile over 30 min). MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated is 1,829.97, found 1,829.71. Yield is 46% with a purity >95%.

(R6/W2)-[Pra-Gal(CH₂)₃(OAc)]-SH, **24** RP-HPLC retention time is 10.5 min (20–60% acetonitrile over 30 min). MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated is 1,998.00, found 1,997.60. Yield is 80% with a purity >95%.

Deacetylation of acetylated galactose [60] and reduction of the *t*-buthio disulfide

Peptides containing acetylated galactose were dissolved in THF/MeOH/(28%)NH₄OH (2/2/1, concentration ~5 mM). The reaction was followed until its completion by RP-HPLC using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). RP-HPLC showed two main products corresponding to the deacetylated peptides with different amounts of the *t*BuS-SCys and the deprotected cysteine. After evaporation of THF and MeOH and lyophilization of the residual aqueous solution, the mixture of peptides (corresponding to the CysS-*t*Bu and the deprotected cysteine) were dissolved in a degassed solution of Tris-HCl 50 mM (~2 mM). DTT (20 eq.) was added, and the reaction was stirred at RT for 2 h. The final deacetylated free thiol peptides were purified by RP-HPLC on a preparative C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Peptide purity was analyzed by analytical RP-HPLC with a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) and characterized by MALDI-TOF MS.

(R6/W2)-[Pra-Gal(OH)]-SH, **16** RP-HPLC retention time is 8.2 min (15–55% acetonitrile over 30 min). MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated is 1,771.93, found 1,771.95. Yield is 70% with a purity >95%.

(R6/W2)-[Pra-Gal(OH)]₃-SH, **19** RP-HPLC retention time is 8.0 min (0–55% acetonitrile over 30 min). MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated is 1,999.98, found 2,000.15. Yield is 38% with a purity >95%.

Biotin(O₂)-G₄-C(Npys)-KLAKLAKKLAKLAK-NH₂, **25**

Boc-KLAKLAKKLAKLAK-NH₂ was synthesized in a stepwise manner on a 0.5-mmol scale using a (ABI 433-A, Applied Biosystems) synthesizer with standard protocols of Boc chemistry (amino acid activation with DCC/HOBt in NMP). After removal of the Boc group, the resin was neutralized with a solution of DIPEA (10%) in NMP; Boc-(L)-Cys(Npys) (5 eq.) was coupled manually using DCC (5 eq.) activation. After Boc removal, the glycines (10 eq.) and biotin(O₂) (10 eq.) were coupled using HBTU activation and in situ neutralization with DIEA (25 eq.). The resin was washed with NMP, CH₂Cl₂, MeOH, and dried under vacuum. The peptide was cleaved from the resin with anhydrous HF (1 h 15, 0 °C) in the presence of anisole (1.5 ml/g resin) and dimethylsulfide (0.25 ml/g resin). The peptide was purified by RP-HPLC with a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Its purity was analyzed by analytical RP-HPLC with a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) and characterized by MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated 2,266.23, found 2,266.71, [*M*-NpysH]⁺ calculated 2,112.24, found 2,112.78. Retention time is 18.25 min (15–55% acetonitrile over 30 min). Yield is 15% with a purity >97%.

Biotin(O₂)-G₄-C(Acm)-KLAKLAKKLAKLAK-NH₂, **31**

TCEP (2.5 eq.) and iodoacetamide (100 eq.) were added to a solution of peptide **25** (approximately 5 mM) in degassed Tris-HCl (50 mM). Completion of the reaction was determined by HPLC. The peptide was purified by RP-HPLC with a C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Its purity was analyzed by analytical RP-HPLC with a C8 column using a linear acetonitrile gradient; retention time is 20.5 min (10–50% acetonitrile over 30 min). The peptide was characterized by MALDI-TOF MS (*m/z*) [*MH*]⁺ calculated 2,169.26, found 2,169.05.

Syntheses of the carrier-cargo conjugates

The thiol peptides were dissolved in a degassed 10% acetic acid and mixed with a slight excess (1.2 eq.) of peptide Biotin(O₂)-G₄-C(Npys)-[KLAKLAK]₂-NH₂. The reaction was followed by RP-HPLC, and the conjugates were

purified by RP-HPLC on a preparative C8 column using a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Peptides were purified by analytical RP-HPLC using a C8 column with a linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) and characterized by MALDI-TOF MS.

(R6/W3)*S-S-KLAK*, **26** (Previously described herein for the analog with four deuterated glycines) RP-HPLC retention time is 20.0 min (15–35% acetonitrile over 30 min). MALDI-TOF MS (m/z) [MH]⁺ calculated is 3,775.18, found 3,775.28. Yield is 46% with purity >95%.

(R6/W2)-[Pra-Gal(OH)]*S-S-KLAK*, **27** RP-HPLC retention time is 14.0 min (15–55% acetonitrile over 30 min). MALDI-TOF MS (m/z) [(R6/W2)-[Pra-Gal(OH)]SHH]⁺ calculated is 1,771.93, found 1,771.91; [KLAK-SHNa]⁺ calculated is 2,134.24, found 2,133.85. Yield is 28% with purity >99%.

(R6/W2)-[Pra-Gal(CH₂)₃(OH)]*S-S-KLAK*, **28** RP-HPLC retention time is 14.0 min (15–55% acetonitrile over 30 min). MALDI-TOF MS (m/z) [(R6/W2)-[Pra-Gal(CH₂)₃(OH)]SHH]⁺ calculated is 1,829.96, found 1,829.73; [KLAK-SHNa]⁺ calculated is 2,134.24, found 2,134.03. Yield is 33% with purity >95%.

(R6/W2)-[Pra-Gal(CH₂)₃(OAc)]*S-S-KLAK*, **29** RP-HPLC retention time is 3.5 min (20–60% acetonitrile gradient over 30 min). MALDI-TOF MS (m/z) [MH]⁺ calculated is 4,107.22, found 4,107.11; [(R6/W2)-[Pra-Gal(CH₂)₃(OAc)]SHH]⁺ calculated is 1,998.00, found 1,997.51; [KLAK-SHH]⁺ calculated is 2,112.24, found 2,111.71. Yield is 22% with purity >95%.

(R6/W2)-[Pra-Gal(OH)]₃ *S-S-KLAK*, **30** RP-HPLC retention time is 19.5 min (0–55% acetonitrile gradient over 30 min). MALDI-TOF MS (m/z) [MH]⁺ calculated is 4,109.20, found 4,109.92; [(R6/W2)-[Pra-Gal(OH)]₃SHH]⁺ calculated is 1,999.98, found 1,999.78; [KLAK-SHH]⁺ calculated is 2,112.24, found 2,112.05. Yield is 72% with purity >95%.

PenetratinS-S-KLAK, **32** (Previously described herein for the analog with four deuterated glycines) RP-HPLC retention time 19.7 min (15–35% acetonitrile gradient over 30 min). MALDI-TOF MS (m/z) [MH]⁺ calculated is 4,507.62, found 4,507.48.

Cell viability assays

A cell suspension (100 μL, 5,000 CHO cells per well) were seeded in DMEM plus 10% FCS in 96-well microtiter

plates at 37 °C. After 24 h of cell culture, the supernatant was removed, and the peptide in DMEM (100 μL) at concentrations of 1, 10, 20, or 50 μM was added to the cells. After 2 h of incubation at 37 °C, the supernatant was removed, and a solution of CCK8 cell counting kit in DMEM (10%) was used according to the supplier (Dojindo Laboratories). The absorbance was measured at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH) with a reference wavelength at 620 nm.

Fluorescence microscopy

Fluorescence microscopy was performed on an inverted Nikon Eclipse TE2000-S (objective ×100 PLANFLUO). All experiments were performed with fixed cells. Cells were plated on glass coverslips (7 × 10⁴ cells, 1 cm²) and cultivated for 24 h. The cells were washed once with serum-free medium. Biotin-labeled peptide or conjugates (10 or 20 μM) were incubated with CHO-K1 cells in 300 μL fresh serum-free DMEM/F12 medium for 90 min at 37 °C. After washing, the extracellular peptide was quenched with avidin (10 μM, 10 min at 4 °C). Cells were fixed with 4% paraformaldehyde and treated with 0.1% Triton ×100. The intracellular peptide was labeled with Alexa-488-streptavidin (1/2,000 of 1 mg/mL solution) for 30 min. Nuclei were stained with DAPI (1.5 μg/mL; Pierce). For the membrane peptide, avidin washings and Triton permeabilization were omitted. Coverslips were mounted in Vectashield (BioValley) for microscopy observation.

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