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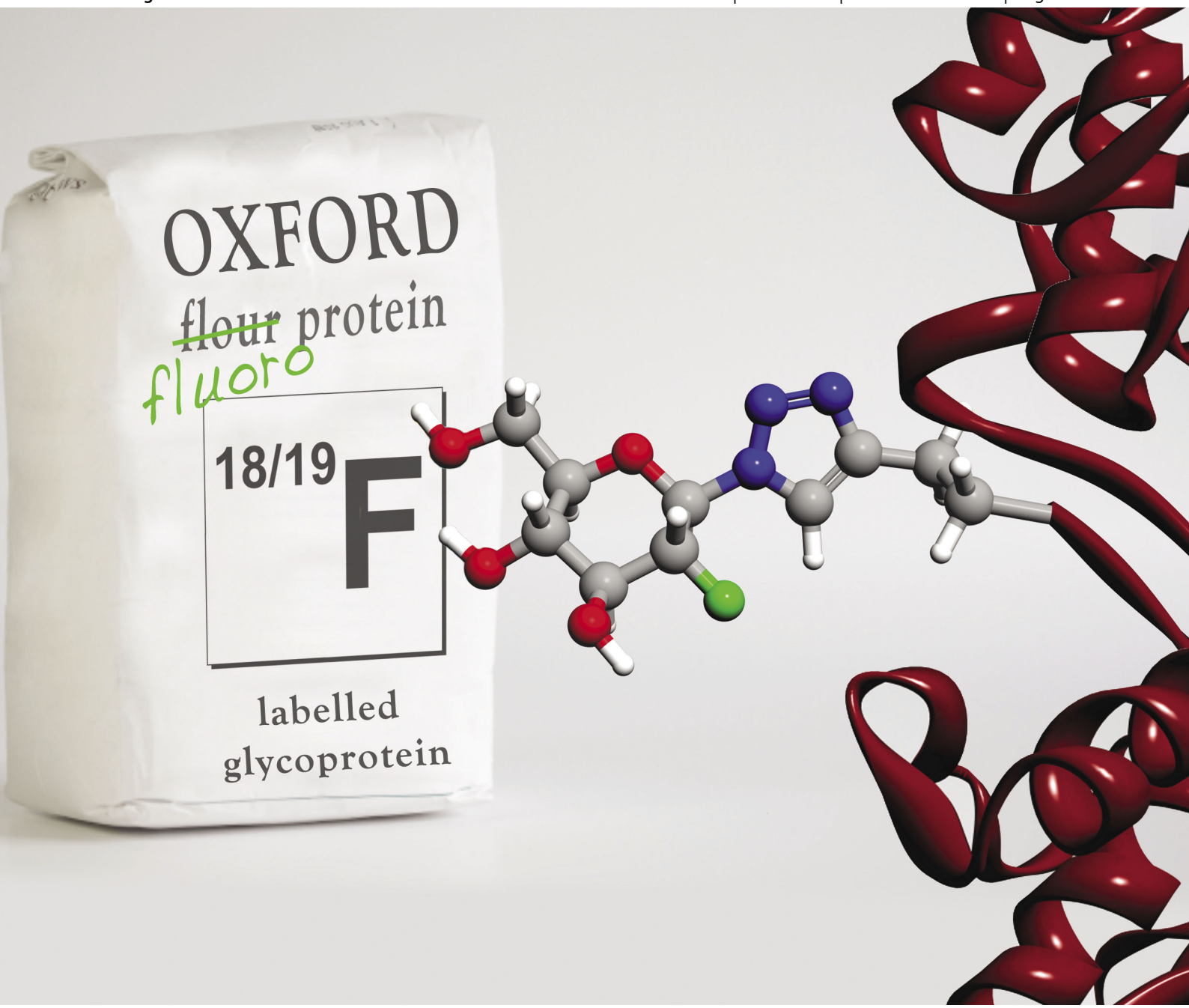

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Fluoroglycoproteins: ready chemical site-selective incorporation of fluorosugars into proteins

Fluoroglycoproteins: ready chemical site-selective incorporation of fluorosugars into proteins†

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A tag-and-modify strategy allows the practical synthesis of homogenous fluorinated glyco-amino acids, peptides and proteins carrying a fluorine label in the sugar and allows access to first examples of directly radiolabelled ([^{18}F]-glyco)proteins.

The study of carbohydrates found in nature,[1,2] either bound to proteins or lipids, can be hampered by difficulties in accessing well-defined glycoconjugates.[3,4] As part of our continuing effort to access pure synthetic glycoproteins[5,6] and to better understand the role of individual glycoforms, we have set out to develop novel strategies for their construction and to delineate glycan function. In this context, there is strong potential for labels (*e.g.*, in both radiolabelled glycoproteins[7–9] and fluorosugars[10–15]) to probe carbohydrate–protein interactions *in vitro* and *in vivo*.[16] In addition, glycosylation of peptides and proteins has been often correlated with enhancement of pharmacokinetic properties and specific cellular uptake.[7–9,17] We therefore considered that a chemical method that would allow simultaneous formation of a well-defined glycoprotein and incorporation of fluoro-labelled sugars (with additional radiolabelling potential) would facilitate such investigations.

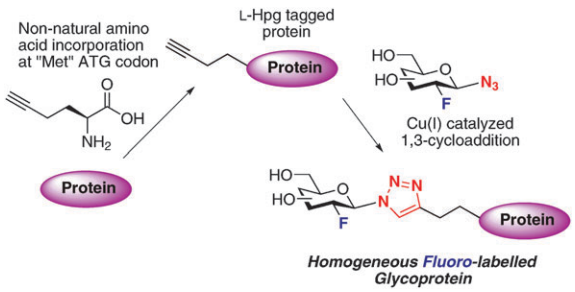
Fluorine is a powerful probe in NMR, MRI, and positron emission tomography (PET) techniques and has found several applications in Chemical Biology and Chemical Medicine.[18–20] Indeed, fluorosugar 2-deoxy-2-[^{18}F]fluoroglucose (^{18}FDG) is the most widely used radiopharmaceutical for imaging with PET.[21] Despite a number of methods for the ^{18}F-labelling of peptides,[22–26] protein labelling is more rare[27–33] and ^{18}FDG itself has only been added directly to proteins using a so-called prosthetic group strategy that generates isomeric mixtures.[34] Many current methods for ^{18}F-labelling of peptides also use reaction conditions and attachment groups that are not suitable for application in proteins; multi-step syntheses with lower overall radiochemical yield (RCY); and can necessitate additional late-stage lipophilic derivatization to enable RP-HPLC.[26] Moreover, existing ^{18}F-protein methods have relied upon modification of natural amino acids (Lys, Cys), reducing (site)selectivity and synthetic flexibility.[35] As a consequence, general strategies for mild, site-*selective* synthesis of pure $^{18/19}$F-labelled glycoproteins are of value.

We describe here a pragmatic, convergent approach for site-selective chemical synthesis of proteins bearing fluorosugars. The strategy, based on a 'tag-and-modify' approach,[36] exploits the introduction into proteins of a non-native amino acid bearing a side chain that contains a functional group that may be chemoselectively addressed.

The method exploits non-natural amino acid incorporation[37] for introduction of L-homopropargylglycine (Hpg) into a protein and triazole formation chemistry,[38,39] under conditions mild enough to retain protein activity (Scheme 1). 1,2,3-Triazoles, formed from reaction of a fluoro-labelled glycosyl azide with an alkynic-amino acid partner, may be considered hydrolytically-stable bioisosteres of the amide bond found in natural Asn-linked glycoproteins.[40] During the course of this work methods were disclosed[26] for attachment to short peptides containing alkynyl-amino acids, although these amino acids cannot yet be introduced into proteins[37] and these methods prove too harsh for application to proteins. The introduction of fluoro-sugars described here therefore represents a variant (FGlyco-CCHC) that expands previous copper-catalyzed Huisgen cycloadditions for introduction of sugars into proteins (Glyco-CCHC).[35,41]

As potential coupling partners for Hpg-containing peptides and proteins, representative fluoroglycosyl azides **1–3** with different configurations (*gluco*, *galacto*, *manno*) were constructed (see ESI†). To survey reaction conditions and demonstrate compatibility, we synthesized and used **4**[35] as a model tag-containing amino acid. FGlyco-CCHC reactions with **1–3** using copper(I) bromide under aqueous conditions afforded corresponding triazole-linked fluoro-glycoamino acids **5–7** with high conversions (>95%) regardless of sugar configuration; more hindered 1,2-*cis* 2-F-Man-β-glycoside proved more sluggish. Importantly, in all cases, β-anomeric configuration was retained after reaction ensuring a single ^{19}F-NMR resonant signal and conjugate product homogeneity.

FGlyco-CCHC was then expanded to peptide substrates. A variant of the 153–161 fragment of model protein subtilisin



Scheme 1 Site-selective protein fluorine-labelling using a tag-and-modify approach coupled with a fluoro-sugar variant of Cu(I)-catalyzed Huisgen-azide–alkyne cycloaddition (FGlyco-CCHC).

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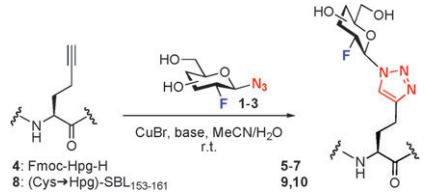
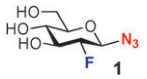
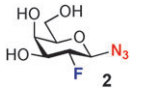
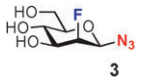
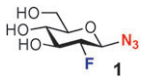
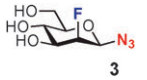
† Electronic supplementary information (ESI) available: Experimental details (^{1}H, ^{13}C, ^{19}F NMR spectra for all new compounds, ESI–MS for proteins, SDS PAGE, radio-HPLC). See DOI: 10.1039/c0cc01576h

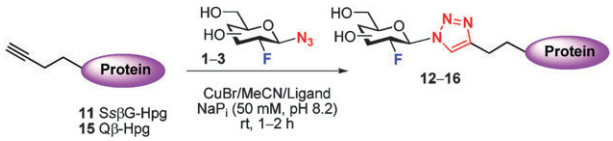
Table 1 Amino acid and peptide FGlyco-CCHC reactions[a]

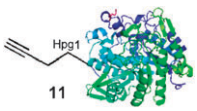
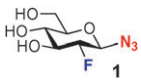
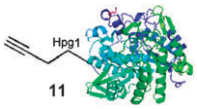
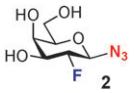
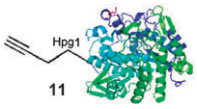
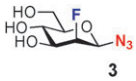
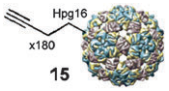
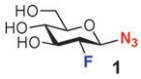


Entry	Amino acid/peptide	Glycan	*t*/h	Product	Conv.[b] (%)
1	**4**	 **1**	18	**5**	>95
2	**4**	 **2**	18	**6**	>95
3[c]	**4**	 **3**	44	**7**	>95
4[d]	**8**	 **1**	2	**9**	>95
5[d]	**8**	 **3**	2	**10**	>95

[a] *General conditions*: see ESI† for details, CuBr, Et_3N, CH_3CN/H_2O unless indicated. [b] Determined by ^{19}F NMR. [c] Addl CuBr at 16.5 h. [d] CuBr with ligand in CH_3CN/sodium phosphate buffer (50 mM, pH 8.2).

Table 2 Protein FGlyco-CCHC reactions[a]



Entry	Protein	Glycan	Product and Conv.[b] (%)	ESIMS found (calcd)
1	 **11**	 **1**	**12** (>95)	57 527 (57 530)
2	 **11**	 **2**	**13** (>95)	57 526 (57 530)
3	 **11**	 **3**	**14** (>95)	57 531 (57 530)
4[c]	 **15** (14 313)[d]	 **1**	**16** (>95)	14 311

[a] *General conditions*: CuBr, ligand in CH_3CN/sodium phosphate buffer (50 mM, pH 8.2) for 1 h at RT unless indicated; see ESI.† [b] By LC–MS. [c] 2 h at RT. [d] Multimer denatured (1 M DTT, 60 °C, 5 min) prior to LC–MS.

from *Bacillus lentus* ((Cys → Hpg)–$SBL_{153-161}$) **8** containing a site-selectively positioned Hpg residue at site 156 as a reactive 'tag' was generated and tested in the FGlyco-CCHC with fluoroglycosyl azides **1**, **3** (Table 1, entries 4 and 5). Reaction was successful in pH 8.2 phosphate buffer at room temperature after 2 h affording corresponding *gluco* **9** and *manno* **10** derivatives in >95%. ^{19}F NMR of fluoroglycopeptide showed the potential of F as a privileged label for creating sharp signals in selective detection and monitoring of glycopeptides with no biological background signal (see SI†).

Next, the FGlyco-CCHC approach was applied to model proteins: a TIM-barrel protein (*Ss*βG),[42] and self-assembled homomultimer virus-like bacteriophage particle Qβ.[43–45] Unnatural amino acid Hpg was site-specifically introduced into these proteins as an alkynic 'tag' through expression of corresponding genes in *E. coli* auxotroph (B834(DE3)).[37] Sequences were designed to create proteins displaying alkynes at sites for which position could be controlled using 'Met' triplet codon ATG. Replacement of wildtype methionine (Met) residues, with near-isosteric amino acid isoleucine (Ile) allows reassignment of Met codons in the gene sequence to allow incorporation instead of Hpg as a 'tag'.

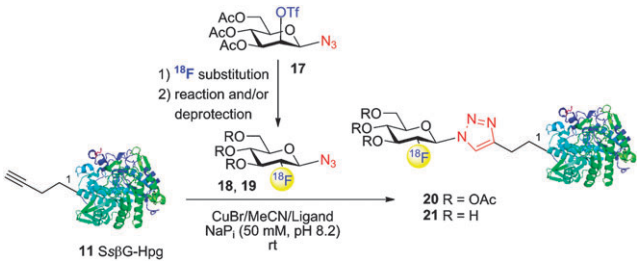
*Ss*βG is an example of a generic protein scaffold, the TIM barrel, that we have previously used to create synthetic glycoprotein probes that may be used both *in vitro* and *in vivo*.[35,46] Gratifyingly, FGlyco-CCHC reaction of *Ss*βG protein containing Hpg at position 1 (**11**) with fluoroglucosyl azide **1** gave good conversion (>95%) to *Ss*βG-1-(2F-Glc) **12** (Table 2, entry 1). Reaction conditions used a pre-formed solution of highly pure Cu(I) stabilized by coordinating triethyl 2,2′,2″-(4,4′,4″-nitrilotris(methylene)tris(1*H*-1,2,3-triazole-4,1-diyl))triacetate ligand in sodium phosphate buffer (50 mM, pH 8.2) for 1 hour at room temperature.

Having established conditions for efficient site-selective incorporation of 2-deoxy-2-fluoroglucose (^{19}FDG) into an alkyne-tagged protein, we explored extension of this approach to other monosaccharides, configurations and proteins. These were equally successful, yielding corresponding fluoroglycoproteins **12–14** (Table 2, entries 1–3) in >95%. Moreover, reaction of fluoroglucosyl azide **1** with virus-like particle multimer of Qβ-Hpg16 **15** afforded F-glycoprotein in >95% and a particle containing 180 site-selectively positioned fluoroglycans (**16**) (Table 2, entry 4). Importantly, and unlike other methods,[26] the conditions were mild and effective for site-selective protein glycosylation whilst maintaining protein function (*e.g.* enzymatic activity) without degradation.

Finally, given the potential utility of combined simultaneous attachment of fluorosugar and radiolabel we extended the method to create ([^{18}F]-glyco)proteins. Radioactive ^{18}FDG was prepared as its glycosyl azide variants **18**,**19** in protected and unprotected forms, respectively, from mannosyl triflate **17**.[47] Reaction with *Ss*βG protein **11** gave good radioconjugations up to 60% for **18** under mild conditions (Table 3, entry 1), although yields with **19** were lower. The complete radiosynthetic procedure, including preparation of the ^{18}F-glycosylating reagent Ac_3–[^{18}F]FGlc–N_3 **18** and protein ligation, was performed under timescales compatible with incorporation of even short half-life radioisotope ^{18}F and provided a decay-uncorrected radiochemical yield of 2.2% after a synthesis time of 135 min.

In summary, combined site-selective FGlyco-CCHC with fluoroglycosyl azides and non-natural amino acid

Table 3 Protein [^{18}F]Glyco-CCHC reactions[a]



Entry	Glycosyl azide (R)	t^b/min	Product	Y^c (%)
1	**18** (Ac)	180	**20**	60
2	**19** (H)	45	**21**	4.1

[a] *General conditions*: **18**, **19** (10–15 MBq), *Ss*βG-1Hpg43Hpg344Ser **11**, CuBr, ligand in CH_3CN/phosphate buffer (50 mM, pH 8.2) at RT see ESI† for full details. [b] [^{18}F]Glyco-CCHC reaction time. [c] Radio-RP-HPLC.

incorporation into proteins is an efficient, pragmatic and benign strategy to label (and radiolabel) proteins whilst maintaining function. Its chemoselectivity should allow use in orthogonal, multi-site modification approaches.[35,41] Simultaneous controlled introduction of both sugar and (radio)label ensures homogeneity; standard non-specific methods for (radio)labeling, if applied subsequently to even pure (glyco)proteins, still lead to heterogenous mixtures that limit the data they generate. Here, we have demonstrated successful application of this approach on amino acids, peptides, proteins and multisite incorporation into virus-like particles. We are currently using modified conjugates in chemoenzymatic syntheses of fluoro-oligosaccharide-bearing proteins. We expect utility from site-specific incorporation of fluorosugars into proteins, combined with use of fluorine as a powerful NMR, MRI, PET probe of *in vitro* and *in vivo* carbohydrate–protein interactions.

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