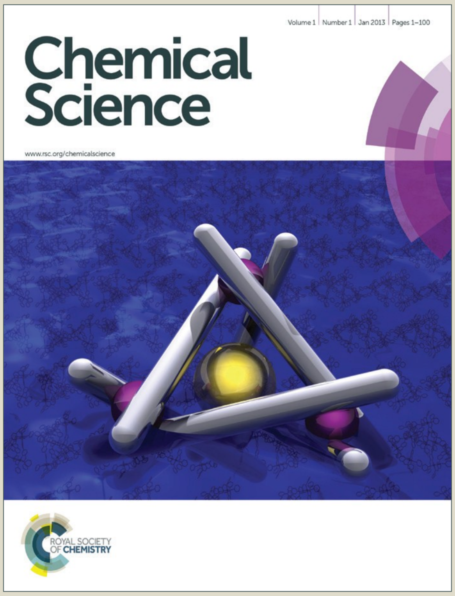

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ARTICLE

Rapid Biocompatible Macrocyclization of Peptides with Decafluoro-diphenylsulfone

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In this manuscript, we describe modification of Cys-residues in peptides and proteins in aqueous solvents via aromatic nucleophilic substitution (S_NAr) with perfluoroarenes (fAr). Biocompatibility of this reaction makes it attractive for derivatization of proteins and peptide libraries comprised of 20 natural amino acids. Measurement of the reaction rates for fAr derivatives by ^{19}F NMR with a model thiol donor (β-mercaptoethanol) in aqueous buffers identified decafluoro-diphenylsulfone (**DFS**) as the most reactive S_NAr electrophile. Reaction of **DFS** with thiol nucleophiles is >100,000 faster than analogous reaction of perfluorobenzene; this increase in reactivity enables application of **DFS** at low concentrations in aqueous solutions compatible with biomolecules and protein complexes irreversibly degraded by organic solvents (e.g., bacteriophages). **DFS** forms macrocycles when reacted with peptides of the general structure X_n-Cys-X_m-Cys-X_l, where X is any amino acid and m=1-15. It formed cyclic peptides with 6 peptide hormones—oxytocin, urotensin II, salmon calcitonin, melanin-concentrating hormone, somatostatin-14, and atrial natriuretic factor (1-28) as well as peptides displayed on M13 phage. Rates up to 180 $M^{-1}s^{-1}$ make this reaction one of the fastest Cys-modifications to-date. Long-term stability of **DFS**-macrocycles and their stability toward oxidation further supports **DFS** as a promising method for modification of peptide-based ligands, cyclization of genetically-encoded peptide libraries and discovery of bioactive macrocylic peptides.

Introduction

Rapid, site-specific modifications of polypeptides are critical for chemical modification of proteins, synthesis of antibody-drug conjugates, and generation of genetically-encoded libraries with unnatural moieties. Biocompatible site-specific cross-linking of two side-chain residues in polypeptides is a rapidly growing field because such modification can increase stability and efficacy of peptide-based therapeutics (for recent reviews see[1-6]). While such cross-linking or macrocyclization can be performed on amino acid side-chains that contain unnatural reactive groups,[7-9] modification of natural-occurring residues is particularly attractive because it can be used for modification of readily-available peptides/proteins and phage display libraries comprised of 20 natural amino acids.[10-16]

In this article, we report rapid, biocompatible modification of Cys residues *via* nucleophilic aromatic substitution (S_NAr) reaction with perfluoroarenes (fAr). We overcome the deficiencies of previously known S_NAr-reagents, such as 1-chloro-2,4-dinitrobenzene (CDNB),[17] which are susceptible to background reactivity with nucleophilic residues in proteins, or perfluorobenzene[18] and perfluorobiphenyl[18, 19] which suffer from low reactivity and poor solubility in water. The latter reagents are effective in organic solvents, such as DMF, but are not suitable for modification of biological entities that are irreversibly deactivated by high concentrations of organic solvents (e.g., proteins, bacteriophage, etc.). While glutathione transferase can be used to catalyze S_NAr-reactions in water,[20] the enzyme acts only on peptides that contain unnatural *N*-terminal-α-Glu.[20, 21] Hence, improved fAr cross-linking agents provide the ability to access diverse chemical modalities through native polypeptide sequences.

Results and discussion

To identify S_NAr derivatives for aqueous bioconjugations, we explored the rate of the reaction of a model thiol donor β-mercaptoethanol (βME) and twelve fAr reagents using ^{19}F NMR (see Supporting Figures S1-S3, examples of ^{19}F NMR traces) in aqueous Tris-buffer pH=8.5 supplemented with CH_3CN as co-solvent (Figure 1A-C). Reactivity of most perfluoroarenes was hampered by both poor solubility and poor reactivity (Figure 1D); many reactions required 80% or more CH_3CN as co-solvent. Similar to studies in organic solvents,[22] the reactivity in aqueous conditions correlates with the electronegativity of the substituent on the aromatic ring (Figure 1C) yielding the

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x fastest reactivity for perfluoronitrobenzene (R=NO_2), decafluorobenzophenone (R=-COC_6F_5), or decafluorodiphenylsulfone (R=-$SO_2C_6F_5$, **DFS**) reagents, or heteroaromatic derivatives such as perfluoropyridine.

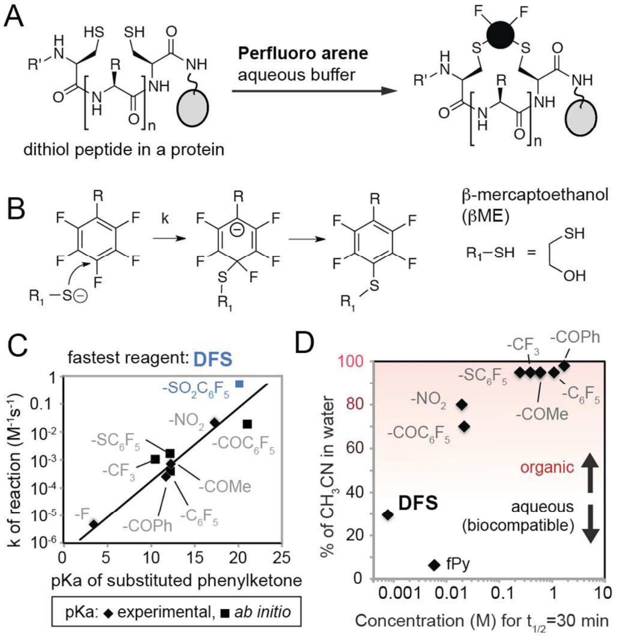

Figure 1. A) Design of reagents for perfluoroarene macrocyclization by cross-linking two Cys-residues in a protein in aqueous conditions. B) Mechanism of S_NAr reaction. C) Rate constants (k) of the reaction between substituted fAr and βME, as measured by ^{19}F NMR, increase with the increasing electronegativity of R group. As a measure of electronegativity, we used pK_a in a series of phenylketones for which many values are known[23] or can be calculated (Supporting Figure S4). D) To find the S_NAr reagents for rapid modification in water, we plotted molar concentration of fAr necessary to reach 50% conversion in 30 minutes (calculated as ln(2)/(1800×k), where k is the rate constant) and % of organic co-solvent necessary to dissolve the fAr at this concentration. Most fAr are poorly reactive and/or too insoluble in aqueous solvents; only **DFS** and perfluoropyridine (fPy) can be used in conditions that require low amount of organic co-solvent.

We then validated the reactivity and specificity of the most reactive substrate (**DFS**) using model peptides H_2N-SWCXC-$CONH_2$, where X= Asp, Ala, Ser or Arg that contained two types of nucleophiles: *N*-terminal amine and thiol (Figure 2A). We were pleased to find that **DFS** reacted remarkably fast with Cys side chains (Figure 2B and Supporting Figures S5-S6) in conditions that required as little as 10% of CH_3CN co-solvent (Figure 1) or as little as 5% DMF co-solvent (Supporting Figure S7). As S_NAr reaction alters the aromatic π-system,[17, 24] we measured rate constants by measuring changes in absorption at 300 nm (Supporting Figures S5-S6, S8-S11) and validated these rates by ^{19}F NMR (Supporting Figures S10-S11) and LCMS (Supporting Figure S7). Conveniently, the reaction was accelerated in solvents with high water content, although the rate decreased in water without any CH_3CN due to limited solubility of **DFS** (Supporting Figure S12). At pH 8.5 and water content of 70-80%, the rate constant was 100–180 $M^{-1}s^{-1}$ for positively charged peptide sequence SWCRC and 50-80 $M^{-1}s^{-1}$ for neutral analog SWCAC (Figures 2B).

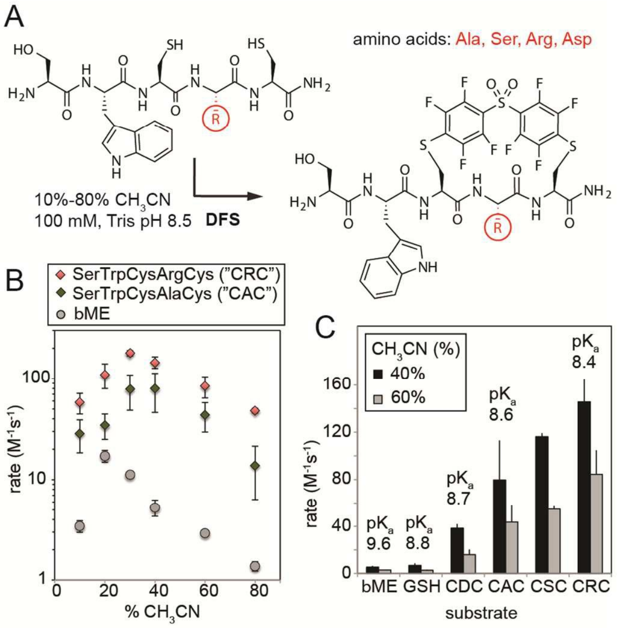

Figure 2. A) S_NAr reaction of **DFS** with peptides is sequence and solvent-dependent. B) Rates in all conditions were measured by monitoring absorbance at 320 nm, and identities of the products were confirmed by NMR and Liquid Chromatography Mass-Spectrometry (LCMS, Supporting Figures S7, S12-S15). C) The rate of the reaction correlates with acidity of the thiol; pK_a were estimated by measuring the absorbance of peptides at 240 nm as described in Supporting Figure S18 and were in accordance with previously reported values for CXC peptides.[25]

The rate of the reaction with **DFS** was sequence dependent (Figure 2B-C and Supporting Figure S5). Positively-charged Arg residue next to Cys accelerated the reaction, negatively charged Asp suppressed it. This observation can be explained by higher basicity of thiol in CRC vs. CDC (Figure 2C). Additionally, the positive charge on the Arg side-chain can stabilize the intermediate carbanion (Figure 1B). We anticipate that analogous strong dependence of the rate constant on the pK_a of thiol (Figure 2C) exist in other S_NAr reactions between peptides and perfluoroarenes,[18, 19] because S_NAr reactions with thiols exhibit large Brønsted coefficients.[20] Substrate-dependence was the most pronounced when solvation was poor (e.g., high CH_3CN content); increased solvation in solutions with high aqueous content masked the effect (Figure 2B and Supporting Figure S6). The pH-dependence of the rate plateaued at pH 8-9 confirming that thiolate is the nucleophile (Supporting Figure S19-20). In acidic pH, the rate dropped exponentially (i.e., decrease of log(k) was linear with pH). Reaction with perfluoropyridine exhibited similar solvent-dependent and substrate-dependent profiles (Supporting Figure S8, S9, and S21), however, the rates were 10-100 times slower.

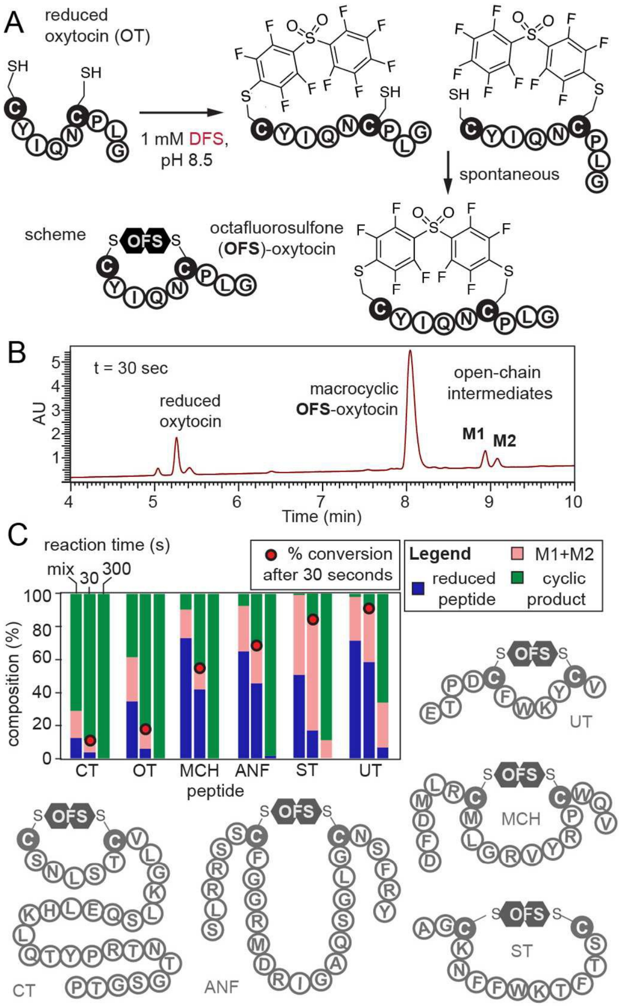

Figure 3. A-B) Cyclization of reduced oxytocin (OT) by **DFS**. Quenching the reaction by 2% TFA at 30 s revealed ~90% consumption of starting material, 80% of product, and 12% of open-chain intermediates M1 and M2. C) Stacked bar representation of the composition of reaction mixture determined by HPLC shows that both M1 and M2 of OT were completely converted to product by 5 min. Analogous studies with five other peptide hormones in the presence of 1 mM **DFS**. Fraction of the reactant, sum of M1+M2 intermediates, and products was determined by integration of the HPLC traces at 215 nm (see Supporting Figure S16, S22-S23 for raw traces).

LCMS confirmed the identity of the products during the reaction and 24-48 hours after its completion (Supporting Figures S12-S15). LCMS confirmed that the reaction was selective for Cys in the presence of free *N*-terminus and large excess of **DFS**. Side-reactivity was observed only after prolonged exposure to **DFS**. Specifically, peptides SWCAC or SWCRC incubated with large excess of **DFS** (10 eq, 2 mM) for 48 hours contained ~20% of the product with two **DFS** moieties. Less than 5% of this by-product was visible after 48 hours in the presence of 0.2 mM **DFS**. In contrast, Cys arylation under analogous conditions was complete in 5-10 minutes (Supporting Figure S6). We observed this by-product in three different peptide sequences (Supporting Figures S13-15) and proposed it to be a product of S_NAr reaction between **DFS** and *N*-terminus. While the side-reaction of *N*-terminal amine is possible, it is $>10^4$ times slower than Cys-arylation. This data still do not completely rule out the possibility that the cyclization could also happen between the thiol and the terminal amine. To show that cyclization occurs exclusively on Cys residues, we treated peptide with **DFS** and then with thiol-specific reagent, biotin-PEG$_2$-iodoacetamide (**BIA**). While **BIA** reacted readily with thiols in starting material, we detected no reaction between **BIA** and octafluorodiphenylsulfone (**OFS**)-peptide conjugate (Figure S17).

To confirm that **DFS** is useful for modification of complex peptides, we modified peptide CYIQNCPLG (oxytocin, OT). Reaction yielded a single product with expected mass and apparent rate constant of 37.2 $M^{-1}s^{-1}$ as measured by LCMS (Figure 3, Supporting Figure S16). To stop the reaction before completion, we quenched it with acid and observed mono-arylated intermediates of oxytocin M1 and M2 in a transient open-chain state (Figure 3A-B). Both intermediates disappeared over the course of the reaction (Figure 3C). To confirm that **DFS** can form cyclic peptides regardless of their ring size, we tested reaction between **DFS** and five other peptide hormones of general structure X$_{N1}$-Cys-X$_M$-Cys-X$_{N2}$, where X is any amino acid other than Cys and M—the number of amino acids flanked by two Cys—ranged from 4 to 15. Urotensin II (UT, M=4), salmon calcitonin (CT, M=5), melanin-concentrating hormone (MCH, M=8), somatostatin-14 (ST, M=10), and atrial natriuretic factor (1-28) (ANF, M=15) formed cyclic peptides in reaction with **DFS** regardless of their ring size. We used HPLC to estimate the rate of cyclization (Figures 3C, S22-S23) by quenching the reaction with acid in the presence of 1 mM **DFS**. After 30 seconds, we detected 10-90% of the final cyclized product and <5-60% of starting material remaining. After 10 min, only urotensin II, which displayed the slowest kinetics, contained ~5% of the unreacted starting material. Other peptides were completely converted to final cyclic products.

We then confirmed reaction of **DFS** with thiols on intact M13 bacteriophage, which is composed of 5 types of proteins and a single-stranded DNA genome. The phage virion is an ideal model for bioconjugation because (i) it can be engineered to display any peptide sequence, (ii) modification can be detected in individual phage particles,[14] (iii) biocompatibility of the modification can be readily quantified by measuring the fraction of M13 particles that can replicate in bacteria; reagents that damage the proteins or DNA disrupt this replication[14] (Supporting Figure S24). We validated the modification on M13 phage using a biotin capture assay developed previously by our group (Figure 4).[14, 26] Briefly, a phage displaying a disulfide-peptide ACPARSPLEC was reduced with TCEP and the resulting thiols were alkylated using **BIA** (Figure 4B-C). The reaction yielded ~95% of the biotinylated phage, as determined by titering the phage before and after exposure to streptavidin-coated beads (Figure 4D). We then used a "pulse-chase" approach[13-16, 26] to quantify reaction of phage with **DFS**. "Pulsing" the reduced phage with 0.5 mM **DFS** in 30% CH$_3$CN-Tris buffer (pH 8.5) for 30 min consumed

~75% phage-displayed thiols. "Chase" of the reaction with **BIA** yielded only 20% of biotinylated phage. In contrast, the phage remained reduced and susceptible to alkylation by **BIA** when it was "pulsed" with buffer that contained no **DFS**. Thus, disappearance of thiol indicates the reaction with **DFS** and is not due to side reactions (e.g., oxidation). The same "pulse-chase" quantification confirmed that peptide ACPARSPLEC displayed on phage reacts with perfluoropyridine at much slower rate than **DFS** (Figure S25). We further validated the "pulse-chase" method on purified peptide SWCDYRC and confirmed that the peptide "pulsed" by **DFS** for 30 minutes forms a product that does not react with **BIA** (Figure S17).

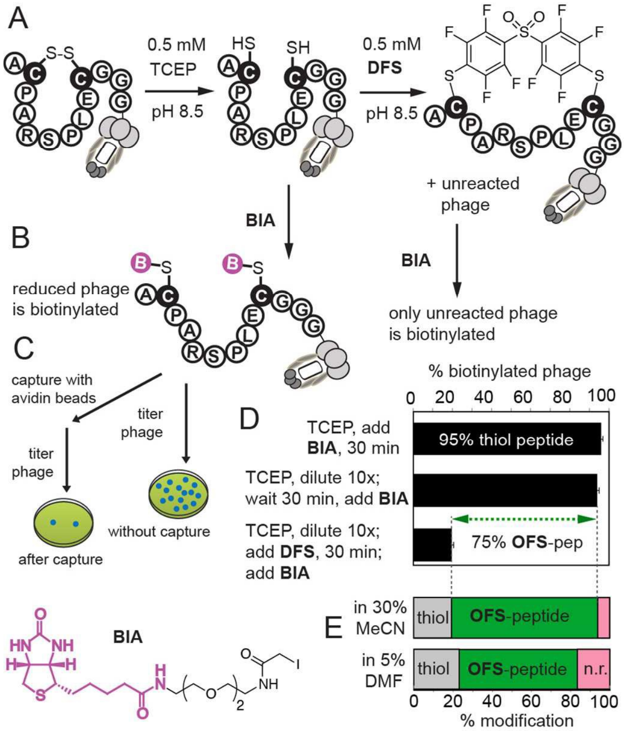
A
0.5 mM TCEP
pH 8.5
0.5 mM DFS
pH 8.5
S-S
HS
SH
+ unreacted phage
BIA
BIA
B
reduced phage is biotinylated
only unreacted phage is biotinylated
C
capture with avidin beads
titer phage
titer phage
after capture
without capture
D
% biotinylated phage
0 20 40 60 80 100
TCEP, add BIA, 30 min
95% thiol peptide
TCEP, dilute 10x; wait 30 min, add BIA
TCEP, dilute 10x; add DFS, 30 min; add BIA
75% OFS-pep
E
in 30% MeCN
thiol
OFS-peptide
in 5% DMF
thiol
OFS-peptide
n.r.
0 20 40 60 80 100
% modification
BIA

Figure 4. A) Quantification strategy for the reaction between **DFS** and M13 phage displaying a peptide ACPARSPLEC.[13] B) Reaction of reduced phage with biotin-iodoacetamide (**BIA**) biotinylates the phage. C) Capture assay measures the yield of biotinylation. D) Capture assay determined that 95% of phage is reduced (reactive with BIA). Diluting the reducing agent 10-fold does not change the number of reduced thiols, but exposing the reduced phage to 0.5 mM **DFS** in 30% MeCN/Tris buffer for 30 min consumes reactive thiols yielding 80% **OFS**-modified phage. Error bars represent standard deviations from two experiments. E) Capture can be summarized as stack bar: bottom bar shows that ~60% of phage is modified by 0.5 mM solution of **DFS** in aqueous Tris buffer containing 5% DMF as co-solvent.

While M13 phage is stable in solutions that contain 30% CH_3CN, in many applications such as protein modifications, such a high amount of acetonitrile might not be tolerated. To this end, **DFS**-mediated cyclization can be performed in aqueous solutions containing only 5% DMF as co-solvent. We observed that solubility of **DFS** in Tris buffer (pH 8.5) containing 5% DMF (v:v) is approximately 500 μM. This concentration is sufficient to perform rapid modifications of synthetic peptides, phage-displayed peptides and phage-displayed libraries of peptides. For example, reaction between 100 μM **DFS** and 100 μM peptide SWCDYRC required approximately 100 seconds to reach 50% conversion and 15 min to reach completion (Supporting Figure S7). LCMS monitoring of this reaction estimated the 2nd order rate constant to be 100-130 $M^{-1}s^{-1}$ (Supporting Figure S7). We then used these conditions to modify phage clone that contained peptide ACPARSPLEC. The modification efficiency by 0.5 mM **DFS** in 5% DMF for 30 min was ~60%, (Figure 4E). Finally, these conditions can also be used to modify commercially available libraries of peptides displayed on phage with 7 random amino acids in between the cysteine residues (New England Biolabs (Ph.D.™-C7C, Figure 5). A decrease in chemical modification efficiency of libraries to 40%, when compared to modification of an individual clone (Figure 4E) is not surprising because library contains 10^9 peptide sequences with diverse reactivity. This decrease in modification is also consistent with our previous reports.[13, 15] **DFS** thus can conveniently convert readily available libraries of peptide-disulfides to genetically-encoded macrocyclic libraries that contain unique perfluoroarene cross-linker. We have employed these libraries in selection of peptide-macrocycle ligands for protein targets but results of these investigations extend beyond the scope of this manuscript and will be described in our subsequent report.

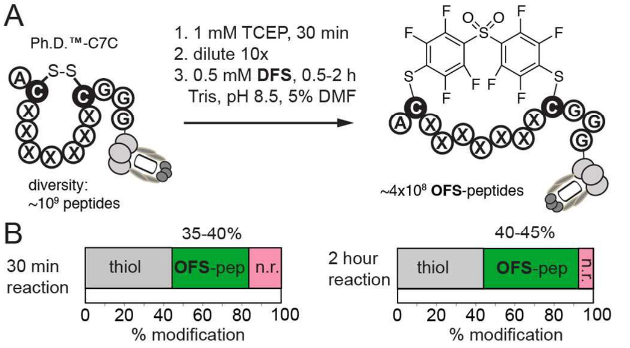
A
Ph.D.™-C7C
S-S
diversity: ~10⁹ peptides
1. 1 mM TCEP, 30 min
2. dilute 10x
3. 0.5 mM DFS, 0.5-2 h Tris, pH 8.5, 5% DMF
~4x10⁸ OFS-peptides
B
35-40%
30 min reaction
thiol
OFS-pep
n.r.
0 20 40 60 80 100
% modification
40-45%
2 hour reaction
thiol
OFS-pep
n.r.
0 20 40 60 80 100
% modification

Figure 5. A) An aqueous solution of **DFS** containing 5% DMF as co-solvent can conveniently convert commercially available phage displayed library of 10^9 disulfide heptapeptides—Ph.D. C7C from New England Biolabs—to a library of **OFS**-macrocyclic peptides. B) Biotin capture, analogous that described in Figure 4C-E, quantified the efficiency of this modification. After 30 min to 2 hours reaction with DFS, 35-45% of the library is modified with DFS yielding ~350-450 million of diverse phage-displayed **DFS**-macrocycles. Data is average from two independent experiments.

Cross-linking of two Cys residues is one of the most common modifications of synthetic peptides and recombinant proteins. To demonstrate advantages of **DFS** over well-established cysteine cross-linkers, we selected α,α'-dibromo-*meta*-xylene (**DBMX**) reagent as a reference (Supporting Figure S26). Reagents structurally and electronically similar to **DBMX** are commonly used to stabilize secondary structure of peptides and synthesize genetically-encoded macrocycle libraries.[27-29] The major advantage of **OFS**-peptide conjugates (perfluoroaryl thioethers) is their higher stability to oxidation when compared to **DBMX**-conjugates (benzyl thioethers). For example, we routinely use $NaIO_4$ to convert *N*-terminal Ser to *N*-

terminal aldehyde.[14, 30, 31] Treatment with $NaIO_4$ readily converted SWCDYRC-**OFS** conjugate to *Ald*-WCDYRC-**OFS**, where "*Ald*-" denotes aldehyde group (Figure 6), and we observed no oxidation of aryl thioethers. As an example of application, we introduced *N*-terminal glycosylation[14, 30, 31] in SWCDYRC-**OFS** conjugate (Figure 6). One-pot two-step reaction quantitatively converted peptide-**OFS** conjugate to *Ald*-peptide-**OFS** and then to glycopeptide-**OFS** conjugate. Such transformation would be challenging to achieve in conjugates that contain thioether bonds derived from benzyl or haloacetyl cross-linkers (Supporting Figure S27). Treatment of SWCDYRC-**DBMX** conjugate by $NaIO_4$ led to concomitant oxidation of serine and benzyl-thioether (Supporting Figure S27D), whereas treatment of SWCDYRC-**OFS** in identical conditions produced no detectable traces of oxidized product (Supporting Figure S27C).

To compare the stability of **OFS**-thioethers towards oxidation with stability of other conjugates, we exposed them to conditions recently used to evaluate stability of diverse thioethers (1 mM H_5IO_6 at 37 °C).[32] Consistent with the published results,[32] we observed that H_5IO_6 rapidly oxidized benzyl-thioether bonds and converted SWCDYRC-**DBMX** conjugate to four oxidized products in 10 minutes (Supporting Figure S27F). In contrast, half-life of oxidation of thioethers in SWCDYRC-**OFS** by H_5IO_6 was 16 hours (Supporting Figure S27E). This value exceeds the reported oxidation half-lives for tolyl- or 4-cyanophenyl-thioether (1 and 6 h, see Supporting Figure S4 in reference[32]). As the rate of oxidation of aryl thioether scales with electronegativity of the aryl substituent,[32] it is not surprising that the peptide-**OFS** macrocycles exhibit high resistance to oxidation. We believe that fast reactivity of **DFS** combined with exceptional oxidative stability of resulting **OFS**-thioethers will make it possible to conduct robust multi-step modification of peptides, which combines Cys cross–linking and *N*-terminal ligations to functionalize natural proteins and diversify genetically-encoded libraries.

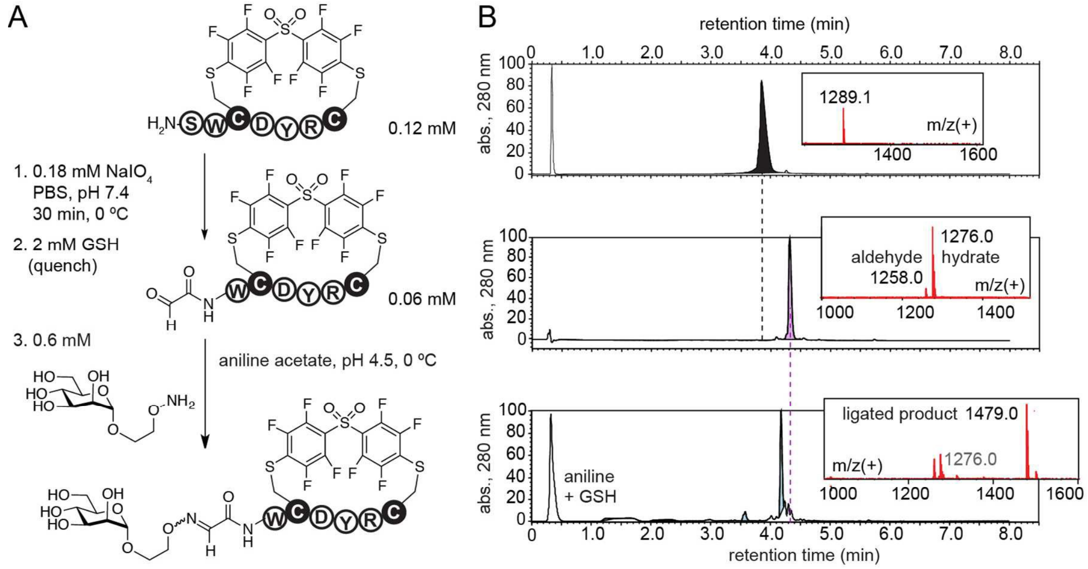

Figure 6. Robust oxidation of SWCDYRC-**DFS** conjugate to peptide-aldehyde and subsequent one-pot conversion to a cyclic glycopeptide.

Conclusions

The **DFS** framework combines fast reactivity up to 180 $M^{-1}s^{-1}$, specificity to Cys-residues, biocompatibility with complex multiprotein complexes (here, bacteriophage), and maximum performance at neutral to mildly-basic pH. Its reactivity significantly exceeds the rates of substitution reactions of Cys with CDNB (k = 1 $M^{-1}s^{-1}$),[17] haloacetamide cross-linkers (k = 10-20 $M^{-1}s^{-1}$ at pH 8.5),[13, 33] bis-bromobenzyl cross-linkers (k = 30 $M^{-1}s^{-1}$ at pH 8.8),[34] bis-methyl-maleimide cross-linkers (k = 1-3 $M^{-1}s^{-1}$)[35] and allenamides[36] (k = 16-30 $M^{-1}s^{-1}$).[16] Recent summaries from the Bode[37] and Prescher[38] groups highlight that in modern bio-orthogonal reactions, very few reach rates of >100 $M^{-1}s^{-1}$ as exhibited by **DFS**. To date only two Cys-reactive reagents, dichlorotetrazene[39] and maleimide (rates up to 800 $M^{-1}s^{-1}$),[37] can rival **DFS** in reactivity, but the products are susceptible to long-term hydrolytic instability.[39-42] In contrast, **DFS** adducts of oxytocin are stable for over 2 weeks at pH=7.4 (Supporting Figure S28). We note that the size of the **DFS** is compatible to the size of many previously reported thiol crosslinkers such as bisbromoxylene,[27-29] decafluorobenzene,[18, 19] benzophenone,[32] bismaleimide, chloroacetamide-azobenzene,[13] and others. Introduction of **DFS** into proteins or peptides will be context dependent: it can provide favourable stabilization of secondary structure but it can also interfere with biological function. In contrast to many known Cys cross-linkers, **DFS** produces conjugates that are stable to oxidation; this stability will enable development of multi-step transformations that convert peptides made of natural amino acids to functionality-rich macrocyclic derivatives. We anticipate that this reaction will serve as a platform for development of new bioconjugation and macrocyclization strategies as well as post-translational diversification of genetically-encoded libraries.

Acknowledgements

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