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Letter

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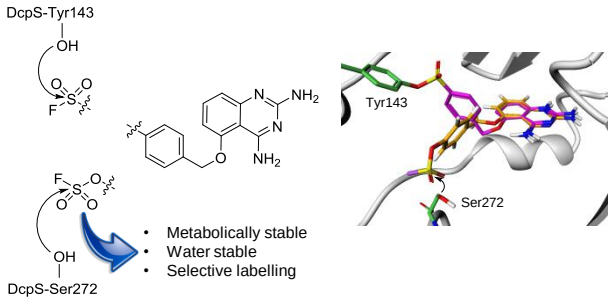

Covalent Enzyme Inhibition through Fluorosulfate Modification of a Non-Catalytic Serine Residue

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Abstract

Irreversible enzyme inhibitors and covalent chemical biology probes often utilize the reaction of a protein cysteine residue with an appropriately positioned electrophile (e.g., acrylamide) on the ligand template. However, cysteine residues are not always available for site-specific protein labeling and therefore new approaches are needed to expand the toolkit of appropriate electrophiles ("warheads") that target alternative amino acids. We previously described the rational targeting of tyrosine residues in the active site of a protein (the mRNA decapping scavenger enzyme, DcpS) using inhibitors armed with a sulfonyl fluoride electrophile. These inhibitors subsequently enabled the development of clickable probe technology to measure drug-target occupancy in live cells. Here we describe a fluorosulfate-containing inhibitor (aryl fluorosulfate probe (FS-p1)) with excellent chemical and metabolic stability that reacts selectively with a non-catalytic serine residue in the same active site of DcpS as confirmed by peptide mapping experiments. Our results suggest that non-catalytic serine targeting using fluorosulfate electrophilic warheads could be a suitable strategy for the development of covalent inhibitor drugs and chemical probes.

Cysteine-targeted irreversible enzyme inhibitors have found considerable utility in drug discovery, particularly in the development of kinase inhibitor drugs and chemical probes.[1] Covalent enzyme inhibitors possess the potential advantages of high potency and enhanced pharmacodynamic duration. However, in cancer there are several examples of cysteine-to-serine mutations causing reductions in efficacy of cysteine-reactive drugs and drug candidates. Also, many enzymes do not possess a targetable cysteine residue within the active site. Drugs that specifically target other amino acid residues could expand the synthetic biology toolbox and significantly enhance the opportunities for covalent inhibitor development.

Our group was the first to rationally design irreversible inhibitors that target tyrosine residues in the active site of an enzyme. Sulfonyl fluoride probes (e.g. aryl sulfonyl fluoride probe (SF-p1), Figure 1)[2,] were designed to react with Tyr113 and Tyr143 in the binding site of the mRNA decapping scavenger enzyme DcpS, a pyrophosphatase responsible for the hydrolysis of the m^7GTP cap from mRNA fragments, and involved in mRNA and microRNA metabolism.[3-4] Recently, inhibitors of DcpS have been under investigation for the treatment of spinal muscular atrophy (SMA),[5-7] all based on a 2,4-diaminoquinazoline (DAQ) template that we used as the basis for probe design, including a clickable SF covalent probe, SF-p1-yne (Figure 1) to report on drug-target engagement in live cells for the first time.[2, 8]

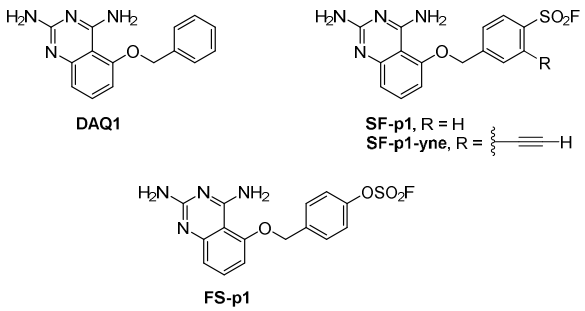


Figure 1. Structures of diaminoquinazoline (DAQ) inhibitors of DcpS.

Sulfonyl fluorides are somewhat privileged electrophiles for use in chemical biology studies as they possess context-dependent protein reactivity balanced with adequate water stability for most applications. Sulfonyl fluorides have been shown to react with a number of amino acid residues (Tyr, Lys, Ser, Thr, Cys, His) in protein binding sites and probes containing this electrophile have found considerable utility in chemical biology research.[9-10]

The application of a fluorosulfate covalent electrophile (or "warhead"), however, has been reported to a much lesser extent.[10] Recent work by the Kelly and Sharpless groups has shown that fluorosulfates are significantly less reactive in a whole cell proteome than the better known sulfonyl fluorides.[11] They showed that an aryl fluorosulfate chemical probe selectively modified a conserved tyrosine residue in the binding site of intracellular lipid binding proteins (iLBPs). A selective covalent inhibitor bearing a biphenyl core structure labeled Tyr134 in retinoic acid binding protein 2 (CRABP2) in cells and inhibited RARα-mediated retinoic acid signaling in a breast cancer cell line.

The presence of two sulfonyl fluoride-reactive tyrosine residues in the DcpS binding site[2] suggested that a fluorosulfate-containing diaminoquinazoline (DAQ) may covalently modify and inhibit enzymatic function. Below we describe the preparation of the fluorosulfate congener (aryl fluorosulfate probe (FS-p1)) of the aryl sulfonyl fluoride probe SF-p1[2] and, surprisingly, it was found to selectively label a non-catalytic serine residue in the mRNA cap-binding site of DcpS, as shown by peptide mapping experiments (Figure S3). Further profiling of FS-p1 suggests that fluorosulfate targeting of serine residues in protein binding sites could be a viable strategy for covalent inhibitor and chemical biology probe design.

Although sulfonyl fluorides such as SF-p1 and the clickable probe SF-p1-yne are useful chemical biology probes, the relatively high reactivity of the sulfonyl fluoride warhead presents a challenge for the development of selective and targeted covalent inhibitor drug candidates. For instance, the instability of SF-p1 prevents the measurement of metabolic turnover rate in human liver microsomes (HLM) and shake-flask LogD using well-established assays developed at Pfizer (Supporting Information, Figure S1).[12-13] We decided to explore an alternative electrophile, aryl fluorosulfate, which would be predicted to be considerably more stable,[11] and thus potentially more suitable as a drug-compatible moiety for targeted covalent inhibitor discovery.

To further assess and compare the intrinsic potential for 'off-target' protein reactivity of aryl sulfonyl fluoride and fluorosulfate electrophiles, the fragments benzene sulfonyl fluoride (BSF) and benzene fluorosulfate (BFS) were exposed to human serum albumin (HSA) and the propensity for adduct formation was measured using LC-MS of intact protein. Neither fragment reacted with HSA even when incubated with the protein for 24 h at 23°C, in the absence of light (1 μM HSA, 10 μM compound), reflecting the lack of broad, non-specific protein reactivity of these electrophiles. However, multiple adducts with BSF were observed when the concentration was increased (1 μM HSA, 300 μM compound, Figure 2, panel a) while BFS showed minimal adduct formation at these concentrations. A similar trend occurred when fluorosulfate probe FS-p1 and sulfonyl fluoride probe SF-p1 were exposed to HSA. Under these conditions, FS-p1 does not label HSA, but some labeling occurred with the SF-p1 probe (Figure 2, panel b). These results suggest that an aryl fluorosulfate-containing ligand may be more discerning in its protein reactivity relative to an aryl sulfonyl fluoride.

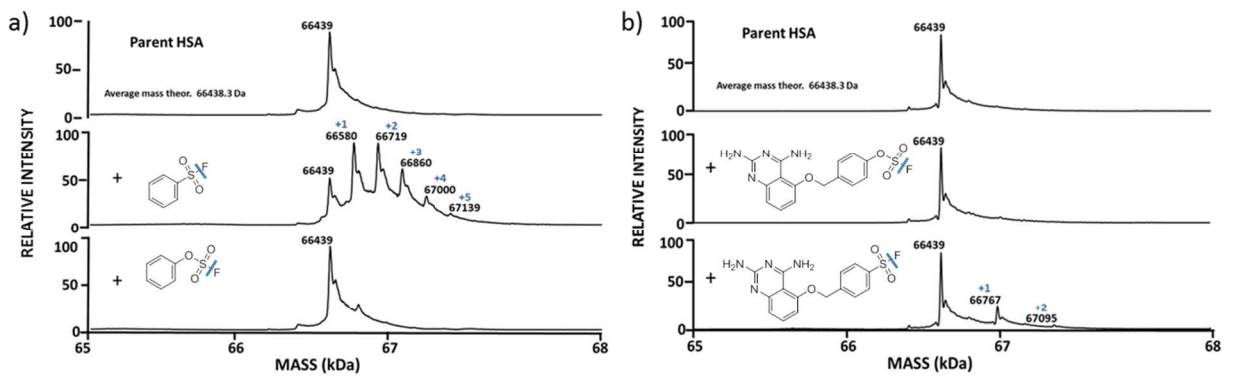
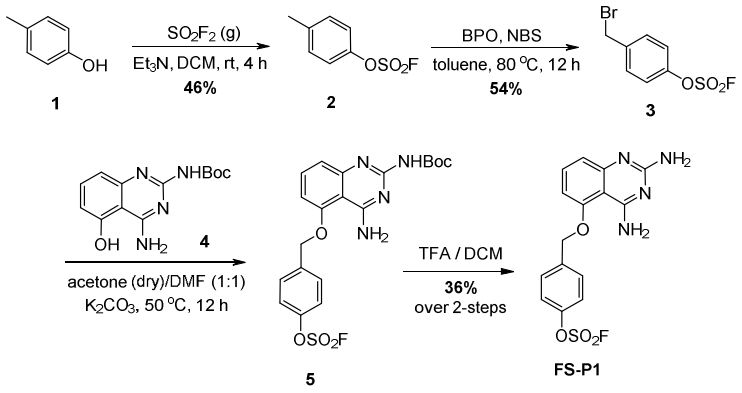


Figure 2. a) Intact protein MS of the reaction of benzene sulfonyl fluoride (BSF, 300 μM) or benzene fluorosulfate (BFS, 300 μM) with human serum albumin (HSA, 1 μM) after 24 hours. b) Intact protein MS of the reaction of FS-p1 (5 μM) or SF-p1 (5 μM) with human serum albumin (HSA, 0.5 μM) after 24 hours.

We therefore decided to explore the potential reactivity of a DAQ inhibitor of the DcpS enzyme bearing the fluorosulfate warhead, in order to compare this with the SF-p1 derivative. The aryl fluorosulfate FS-p1 was prepared using the chemistry outlined in Scheme 1 (details provided in the Supporting Information). The route relies on the recently developed reaction of phenols with sulfuryl fluoride gas to form fluorosulfates.[10] *p*-Cresol (**1**) was converted to aryl fluorosulfate **2**, followed by benzylic bromination to form **3**. Subsequent alkylation of known phenol **4**[2] yielded **5** and Boc-deprotection furnished fluorosulfate probe **FS-p1**.

Scheme 1. Preparation of DcpS Covalent Inhibitor FS-p1.



The superior hydrolytic stability of FS-p1 relative to SF-p1 was confirmed in aqueous buffer solutions. Incubation of SF-p1 in phosphate buffered saline (pH 7.4) for just 2 hours resulted in considerable hydrolysis (measured by HPLC) while FS-p1 was completely intact after 24 hours (Figure 3).

Hydrolysis of FS-p1 and SF-p1

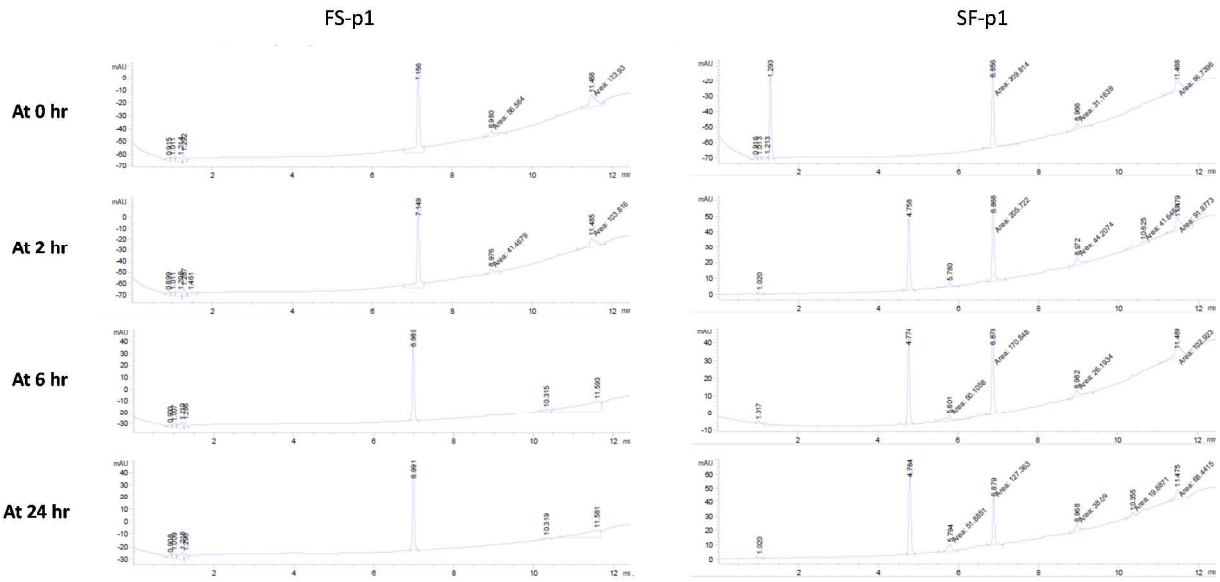


Figure 3. HPLC traces showing the hydrolytic stability of FS-p1 and SF-p1. Compounds were dissolved in 100 μM PBS (pH 7.4): 5 μL of 30 mM DMSO stock solution was added to 895 μL of PBS solution. HPLC: 12.5 minute run; 5 μL aliquot; MeCN/H_2O with 0.1% TFA; traces at 254 nm.

FS-p1 was screened in a DcpS ELISA assay using a biotinylated cap substrate (m^7GpppA),[2] and was found to inhibit enzymatic function with an IC_{50} = 3.2 nM (Table 1). This value is comparable to the unsubstituted derivative 5-benzyloxy-2,4-diamino quinazoline (compound DAQ1, Table 1),[2, 14] although FS-p1 was significantly less potent than the more reactive covalent inhibitor SF-p1 in this assay (IC_{50} < 0.02 nM).[2] Fluorosulfate FS-p1 was incubated with DcpS for 24 hours and intact mass LC-MS analysis confirmed the presence of a small molecule-protein adduct (with a concomitant loss of fluoride, Figure 4b and Supporting Information). However, the main species detected by MS had lost 18 mass units, corresponding to a protein dehydration event (Figure 4d). The mass of another minor product agreed with a combination of the potential dehydration as well as adduct formation with FS-p1 (and loss of fluoride), that is Dha-DcpS undergoing an additional reaction with FS-p1.

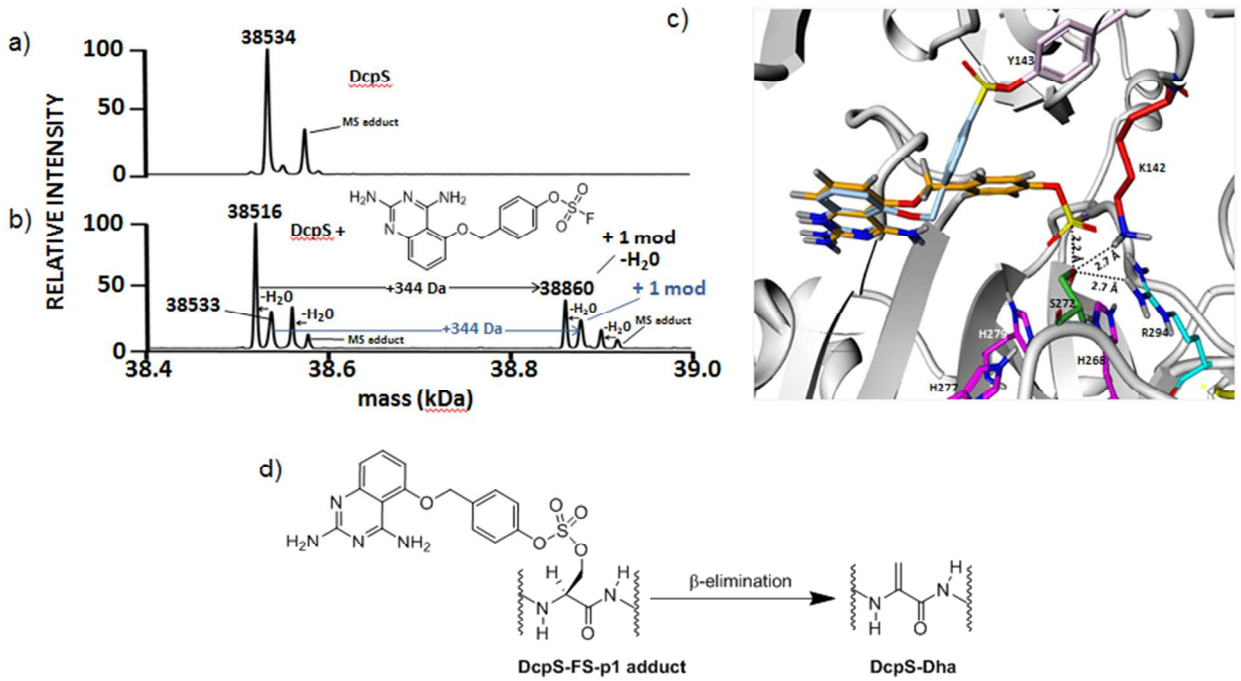


Figure 4. a) LC-MS of DcpS (MS adduct is acetonitrile clusters of DcpS (adduct mass = 41.02655) seen in the LCT Premier mass spectrometer) and b) DcpS treated with FS-p1 (0.5 μM protein/5 μM probe) for 24 h at 23°C, in the absence of light. c) Crystal structure of SF-p1 (blue) and DcpS (PDB 4QDV) showing reaction with Tyr143 (pink). FS-p1 (gold) docked into the DcpS binding site (based on 4QDV structure) where the diaminoquinazoline motif was constrained to the SF-p1 binding conformation and the remainder of the ligand was energy minimized in the pocket. The proximity of the fluorosulfate electrophile to Ser272 (green) is apparent. Key basic residues proximal to Ser272 are also highlighted: histidine triad (magenta); Arg294 (cyan); Lys142 (red). d) DcpS-Dha formation via β-elimination of DcpS-FS-p1 adduct.

Interested by these observations, we decided to further elucidate the site of protein modification using peptide mapping experiments. The DcpS/FS-p1 adduct was treated with trypsin and the digested peptides were analyzed by LC-MS (Supporting Information, Figure S2). This work confirmed that the major species following reaction with FS-p1 and MS analysis corresponded to dehydration of the Ser272 residue, resulting in the formation of dehydroalanine at this position. These results are reminiscent of the chemical mutagenesis conversion of serine

to cysteine hydrolases using sulfonyl fluorides.[15] This reaction creates a sulfonate ester that at higher pH eliminates to form an intermediate dehydroalanine species (Figure 4d).

It is possible that the presence of the Dha272 is an artifact of the analytical technique, and we therefore further investigated the potential covalent modification of DcpS using native electrospray ionization (ESI) MS. This milder technique showed that DcpS exists as a dimer (as revealed previously)[16] and the major species following reaction with FS-p1 corresponded to an adduct with 2:2 FS-p1/DcpS stoichiometry (minus 2 fluorides, Figure S3). A minor species was detected that corresponded to dimer with one intact FS-p1 adduct and one dehydrated DcpS species that increased over time (Figure S3). Although more studies are required to understand these observations, it is possible that the acidic nature of the LC-MS mobile phase accelerates dehydroalanine formation, and that elimination can also occur slowly in solution at pH 7.4.

The labeling of Ser272 is an important finding as this residue is known to contact the β-phosphate of the m^7GTP cap but is not involved in catalytic activity since S272A retains the same activity as wild-type enzyme.[16] Ligand-mediated covalent modification of a non-catalytic serine residue in a protein binding site is extremely rare, whilst labeling of the activated catalytic serine in serine hydrolases is well-known.[17] To our knowledge, the only other confirmed example is that of irreversible inhibition of cyclooxygenase enzymes COX-1 and COX-2 by aspirin through acetylation of Ser530 near the active site.[18] In this example, there is a charge interaction between the aspirin carboxylate and Arg120, and hydrogen bonding between Tyr385 and the acetyl group that facilitate the transacylation reaction (the carboxylate also acting as a general base to abstract the proton from the Ser530 hydroxyl substituent).[19-20]

Similarly, a closer inspection of DcpS crystal structures with DAQ ligands provides some insight into our results. The very low intrinsic reactivity of aryl fluorosulfates puts a strong

emphasis on the need for an appropriate orientation of the ligand to facilitate labeling with a suitably reactive amino acid in the binding site. In our previous work, the rational design of sulfonyl fluoride-containing DAQs optimally placed the electrophile proximal to the nucleophilic Tyr113 and Tyr143 residues.[2] The addition of an oxygen atom tether from the aromatic ring to the electrophilic sulfur atom of the fluorosulfate hinders a conformation that could provide a similar trajectory for reaction with the same tyrosine residues. Interestingly, Ser272 contacts a DAQ reversible inhibitor, D156844 (PDB 3BL7, Figure S4),[5] and work from our group has confirmed that this region of the binding site can accommodate a wide variety of alternative ligand substituents.[21] Docking of FS-p1 into the DcpS binding site suggests that a desirable conformation of the electrophilic aryl fluorosulfate and nucleophilic serine residue is attainable (Figure 4c). Ser272 is also in proximity to basic amino acid residues (Arg294, Lys142) and is located in the vicinity of the histidine triad motif (His268, His277, His279 – Figure 4c and S6). As a result, this region of the binding pocket not only accommodates small molecule ligands, but may also perturb the pK_a of the serine hydroxyl and/or enhance the leaving group ability of the fluoride ion from FS-p1 through hydrogen-bonding interactions[10] – further work is warranted to explore the possible mechanisms at play. In order to obtain information about the extent and rate of reaction between DcpS and FS-p1, time course experiments were conducted using an MS readout. The reaction mixture of 0.5 μM DcpS and 5 μM FS-p was stored at room-temperature and continuously analyzed after defined time intervals. Figure S5 shows the rate of reaction of DcpS with FS-p1 (Figure S5) is slower than with SF-p1 (which is complete within 1 hour),[2] as one might expect based on the higher intrinsic reactivity of sulfonyl fluorides.[11] These results may also explain why SF-p1 is considerably more potent than FS-p1 in the DcpS ELISA assay, where the compounds were incubated with the enzyme for 45 minutes (see SI). Since FS-p1 was

not specifically designed to react with Ser272, further optimization of the equilibrium binding component of aryl fluorosulfate DcpS inhibitors could potentially enhance the templated conjugation rate with Ser272. Consistent with non-covalent interactions catalyzing the conjugation, we found that fragments BSF and BFS, which would not be expected to possess significant binding affinity for DcpS, did not react with the protein under these conditions (0.5 μM DcpS, 10 μM probe, 18 hours incubation).

Table 1. Potency, metabolic stability and cell permeability of diaminoquinazoline DcpS inhibitors

Compound	**DAQ1**	**FS-p1**
DcpS IC_{50} (nM)	1.0	3.2
MW	266	364
SFLogD	2.0	2.7
HLM (μL/min/mg)	20	10.6
MDCK/MDR1 AB (10^{-6} cm/s)	7.9	2.0
MDCK/MDR1 BA (10^{-6} cm/s)	12	6.0

Finally, we wanted to ascertain the suitability of the fluorosulfate electrophile as a motif that may be used in the design of small molecule drugs, beyond its utility in chemical biology probes. Metabolic stability was assessed in human liver microsomes (HLM)[13] and the rate of turnover of FS-p1 was lower than the unsubstituted derivative DAQ1, even though the lipophilicity of FS-p1 is higher (ΔLogD 0.7 units, Table 1). FS-p1 also possessed adequate membrane permeability, as measured in the Madin Darby Canine Kidney (MDCK) epithelial cell assay (transfected with the P-glycoprotein efflux protein MDR1),[22] although flux was found to be lower than DAQ1, and there appeared to be evidence of efflux (Table 1). The high hydrogen bond donor capacity of the

DAQ head group, combined with the additional H-bonding groups present in FS-p1 are the likely reasons for these observations.[23]

In conclusion, the selective labeling of a non-catalytic serine residue in the DcpS binding site by FS-p1 combined with its excellent metabolic and chemical stability, illustrate that the fluorosulfate motif is suitable for incorporation into covalent drug design strategies. It will be interesting to assess the in vivo pharmacodynamic and pharmacokinetic profiles of such compounds in the future. The electropositive nature of the microenvironment of the reactive Ser272 in DcpS and the proximal nature of basic amino acid residues may provide insight into the propensity of other non-catalytic serine residues in proteins to be targeted with fluorosulfate electrophiles.

The identification of a dehydrated (dehydroalanine) species is intriguing as it suggests that elimination of the intermediate sulfate adduct could be optimized, potentially through further design of the ligand. From the perspective of synthetic biology, the ability to site-specifically convert a non-catalytic serine residue to a dehydroalanine amino acid using fluorosulfate chemistry is tantalizing as it could provide a route to incorporate a general chemical mutagenesis handle in proteins.[15] We have explored selective fluorosulfate and sulfonyl fluoride labeling of a number of proteins and the presence of a dehydroalanine species in the LC-MS is diagnostic of adduct formation with serine or threonine residues. A future direction for this work could therefore be the site-specific formation of dehydroalanine derivatized proteins in live cells that may provide useful avenues for biological engineering and chemical biology research.

The results presented here, and recent advances by other groups,[11, 24-29] suggest that a vast unchartered opportunity space lies ahead for the development of next-generation covalent modulators that target natural protein residues beyond cysteine.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

All authors were employees and shareholders of Pfizer when the research described in this manuscript was performed. Research was conducted in accordance with all acceptable Pfizer policies including IRB/IEC approval.

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ASSOCIATED CONTENT

Supporting Information. Metabolic stability of FS-p1 in human liver microsomes; human DcpS biochemical assay; DcpS intact LCMS, peptide mapping and native ESI MS experiments; analysis of DcpS Ser272 microenvironment; time course of the reaction of FS-p1 with DcpS;

synthetic methods and preparation of FS-p1. This material is available free of charge via the Internet at http://pubs.acs.org.

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