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A Study of the Reactivity of $S^{(VI)}$-F Containing Warheads with Nucleophilic Amino-Acid Side Chains Under Physiological Conditions†

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Sulfonyl fluorides (SFs) have recently emerged as a promising warhead for the targeted covalent modification of proteins. Despite numerous examples of the successful deployment of SFs as covalent probe compounds, a detailed exploration of the factors influencing the stability and reactivity of SFs has not yet appeared. In this work we present an extensive study on the influence of steric and electronic factors on the reactivity and stability of the SF and related S^{VI}-F groups. While SFs react rapidly with *N*-acetylcysteine, the resulting adducts were found to be unstable, rendering SFs inappropriate for the durable covalent inhibition of cysteine residues. In contrast, SFs afforded stable adducts with both *N*-acetyltyrosine and *N*-acetyllysine; furthermore, we show that the reactivity of arylsulfonyl fluorides towards these nucleophilic amino acids can be predictably modulated by adjusting the electronic properties of the warhead. These trends were largely conserved when the covalent reaction occurred within a protein binding pocket. We have also obtained a crystal structure depicting covalent modification of the catalytic lysine of a tyrosine kinase (FGFR1) by the ATP analog 5′-*O*-3-((fluorosulfonyl)benzoyl)adenosine (m-FSBA). Highly reactive warheads were demonstrated to be unstable with respect to hydrolysis in buffered aqueous solutions, indicating that warhead reactivity must be carefully tuned to provide optimal rates of protein modification. Our results demonstrate that the reactivity of SFs complements that of more commonly studied acrylamides; and hope that this work spurs the rational design of novel SF-containing covalent probe compounds and inhibitors, particularly in cases where a suitably positioned cysteine residue is not present.

Introduction

The covalent modification of proteins by small molecules is an important and active area of research in both medicinal chemistry and chemical biology. Indeed, within drug discovery targeted covalent inhibitors (TCIs) have undergone a resurgence in recent years.[1] Historically, the development of covalent inhibitors as drugs was discouraged due to the belief that reactive functional groups would lead to promiscuous modification, and result in idiosyncratic toxicity. Recent advances, however, have led to the understanding that careful tuning of warhead reactivity can allow for the generation of highly selective TCIs. TCIs are now emerging as valuable therapeutic agents,[2] spurring the development of novel approaches for the covalent modification of target proteins.[3,4]

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Despite renewed interested in TCIs, the ability to modify the target protein is often limited by the serendipitous placement of a nucleophilic cysteine residue in, or near, the binding site of the small molecule. This limitation vastly reduces the number of targets that can utilize covalent modification; warheads which expand the range of protein residues that can be modified are thus highly desirable. In this regard, the sulfonyl fluoride (-SO_2F) group, originally described by Baker,[5] has recently reemerged as a promising warhead for the covalent modification of proteins. Covalent inhibitors and probe compounds containing sulfonyl fluorides (SFs) and related S^{VI}-F groups that address a wide range of protein targets have been described in the literature,[4] and several have been utilized in activity-based protein profiling (ABPP) experiments.[6,7,8] The reason for the rapid adoption of these SF-based warheads is due to several attractive features, including: 1) their ability to covalently modify a range of nucleophilic amino acids under physiological conditions, including tyrosine,[9,10,11] lysine,[12] proline,[13] cysteine,[14,15] histidine,[16,17] catalytic serine[6,18] and threonine;[19] 2) their ease of incorporation into complex molecules, owing to the stability of this group towards a wide range of synthetic transformations;[20] 3) the biologically benign nature of the fluoride leaving group; and 4) the stability of the resultant sulfonate and sulfonamide linkages.[21] Recently, Sharpless and coworkers have expanded such S^{VI}-F-derived scaffolds into the "third dimension" through the use of SOF_4 as a versatile building block.[22] Despite the wealth of literature on the synthesis and applications of the SF group, a comprehensive study of the factors contributing to the reactivity of this functionality has not yet been reported. Similarly, descriptions of the stability of sulfonyl fluorides in aqueous solutions are limited to a single report of a small panel of ^{18}F-labelled sulfonyl fluorides.[23] Herein, we describe our efforts to profile the reactivity of the SF group and other closely related warheads in order to generate an understanding of the factors that influence its ability to modify nucleophilic amino acid residues. In these experiments we have examined the reaction between the warheads and free amino acids, as well as the factors affecting the hydrolytic, plasma, and metabolic stability of compounds incorporating the SF group. Finally, we have studied the interplay between these factors when the SO_2F group is bound within a kinase active site. Overall, our data suggest that sulfonyl fluorides and related S^{VI}-F groups represent versatile warheads for the development of novel covalent inhibitors and biochemical probe compounds.

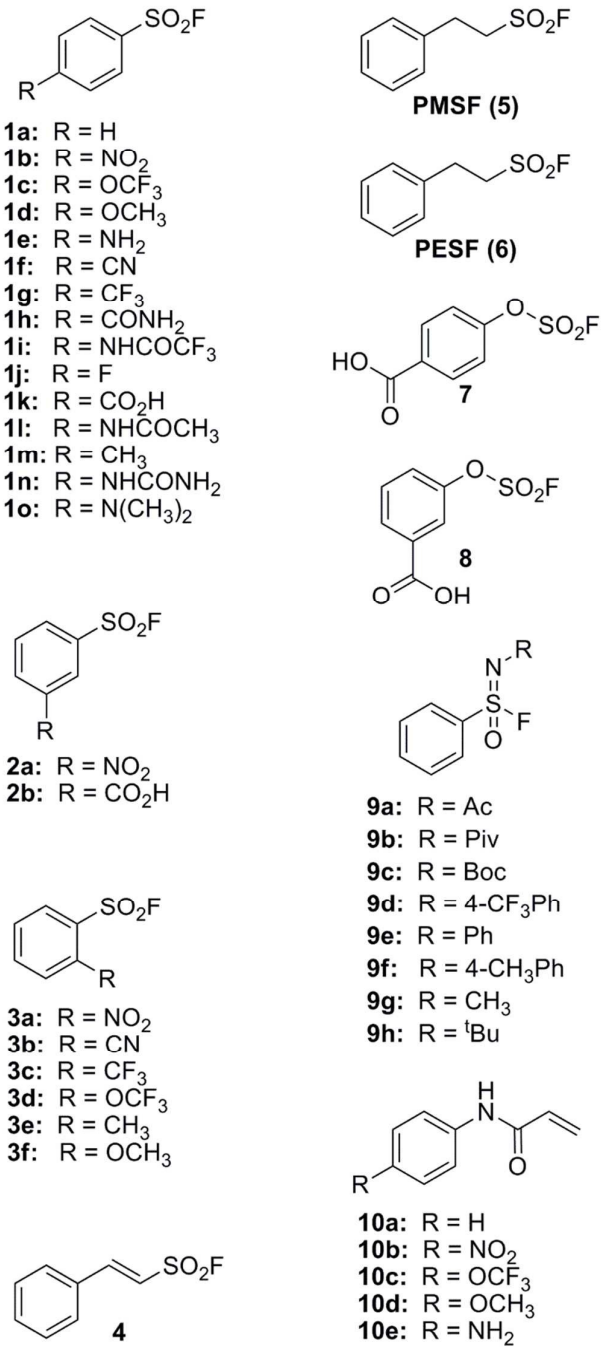

Fig. 1 Structures of sulfonyl fluorides and related S^{VI}-F warheads examined in this study. For comparison, a small panel of *N*-arylacrylamides (**10a – e**) was also synthesized and evaluated.

Results and Discussion

Synthesis of Sulfonyl Fluorides and Closely Related Warheads.

To probe the influence of various structural and electronic factors on the reactivity and stability of the SF moiety, we first synthesized a panel of monosubstituted aryl SFs (Fig. 1, **1 – 3**). Generally, these compounds were prepared in high yields and purity from the corresponding commercially available sulfonyl chlorides, utilizing potassium bifluoride under the biphasic conditions previously described by Sharpless and co-workers.[24] An exception was 4-(dimethylamino)benzenesulfonyl fluoride **1o**, which was synthesized from 4-fluorobenzenesulfonyl fluoride **1j** and dimethylamine in THF. The reaction proved selective for the

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desired S_NAr product over the isomeric sulfonamide (>9:1). However, when water was the reaction solvent, the sulfonamide was the exclusive product, highlighting the previously reported sensitivity of the sulfonyl fluoride group towards activation by hydrogen bond donors.[24,25] In addition to this aryl SF library, several other closely related warheads (**4 – 9**) and a range of electronically diverse *N*-arylacrylamides (**10a – e**) were synthesized (see Supporting Information).

Comparison between the Reactivity of Sulfonyl Fluorides and *N*-Arylacrylamides with Cysteine.

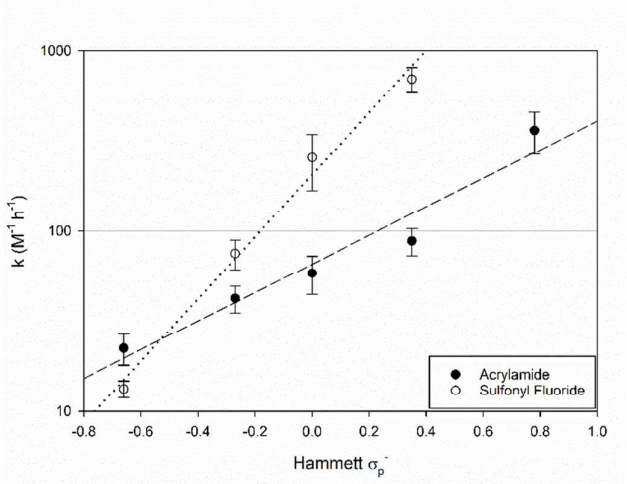
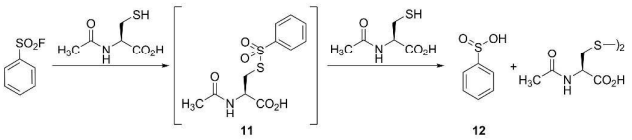


Fig. 2 Plot depicting the second-order rate constants for the reaction of *N*-acetylcysteine with *N*-arylacrylamides (filled circles) or aryl SFs (open circles) vs. the Hammett σ_p^- parameter of the substituent. The dashed and dotted lines indicate best fit lines for the acrylamide (ρ = 0.79, R^2 = 0.947) and SF (ρ = 1.72, R^2 = 0.989) rate data, respectively. Error bars represent the mean ± standard deviation of three independent experiments.

The reaction of a cysteine residue with an *N*-arylacrylamide is by far the most commonly used methodology for the site-specific covalent inhibition of proteins.[26] As such, this reaction has been extensively studied under physiological conditions, and its rate has been demonstrated to follow a log-linear relationship with respect to the Hammett value of the attached aromatic ring.[27,28] Therefore, we began our enquiries by determining the relative rates of reaction of monosubstituted *N*-arylacrylamides **10a – e** (10 μM) with *N*-acetylcysteine (100 μM) in phosphate buffer (100 mM) containing NaCl (1.0 M) and 5% CH_3CN at pH 7.5 at 37 °C (All subsequent studies described below utilized identical conditions, but substituted a different electrophile or nucleophile as needed – for detailed experimental procedures and example plots see Supporting Information). Under these assay conditions we recapitulated the previously observed log-linear relationship between the Hammett values of the aryl ring substituents of the *N*-arylacrylamides and their second order rate constants (Fig. 2, black closed circles). With these benchmarking experiments in hand, we sought to compare the reactivity of our SFs with that of the *N*-arylacrylamides. We first noted, however, that when the SF library was screened under identical (pseudo-first order) conditions we observed the formation of a small amount of the corresponding sulfonic acid. Due to the magnitude difference between the second-order rate constants for the two processes (see Supporting Information – Appendix 1: Table S2-6), we did not correct the subsequent experiments for the hydrolysis. In addition, in the case of *N*-acetylcysteine, the initial adduct expected, sulfonothioate ester **11**, was not observed (Scheme 1). Instead, **11** was subsequently reduced to the corresponding sulfinic acid **12** via attack of another cysteine, with concomitant formation of *N*-acetylcysteine disulfide.[29] While this second step is clearly favored under our assay conditions due to the presence of excess *N*-acetylcysteine, it should be noted that intermediate **11** was never observed by MS analysis, even when less reactive warheads (vide infra) were assayed or when the reaction mixtures were analyzed at early time points. Similarly, attempts to observe **11** by increasing the ratio of SF to *N*-acetylcysteine were also unsuccessful; reaction mixtures containing either equimolar amounts of the two reactants or a fivefold excess of SF relative to *N*-acetylcysteine both afforded the corresponding sulfinic acid and *N*-acetylcysteine disulfide as the only observable products.



Scheme 1 Reduction of the SF group by *N*-acetylcysteine. Proposed intermediate **11** was not observed under our assay conditions.

Taken together, our results suggest that utilizing the SF group to stably modify a target protein via reaction with a cysteine residue should be undertaken with caution due to the inherent instability of intermediates of type **11**. Thus, while sulfonyl fluorides have been demonstrated to react with cysteine residues by us (vide supra) and others,[14,15] it is instructive to note that crystal structures exhibiting SF-modified cysteine residue(s) remain elusive. Similarly, when SFs are used as probe molecules in biochemical contexts, we anticipate that SF-cysteine adducts will almost certainly be cleaved by standard reducing agents used in sample preparations, possibly leading to misleading selectivity data. We do note, however, that targeting cysteine residues with SF-based compounds can in certain cases lead to durable inhibition, if the intermediate thiosulfonate ester is subsequently captured intramolecularly by a nucleophilic side chain.[14,15]

The rapid cleavage of intermediate **11** and the fact that it does not detectably accumulate indicates that its formation is rate-limiting, allowing us to measure the rate of the initial reaction of the thiol with SF by monitoring formation of the corresponding sulfinic acid **12**. A subset of our SF library (**1a – e**) containing identical substituents to the *N*-arylacrylamides

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was exposed to the *N*-acetylcysteine reactivity assay described above. The results showed that the SFs (open circles), too, displayed a log-linear relationship to the Hammett σ_p^- parameter, and that the SFs generally reacted faster than the corresponding *N*-arylacrylamides (filled circles) under these conditions. Indeed, the rate of reaction of nitro derivative **1b** was ≥16-fold greater than that of the corresponding acrylamide, and was too rapid to be accurately determined. The reactivity of the SFs (ρ ~ 1.7) is more sensitive to aryl substituents than is the case for the arylacrylamides (ρ ~ 0.8), presumably at least in part because in the SFs the electrophilic sulfonyl sulfur atom is directly connected to the aromatic ring. Thus, while SFs do not afford stable adducts with cysteine residues, their reactivity can be tuned over a wide range by substitution on the aromatic ring; furthermore, this variability encompasses the absolute range of reactivities measured for the *N*-arylacrylamides. The cysteine-acrylamide interaction is known to have a kinetic profile that is suitable for use in TCI drugs. The above results suggest that a similarly useful kinetic profile is, in principle, achievable with a suitably functionalized SF warhead.

Evaluation of Sulfonyl Fluoride Reactivity against Tyrosine, Lysine and Serine

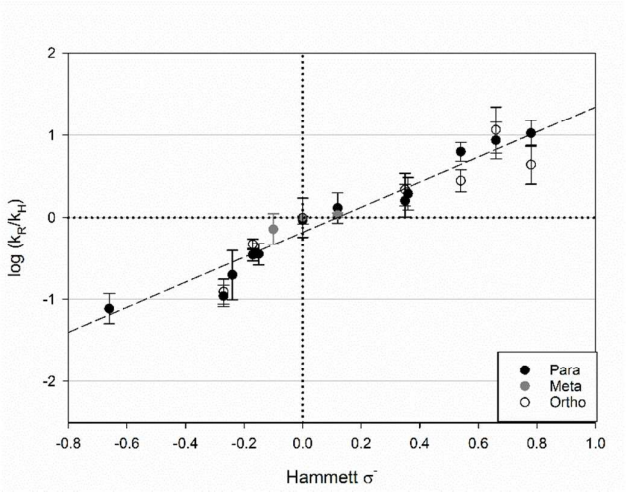

Fig. 3 Hammett plot showing the dependence of the rate of modification of *N*-acetyltyrosine by monosubstituted aryl SFs on the σ_p^- (ortho and para substituents) or σ_m^- (meta substituents) parameter. The dashed line indicates the best linear fit for the data (ρ = 1.53, R^2 = 0.913). For rate data in tabular format, refer to Table S1 (Supporting Information). Error bars represent the mean ± standard deviation of three independent experiments.

Having established that the rate of modification of cysteine by SFs is more susceptible to electronic effects than that of the *N*-arylacrylamides, we next chose to examine the ability of our electronically diverse SFs to modify other amino-acid side chains. It is well documented that SFs can be utilized to modify tyrosine residues in proteins in a site-specific manner.[11,30] This capability is valuable in the context of targeted covalent modification, because it may expand the number of residues in proteins amenable to covalent modification. In particular, tyrosine residues are often enriched at protein-protein interfaces,[31] thus SFs may represent promising warheads for these challenging targets. We therefore decided to test our broad panel of substituted aryl SFs using the LC-MS assay described above, but using *N*-acetyltyrosine as the nucleophile. As a first point of reference, we observed that the initial reaction rate of parent SF **1a** was 3.4-fold smaller when *N*-acetyltyrosine was the nucleophile relative to *N*-acetylcysteine. A Hammett analysis of the resulting initial rate data revealed a strong correlation between the electron-withdrawing properties (as measured by the σ_p^- parameter) of the substituent[32] and the rate of reaction (Fig. 3, filled circles). Ortho-substituted sulfonyl fluorides (**3a – f**, open circles) exhibited roughly the same correlation between reaction rates and the σ_p^- parameter, suggesting that electronic rather than steric factors largely determine the reactivity of this family of compounds (ρ_{ortho} = 1.52, ρ_{para} = 1.63). We also note the possibility of stereoelectronic interactions between the SF group and an ortho substituent, and observe some evidence for this in *ortho*-methoxy containing **3f**.[33]

We also note that *N*-arylacrylamide **10b**, which exhibited a rate of reaction with *N*-acetylcysteine comparable to that of SF **1a**, did not afford any 1,4-addition product when screened against *N*-acetyltyrosine. Lastly, control experiments revealed that the reaction was susceptible to general acid-base catalysis; thus, the concentration and identity of the reaction buffer did have an effect on the measured rates of reaction with *N*-acetyltyrosine (see Supporting Information for details).

We next turned our attention to the reaction between the SF warhead and lysine. At physiological pH, lysine side chains exist predominately in the protonated form, and therefore usually only pKa-perturbed lysines can be successfully targeted with covalent warheads.[12] Nonetheless, solvent-exposed lysine side chains can be covalently modified by highly reactive warheads, which can react rapidly with the small fraction of unionized amine present at equilibrium to form the covalent linkage. These reactions typically require that the warhead is appended to a high affinity small molecule, ensuring that the warhead experiences a long residence time in close proximity to the nucleophilic lysine, and/or long reaction times.[34] We were therefore intrigued to study the reaction of aryl SFs with non-pKa perturbed lysines. Using a subset of our panel of aryl SFs (**1a – e**), we again repeated our reactivity assay, employing N^{α}-acetyllysine as the nucleophile (Supporting Information, Table S2). Under our assay conditions, each of the five SFs afforded the corresponding sulfonamide, though in all cases this reaction was slower than the corresponding reactions of *N*-acetyltyrosine (2.9-fold rate reduction for **1a**) and *N*-acetylcysteine (10-fold rate reduction for **1a**). This result suggests that it may be possible to design small molecules with highly reactive SF warheads to modify lysines when more suitable residues are not available, but slow reaction rates and

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competing warhead hydrolysis (vide infra), may represent significant challenges to this approach. We additionally sought to explore the potential utility of SF warheads to covalently modify unactivated serine residues; however, only sulfonic acid formation (hydrolysis) was observed when *N*-acetylserine was utilized as the nucleophile under our assay conditions, even with the most electron deficient SFs.

Effects of pH and Buffer on the Rate of Reaction

Having demonstrated that aryl SFs react with cysteine, tyrosine, and lysine at rates dependent on the reactivity of the amino acid side chain as well as the electronic substitution of the aryl ring, we next tested the effect of pH on the reaction rates. Using a subset of SFs (**1a – e**), we performed the same MS based assay at two additional pH values for all three amino acid nucleophiles. As expected, increasing the reaction pH to 10.0 greatly increased the reaction rate in all three cases (Supporting Information, Table S2). This rate acceleration was so large that no reliable rate data could be obtained for reactions involving *N*-acetylcysteine. For the other two amino acids, only strongly deactivated warheads **1d** and **1e** afforded reliable data, showing rate accelerations of 95- and 59-fold, respectively, for reactions with *N*-acetyltyrosine, and 161- and 201-fold, respectively, for reactions with N^{α}-acetyllysine. Predictably, lowering the reaction pH to 5.0 had the opposite effect. In this case, neutral or deactivated SFs **1a**, **1d**, and **1e**, failed to react with *N*-acetylcysteine or *N*-acetyltyrosine, and **1a** and **1c – e** were all inert towards N^{α}-acetyllysine at this pH. For strongly activated SF **1b**, we observed 26-, 44-, and 89-fold decreases in reaction rate for *N*-acetylcysteine, *N*-acetyltyrosine, and N^{α}-acetyllysine, respectively. Similar trends were observed when the hydrolytic stabilities of **1a – e** were examined at varying pH (Table S3, Supporting Information). These data indicate that, at physiological pH, the reaction of SF with each of the three nucleophilic residues is subject to specific base catalysis, presumably due to the need to deprotonate the nucleophile (-SH, -OH or $-NH_3^+$) for attack on the SF electrophile. The rate would be expected to become pH independent at values above the pKa of the nucleophile. However, the highest pH tested here, pH 10, is below the pK_a of the nucleophilic amine of lysine and of the phenolic oxygen of tyrosine, and for *N*-acetylcysteine (pKa ~9.5) the reaction at pH 10 was too fast for the rate to be accurately measured using our MS-based assay method. The observation that reaction at pH 7.5 involves specific base catalysis is consistent with our observation that the reaction is also subject to general acid-base catalysis by buffer, as described above (Fig. S1, Supporting Information). We speculate that this buffer catalysis similarly involves deprotonation of the nucleophile by basic buffer components. In the context of TCIs, these findings suggest that rate of covalent modification of proteins by SF-containing inhibitors could be enhanced if the target residue is located near an appropriately-positioned basic side chain. The presence of such a nucleophilic site diad could provide new strategies for the rational design of highly selective or highly efficient covalent probe molecules.

Hydrolytic, Plasma and Metabolic Stability of Aryl Sulfonyl Fluorides

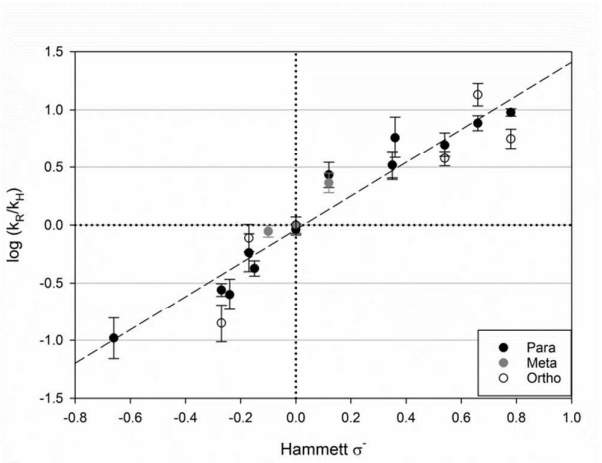

Fig. 4 Hammett plot showing the dependence of the rate of hydrolysis of monosubstituted aryl SFs on the σ_m^- (meta substituent) or σ_p^- (ortho and para substituents) parameter. The dashed line indicates the best linear fit for the data (ρ = 1.45, R^2 = 0.905). Complete tabulated data is provided in the Supporting Information (Table S1). Error bars represent the mean ± standard deviation of three independent experiments.

With an understanding of the relative reactivities of the aryl SFs, we next focused our attention on their hydrolytic stability. Modifying our previously described assay, by leaving out any nucleophilic amino acid and instead including *N*-acetylphenylalanine as an inert internal standard, we monitored the formation of the corresponding sulfonic acid by MS. As expected, the rate of hydrolysis also displayed a strong correlation with the electronics of the aryl ring (Figure 4). Strongly electron-deficient SFs **1b**, **1c**, **1f**, and **1g** possessed half-lives on the order of 5 – 15 minutes under these conditions, while strongly electron donating substituents greatly improved the stability of the SF; compounds **1e** and **1o** were stable for several days under these conditions. Interestingly, the rates of hydrolysis of **3b** and **3f** deviated from the generally observed trends; this observation mirrors their behavior in reactivity assays employing *N*-acetyltyrosine and supports the hypothesis that a stereoelectronic interaction between their ortho-substituents and the $-SO_2F$ group influences the reactivity of these two compounds. Lastly, we note that the acrylamides did not undergo appreciable hydrolysis under our assay conditions, even when the reaction was monitored for several days.

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When a subset of our SF library was subjected to a rat plasma stability assay in which the loss of SF was recorded over time (it was not possible to determine if the compounds had hydrolyzed or reacted with the constituent parts of the rat plasma), the same correlation was observed (Fig. 5). Although compound **1o** showed remarkable stability ($t_{1/2}$ = 25.1 h), we note that it remains relatively unstable when compared to the *N*-arylacrylamides, which do not undergo appreciable reaction under these conditions (data not shown). Taken together, our results indicate that the electronic parameters of a given SF warhead must be carefully tuned based on its intended application. In particular, highly reactive SF probes may exhibit reduced rates of covalent modification due to competing hydrolysis under physiological conditions (vide infra).

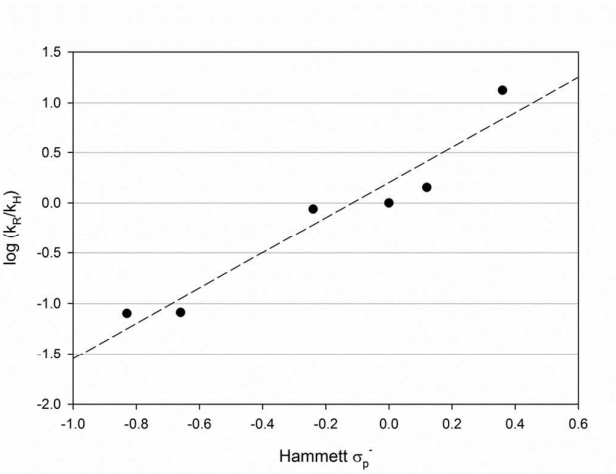
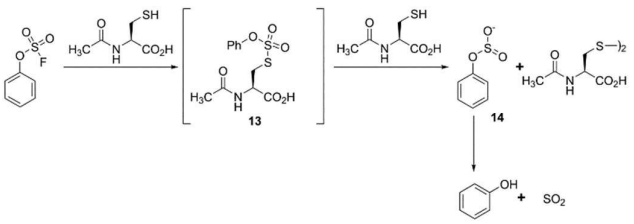


Fig. 5 Plot showing the dependence of the rate of decomposition of monosubstituted aryl SFs in rat plasma on the Hammett σ_p^- parameter of the aryl substituent. The dotted line indicates the best linear fit for the data (ρ = 1.75, R^2 = 0.927).

Finally, we were interested in investigating the metabolic stability of our library. Unfortunately, the low molecular weight and poor ionization in MS signal of a number of the library members prevented accurate measurements in standard pharmacokinetic assays. Nevertheless, three compounds, **1k**, **1n**, and **1o** afforded robust clearance data from rat hepatocytes (165, 24, and 170 µL / 10^6 cells min, respectively). On the other hand, our library of FSBA analogues **19** (vide infra) afforded robust microsomal stability data. Within this series of compounds we did not observe a strong correlation between the Hammett value of the substituent and the rate of metabolism, suggesting that the metabolism of SF-containing molecules does not primarily depend on the intrinsic reactivity of the SF group (Supporting Information, Fig. S3A). In contrast, a stronger correlation existed between the measured logD of the compounds and their rate of metabolism, in agreement with well-known relationships in medicinal chemistry (Supporting Information, Fig. S3B).[35]

Investigation of Closely Related Warheads

We next studied the reactivity and stability of related S^{VI}-F groups, including vinyl sulfonyl fluorides, aliphatic sulfonyl fluorides, aryl fluorosulfates, and aryl sulfonimidoyl fluorides. To this end, compounds **4 – 9** were synthesized and their rates of reaction with tyrosine and rates of hydrolysis were determined (Supporting Information, Table S4). 2-Phenylethenesulfonyl fluoride **4** was found to be approximately 9-fold less reactive towards *N*-acetyltyrosine than **1a**, and, as expected, this decrease in reactivity correlated with an increase in hydrolytic stability. Despite the presence of a strongly electron-deficient olefin, 1,4-addition of *N*-acetyltyrosine to the vinylsulfonyl fluoride moiety of **4** was not observed in any assay. Phenylmethanesulfonyl fluoride (PMSF, **5**), a protease/esterase inhibitor widely used in the biological sciences,[36] was observed to rapidly hydrolyze at pH 7.5, in agreement with previous reports.[37] However, in contrast to our previous experiments with aryl SFs, this decrease in hydrolytic stability did not correlate with an increase in reactivity towards *N*-acetyltyrosine. This observation is consistent with hydrolysis occurring through the intermediacy of a sulfene-like intermediate, rather than via direct reaction at sulfur. The hypothesis that aliphatic SFs primarily hydrolyze via sulfene-like intermediates was further supported when we observed that the rate of hydrolysis of 2-phenylethanesulfonyl fluoride (PESF, **6**) was roughly 13-fold slower than that of **5**, presumably due to the decreased acidity of the α-protons of **6**. Despite this increased stability, we still did not observe sulfonylation of tyrosine by **6** under our standard assay conditions, suggesting that sulfene formation and subsequent hydrolysis were still much faster than nucleophilic addition to the sulfonyl group. On the other hand, fluorosulfates **7** and **8** (in which the carboxylic acid groups were required for solubility reasons) were found to be extremely stable under these conditions; no hydrolysis was observed at pH 7.5 over 24 hours at 37 °C, nor did either compound afford substitution products with either *N*-acetyltyrosine or *N*-acetylcysteine. On extended incubation with *N*-acetylcysteine (4 days), however, we did observe the formation of the corresponding phenol. These phenols are thought to arise from initial formation of a sulfothioate **13**, which is rapidly reduced by *N*-acetylcysteine to sulfite ester **14**. Expulsion of SO_2 from the sulfite ester then affords the observed phenols (Scheme 2).



Scheme 2 Reaction between a generic aryl flurosulfonate and *N*-acetylcysteine. As with the sulfonyl fluorides, intermediates **13** and **14** were not observed.

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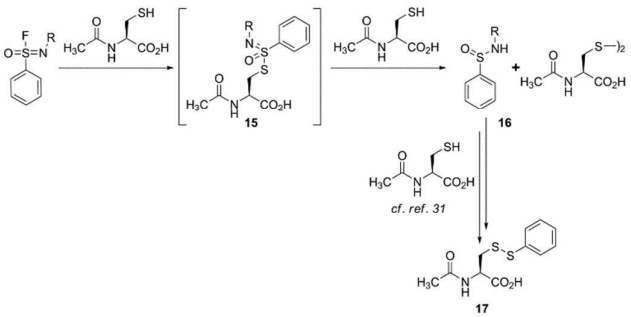
Due to the possibility of substitution on nitrogen, the sulfonimidoyl fluoride group [R-SO(=NR')F], may represent a more versatile covalent warhead than the -SO_2F group. Indeed, we recognized that not only does the *N*-substituent represent a novel way to modulate the reactivity of the sulfonimidoyl fluoride warhead, but it also offers a new vector with which to attach the covalent warhead to a larger molecule. Despite the potential advantages afforded by sulfonimidoyl fluorides, which first appeared in the literature in 1983,[38] reports of their applications remain scarce. We thus synthesized a small panel of *N*-substituted benzenesulfonimidoyl fluorides to determine the effects of *N*-substitution on the stability and reactivity of this class of compounds.

Of the eight sulfonimidoyl fluorides, **9a – h**, tested, only the four most electron deficient, **9a – d**, afforded adducts with *N*-acetyltyrosine at pH 7.5 at 37 °C. In particular, sulfonimidoyl fluorides bearing *N*-acyl (**9a**, **9b**) substituents were found to label *N*-acetyltyrosine more rapidly than $PhSO_2F$ (relative rates = 2.5 and 1.2, respectively), whereas the rate of reaction of *N*-Boc substrate (relative rate = 0.98) was similar to that of $PhSO_2F$, and that of *N*-(4-(trifluoromethyl))phenyl warhead **9d** (relative rate = 0.05) was substantially reduced (Supporting Information, Table S5). In contrast to our observations of sulfonyl fluorides, however, we found that variation of the *N*-substituent could improve hydrolytic stability without compromising reactivity – thus **9b** (R = Piv) and **9c** (R = Boc) were both found to be more stable towards hydrolysis than $PhSO_2F$. Possibly, increasing the steric bulk of the *N*-substituent increases the hydrolytic stability of these warheads, while having a less pronounced effect on the reactivity of this group towards weaker nucleophiles, though how this might occur is unclear. Alternatively, it is possible that sulfonimidoyl fluorides react through different mechanisms depending on the nature of the *N*-substituent. Notwithstanding these open questions, we regard sulfonimidoyl fluorides bearing *N*-carboxyl and *N*-acyl groups as promising, yet underexplored scaffolds for the development of novel tyrosine-targeting covalent probe compounds, though further experiments are undoubtedly required to fully elucidate the reactivity and utility of these groups.

In contrast, other *N*-aryl warheads **9e** and **9f** were observed to rapidly decompose under assay conditions (rates of decomposition relative to **1a** = 6.6 and 5.3, respectively); we hypothesized that this reduction in stability may be due to an alternate pathway for decomposition,[39] possibly involving oxidation of their electron-rich arene rings. *N*-alkyl compounds **9g** and **9h** were inert under these conditions. Remarkably, these two compounds were found to be exceptionally stable even at pH 10.0, with no decomposition observed after 8 h at 37 °C. Attempts to access the substitution chemistry of **9e – h** using *N*-acetylcysteine as the nucleophile were similarly unsuccessful; **9g** and **9h** remained inert, while decomposition of **9e** and **9f** still outcompeted substitution pathways. Sulfonimidoyl fluorides **9a – d** did react with *N*-acetylcysteine, though as before, the direct substitution products were never observed. Rather, all four compounds ultimately afforded a mixture of products, with phenyl *N*-acetylcysteine disulfide **17** as the major component. We propose that these reactions initially afford S-sulfonylated substitution products **15**, which are rapidly reduced to the corresponding sulfinamides **16** and *N*-acetylcysteine disulfide, in an analogous reaction to that of the sulfonyl fluorides and fluorosulfates. The sulfinamides, unlike the deprotonated sulfinic acids produced in the sulfonyl fluoride reaction, are then further reduced by additional cysteine, ultimately affording a mixture of disulfides (Scheme 3). We note that similar reactions of sulfinamides and thiols have previously been described in the literature.[40]



Scheme 3 Formation of *N*-acetylcysteine phenyl disulfide from the reaction of *N*-acetylcysteine and benzenesulfonimidoyl fluorides.

"In-Pocket" Reactivity of Sulfonyl Fluorides.

The reactivity profiling experiments described above demonstrated that the reactivity and stability of substituted aryl SFs was determined almost entirely by their electronic properties. We recognized, however, that such a simplified system may not accurately predict the reactivity of the sulfonyl fluoride group and the target residue when covalent bond formation is occurring in the context of a protein-inhibitor complex. In order to assess the reactivity and stability profiles of the SF group in such a system, we turned to two sulfonyl fluoride-containing ATP analogs, 5'-*O*-(4-fluorosulfonyl)benzoyl adenosine (p-FSBA, **18**)[41] and its more recently disclosed isomer m-FSBA (**19a**, Figure 6).[42] **18** is a non-selective kinase inhibitor, which has been demonstrated to covalently modify the catalytic lysine residue[43] (and potentially, in some cases, other proximal residues)[44] within the ATP binding pocket; m-FSBA has been hypothesized to react in an analogous manner.[42] In order to probe the generality of the FSBA-kinase interaction, we selected two kinases, FGFR1 and SYK, as targets for covalent modification. In particular, an analysis of previously published crystal structures of tyrosine kinase FGFR1,[45] suggested that it may be possible to incorporate various electronically-modulating substituents ortho to the SF on the aryl ring of m-FSBA without interfering with ligand binding. A library of electronically diverse m-FBSA analogues would subsequently allow us to test whether our previously determined structure-reactivity relationships were conserved in more complex systems.

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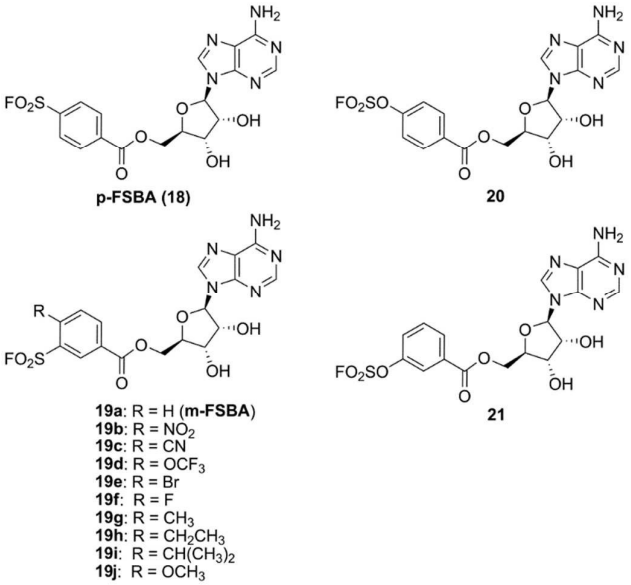
Fig. 6 Structures of p- and m-FSBA, novel m-FSBA analogs, and FSBA-derived fluorosulfates.



To accomplish this aim, we first sought to obtain evidence that substituents on the aryl ring of the FSBA analogs could be accommodated within the ATP binding pocket of FGFR1. To this end, we obtained two X-ray crystal structures of m-FSBA analogs (**19a** PDB: 5O49 and **19h** PDB: 5O4A) covalently bound to the catalytic lysine (Lys^{514}) of FGFR1 (Fig. 7). Both structures contain two molecules in the asymmetric unit of the crystal structure, with minimal differences in ligand binding. As expected, the adenosine moiety of forms H-bond interactions with the hinge of the kinase in both structures. The phenyl ring occupies the space underneath the glycine rich loop and positions the $–SO_2F$ group in close proximity to Lys^{514}. The glycine-rich loop appears to be flexible and partially disordered in both structures, as indicated by weaker electron density within this region. In spite of the inherent flexibility of this loop, a covalent bond is clearly visible between Lys^{514} and the sulfur atom of **19a**. In contrast, multiple binding modes were observed within the crystal structure containing **19h**. In one of the two binding sites within the asymmetric unit of the FGFR1-**19h** complex, the covalent bond is clearly visible and the ligand binding mode is essentially identical to that of **19a** (Fig. 7). However, the electron density around the ethyl group was weak, and thus the precise orientation of this group could not be determined unambiguously. In the other binding site within the asymmetric unit cell, the electron density indicated a mixture of two species in a roughly 1 : 1 ratio. In the first of these, the covalent bond had formed between the sulfur atom of **19h** and Lys^{514} of FGFR1. The second species depicted the apparent noncovalent interaction between **19h** and FGFR1, thus the sulfonyl fluoride group was intact and was located further away from the key lysine residue. Nevertheless, given the overall degree of similarity of both the ligand binding mode and the FGFR1 binding site between the co-crystal structures of **19a** and **19h**, we were confident that substitution at the 4'-position of the m-FSBA analog would largely be tolerated.

This structure conclusively established that m-FSBA specifically modifies the catalytic lysine of FGFR1; only a single modification of FGFR1 was ever observed by MS. It also suggested that substitution at the 4-position of the aryl ring can be accommodated, as the nearest protein residue appears to be greater than 5 Å away. Confident that ortho substitution on m-FSBA would not significantly disrupt binding of the small molecule to the kinase domain of FGFR1, we synthesized a panel of m-FSBA analogs (**19a – j**, Scheme 4). Briefly, 4-substituted benzoic acids **22** were treated with hot chlorosulfonic acid, then the resulting crude sulfonyl chlorides were directly converted to the corresponding sulfonyl fluorides **23** with potassium bifluoride under biphasic conditions. Utilizing a modified literature procedure, the crude benzoic acids were then converted to the corresponding acid chlorides, which were treated with adenosine and DMPU in the absence of base to afford the substituted FSBA analogs **19** in low to moderate yields.

Fig. 7 X-Ray structures of m-FSBA analogs **19a** (purple - PDB: 5O49) and **19h** (blue - PDB: 5O4A) covalently bound to the catalytic lysine residue (Lys^{514}) of FGFR1.

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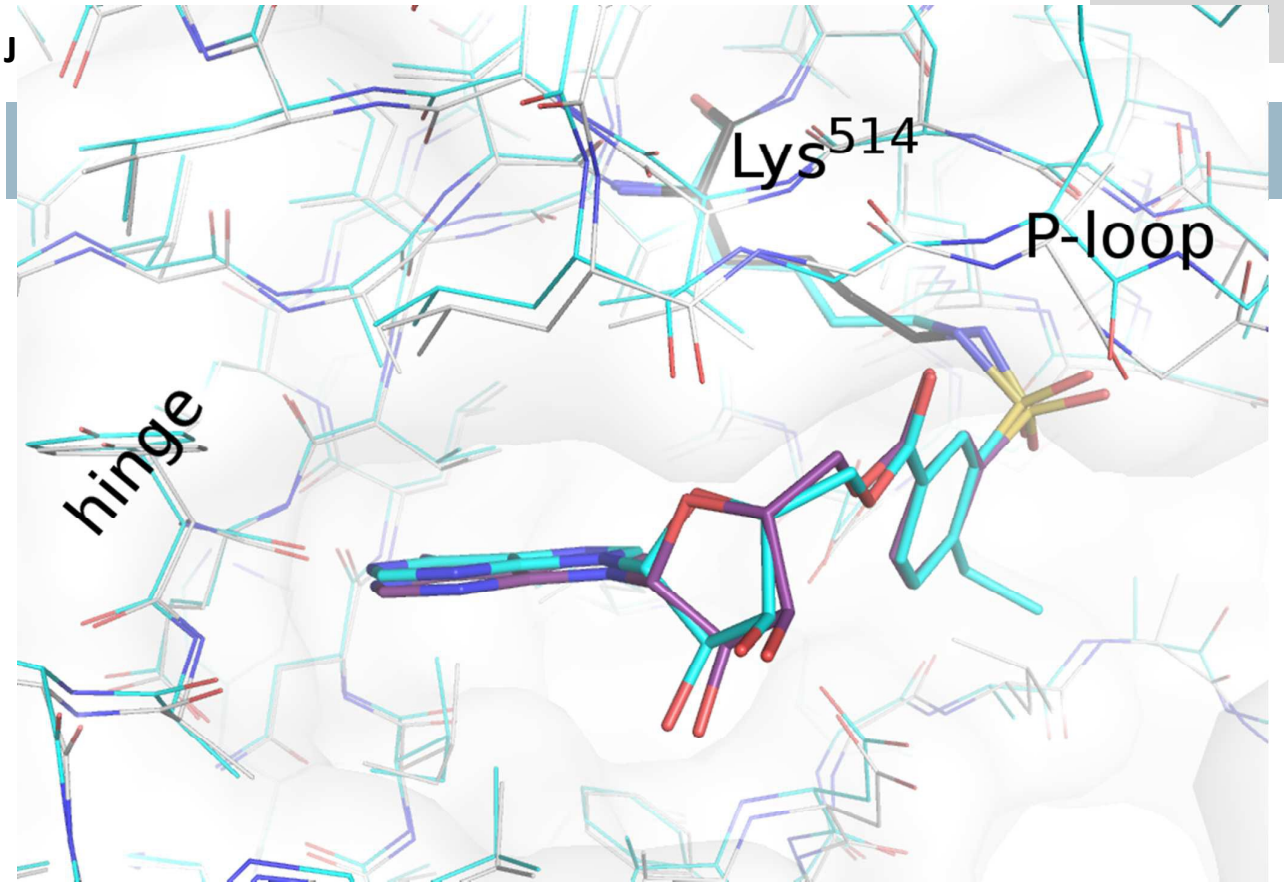
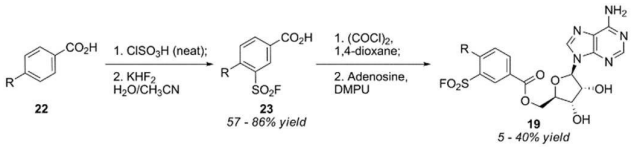


Scheme 4 Synthesis of FSBA analogs.



We subsequently determined the rates of covalent modification of the kinase domains FGFR1 and SYK for our panel of FSBA analogues using an LC-MS protocol (see Supporting Information). We employed a large excess of the FSBA analogs relative to the kinase to fully saturate the kinase ATP binding site, thus enabling us to decouple the rate of (irreversible) covalent modification from the non-covalent equilibrium reaction. These experiments revealed an interesting correlation between the rate of covalent modification and the electronic properties of the FSBA analogues (Figure 8). In line with our hypothesis that ortho substituents would have minimal effects on the binding of the m-FSBA analogues to FGFR1, analogs **19g – i** (which all possess similar Hammett parameters) had essentially identical reaction rates, despite a large difference in steric bulk in close proximity to the reactive warhead. A fairly robust correlation was observed between the rate of modification of the kinases and the σ_p^- parameter of the ortho-group, with the exception of analogs **19b** (R = NO_2) and **19j** (R = OCH_3). The Hammett ρ values for these reactions were determined to be 0.77 and 1.12 for FGFR1 and SYK, respectively, in reasonable agreement with the results obtained using isolated amino acids. FSBA analog **19b**, however, did not afford a covalent adduct with either kinase domain. Indeed, **19b** was fully converted to the corresponding sulfonic acid within 10 min under our assay conditions (LC-MS analysis). On the other hand, FSBA analog **19j** was observed to react with the kinases far slower than would be expected based on electronic properties alone, suggesting that the electronic differences associated with the methoxy group was not well tolerated. We also monitored the rate of modification of FGFR1 by p-FSBA, and determined it to be slower than that of m-FBSA (relative rate = 0.65) despite electronic activation from the para-ester group. Here, we attributed this difference to poor overlap between the incoming amine and the sulfur center. For completeness, we synthesized the two fluorosulfonate analogues **20** and **21**. These two compounds showed only traces of modification of FGFR1, even after extended reaction times, in line with our expectations based on profiling experiments involving the fluorosulfonate group.

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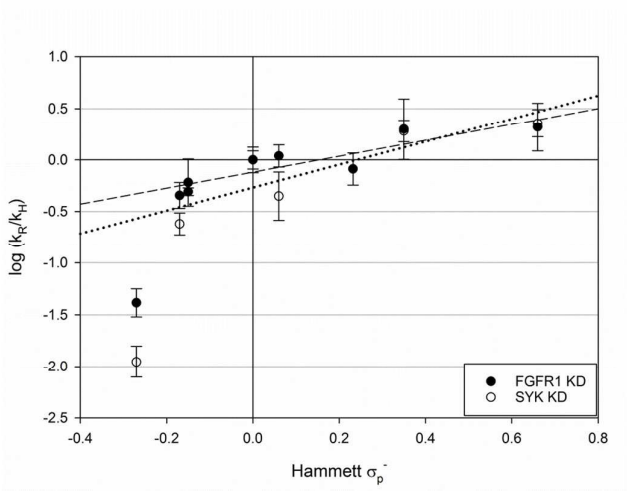

Fig. 8 Hammett plot showing the dependence of the rate of modification of FGFR1 and SYK kinases domain by substituted FSBA analogs on the σ_p- parameter of the substituent. Best linear fits for FGFR1 (dashes, ρ = 0.77, R^2 = 0.801) and SYK (dots, ρ = 1.12, R^2 = 0.771) are shown, excluding the point where R = OCH_3 (**19j**, σ = -0.27, see text). Note that FSBA analog **19b** (R = NO_2) did not afford and adduct with either kinase under assay conditions.

Conclusions

We have completed an extensive study of the effects of electronic and steric factors on the reactivity and stability of the sulfonyl fluoride and closely related S^{VI}-F groups. While alkylsulfonyl fluorides are typically too unstable to effect the modification of amino acids, we found that the reactivity of arylsulfonyl fluorides can be predictably adjusted by modulating the electronic properties of the warhead, enabling the rational design of covalent compounds targeting a given residue. Furthermore, sulfonimidoyl fluorides bearing electron-withdrawing groups on nitrogen were found to exhibit comparable rates of reaction with tyrosine, relative to SFs, while in certain cases also displaying enhanced stability, and may thus represent an even more promising starting point for the development of novel TCIs or other probe compounds. Although the hydrolytic stability of the sulfonyl fluoride moiety proved to be a liability when highly reactive SF warheads were incorporated into probe molecules, it is the less reactive SFs whose reactivity matches that of the well-validated *N*-arylacrylamide class of TCI warheads. Moreover, this trend towards reduced stability for the more reactive SFs was not observed when the metabolic stability of these compounds was examined in rat hepatocytes. This indicates that the intrinsic reactivity of the sulfonyl fluoride group may not necessarily be a useful predictive marker when considering the metabolic clearance of SF-containing probe molecules.

In many ways, the reactivity of the SF moiety complements that of the *N*-arylacryamide group due to its ability to stably modify nucleophilic residues other than cysteine, especially tyrosines. Unfortunately, this aspect of SF chemistry comes at the expense of reduced stability in aqueous media. Our results provide a framework for the rational design of SF-based warheads with specific kinetic profiles. These SF-based covalent probes should be especially useful in cases where a proximal cysteine residue is not present, thereby eliminating the potential use of acrylamides and related groups. In particular, we note that the rates of reaction measured for SF warheads span the same range as those obtained in assays employing *N*-arylacrylamides. Overall, the data reported herein demonstrate that SFs possess predictable reactivity and stability profiles and are able to engage numerous amino acids in a covalent manner; we thus consider SFs and related groups to be valuable warheads for the development of novel TCIs and probe compounds.

Experimental

General Procedure for LC-MS Assays for $S^{(IV)}$-F Reactivity Profiling

Reactions were conducted in 2-mL glass vials. Each vial was charged with 250 µL water, 100 µL sodium hydrogen phosphate / sodium dihydrogen phosphate buffer (1.0 M, pH 7.5), 400 µL *N*-acetyltyrosine solution (25 mM), 200 µL NaCl solution (5.0 M), and 40 µL CH_3CN. The reaction buffer was prewarmed to 37 °C, then the reactions were initiated by the addition of sulfonyl fluoride (10 µL, 100 mM in CH_3CN). The reaction vials were immediately placed in the preheated (37 °C) autosampler compartment of the mass spectrometer and spectra were obtained at regular intervals (minimum sampling interval ~2.5 minutes) using the same LC-MS conditions as listed in General Experimental Considerations. For reactions conducted with other amino acids, the corresponding *N*-acetyl amino acid was used in place of *N*-acetyltyrosine. For stability profiling, *N*-acetylphenylalanine was used in place of *N*-acetyltyrosine. For reactions conducted at other pH values, the sodium phosphate buffer was replaced with either sodium acetate/acetic acid buffer (1.0 M, pH 5.0) or sodium bicarbonate/sodium carbonate buffer (1.0 M, pH 10.0). Reaction products were identified by mass spectrometry (ES- ionization); sulfonyl fluorides which did not yield ions under these conditions were identified by comparison of their LC UV chromatogram with that of a purified sample. Samples were quantified by integration of the UV traces. For stability studies, extent of hydrolysis was determined, where possible, by UV quantification of the corresponding sulfonic acid. In certain cases, however, the sulfonic acid eluted within the first 0.30 minutes of the LC run; this interval could not reliably be quantified due to the presence of inorganic salts in the reaction mixture. In these cases, extent of hydrolysis was determined by the loss of parent sulfonyl fluoride signal in comparison to *N*-acetylphenylalanine. In all cases, the reaction rates reported in the text were obtained by measuring the formation of product over time in the interval where this rate was linear (typically <10% product formation). For example plots see **Figure S1**.

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Rat Plasma Stability Assay

Each well of a deep well 96-well plate was charged with 200 µL acetonitrile. Blank samples were prepared by adding 50 µL rat plasma (purchased from BioreclamationIVT (Westbury NY). Part number was RATPLEDTA3-WH. (Wistar Hanover rats, plasma in K3 EDTA)) to the acetonitrile. The plate was then incubated on ice, protected from light. Assay samples were prepared by adding 5 µL of each of the indicated sulfonyl fluorides (4 mM in DMSO) to 995 µL rat plasma at room temperature. The samples were mixed thoroughly, then were allowed to incubate at room temperature. At intervals of 0, 15, 30, 60, 120, 240, and 360 minutes, a 50 µL aliquot of the reaction mixture was removed and added to the deep well plate containing cold acetonitrile. The plate was mixed using a plate shaker (ca. 5 s) to ensure complete mixing after each addition of plasma solution. After the final aliquot of plasma was added to the plate, the plate was centrifuged (4 000 x *g*, 5 min). The supernatant was withdrawn from each well and subjected to LC-MS/MS analysis using an Agilent 1290 UHPLC coupled to an Agilent 6490 triple quad mass spectrometer. The MS data was integrated using the MRM peak area for each sulfonyl fluoride. To obtain the reported t1/2 values, ln (S_t – B_t), where S_t = sulfonyl fluoride peak area at time t and B_t = blank area at time t, was plotted against time, affording slope m. The $t_{1/2}$ values were then calculated (assuming first-order kinetics) as $t_{1/2}$ = -ln (2)/m.

Profiling the Reactivity of FBSA Analogues against SYK or FGFR1

Reactions were performed in 2-mL glass vials fitted with 0.2-mL polypropylene inserts. Each insert was charged with 100 µL reaction buffer (described above) containing FGFR1 or SYK kinase domain (10 µM). The reaction vials were preheated to 37 °C, then the reactions were initiated by the addition of a DMSO solution of FSBA analogue (10 mM, 1.0 µL, 10 eq. relative to kinase). The reaction vials were immediately placed in the preheated (37 °C) autosampler compartment of the mass spectrometer. Mass spectra were obtained at regular intervals (minimum sampling interval ~6 minutes) using a Waters UPLC linked to a Waters SQD mass spectrometer (Column temperature = 27 °C, UV = 210-400 nm; positive electrospray ionization) equipped with a Phenomenex Jupiter 5u C_4 300A column (5 µm, 2.0 x 50 mm), eluting with 2 – 98% solvent B in solvent A over 4.5 minutes followed by a 1 minute hold at 98% solvent B, where solvent A = water containing 0.1% formic acid and solvent B = CH_3CN containing 0.1% formic acid. The mass spectra corresponding to the proteins were averaged and deconvoluted; the deconvoluted spectra were then integrated to afford the initial rate data.

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