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# Simple One-Pot Synthesis of Disulfide Fragments for Use in Disulfide-Exchange Screening

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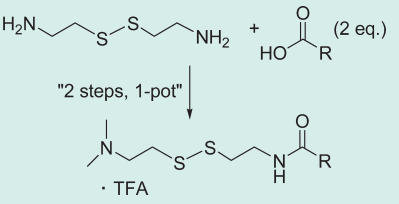
Supporting Information

**ABSTRACT:** Disulfide exchange screening is a method for evaluating the binding of small molecule fragments to proteins that have at least one accessible cysteine. While operationally simple, it does require a large library of small fragment molecules bearing disulfide-containing side chains. These specialized fragments are not available commercially and this has limited the adoption of the method. We report here a convenient one-pot procedure that enables facile preparation of disulfide screening fragments while also producing less of an environmental impact. The new synthetic method involves the initial formation of symmetric disulfides, followed by a disulfide exchange reaction in which the symmetrical dimer is converted into the final screening fragment by introduction of a solubilizing 'cap'. The method is amenable to parallel synthetic methods and can be carried out in air without the need for the specialized equipment typically required for performing organic synthesis.




**KEYWORDS:** disulfide synthesis, Tethering, fragment synthesis, disulfide exchange

Biophysical screening of small-molecule "fragments" is an increasingly important method in pharmaceutical lead discovery.[1,2] Fragment screening can be carried out by X-ray, NMR, or surface plasmon resonance (SPR) methods among others, and is often applied as an orthogonal approach to traditional high-throughput screening. Fragments are usually defined as drug-like small molecules with a molecular weight of less than 250 Da. Because fragments are much smaller than the molecules in typical HTS libraries, they are better able to form effective binding contacts with diverse targets, and thus typically bind with greater "ligand efficiency" than do HTS compounds. Structural characterization of fragments bound to their target is usually a prelude to further optimization through structure-informed medicinal chemistry.

One of the inherent difficulties of fragment screening is detection of the low affinity binding events. Disulfide-exchange screening or Tethering[3] is a mass-detected screening method that involves equilibrium exchange of disulfide-containing fragments with native or introduced cysteine residues on a protein target of interest.[4,5] Those fragments that form effective non-covalent surface contacts in addition to the covalent disulfide bond are sufficiently stable to persist and be detected by mass spectrometry. This method has been used to identify drug leads[6] and has helped to identify novel allosteric sites in therapeutically relevant targets such as caspases.[7−9]

A typical disulfide fragment is shown below (**1**, Figure 1) and consists of a small drug-like organic fragment attached via an amide bond to a disulfide bearing a basic amine function for improved solubility. After screening a target by disulfide exchange, it is generally necessary for fragment hits to be resynthesized for further validation studies. Structure−activity relationships can then be explored by preparing and screening focused libraries of

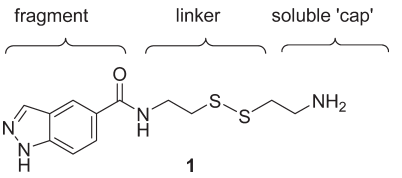



**Figure 1.** Chemical structure of a typical disulfide fragment.

disulfide analogs, in an iterative process very much like traditional medicinal chemistry optimization.

Previously, disulfide fragments had been synthesized via an approach that proceeded through a "cap protected" linker-cap moiety, as in the mono Boc-protected cystamine **3** (Scheme 1).[4,10] While these can be synthesized in bulk, they are typically soft crystalline waxes, tend to be hydroscopic and, in general, are difficult to handle and purify. The protected linker-caps were then coupled to activated acids on the solid phase[11] or via standard peptide coupling techniques to yield the Boc protected disulfide fragment (**4**) which could be purified at this stage via silica chromatography if needed. The Boc group was removed by treatment with trifluoroacetic acid (TFA) and the crude product **5** was typically used without further purification. Though not difficult, this chemistry requires experience in organic synthesis, and access to a fully equipped organic synthesis laboratory. We therefore sought an improved procedure that did not require special expertise or equipment, which in turn could enable broader adoption of disulfide exchange screening methods. These requirements, together with our need to maintain and

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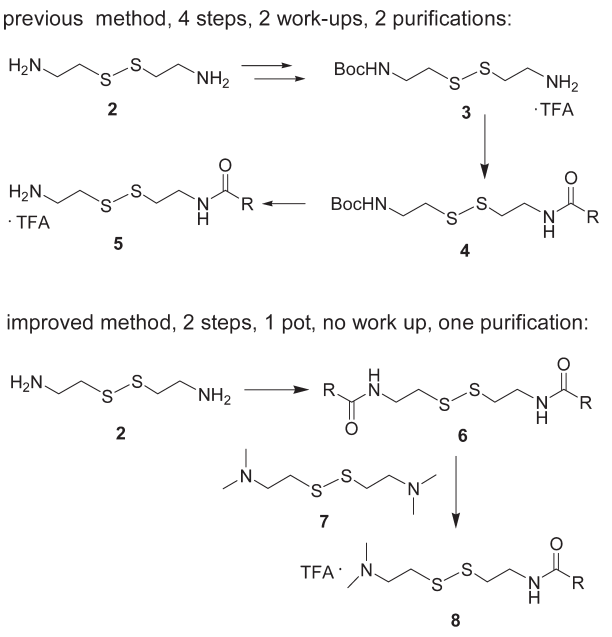
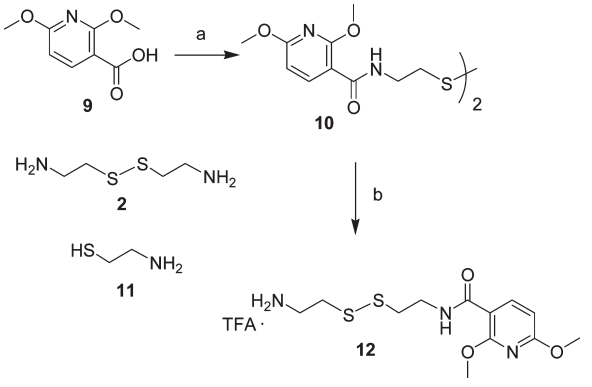
**Scheme 1. Previous and Improved Approaches for Disulfide Fragment Synthesis[a]**




[a] The new one-pot method eliminates all work ups and requires only a single reverse-phase chromatographic purification step.

**Scheme 2. Synthesis of a Disulfide Fragment[a]**



[a] Conditions: (a) **2**·2HCl, EDC, HOBt, $CH_2Cl_2$; (b) 3 equiv. **2**·2HCl, 0.1 equiv. **11**·HCl, $Et_3N$, DMF.

grow our existing disulfide fragment library, drove us to develop just such a synthetic manifold. Herein, we report a very simple, one-pot synthetic methodology (Scheme 1) that can be performed on the benchtop, in a parallel fashion without the use of air-free techniques, significantly reducing the use of toxic or hazardous materials, such as TFA, and generating a very modest waste stream.

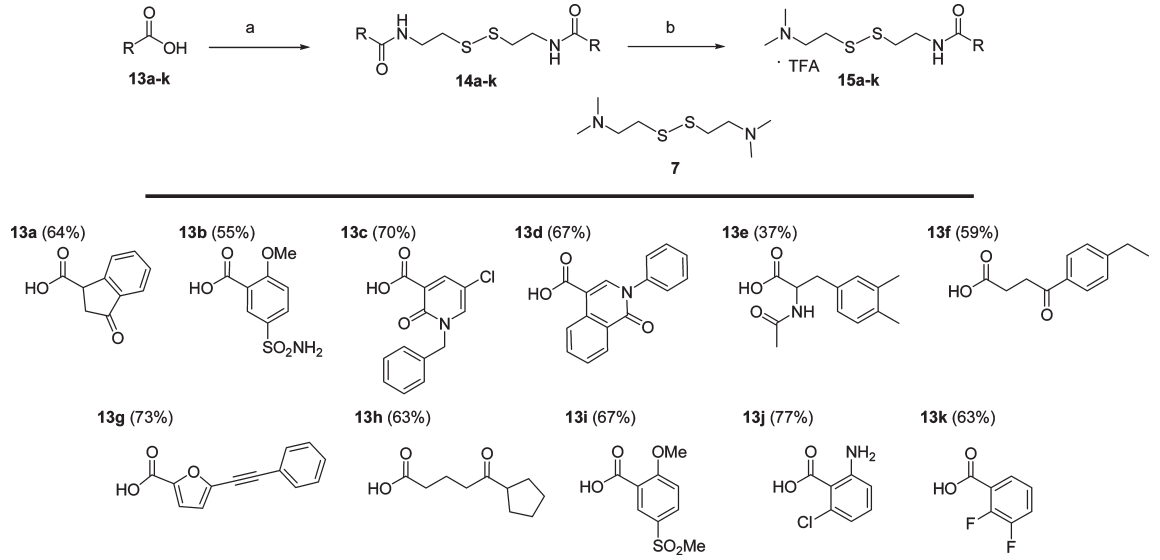
In the original synthesis of disulfide fragments, scrambling of disulfide intermediates and side products was a significant problem that had to be carefully monitored. We reasoned that this liability might be used to advantage by altering the synthetic approach. Hence, rather than coupling a partially protected (and non-symmetric) disulfide (e.g., **3**) to the acid building block, we envisioned coupling the acid to both ends of a symmetric disulfide (e.g., **2**). The desired fragments would then be obtained by promoting free exchange between the symmetrical fragment disulfide **6** and a second symmetrical disulfide **7** containing the hydrophilic 'capping' group (amino or dimethylamino). By using an excess of the "capping" disulfide **7**, the equilibrium would be driven to favor formation of the desired disulfide fragment **8** bearing the soluble cap. This material would then be separated chromatographically (HPLC) from the much more hydrophilic diamine homodimer **7** and any remaining fragment homodimer (**6**).

The two steps of the proposed synthetic route were explored separately before attempting the desired one-pot reaction protocol. Hence, we started by coupling 2,6-dimethoxy nicotinic acid (**9**) to cystamine dihydrochloride salt (**2**·2HCl), using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) in methylene chloride (Scheme 2). The desired dimeric fragment **10** was purified by flash chromatography and isolated in reasonable yield (57%). Our initial attempts at effecting disulfide exchange involved reacting purified disulfide **10** with an excess of the dimeric capping reagent **2** in the presence of a free thiol initiator (cysteamine·HCl, **11**, 0.5 equiv) and an excess of either diisopropylethylamine or sodium hydroxide, in DMF or DMSO. We were happy to find that each of these procedures indeed produced a reasonable mixture of hetero and homo disulfides after stirring overnight. Three disulfide species (**2**, **10**, and **12**) could be identified by LC/MS analysis and these were readily separated via preparative HPLC. Ultimately we found that increasing the amount of capping disulfide (to 5 equiv[12]) gave improved conversion to the desired disulfide (**12**), that use of 0.1 equivalents of thiol initiator was sufficient, and that triethylamine was as effective as diisopropylethylamine or aqueous NaOH.

To operationally simplify the procedure, we next explored the feasibility of performing the exchange chemistry directly on the crude reaction mixture obtained in the initial coupling. In fact, this two step, one-pot procedure worked well after some optimization of the reaction conditions. We switched to dimethylformamide (DMF) for the coupling reaction, which in turn required that a small amount of water be included to dissolve the cystamine dihydrochloride salt. And although most of the coupling reactions proceed to completion with only a small excess of EDC, we found that using 2 equiv gave more consistent results over a broader array of substrates and this, significantly, allowed us to eliminate monitoring of individual reactions prior to initiating the exchange reaction. The inclusion of water in both the coupling and exchange reactions also insures that any excess EDC is quenched prior to chromatographic purification. We also switched from a cysteamine (ethylamino) cap (as in **5**) to a dimethylamino cap (as in **8**). Not only does this improve chromatographic separations, it eliminates a nucleophilic primary amine from the reaction sequence. A significant improvement to the exchange step was realized by eliminating the exchange initiator cysteamine **11**, which is prone to air oxidation over time, and therefore requires either careful handling or use of fresh reagent for the most consistent results. In the improved procedure, a reactive thiol is produced in situ by including the water-soluble phosphine tris-(2-ethylcarboxy)phosphine hydrochloride (TCEP, 0.1 equivalent) in the exchange reagent solution containing bis([2-(*N*,*N*-dimethylamino)ethyl]disulfide **7**. This modification eliminates the formation of multiple disulfide products as is observed when using cysteamine as the initiator.

The bottleneck in most parallel synthesis procedures is the workup and/or purification of the final products. We therefore

**Scheme 3. Improved One-Pot Synthesis and Representative Library Members**[a]




[a] Conditions: (a) **2**·2HCl, EDC, HOBt, $H_2O$, DMF, (THF); (b) 5 equiv. **7**·2HCl, 0.1 equiv. TCEP, $Et_3N$, $H_2O$, DMF.

examined whether the crude reaction mixtures could be directly subjected to preparative reverse phase HPLC purification to yield the desired product (e.g., **8**) with no time-consuming and wasteful workup. In fact, this approach works quite well. The crude reaction mixtures are first diluted slightly to a total volume of 1 mL using a 2:1 DMSO−water solution,[13] (typically 0.3 mL added). The aqueous DMF/DMSO solution is then loaded directly on a 19 × 50 mm C18 reverse phase HPLC column and eluted using water−methanol or water−acetonitrile mobile phases with gradient elution. Our experience after having performed hundreds of such separations is that the three disulfide species present in the reaction mixture (e.g., **6**−**8**) are quite distinct chromatographically, and that other side products arising from EDC, HOBt, and TCEP do not coelute with the desired disulfide fragments.

Shown below (Scheme 3) is a representative set of structurally diverse carboxylic acids that have been successfully converted into the corresponding disulfide fragments. Yields are based on the cystamine (**2**) starting material and are normalized to account for the fact that two equivalents of disulfide fragment are produced from each equivalent of cystamine. Generally speaking, any carboxylic acid that can be successfully coupled to a primary alkyl amine should be a suitable candidate for disulfide fragment synthesis using the methodology described herein. Beyond improved efficiency and experimental simplicity, the improved method has a much reduced chemical footprint. This improvement stems from the elimination of (1) protection/deprotection steps, (2) the need to synthesize starting materials in bulk, and (3) the use of TFA, and 4) the elimination of all workup procedures. This new simplified and streamlined synthesis of disulfide fragments enables facile parallel library production, as well as resynthesis of fragment hits and analogs for SAR studies.

We are presently working to automate this method and to expand its scope by exploring other linkages (e.g., "reverse" amides, 1,2,3-triazoles) that can be prepared using analogous two-step, one-pot methods. The results of this work will be described in subsequent communications.

## ■ ASSOCIATED CONTENT

**Supporting Information.** This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Using 5 equiv of the capping disulfide leads to a 1:10:25 mixture of coupled dimer, monophore, and cap dimer, assuming the statistical equilibrium is reached. Maximum theoretical yield is 91%.

(13) Addition of some water helps to dissolve the amine salt byproduct, the total volume is largely dependent on the preparative HPLC's injection volume.