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Tyrosylprotein Sulfotransferase Inhibitors Generated by Combinatorial Target-Guided Ligand Assembly

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Abstract—Tyrosylprotein sulfotransferases (TPSTs) catalyze the sulfation of tyrosine residues within secreted and membrane-bound proteins. The modification modulates protein–protein interactions in the extracellular environment. Here we use combinatorial target-guided ligand assembly to discover the first known inhibitors of human TPST-2.

Protein tyrosine sulfation is emerging as a widespread post-translational modification that modulates protein–protein interactions in the extracellular environment.[1] Tyrosine sulfate has long been known to occur in a number of human proteins, but recent reports describing tyrosine sulfate in P-selectin glycoprotein ligand (PSGL)-1[2–4] and the chemokine receptors CCR5, CXCR4, and CCR2[5,6] have dramatically increased interest in this modification. In these cases, sulfation of tyrosine residues increases the affinity of the respective protein–protein interactions, just as phosphorylation of tyrosine residues can modulate interactions among intracellular proteins. For example, PSGL-1 loses the ability to bind P-selectin if the three sulfated tyrosine residues are mutated to phenylalanine.[3,4] The role of CCR5 as a coreceptor for HIV infection is well known, and in vitro assays indicate that the sulfotyrosine residues on CCR5 are recognized by gp120.[7]

A major breakthrough in the study of tyrosine sulfation occurred in 1998 with the cloning of tyrosylprotein sulfotransferases (TPSTs)-1 and -2 from both mouse and human.[8–10] The enzymes catalyze the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a tyrosyl residue within the target protein (Fig. 1). Both are membrane-bound Golgi enzymes with lumenal catalytic domains, a single transmembrane span, and a short cytosolic tail. As no consensus sequences for tyrosine sulfation have been identified, the complete list of sulfated proteins is unknown. Furthermore, there is no straightforward means to determine if a given protein–protein interaction requires tyrosine sulfate. Cell-permeable small molecule inhibitors of the TPSTs would facilitate the identification of interactions modulated by tyrosine sulfate.

We applied the recently described technique of combinatorial target-guided ligand assembly, developed by Ellman and coworkers, to generate the first inhibitors of human TPST-2 (hTPST-2).[11] The technique is well-suited for targets that lack lead compounds and structural information, such as the TPSTs. Furthermore, the approach was highly effective in generating an inhibitor of the tyrosine kinase c-Src,[11] an enzyme that catalyzes a similar anionic group transfer reaction. The strategy

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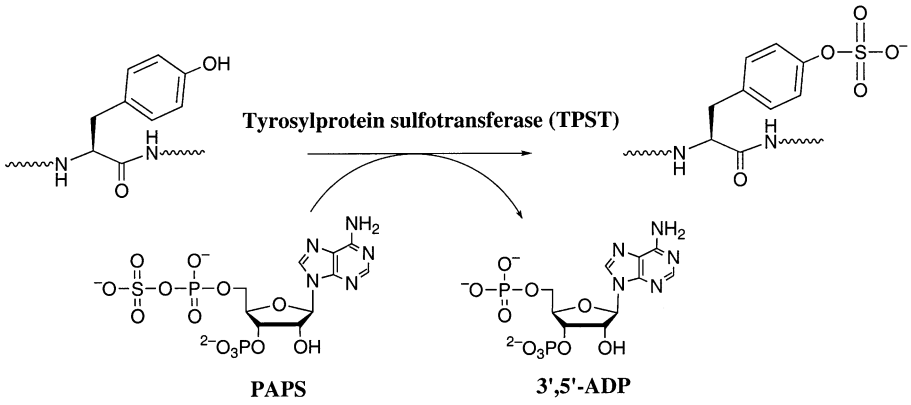

Figure 1. The tyrosylprotein sulfotransferases catalyze the addition of sulfate to tyrosine residues within polypeptide chains.

begins with assembly of a library of compounds, or 'monomers,' that carry a common chemical handle. The monomers are screened against the target at concentrations of 1 mM or higher. Compounds that demonstrate inhibitory activity are then used to construct a library of 'dimers' via the common chemical handle, and the library of dimers is screened for inhibitors. This two-step process applied to hTPST-2 produced two active dimers with IC_{50} values of 30 and 40 μM.

High-Throughput Assay for Inhibitor Screens

We first developed a high-throughput assay for TPST inhibitors. Briefly, a biotinylated peptide substrate and ^{35}S-labeled PAPS were incubated with the enzyme and inhibitor candidates in a 96-well microtiter plate. The radiolabeled peptide products from each well were quantified by capture on a streptavidin-impregnated membrane (SAM^2 biotin capture membrane, Promega) followed by phosphorimaging analysis. The interaction between biotinylated peptide and streptavidin-modified membrane is stable to high salt and low pH, such that the excess radiolabeled PAPS can be washed away prior to analysis. The peptide substrate used in the assay, generated by Fmoc-based solid-phase peptide synthesis, was modeled on the N-terminal tyrosine sulfation sites of PSGL-1 (underlined) and possessed the following sequence: biotin–NH–RDRRQATEYEYLDYDFLPE-TEPPP–CO_2H. ^{35}S-Labeled PAPS was generated according to Cook et al.[12] For inhibitor screens, a soluble, truncated form of hTPST-2 comprising the catalytic domain was secreted from stably transfected Chinese Hamster ovary (CHO) cells.[8] Conditioned, serum-free media was used without any further purification. The reaction contained 30 μM substrate peptide, a mixture of [^{35}S]PAPS and PAPS at a total concentration of 10 μM (compared to a K_m of 12 μM) and buffers as previously described.[8] Inhibitor candidates were added in DMSO, to give a final DMSO concentration of 5%.

The oxime linkage was chosen for compound assembly based on precedents established by Maly et al.[11] Thus, dimeric inhibitors would eventually be formed from two aldehyde-bearing monomers and an *O,O′*-diaminoalkanediol linker. A diverse set of 305 aldehydes was chosen for construction of the monomer library. The aldehydes were converted to *O*-methyl oximes prior to screening in order to better approximate the structures of the eventual dimers. Initial screens against hTPST-2 were performed at an inhibitor concentration of 1 mM. Eighteen compounds with at least 25% inhibitory activity (Fig. 2) were chosen to construct a library of dimers.

Using five straight-chain *O,O′*-diaminoalkanediol linkers ($n = 2$–6; Fig. 3), we constructed a 171-well library of all possible dimer combinations from the 18 monomers in Figure 2. Two aldehydes and five linkers were added to each well to produce a statistical mixture of 15 different molecules. These pools were then screened at a total concentration of 100 μM. We selected two of the most active pools for further characterization. To identify the most active species, the relevant monomer pairs were reacted with a series of individual linkers ($n = 2$–6), and the products were purified and re-screened. The dimers shown in Figure 4, compounds **1** and **2**, were revealed as the most active compounds, with IC_{50} values of 30 and 40 μM, respectively (Table 1). These dimers are composed of monomers **3**, **4**, and **5** (Fig. 4); both dimers share monomer **5** as a common element. Importantly, the dimers are significantly more active than their component monomers. Compound **3** has an IC_{50} value of 425 μM, whereas compounds **4** and **5** have IC_{50} values over 1 mM (Table 1).

Table 1. In the assay of NodST, PAPS was used at 5 μM, the value of its K_m, while chitobiose was used at 5 mM ($K_m = 250$ μM). In the HEC GlcNAc-6-ST assay, PAPS was used at 2 μM ($K_m = 10$ μM) and β-benzyl GlcNAc was used at 1 mM ($K_m = 500$ μM). For EST, both estrone and PAPS were used at concentrations equal to their K_m values, 50 nM and 3 nM, respectively

IC_{50} values against hTPST-2, micromolar				
1	**2**	**3**	**4**	**5**
30	40	425	> 1000	> 1000

Percent inhibition at 100 micromolar		
	1	**2**
NodST	66	19
HEC GlcNA-6-ST	30	7
EST	100	100

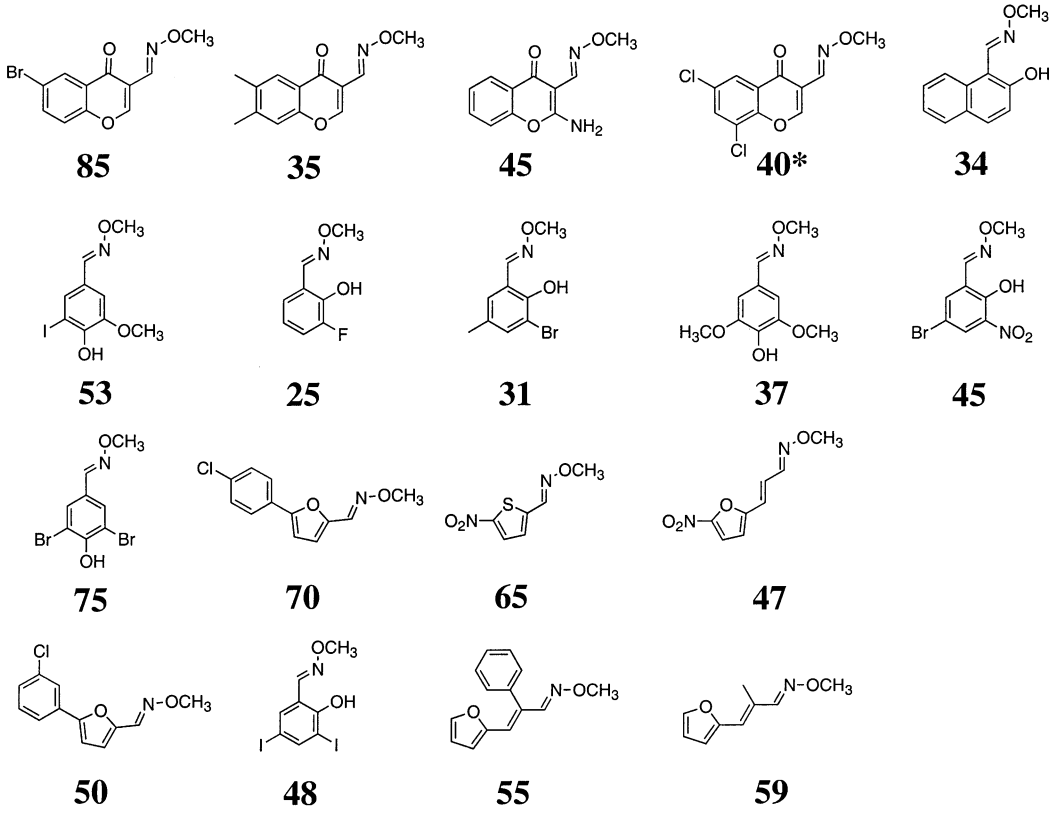

Figure 2. The 18 aldehyde monomers that were used to construct the library of dimers. The numbers given are percent inhibition at 1 mM, except for the value marked with an asterisk, which is the percent inhibition at 800 μM.

Compounds **1** and **2** are relatively large and hydrophobic, raising the concern that they might inhibit hTPST-2 in a non specific fashion. We repeated the IC_{50} determination in 1% fetal bovine serum, so that a large excess of protein other than hTPST-2 would be present. Thus, non-specific binding to hTPST-2 should be minimized. Under these conditions, the IC_{50} values were essentially unchanged. If the primary mechanism of inhibition is nonspecific binding, then addition of fetal calf serum should result in a significant increase in the IC_{50} value.

We also considered the possibility that **1** and **2** are irreversibly inhibiting hTPST-2. To address this issue, we incubated hTPST-2 with **1** or **2** at 40 μM for 5 h and then dialyzed the enzyme against buffer overnight. Compound **1** showed completely reversible inhibition, while compound **2** showed 50% inhibition after dialysis (Fig. 5). It is tempting to speculate that **2** is binding

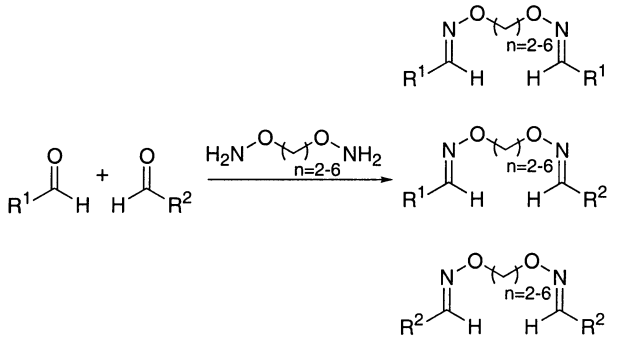

Figure 3. The library was synthesized in 96-well plates via a one-step reaction.

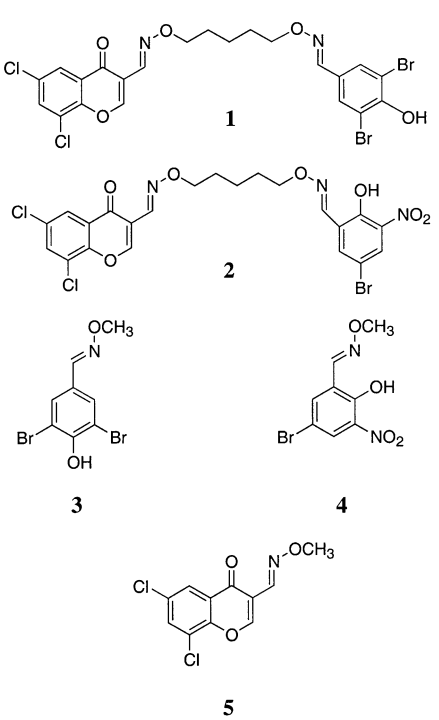

Figure 4. Compounds **1** and **2** are hTPST-2 inhibitors with IC_{50} values of 30 and 40 μM, respectively. Their component monomers, compounds **3**, **4**, and **5**, are shown as well.

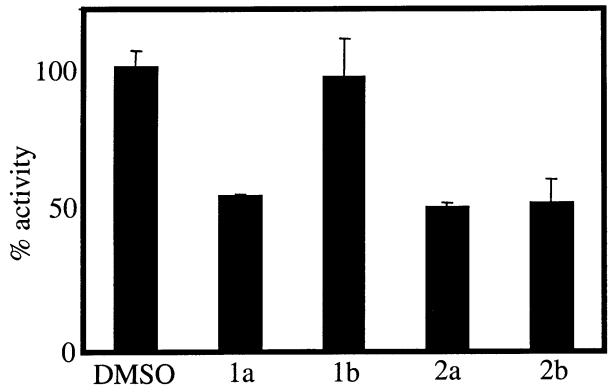

Figure 5. Compound **1** is a reversible inhibitor of hTPST-2, while compound **2** is not. hTPST-2 was incubated with DMSO, **1**, or **2** and dialyzed against buffer overnight. Enzyme activity was then assayed. The activity after incubation with DMSO is defined as 100%. Compound **1** shows significant inhibition at 40 μM (**1a**), but after dialysis the enzyme activity is restored (**1b**). Compound **2** also inhibits TPST-2 at 40 μM (**2a**), but the enzyme remains inhibited after dialysis (**2b**). The data shown are a compilation of multiple experiments.

covalently to hTPST-2; confirmation of this will require the generation of significant quantities of the enzyme.

The TPSTs are members of a sulfotransferase superfamily that includes numerous carbohydrate sulfotransferases.[13] We were curious about the selectivity of compounds **1** and **2** with respect to other members of the family. We first investigated their inhibitory potential against the bacterial GlcNAc-6-sulfotransferase NodST[14] and against the human high endothelial cell (HEC) GlcNAc-6 sulfotransferase (GlcNAc-6-ST).[12] At a concentration of 100 μM, compound **1** showed measurable inhibition of both NodST and HEC GlcNAc-6-ST (Table 1). Although a weaker inhibitor of HEC GlcNAc-6-ST than of hTPST-2, compound **1** was a more potent inhibitor of NodST ($IC_{50} = 15$ μM). Compound **2** showed weaker activity against both carbohydrate sulfotransferases than against hTPST-2.

We also determined the inhibitory activity of compounds **1** and **2** against estrogen sulfotransferase (EST), a human cytosolic sulfotransferase. Unexpectedly, both compounds **1** and **2** were highly active (100% inhibition at 100 μM, Table 1), with IC_{50} values of 250 nM and 3 μM, respectively. It is interesting to note that the small structural differences between compounds **1** and **2** correspond to a difference in IC_{50} values versus EST of over 10-fold. By contrast, these structural distinctions have negligible impact on hTPST-2 inhibition. The apparent efficacy of compound **1** against EST is somewhat undermined by the fact that EST binds PAPS and a wide range of aromatic substrates with high affinity (K_m values in the nM range).[15]

All four enzymes share similar sequence motifs that are thought to be involved in recognition of their common substrate PAPS. It is possible that the cross-reactive compound **1** interacts with the active sites of these enzymes in a manner similar to PAPS. Further kinetic investigations are ongoing.

In conclusion, we have identified inhibitors of hTPST-2 by applying combinatorial target-guided ligand assembly. The two compounds identified represent the first TPST inhibitors to be reported. Optimized inhibitors will be useful tools for biological studies of tyrosine sulfation.

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