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Chemical tagging of a drug target using 5-sulfonyl tetrazole

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ABSTRACT

Irreversible modification is one of the most promising strategies to identify cellular receptors of bioactive small molecules. Here we report that receptor proteins can be chemically tagged using a 5-sulfonyl tetrazole probe. 5-Sulfonyl tetrazole easily accepted nucleophilic attack of thiol groups, while 5-sulfinyl tetrazole did not. These functional groups were introduced into probe molecules of a natural product. Cyclosporine A, an immunosuppressant produced by a microbe, was derivatized to possess 5-sulfonyl tetrazole and a tag group, which enabled chemical tagging of cyclophilin A, the cellular receptor of cyclosporine A. Cyclosporine A derivative possessing 5-sulfinyl tetrazole could not tag cyclophilin A. This technique will allow efficient identification of cellular receptors of bioactive small molecules.

Small molecules often exhibit potent biological activities and sometimes they are used as pharmaceuticals. To develop effective and safer drugs, we have to understand modes of action of currently-used drugs and newly discovered bioactive small molecules at a molecular level. Taking advantage of genome-wide genetic materials and advances in informatics analysis, pathways targeted by small molecules can often be predicted.[1,2] However, identification of the receptor molecules to which small molecules directly bind remains to be challenging, especially when the binding affinity is low or the amount of receptor molecules is not abundant in a cell.

Irreversible modification is a powerful strategy to detect binding partners of a molecule of interest. In fact, small molecules naturally possessing reactive functional groups often exhibit potent biological activities and their cellular receptors have been successfully identified.[3–5] Reactive groups are usually electrophilic, accepting nucleophilic attack by amino acids or nucleotides to form covalent bonds in the active site of the receptors. On the other hand, technologies based on multifunctional probe molecules containing reactive groups and bioactive small molecules have been developed, for example, affinity-based protein profiling[6] and ligand-directed tosyl chemistry.[7] Through this technology, specific tagging of cellular receptors can be achieved in the proteome and the activity of the receptor proteins can be detected. For these purposes, probe molecules are made to contain reactive groups, for example, fluorophosphonate, vinylsulfone, sulfonate ester, epoxide, and so on.[8,9] We can choose the functionality from this repertoire. However, the chemical tagging technology is not mature; we do not fully understand the potential and limitation of this technology. It is still necessary to explore other functions with distinct chemical properties for providing opportunities to analyze mode of action of small molecules and develop new pharmaceuticals. Here, we demonstrate that 5-sulfonyl tetrazole is an alternative to chemically tag receptor proteins using a bioactive small molecule as a ligand (Fig. 1).

5-Sulfonyl tetrazole is known to be reactive toward nucleophilic attack by an addition-elimination pathway.[10–12] First we evaluated the reactivity of 5-sulfonyl tetrazole toward thiol, one of the most abundant nucleophiles in biological systems, using three model compounds **1**, **2** and **3** possessing 5-sulfanyl tetrazole, 5-sulfinyl tetrazole and 5-sulfonyl tetrazole, respectively (Fig. 2a). All these molecules were designed to possess a tosylate group to compare the reactivity. When incubated with *n*-pentanethiol (1 equiv) in the presence of base, 5-sulfanyl tetrazole in **1** remained intact, while 5-sulfonyl tetrazole in **3** was completely converted to 5-sulfanyl tetrazole (Fig. 2b). A part of **1** was converted to **5**, indicating that tosylate was replaced with *n*-pentanethiol. In contrast, **3** was converted to **4**, where only 5-sulfonyl tetrazole was attacked by the thiol. Compound **2** possessing 5-sulfinyl tetrazole exhibited moderate reactivity to produce **4**. If only the tosylate group in **1** or **2** was attacked by *n*-pentanethiol, compounds **6** and **7** would be produced, respectively. However, we could not detect any peaks other than the starting compound and the major product in which only 5-sulfonyl/sulfinyl tetrazole was attacked by *n*-pentanethiol. These

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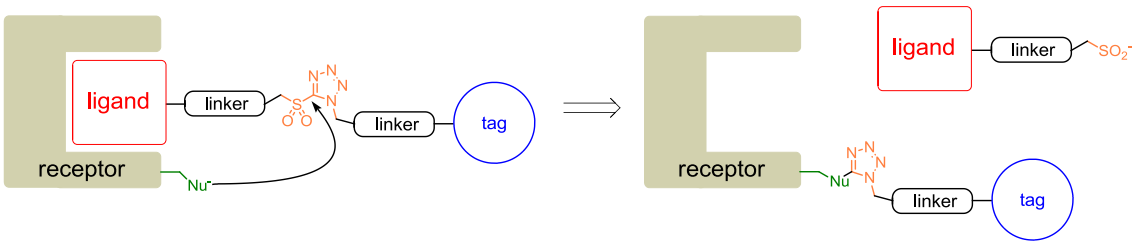


Figure 1. Chemical tagging of a receptor protein using a 5-sulfonyl tetrazole probe. The probe molecule consists of three functions: a ligand for the receptor protein (red), a tagging function that reacts with nucleophiles in the receptor protein (orange), and a tag group for detection (blue). When the probe molecule is directed to the receptor protein and the tagging function, that is, 5-sulfonyl tetrazole, is attacked by a nucleophile (green), chemical tagging of the receptor protein will be achieved.

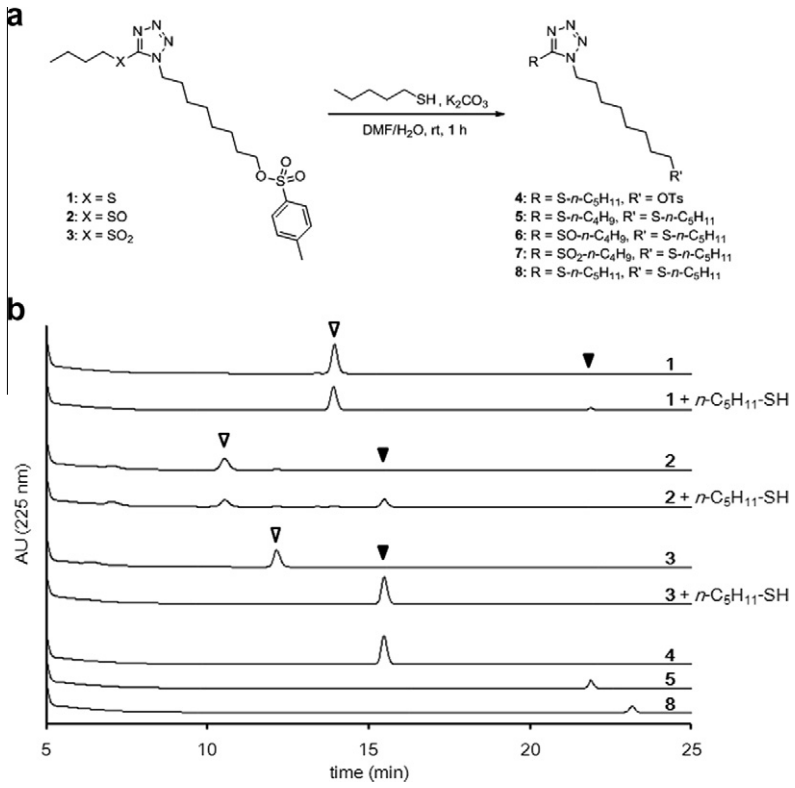


Figure 2. Reactivity of 5-sulfonyl tetrazole. (a) Reaction scheme of model compounds **1**, **2** and **3** with pentanethiol. (b) Reaction properties of **1**–**3**. Model compounds were mixed with pentanethiol in the presence of base, and the reaction mixture was analyzed on ODS-HPLC. Arrowheads with white interior indicate the model compounds **1**, **2**, or **3**. Black arrowheads indicate products. Compounds **4**, **5** and **8**, synthesized authentic samples, were also analyzed in the same condition.

results indicated that the oxidation state of the sulfur atom was important for the reactivity and that 5-sulfonyl tetrazole was the most reactive. It is likely that the reactivity depends on the kind of electron withdrawing groups substituted at C5 of the tetrazole ring. In addition, we found that tosyl ester, one of the well-utilized leaving groups in organic synthesis, was not replaced by alkyl thiol in the presence of 5-sulfonyl tetrazole when 1 equiv of *n*-pentanethiol was used, indicating the higher reactivity of 5-sulfonyl tetrazole. Next, we designed and synthesized probe molecules that enable chemical tagging of receptor proteins.

Probe molecules for chemical tagging consist of three functions (Fig. 1); a ligand that recognizes specific receptor proteins, a tagging function that links the probe to the receptor protein, and a tag group that makes detection or concentration of the receptor molecules easy. 5-Sulfonyl tetrazole probes were prepared as follows (Fig. 3); first, simple heating of neat toluenesulfonyl cyanide with an alkyl azide gave 1-alkyl-5-sulfonyl tetrazole through [2+3] cycloaddition (step 1);[13] second, substitution of the *p*-toluenesulfonate with a thiol followed by oxidation furnished the core structure of a tagging part (step 2); probe synthesis is accomplished by conjugation of a ligand and a tag group to the tagging part (step 3). In this study, the tagging function was 5-sulfonyl tetrazole. We chose cyclosporine A (CsA) as a ligand and biotin as a tag. CsA, a peptidic fungal metabolite,[14] is an immunosuppressant drug widely used in organ transplantation to prevent rejection. CsA binds to cyclophilin A (CyPA) with high affinity, and the heterodimer binds to calcineulin to inhibit its phosphatase activity, leading to suppression of the expression of inflammatory cytokine genes.[15–17] We expected that a linker chain can be elongated from the side chain of the (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt) residue since this side chain seems not to be involved in the binding of CsA to CyPA.[18] In fact, fluorescein-labeled CsA analog in which MeBmt residue was modified, was reported to bind CyPA.[19] Conjugation of CsA, 5-sulfonyl tetrazole and biotin was carried out as shown in Scheme 1. Briefly, tosyl cyanide **9** and alkyl azide **10** was heated to obtain **11**. The tosyl group in **11** was exchanged with thiopropionate in the presence of base, followed by oxidation to obtain **12**. A linker part was condensed with

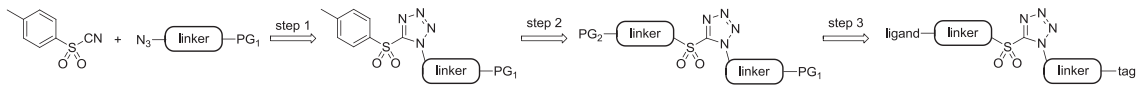
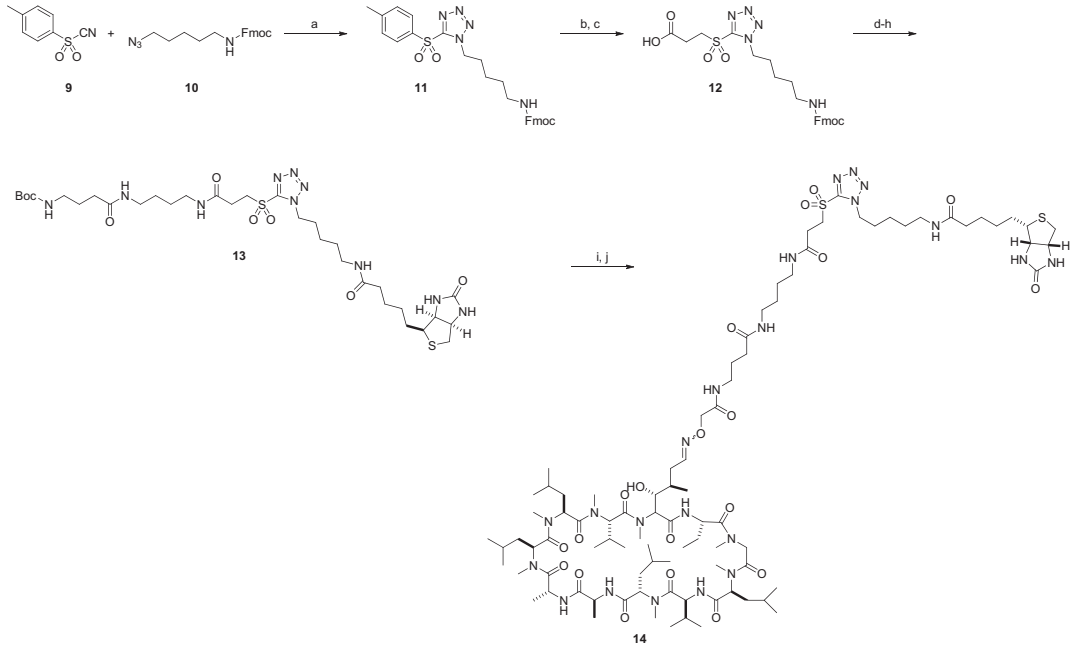


Figure 3. Schematic drawing of preparation of 5-sulfonyl tetrazole probes.



Scheme 1. Preparation of cyclosporine probe **14**. Reagents and conditions: (a) 100 °C, 4 h (quant); (b) $HSCH_2CH_2COOH$, K_2CO_3, CH_2Cl_2, rt, 10 h (quant); (c) *m*CPBA, CH_2Cl_2, 0 °C to rt, 4 h; (d) $BocNH(CH_2)_4NH_2$, DIEA, HBTU, DMF, rt, 1 h (45% in two steps); (e) TFA, CH_2Cl_2, 0 °C to rt, 1 h; (f) $BocNH(CH_2)_3COOH$, DIEA, HBTU, DMF, rt, 1 h (67% in two steps); (g) DBU, DMF, rt, 3 d; (h) biotin, DIEA, HBTU, DMF, rt, 1 h (48% in two steps); (i) TFA, CH_2Cl_2, 0 °C to rt, 1 h; (j) CsA-COOH,[20] DIEA, HBTU, DMF, rt, 1 h (66% in two steps).

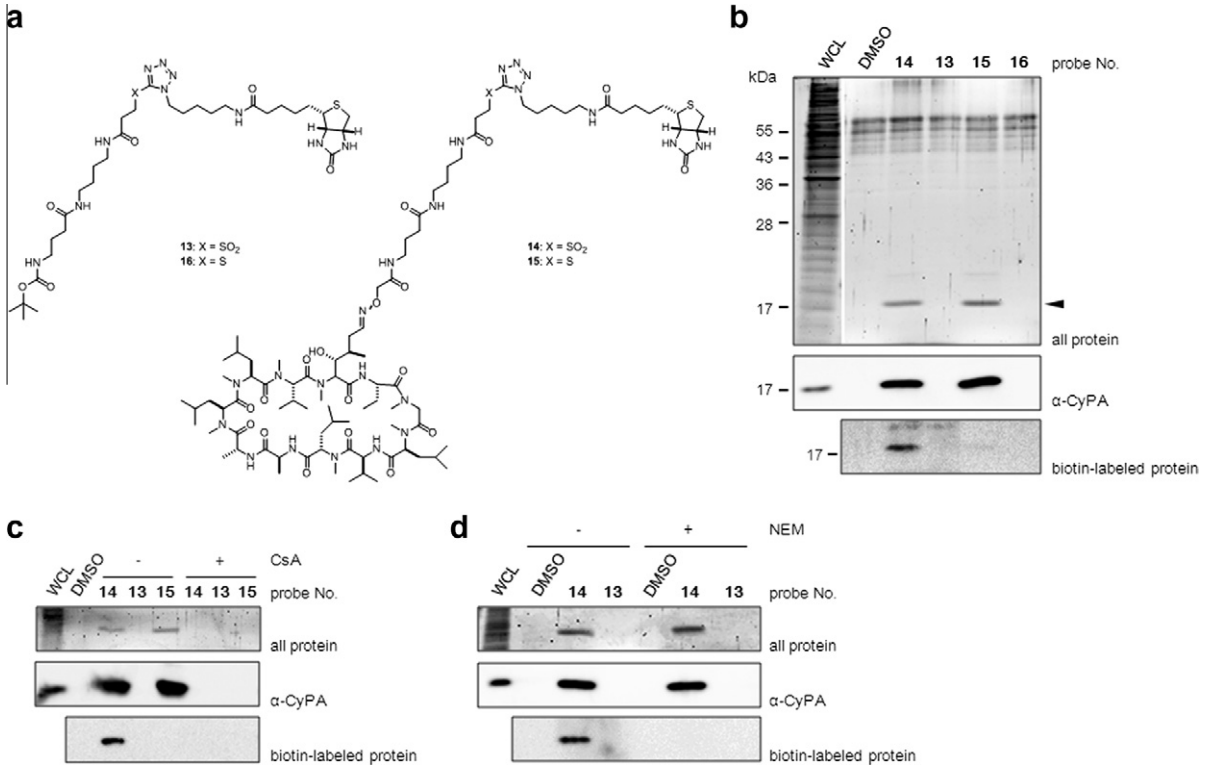


Figure 4. Chemical tagging of cyclophilin A using cyclosporine A probe. (a) Chemical structures of CsA probes **14** and **15**, and control probes **13** and **16**. (b) Binding of CsA probes to CyPA and tagging of CyPA by biotin. The arrowhead indicates the protein bands specifically detected for the CsA probes. (c and d) Specificity of the tagging. Chemical tagging was examined in the presence of CsA (c) or NEM (d). Images around the 18 kDa bands are shown. WCL, whole cell lysate.

the carboxylic acid of **12**, while biotin was conjugated to the amino group, yielding **13**. Finally, deprotection and condensation with a CsA derivative[20] furnished CsA-5-sulfonyl tetrazole probe **14**. The sulfanyl tetrazole probe **15** was prepared in the same manner (Fig. 4a).

We first examined if CsA probes **14** and **15** can interact with CyPA. Probe molecules were incubated with a lysate of Jurkat cells (human leukemia T cell line), which was mixed with streptavidin beads to pull down proteins that have affinity to CsA probes, and proteins captured on the beads were subjected to SDS–PAGE analysis. Protein bands with 18 kDa were detected when cell lysate was incubated with CsA probes **14** and **15** (Fig. 4b). In the case of probe molecules lacking CsA, that is, **13** and **16**, no protein signal was detected around 18 kDa. These 18 kDa bands were confirmed to be CyPA by Western blotting analysis (Fig. 4b), indicating that the prepared CsA-probes bound to CyPA. The binding did not depend on the oxidation state of the sulfur atom adjacent to the tetrazole ring. Next we tested if CyPA was tagged by biotin. As we expected, although the signal was weak, we could repeatedly observe the biotin signal when incubating with the probe molecule **14** (Fig. 4b).[22] As in the case of the model experiment examined in organic solvent (Fig. 2), 5-sulfonyl tetrazole in **14** was likely attacked by some nucleophiles in CyPA to form covalent bonds between the tag group and CyPA (Fig. 1). In contrast, probe molecule **15** could not tag CyPA. This indicated that the reactivity of 5-sulfanyl tetrazole was not enough to be attacked by nucleophiles in proteins although it is known that nucleophilic substitution can occur in harder conditions.[23] The biotin and CyPA signals were not detected in the presence of excess amount of CsA, confirming that the biotin modification occurred on CyPA molecules (Fig. 4c). When cell lysate was treated with *N*-ethylmaleimide (NEM) before addition of the CsA probes, the biotin signal of CyPA disappeared while the affinity between **14** and CyPA was not lost (Fig. 4d). As NEM preferentially reacts and inactivates thiol groups, Cys residues in CyPA seem to attack 5-sulfonyl tetrazole in the probe **14**. Alternatively, nucleophiles other than thiol group that reacted with NEM could be tagged by the CsA probe. 5-Sulfonyl tetrazole has been reported to react with various nucleophiles including imidazole and alcohol in the presence of base.[11,12] Currently, however, the modified residue remains to be identified.

In summary, we have demonstrated that chemical tagging of cellular receptors of a bioactive small molecule can be achieved using probe molecules containing 5-sulfonyl tetrazole. When 5-sulfonyl tetrazole was conjugated with CsA and biotin, and it was incubated with a Jurkat cell lysate, the cellular receptor of CsA, CyPA, was tagged with biotin. This technology will offer advantages in the drug target identification process. First, preparation of the probe molecule is straightforward and can be carried out quantitatively.[13] Second, 5-sulfonyl tetrazole exhibited high reactivity toward thiol. Even in the presence of tosyl ester, 5-sulfonyl tetrazole preferentially reacted with thiol. Different reactivity will make opportunities to gain new insights into the interaction between drugs and receptor molecules. In addition, 5-sulfonyl tetrazole probes may not kill the protein function. It is likely that the ligand is released after 5-sulfonyl tetrazole is attacked by a nucleophile, in a similar way to that of the tosyl-chemistry probes.[24] Enrichment of chemical tagging methodologies that are complimentary to traditional genetic tagging methods will be beneficial for functional analysis of biological macromolecules.[25]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.092.

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