Selective Formation of Homo- and Heterobivalent Peptidomimetics

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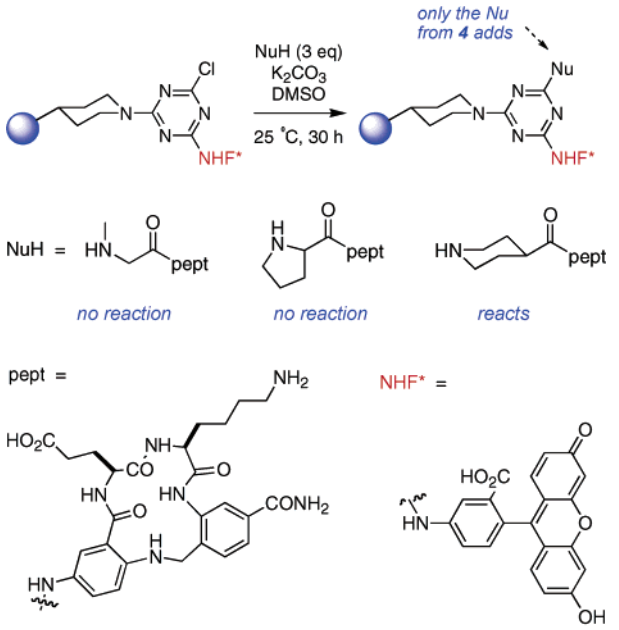
Abstract: Methodology is presented for assembling fluorescently labeled bivalent molecules from monovalent constituents, without side chain protection or coupling agents. To illustrate the procedure, a series of bivalent peptidomimetics directed toward the Trk receptors were prepared and screened via fluorescent activated cell sorting scan assays.

Many signaling processes are associated with conformational changes of cell surface receptors induced by dimeric protein ligands.[1−3] *Synthetic* bivalent ligands therefore can be valuable mimics of these ligand−receptor interactions.[4−7] Combinatorial chemistry is ideally suited to formation of bivalent molecules because $n(n + 1)/2$ products could be prepared by combining n monovalent ones with themselves, providing rapid access to large libraries.

The main obstacles to combining monovalent molecules with themselves to form libraries of bivalent products relate to the chemistry used to assemble them. Formation of bivalent compounds can be efficient if the monovalent starting materials are not protected, but if they are, it is almost always necessary to purify each deprotected compound prior to testing. An ideal method would assemble small amounts of two samples into heterobivalent dimers on mixing, without recourse to protecting groups or reagents that give contaminating byproducts. Practically, efficient coupling of molecules with reactive, unprotected side chains is nontrivial. There are no known methodologies that allow selective formation of heterobivalent compounds from starting materials with unmasked reactive functionalities (e.g., typical pharmacophores such as amines and carboxylic acids). This communication describes methodology that, unlike previous routes to bivalent compounds,[8−15] facilitates this and simultaneously labels every single dimer with a fluorescent label.

Our method arose out of a subtle feature of chemoselective reactions of amines with triazines that is illustrated in the trial reactions shown in Scheme 1. A supported aminofluorescein−chlorotriazine adduct was exposed to three different unprotected peptidomimetics in solution for extended reaction times. The solution-phase peptidomimetics all had primary amine functionality (from Lys) and a secondary amine group from

Scheme 1[a]



[a] Only the piperidine-based nucleophile adds to the chlorotriazine; sarcosine, proline, and the Lys side chain are unreactive.

sarcosine, proline, or 4-pipicolic acid. Only the latter combined with the supported triazine to any appreciable extent. This series of experiments indicated that piperidine-based amines react with 2,4-diamino-6-chlorotriazines at ambient temperature whereas other secondary amines and the Lys side chain do not. We inferred that it might be possible to tag monovalent peptidomimetics with a piperidine functionality that would later serve as a handle for selective dimerization reactions.

Solid-phase approaches tend to require large excesses of one reactant, and this may not be practical if that is a valuable peptidomimetic. Consequently, our studies focused on exploiting the implications of the experiments in Scheme 1 to develop a way of combining near-equimolar amounts of two components in solution. Scheme 2 shows the approach that worked.

A library of 12 peptidomimetics **1** was prepared using solid-phase syntheses developed in this group.[16−19] Each sample was divided into two portions; one was cleaved from the resin, and the other was treated with dichlorotriazinyl-5-aminofluorescein in each case. Compounds **2** and **3** were purified (RP-HPLC). Finally, aliquots of each unlabeled sample **2** were systematically combined in separate vessels with near-equimolar amounts of each labeled **3**, as indicated in Scheme 2 (with elaboration of structure information in Table 1).

Figure 1 shows the crude purities of the bivalent molecules. We used 85% (UV) purity as a threshold for testing. Compounds **4** that formed with inferior purities were purified (RP-HPLC), but most met our purity threshold and were tested "as is". The bivalent molecules that were formed in less than 85% purity were clustered around monovalent constituents **i** and **j**; we believe that this may be attributed to slight decomposi-

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Scheme 2. General Approach to Bivalent Compounds

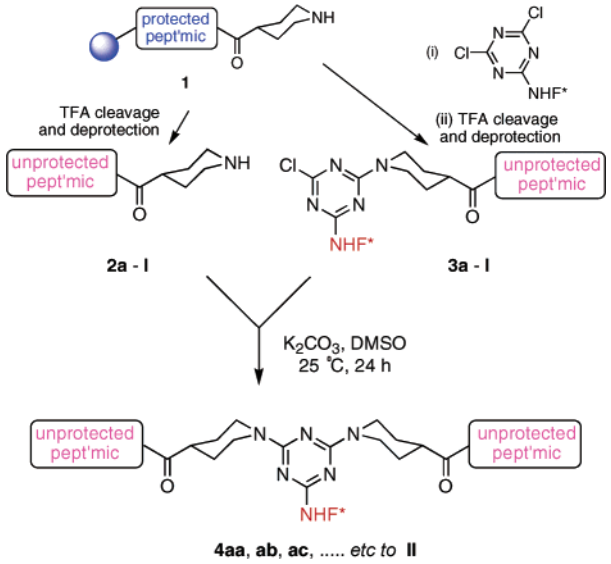
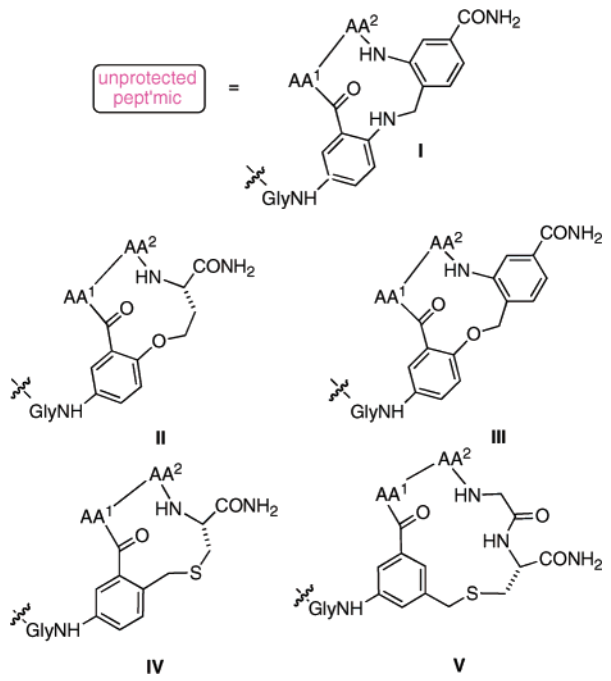


Table 1.



dimerizing partners	macrocyclic structure	AA^1	AA^2
a	**I**	Glu	Lys
b	**I**	Lys	Ser
c	**I**	Ile	Lys
d	**I**	Ile	Arg
e	**II**	Glu	Lys
f	**I**	Ser	Lys
g	**III**	Gly	Lys
h	**III**	Ile	Lys
i	**III**	Ile	Arg
j	**IV**	Lys	Tyr
k	**V**	Ile	Arg
l	**V**	Ile	Lys

tion of these starting materials before the dimerization reaction rather than to failure of the key transformation.

The fluorescein label in **4** serves dual roles. First, it facilitates *direct* binding assays of the bivalent molecules. This is of vital importance. If every compound in a library is labeled, it becomes possible to detect even

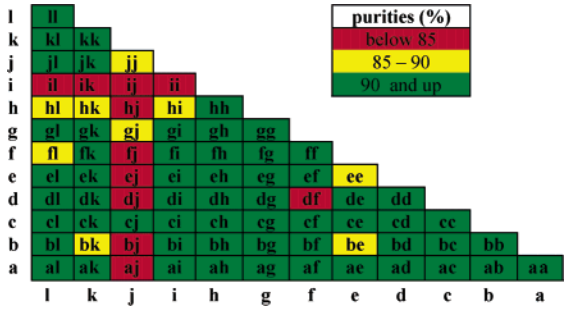


Figure 1. HPLC purities of the bivalent turn mimics **4** as assessed by UV detection.

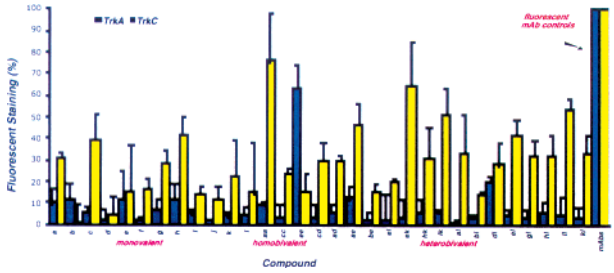


Figure 2. FACS data for **3** and the most strongly bound bivalent ones **4**.

weak binding. Conversely, *competitive* binding assays using labeled, natural ligands and unlabeled libraries would not reveal weak binders if the affinity of the natural ligand is high, and it usually is. We believe that the importance of "universally labeled" combinatorial libraries in binding assays to detect small molecules that mimic or disrupt protein−protein interactions is underappreciated in the field. However, such libraries are easily prepared by the methodology shown in Scheme 2. The second advantage of including a fluorescein label is that it can help to improve the water solubilities of some peptidomimetics.

Most of the compounds **4** were designed as mimics of two neurotrophins: nerve growth factor (NGF) and neurotrophin-3 (NT-3). We have previously reported that some monovalent compounds in the series **I**−**V** can bind to their high-affinity receptors, TrkA and TrkC, respectively, and initiate functional responses.[19,20] Normally, our "first-pass" assay to detect small molecules that bind the Trk receptors is a functional one based on rescuing cells from apoptotic cell death that otherwise ensues if the cells are placed in a medium without growth factors. However, in this work, **3** and **4** were screened in a fluorescent activated cell sorting (FACS) assay using transfected cells that overexpress TrkA receptors. The parent nontransfected cell lines were used as negative controls for nonspecific binding to the cells, and monoclonal antibodies (mAb's) specific to TrkA and TrkC (K_d = 2−8 nM)[19,21] were used as positive controls. Figure 2 shows the data obtained for all the monomers **3** and the most active dimers **4**.[22]

Strong staining for homodimer **ee** was particularly interesting because the monovalent unit **e** closely resembles a NGF mimic we reported previously.[20] However, most of the actives were selective to TrkC binding; this was expected because most of the monomeric units used were NT-3 mimics.[23] The screen clearly shows that some of the bivalent molecules, compound **aa** for instance, give levels of fluorescence staining that

approach those obtained from the monoclonal antibodies that bind the Trk receptors strongly. The next step in this study will be to prepare analogues of compounds such as **aa** without the fluorescent label for testing in competitive binding assays with radiolabeled NT-3 (a reagent that most laboratories would consider too expensive to use in high-throughput assays of compound libraries) and for functional assays. In that case, the group that is substituted for fluorescein could be used to adjust the physical properties of the bivalent molecule in some favorable way (e.g., to improve the water solubility). Some other data shown in Figure 2 merit further investigation. For instance, dimer **ee** strongly stains the TrkA receptor, but the heterodimer **ek** unexpectedly stained TrkC with high selectivity. This is a very surprising result. Conversely, some cases where strong staining might have been expected but was not observed can be explained in terms of inappropriate linker geometries and/or steric effects caused by the linker and fluorescein label.

In summary, we have developed a method for selectively forming fluorescently labeled homo- and heterobivalent molecules. It enables small quantities (e.g., 1 mg amounts) of unprotected peptidomimetics with reactive side chains to be coupled with high efficiencies. No protecting groups or coupling agents are required, so the products are isolated in high states of purity, often enough to be used in a preliminary screen. All the bivalent molecules prepared were fluorescently labeled so that they could be tested in direct binding assays via FACS. Such direct assays facilitate detection of weak binders that might be missed in competitive assays using labeled natural ligands that bind with very high affinities. FACS assays are convenient and allow a reasonably high throughput of compounds to be tested,. They also tolerate higher DMSO levels than a standard cell survival assay for screening growth factor mimics; hence, more poorly water-soluble compounds can be tested. In the case of neurotrophin peptidomimetics binding the Trk receptors, this work has exposed some interesting homo- and heterobivalent molecules for further investigation.

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Supporting Information Available: Details of the compound purities, procedure for syntheses of compounds **2**−**4**, and characterization of representative dimers. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) Both the linker region and the fluorescent label of the bivalent molecules could conceivably bind to cells via nonspecific interactions. However, the FACS scan assay described has an in-built control to test for this possibility. All the compounds have the same linker and label; yet many did not bind the cells. Of the compounds that do bind the cells, most showed selectivity for either the cells that overexpress TrkA or TrkC even though both types of transfectants were formed using the same types of cells. These observations indicate that the linker and label are not playing a significant role in the binding event.
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