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# A Combinatorial Method for Solution-Phase Synthesis of Labeled Bivalent $\beta$-Turn Mimics

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***Abstract:*** Piperidine-functionalized, 1,4-disubstituted-1,2,3-triazoles of generic structure **1** were conceived as "minimalist" mimics of peptidic $\beta$-turn structures. Key features of these molecules include (i) the possibility of incorporating amino acid side chains corresponding to many of the protein amino acids; (ii) a close correspondence of separations of these side chains to $i+1$ to $i+2$ residues in turns; (iii) facile adjustment of the side-chain vectors on docking while only influencing two critical degrees of freedom; and (iv) some electrostatic polarity. Fifteen monomers of this type were made via copper-mediated cycloaddition reactions. Solution-phase methodologies were devised to assemble these monomers into bivalent compounds in high purity states (typically >85%) so that they could be used in first-pass biological assays without further purification. The skeleton for forming these bivalent compounds is triazine-based. There is a third site which allowed for introduction of a fluorescent label (library of compounds **2**) or an alkyne-functionalized triethylene glycol chain (library of compounds **3**) included to promote water-solubility and to allow incorporation of probes via copper-mediated cycloaddition reactions. In the event, two 135-membered libraries were prepared, one consisting of compounds **2** and the other of **3**. No protecting groups or coupling agents were required; these attributes of the method were important to allow most of the products to be obtained in over 85% purities. The fluorescein-tagged library of compounds **2** was screened in a fluorescence-activated cell sorting (FACS) assay using cells transfected to overexpress one of the following neurotrophin receptors: TrkA, TrkC, and p75. Preliminary findings indicate four compounds **2gm**, **2gn**, **2gi**, and **2gj** bound the TrkA receptor selectively; all of these contain a threonine−lysine turn mimic. Thus, a pharmacological probe for the TrkA receptor has been developed.

## Introduction

Many proteins interact via two or more contacts that account for most of the binding energy, that is, hot-spots.[1] Molecules that bring together two pharmacophores to mimic hot spots of one protein component[2] may therefore be referred to as "bivalent".[3−6] The prevalence of such bivalent molecules in the literature underscores their biological importance,[7−12] especially in the field of protein−protein interactions.[13−15]

Combinatorial methods are ideally suited for the syntheses of bivalent products because of the numerical advantages of combining libraries with themselves.[16] If a library of $n$ monovalent compounds were assembled into every different possible bivalent compound, then $n(n+1)/2$ products would result. Thus, there is a "combinatorial advantage"[17] to this type of strategy, that may be defined as

$$\textit{combinatorial advantage} = \frac{\text{time taken to make bivalent compounds } \textit{de novo}}{\text{time taken to make bivalent compounds via selective reactions}}$$

The combinatorial advantage is small for small values of $n$ but increases rapidly as this number of monovalent compounds increases (Figure 1a).

Three main obstacles must be overcome to prepare libraries of discrete bivalent molecules from the corresponding monova-

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(1) Fuh, G.; Li, B.; Crowley, C.; Cunningham, B.; Wells, J. A. *J. Biol. Chem.* **1998**, *273*, 11197−11204.
(2) Boger, D. L.; Desharnais, J.; Capps, K. *Angew. Chem., Int. Ed.* **2003**, *42*, 4138−4176.
(3) Zhang, A.; Liu, Z.; Kan, Y. *Curr. Top. Med. Chem.* **2007**, *7*, 343−345.
(4) Decker, M.; Lehmann, J. *Curr. Top. Med. Chem.* **2007**, *7*, 347−353.
(5) Peng, X.; Neumeyer, J. L. *Curr. Top. Med. Chem.* **2007**, *7*, 363−373.
(6) Haviv, H.; Wong, D. M.; Silman, I.; Sussman, J. L. *Curr. Top. Med. Chem.* **2007**, *7*, 375−387.
(7) Wong, D. M.; Greenblatt, H. M.; Dvir, H.; Carlier, P. R.; Han, Y.-F.; Pang, Y.-P.; Silman, I.; Sussman, J. L. *J. Am. Chem. Soc.* **2003**, *125*, 363−373.
(8) Wittmann, V.; Datta, A. K.; Koeller, K. M.; Wong, C.-H. *Chem.—Eur. J.* **2000**, *6*, 162−171.
(9) Tamiz, A. P.; Zhang, J.; Zhang, M.; Wang, C. Z.; Johnson, K. M.; Kozikowski, A. P. *J. Am. Chem. Soc.* **2000**, *122*, 5393−5394.
(10) Profit, A. A.; Lee, T. R.; Lawrence, D. S. *J. Am. Chem. Soc.* **1999**, *121*, 280−283.
(11) Rao, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1997**, *119*, 10286−10290.
(12) Halazy, S.; Perez, M.; Fourrier, C.; Pallard, I.; Pauwels, P. J.; Palmier, C.; John, G. W.; Valentin, J.-P.; Bonnafous, R.; Martinez, J. *J. Med. Chem.* **1996**, *39*, 4920−4927.
(13) Klemm, J. D.; Schreiber, S. L.; Crabtree, G. R. *Annu. Rev. Immunol.* **1998**, *16*, 569−592.
(14) Ho, S. N.; Biggar, S. R.; Spencer, D. M.; Schreiber, S. L.; Crabtree, G. R. *Nature* **1996**, *382*, 822−826.
(15) Belshaw, P. J.; Ho, S. N.; Crabtree, G. R.; Schreiber, S. L. *Proc. Natl. Acad. Sci.* **1996**, *93*, 4604−4607.
(16) Boger, D. L.; Goldberg, J.; Jiang, W.; Chai, W.; Ducray, P.; Lee, J. K.; Ozer, R. S.; Andersson, C.-M. *Bioorg. Med. Chem.* **1998**, *6*, 1347−1378.
(17) Reyes, S. J.; Burgess, K. *Chem. Soc. Rev.* **2006**, *35*, 416−423.


10.1021/ja074717z CCC: $40.75 

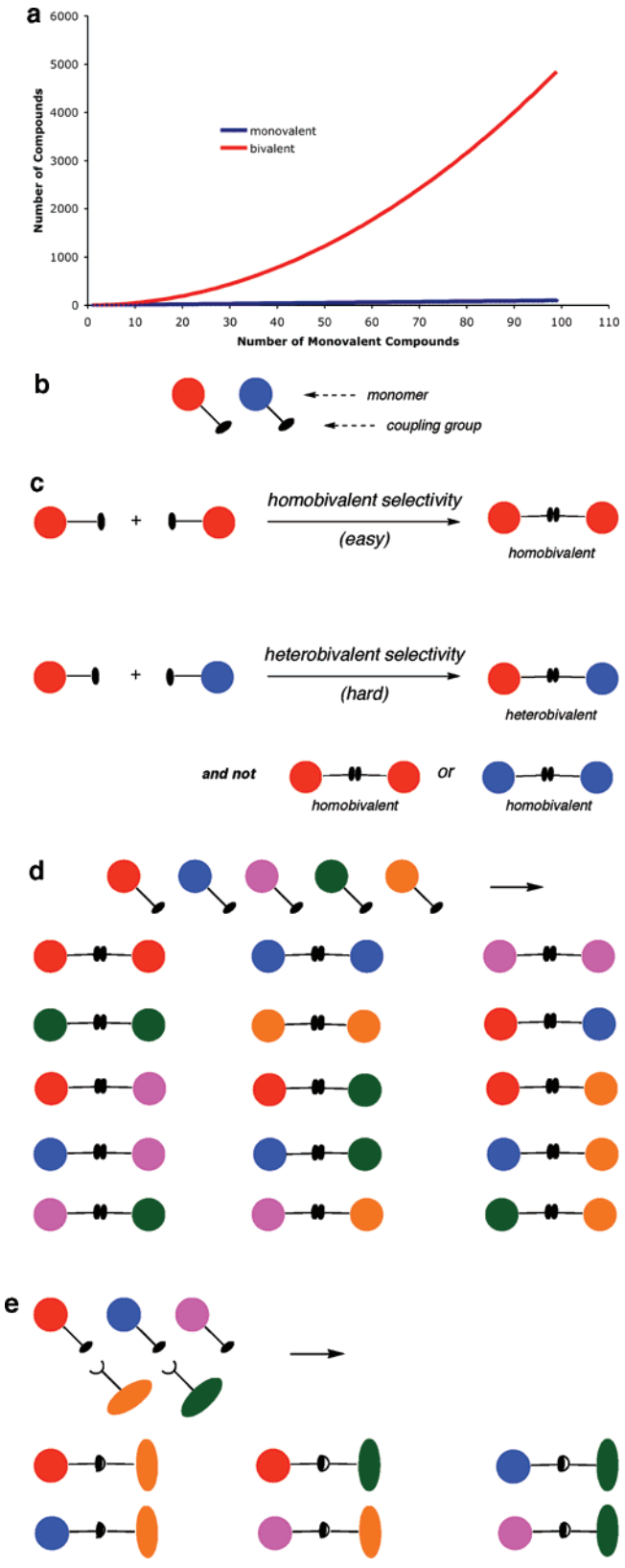



***Figure 1.*** (a) The number of isolated bivalent molecules rises rapidly with the number of monovalent starting materials; (b) monovalent starting materials each having identical coupling groups; (c) formation of homobivalent compounds is easy, but selectivity for heterobivalent ones is hard; (d) perfect heterobivalent selectivity allows assembly of all permutations of the monomers; and (e) given the same number of monomers, fewer compounds can be made by combining two libraries with different coupling groups.

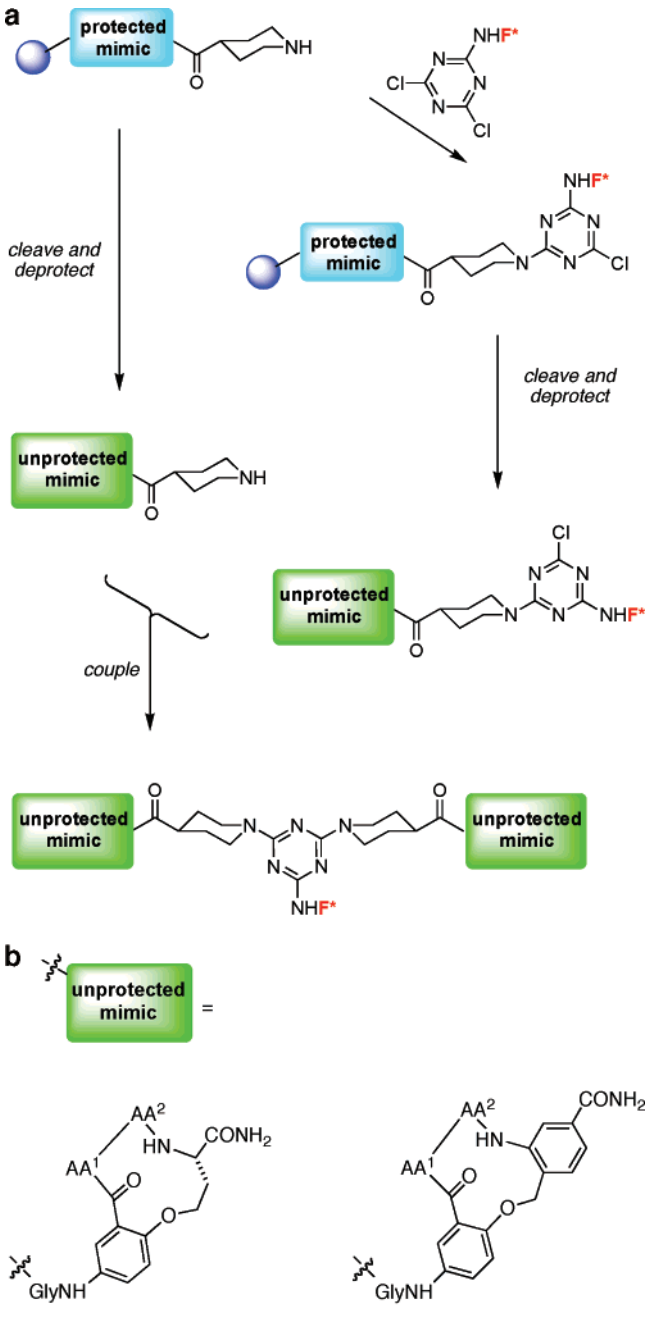



***Figure 2.*** (a) The divergent strategy featuring solid-supported peptidomimetics used in preliminary studies, and (b) the types of monovalent starting materials used.

lent ones. First, a method must be available to combine two different building blocks to form "heterobivalent" products (Figure 1 parts b and c); this is hard to achieve selectively if both building blocks are similar, especially if they also have reactive, unprotected, side chains. Second, it is desirable to form the bivalent products in high states of purity, preferably so that they do not need to be chromatographed before a first-pass biological assay. This means that any coupling agents that are used should give only residues that are readily removed, and that removal of protecting groups after the dimerization step is unlikely to be satisfactory. Ideally, the library would be formed by pipetting in aliquots of each monovalent component into each well, without coupling agents and protecting group chemistry,

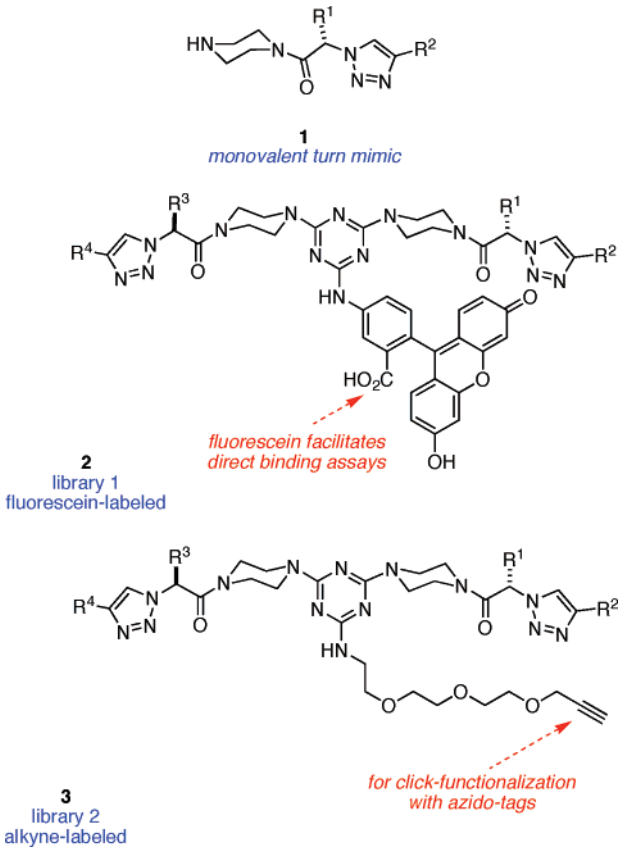
and have them covalently assemble to give highly pure products. Third, and particularly important for medicinal chemistry applications, the coupling chemistry should not interfere with pharmacophore groups on the monovalent compounds. If these criteria can be satisfied then construction of a library according to Figure 1d is extremely efficient.

It is relatively easy to make heterobivalent products from two libraries wherein each compound contains a functional group that reacts exclusively with the other (Figure 1e).[18,19] However, logistically this is a less effective strategy, and it tends to be more work in practice. The strategy is logistically less effective because members of one library cannot be reacted with each other, so less bivalent molecules can be made from the same number of monomers. Mathematically speaking, all combinations are allowed but not all permutations. It is less practical because two different libraries must be made (Figure 1e). It tends to be more work than combining two similar libraries because two different ones must be prepared.

One approach toward achieving selectivity for heterobivalent products is to generate a library of a set of monovalent molecules on a solid phase, divide each sample into two, then treat each portion differently. One part might be cleaved into solution, while the other would be chemically transformed into entities that would react with the samples liberated into solution (Figure 2a). In preliminary work, this strategy was followed in our group using peptidomimetics like the two shown in Figure 2b.[20] Those compounds were designed to mimic $\beta$-turns in proteins, both conformationally, and because relevant amino acid side chains (e.g., Arg, Lys, Glu) could be employed, not just the unreactive ones (e.g., Glu, Ala, Val, Ile).[20]

A total of 78 bivalent compounds prepared via the method shown in Figure 2a were obtained in less than 3 mg amounts.[20] This library was restricted to 78 compounds because it was difficult to obtain the monovalent compounds in sufficient quantities to make more bivalent products. Further, resynthesis of any bivalent molecule showing activity was time-consuming and scale-up was difficult. Both of these problems can be attributed to solid-phase syntheses; such approaches are expensive and inconvenient for preparation of more than 10 mg of material, especially if HPLC purification is required as the final purification. Thus next steps in the project were clear. To obtain larger libraries and to facilitate resynthesis it was necessary to use (i) scalable, solution-phase syntheses of monomers and (ii) solution-phase assembly into bivalent compounds.

This paper describes the design and syntheses of a series of $\beta$-turn mimics **1**. These were made via scalable, solution-phase syntheses, typically in several gram amounts. The solid-phase approach outlined in Figure 2a was adapted so that libraries of bivalent molecules **2** and **3** could be assembled from these turn mimics in solution. In one library, featuring the bivalent compounds **2**, all the constituent members are fluorescein labeled to facilitate direct binding studies. In the other, each compound **3** was nonfluorescent so that the bivalent compounds could be screened in assays designed for such substrates; however, each constituent of that library contained a terminal alkyne so that labels could be added to derivatives for binding studies performed after hits were identified.




## Results and Discussion

**Design of the Monovalent-Turn Mimics.** Our design of the $\beta$-turn mimics **1** was based on two considerations regarding the relative importance of main-chain amides and side-chain functionalities. First, studies of protein complexes crystallographically have shown that main-chain carbonyl groups are involved in only about 11% of protein−protein interface regions, whereas side chains contribute about 80%.[21] Thus we concluded it is far more important to have amino acid side chains represented in the mimics, than main-chain amides. This is consistent with designs by, for instance, Hirschmann and Smith.[22−24] Second, syntheses of the mimics must allow for incorporation of the side chains that occur most frequently at hot-spots, that is, those of Trp, Arg, Tyr, Lys, Glu, Ser, Asn, Leu;[21] syntheses of $\beta$-turn mimics that do not allow this are completely unsatisfactory for medicinal chemistry. Unfortunately, it is actually quite difficult to devise syntheses of mimics that do satisfy this criterion because of the diversity of amino acid side-chain functionalities involved.

Conformational and structural issues also factored into our design considerations for turn mimics **1**. Using the arbitrary

(18) Maly, D. J.; Choong, I. C.; Ellman, J. A. *Proc. Natl. Acad. Sci.* **2000**, *97*, 2419−2424.
(19) Su, S.; Acquilano, D. E.; Arumugasamy, J.; Beeler, A. B.; Eastwood, E. L.; Giguere, J. R.; Lan, P.; Lei, X.; Min, G. K.; Schaus, S. E.; Porco, J. A. *Org. Lett.* **2005**, *7*, 2751−2754.
(20) Pattarawarapan, M.; Reyes, S.; Xia, Z.; Zaccaro, M. C.; Saragovi, H. U.; Burgess, K. *J. Med. Chem.* **2003**, *46*, 3565−3567.
(21) Conte, L. L.; Chothia, C.; Janin, J. *J. Mol. Biol.* **1999**, *285*, 2177−2198.
(22) Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Salvino, J.; Leahy, E. M.; Sprengeler, P. A.; Furst, G.; Smith, A. B., III. *J. Am. Chem. Soc.* **1992**, *114*, 9217−9218.
(23) Hirschmann, R.; Sprengeler, P. A.; Kawasaki, T.; Leahy, J. W.; Shakespeare, W. C.; Amos, B.; Smith, I. *J. Am. Chem. Soc.* **1992**, *114*, 9699−9701.
(24) Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Leahy, E. M.; Salvino, J.; Arison, B.; Cichy, M. A.; Spoors, P. G.; Shakespeare, W. C.; Sprengeler, P. A.; Hamley, P.; Smith, A. B., III; Reisine, T.; Raynor, K.; Maechler, L.; Donaldson, C.; Vale, W.; Freidinger, R. M.; Cascieri, M. R.; Strader, C. D. *J. Am. Chem. Soc.* **1993**, *115*, 12550−12568.

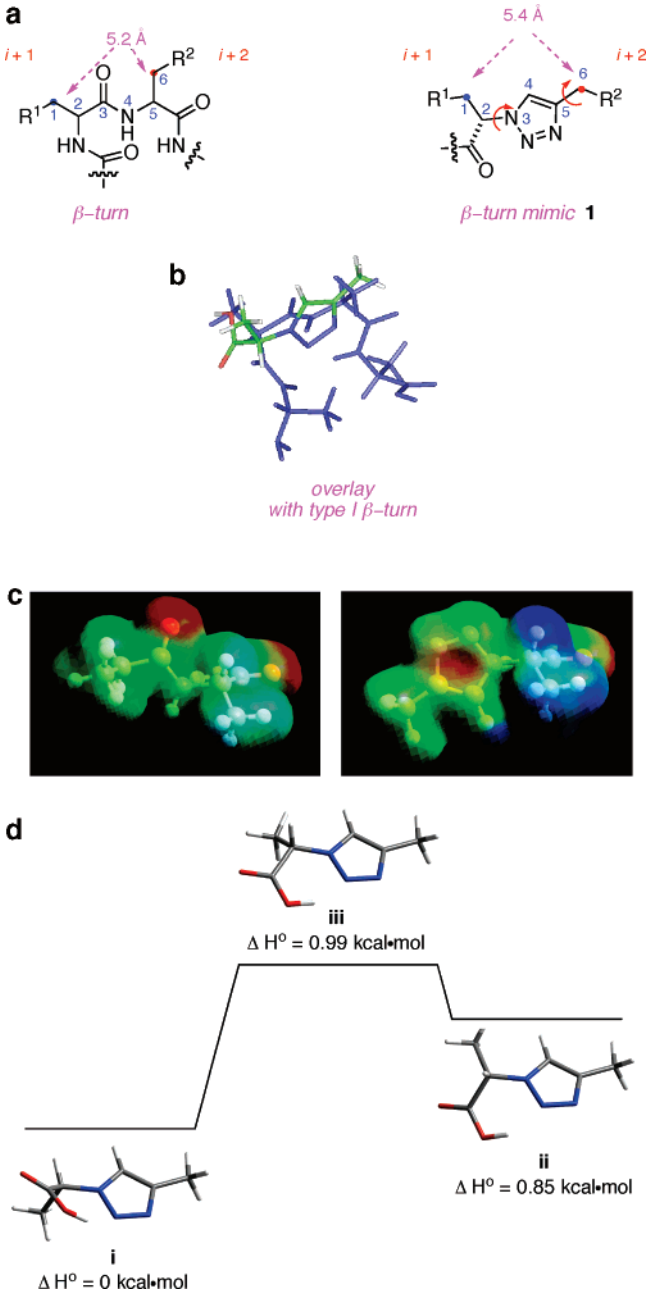
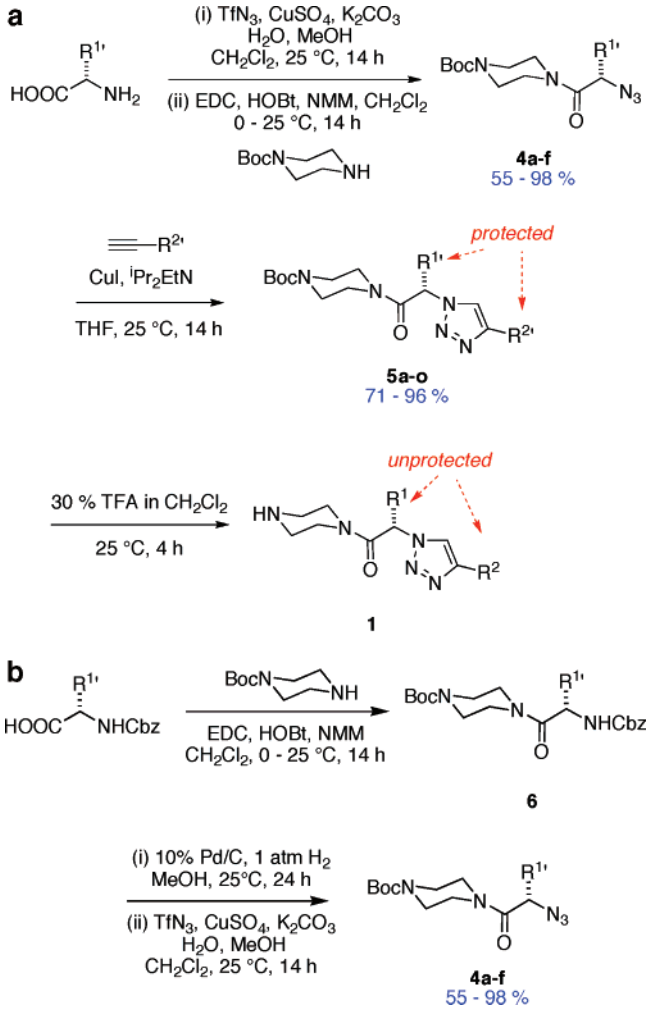



***Figure 3.*** (a) The key distance of $C^{\beta}$-separations of the $i + 1$ to $i + 2$ residues of a type 1 $\beta$-turn and of the monovalent turn mimics featured here; (b) an overlay of the mimics onto a type 1 $\beta$-turn; (c) a comparison of their electrostatic charge separations for these two entities; and (d) B3LYP calculated pathway for rotation around the $C^2$-$N^3$ dihedral for **1** showing the minimum energy conformation **i**, the next highest energy conformation **ii**, and the transition state **iii** that separates them.

***Scheme 1.*** Two Methods for Preparing Monovalent Mimics **1**[a]

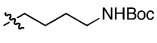
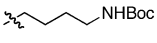
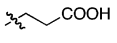
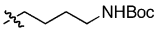
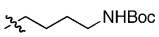
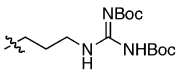
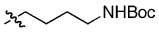
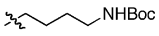
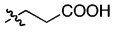
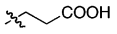
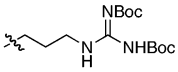
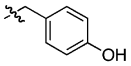
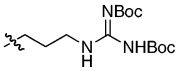



[a] Generally the least expensive and most direct route used is route a featuring unprotected amino acid starting materials (except for Lys which was side-chain protected). Route b uses protected amino acids.

numbering shown in Figure 3a, the bond vectors 1−2, 2−3, 3−4, 4−5, and 5−6 populate relatively well-defined regions of space, whereas rotation about the 1−2 and 5−6 bonds in $\beta$-turns are relatively free.[25,26] To draw an analogy, conformations of $\beta$-turns vaguely resemble "molecular aeroplanes" with the $R^1$ and $R^2$ substituents spinning like propellers on a reasonably rigid framework. Thus we reasoned that good mimics should be able to access conformations with $C^1$−$C^6$ separations that are close to those of $\beta$-turns. Of the several different types of $\beta$-turn conformations, type-1 is the most common (as in Figure 3a).[27] However, in solution, most $\beta$-turns probably are somewhat flexible; good mimics will reflect that characteristic without being so flexible that the entropic cost of binding at a protein interface is prohibitively high. Of course, the turn mimic **1** does not always reside in the conformation indicated in Figure 1b, and this is *not*, in fact, the low-energy conformation. However, the conformational state in Figure 1b is readily accessible at room temperature since the enthalpic barrier to rotation about $C^2$−$N^3$ dihedral is low as shown in Figure 3d. The transition state (Figure 3d, **iii**) and minima (Figure 3d, **i** and **ii**) were fully optimized at the B3LYP/6-311++G(d,p) level of theory, and each stationary point was verified with a frequency calculation (see Supporting Information). The lowest energy conformer that was identified, **i**, has a $C^2$−$N^3$ dihedral angle of 50° which is 140° away from the targeted turn conformation, but the second lowest energy conformer, **ii**, was only 0.85 kcal·mol$^{-1}$ higher in energy and had a $C^2$−$N^3$ dihedral angle of −57° which matched the target turn conformation much closer (deviation = 33°). The calculations showed that the

(25) Garland, S. L.; Dean, P. M. *J. Comput.-Aided Mol. Des.* **1999**, *13*, 469−483.
(26) Garland, S. L.; Dean, P. M. *J. Comput.-Aided Mol. Des.* **1999**, *13*, 485−498.
(27) Ball, J. B.; Hughes, R. A.; Alewood, P. F.; Andrews, P. R. *Tetrahedron* **1993**, *49*, 3467−3478.

***Table 1.*** Peptidomimetics **5** Prepared via Method A

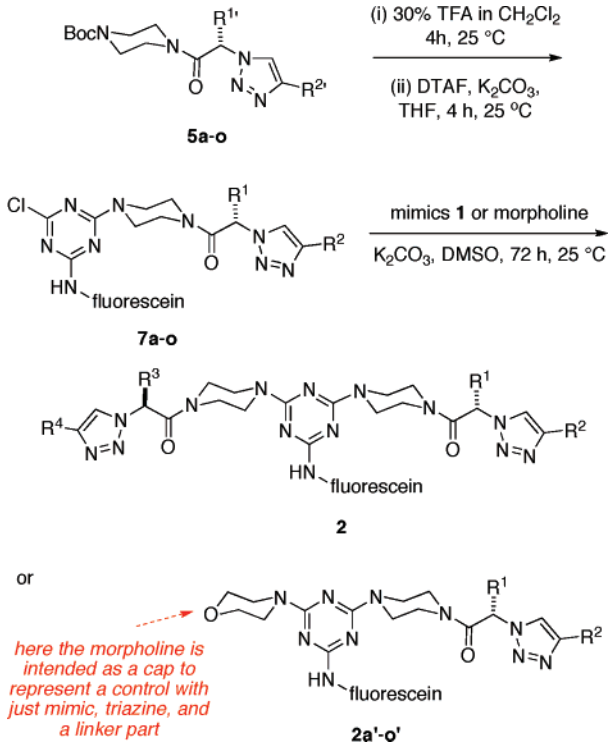
| compound **5** | $R^{1'}$ | $R^{2'}$ | yield (%)[a] |
|---|---|---|---|
| **a** |  |  | 78 |
| **b** | H |  | 79 |
| **c** |  |  | 80 |
| **d** | H |  | 94 |
| **e** | H |  | 96 |
| **f** |  |  | 80 |
| **g** |  |  | 71 |
| **h** |  |  | 92 |
| **i** | Me |  | 77 |
| **j** |  |  | 94 |
| **k** |  |  | 87 |
| **l** | Me |  | 87 |
| **m** | H |  | 86 |
| **n** |  |  | 89 |
| **o** |  |  | 96 |

[a] From intermediate **4** after flash chromatography.

molecule only needs to surmount an enthalpic barrier of 0.99 kcal·mol$^{-1}$ to reach the target turn conformation; such barriers are readily overcome at room temperature. In summary, the turn conformation of peptidomimetics **1** is energetically accessible, and it represents a low-energy conformation that is only slightly less stable than the minima. The energy necessary to reach this conformation is available at ambient temperatures, and the slight increase of energy over the lowest minimum is likely to be easily compensated for via induced fit binding to a protein surface.

All the considerations outlined above are satisfied for the monovalent mimics **1**. The separations between $C^{\beta}$ atoms that correspond to the $i + 1$ and $i + 2$ amino acid side chains (i.e., $C^1$ and $C^6$) in type-1 $\beta$-turns and in mimics **1** are close in an energetically accessible conformation of **1**. $\beta$-Turns have two bonds that are essentially freely rotating (1−2 and 5−6). The core of molecules **1** has three rotatable (1−2, 2−3, and 5−6), and only one of these affects the $C^1$-to-$C^6$ distance (the 2−3 rotation). Consequently, compounds **1** can access appropriate conformations to mimic the $C^1$-to-$C^6$ spacing in $\beta$-turns simply via rotation around the 2−3 bond (Figure 3 panels a and b). The polar triazole part of the mimics also introduces electrostatic polarity that can also resemble turn conformations. Thus, we were satisfied by the match of the mimics to the target secondary structure.

***Scheme 2.*** Solution-Phase Method for the Preparation of the Library of Compounds **2**




**Syntheses of the Monovalent-Turn Mimics.** Two methods were used to prepare the monovalent building blocks **1**. The most economical and direct route (Scheme 1a) begins with amino acids that were converted to the corresponding azides via known azo-transfer reactions,[28] coupled with Boc-piperazine, and combined with appropriately substituted alkynes via copper-mediated cycloadditon processes.[29−31] Only the final products **5** were purified via flash chromatography; the intermediates were isolated via aqueous workup (crude yields of **4** are shown) and used in the next step without further purification. This route was successful for all the amino acid/alkyne combinations tested, that is, those shown in Table 1. Scheme 1a uses $R^{1'}$ and $R^{2'}$ to denote protected side chains (and $R^1$ and $R^2$ to indicate deprotected ones). In fact, the only side chain of the amino acid starting materials ($R^1$ shown in Table 1) that had to be protected was the amino group of Lys (Boc-protected to mask it during the azo-transfer step).

Method B in Scheme 1 is a more conservative approach that begins with Cbz-protected amino acids, and requires an additional deprotection step. This was our original approach, but as the work evolved, it was found that this route had no advantages over method A for the amino acids shown in Table 1. However, method B is shown here just in case the work is later expanded to other amino acids for which it might have some merit.

(28) Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029−6032.
(29) Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057−3064.
(30) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596−2599.
(31) Bock, V. D.; Hiemstra, H.; van Maarseveen, J. H. *Eur. J. Org. Chem.* **2006**, 51−68.

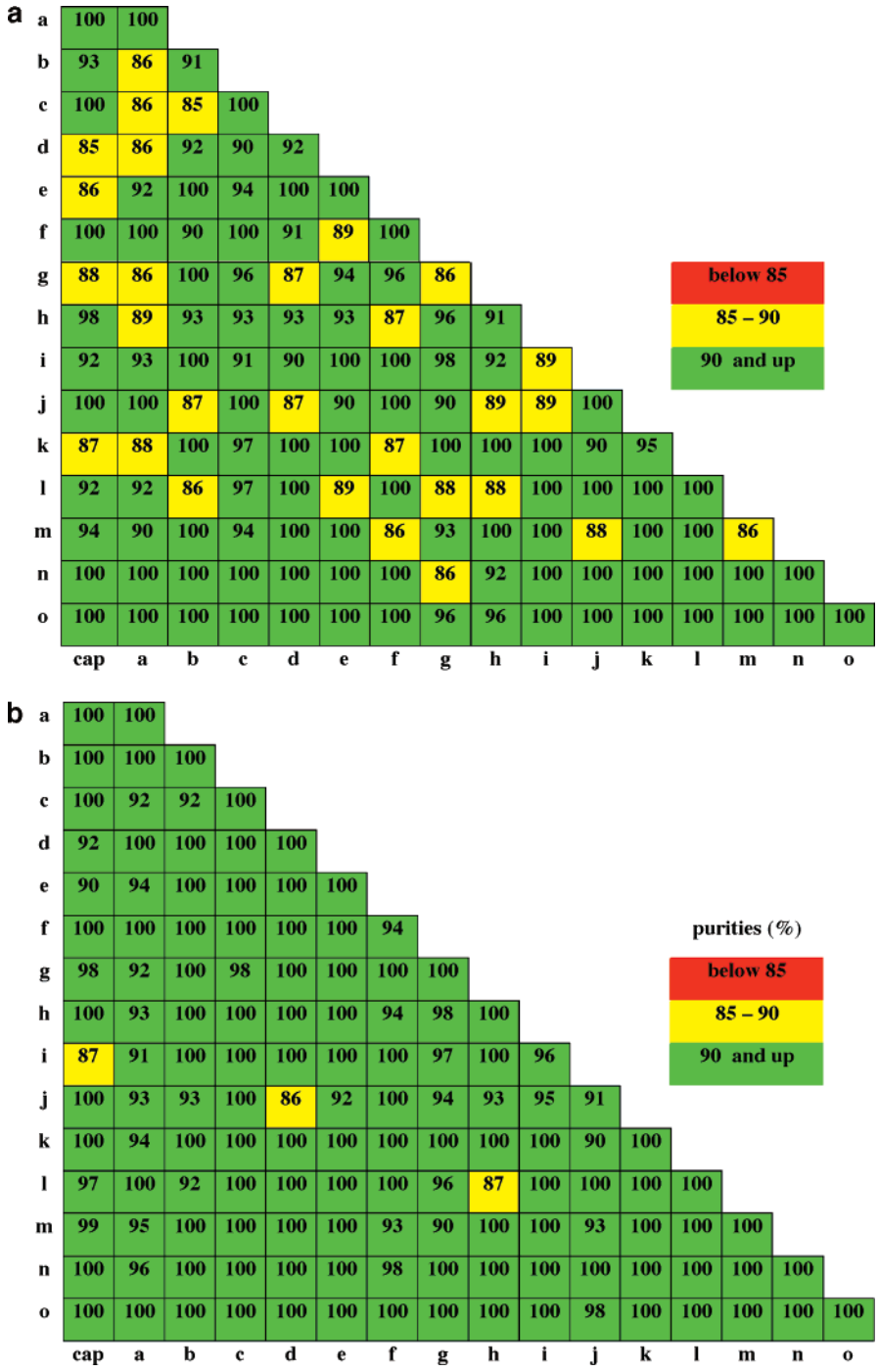
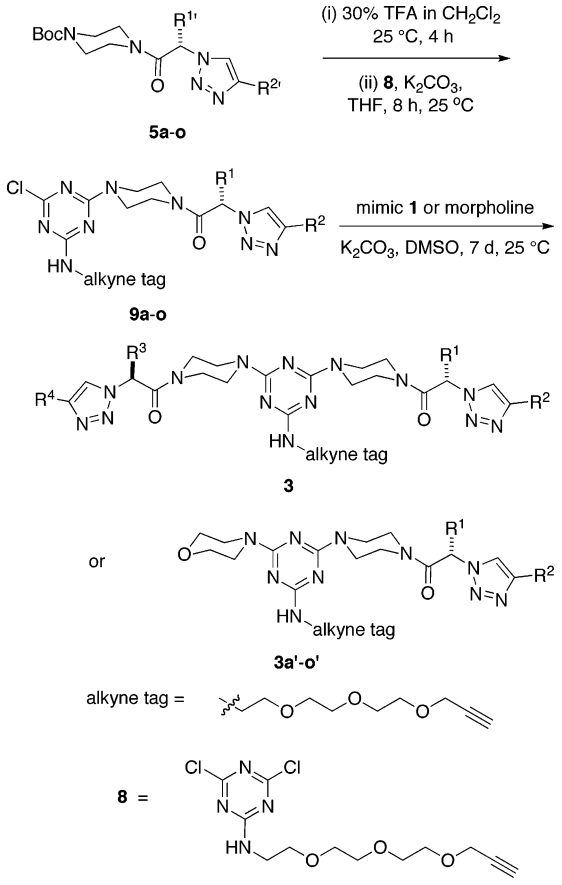



***Figure 4.*** Purities of the library of compounds **2** where the detection method was (a) UV set at 254 nm, and (b) SEDEX detection. The term "cap" is used for morpholine.

**Adaptation of the Dimerization Method to Solution Phase.** After considerable optimization studies, the solution-phase method for assembly of the bivalent compounds shown in Scheme 2 was developed. The protected compounds **5** were unmasked to give mimics **1** and then reacted with a dichlorotriazine derivative of aminofluorescein (i.e., DTAF) to give the electrophiles **7**. These were then reacted with second aliquots of the mimics **1**, in all permutations, to give the library of bivalent mimics **2**. Morpholine was also used in the second couplings. This resulted in syntheses of the specific mimics **2a**−**o** that represent controls for testing which contain only one mimic, triazine with the fluorescein label, and the morpholine part which represents the linker.

Some attributes of the method in Scheme 2 are as follows. Our goal was to prepare compounds **2** in over 85% purity, so the threshold of acceptable purity applied to the intermediates **7** had to be above this. The crude intermediates (chlorotriazines **7**) were assayed by reverse phase HPLC (UV and SEDEX detection) and found to be above 85% purity, so no chromatographic isolation was necessary. In the first coupling, THF facilitated the addition of just one monomer, giving monochloro-intermediates **7** in satisfactory purities for use in the next stage without further purification. Removal of THF after the synthesis of the intermediates **7** is convenient because it is volatile relative to some other solvents. It seems that DMSO facilitates faster coupling of nucleophiles to triazines than THF. Indeed, we found

***Scheme 3.*** Solution-Phase Method for the Preparation of the Library of Compounds **3**




that utilizing DMSO as solvent for coupling the second monovalent compounds was critical; other solvents tested gave unsatisfactory results. The bivalent molecules formed in good overall purities, even though the monovalent building blocks used to assemble them were completely unprotected. The reactions were typically performed on a small scale to form a few milligrams of product. However, no problems were encountered on scaling-up syntheses of some selected bivalent compounds to give 100 mg of product.

Briefly, the exploratory work that led to the method shown above illustrated several valuable conclusions. The solvents tested for the coupling reactions were THF, MeCN, MeOH, DMF, and DMSO; THF proved to be best for the first addition and DMSO for the second (see above). When DMF or DMSO was used for the first coupling reaction, the major products tended to be homobivalent molecules. Methanol was unsuitable for the first coupling because it tends to react with the DTAF. Acetonitrile gave similar results to THF in the first coupling, but it is more toxic and slightly harder to remove. For the second addition, the reaction went very slow in THF, MeCN, and MeOH at 25 °C and could not be completed in a reasonable time period. It was harder to remove DMF than DMSO, but it seems to be an equally good solvent for the second coupling in all other respects. Use of $(NH_4)_2CO_3$ as a base was investigated but it gave incomplete coupling reactions. This is particularly unfortunate because ammonium salt residues are volatile, whereas potassium chloride residues are not and have to be removed using differential solubilities.

Choice of the least polar solvent (THF) for the first coupling reaction, and the most polar one (DMSO) for the second coupling reaction is entirely logical.[32−35] Nucleophilic aromatic substitution reactions involve charged intermediates. It is desirable to destabilize these intermediates in the first coupling step to prevent two couplings occurring to the first peptidomimetic. However, in a polar solvent it is desirable to send the second coupling to completion.

**Application of the Solution-Phase Procedure To Make a Library of Fluorescently Labeled Bivalent Mimics.** The method outlined in Scheme 2 was applied to the synthesis of a library of 135 fluorescently labeled bivalent mimics. Thus, the monovalent compounds **5** were deprotected with TFA; no scavenger was required. The resulting unmasked intermediates **1** dissolved in THF were reacted with equimolar amounts of DTAF and excess $K_2CO_3$ for 4 h to give the intermediates **7**. The THF was removed, then these intermediates were dissolved in DMSO, split into small portions, then coupled with another equivalent of the deprotected monomers **1** to give the bivalent compounds **2**. Alternatively, to generate a set of control compounds, mophorline was used instead of the deprotected monomers as a capping group to give compounds **2a′**−**o′**.

The purities of intermediates **7** were monitored by analytical HPLC, and they were judged to be high enough to use in the next step without purification. After the second coupling, a major practical issue was the removal of inorganic salts; two procedures were developed for this. Most of the bivalent products were not very water soluble so they could be precipitated from the basic solutions by adding 5% HCl; presumably the acid protonates the fluorescein labels transforming them into their less soluble, ring-closed forms. However, some of the bivalent molecules have side chains that promote solubilities in slightly acidic aqueous media (e.g., those in the **g**, **h**, and **k** series). Bivalent molecules in this second category were separated from most of the inorganic salts by removing the solvent and extracting them into methanol. After these procedures, more than 80% of the bivalent compounds **2** were produced with a minimum of 85% purity. It appears that the other 20% were not as pure owing to pipetting errors because all gave purities above 85% when they were reprepared. HPLC and MALDI analyses were performed on all the compounds. Both UV and SEDEX detectors were used to determine the purities shown in Figure 4, which includes the ones that were reprepared. Purity data before and after the repreparation procedures are given in the Supporting Information. In summary, this new methodology allows the monovalent compounds to combine with each other cleanly in a one-compound-per-well format.

**Application of the Solution-Phase Procedure To Make a Library of Alkyne-Tagged Bivalent Mimics.** Libraries of labeled compounds are ideal for direct binding assays, but for others the fluorescent label may lead to unnecessary complications. For instance, it might be desirable to screen the compounds for their positive or negative influence on apoptosis in live cell lines; in this case a superfluous fluorescent label might alter the bioactivity or obscure readings of the outcome. However, fluorescent labeling might subsequently facilitate

(32) Miller, J.; Parker, A. J. *J. Am. Chem. Soc.* **1961**, *83*, 117−123.
(33) Isanbor, C.; Emokpae, T. A.; Crampton, M. R. *J. Chem. Soc., Perkin Trans. 2* **2002**, 2019−2024.
(34) Bartoli, G.; Todesco, P. E. *Tetrahedron Lett.* **1968**, *47*, 4867−4870.
(35) Emokpae, T. A.; Atasie, N. V. *Int. J. Chem. Kinet.* **2005**, *37*, 744−750.

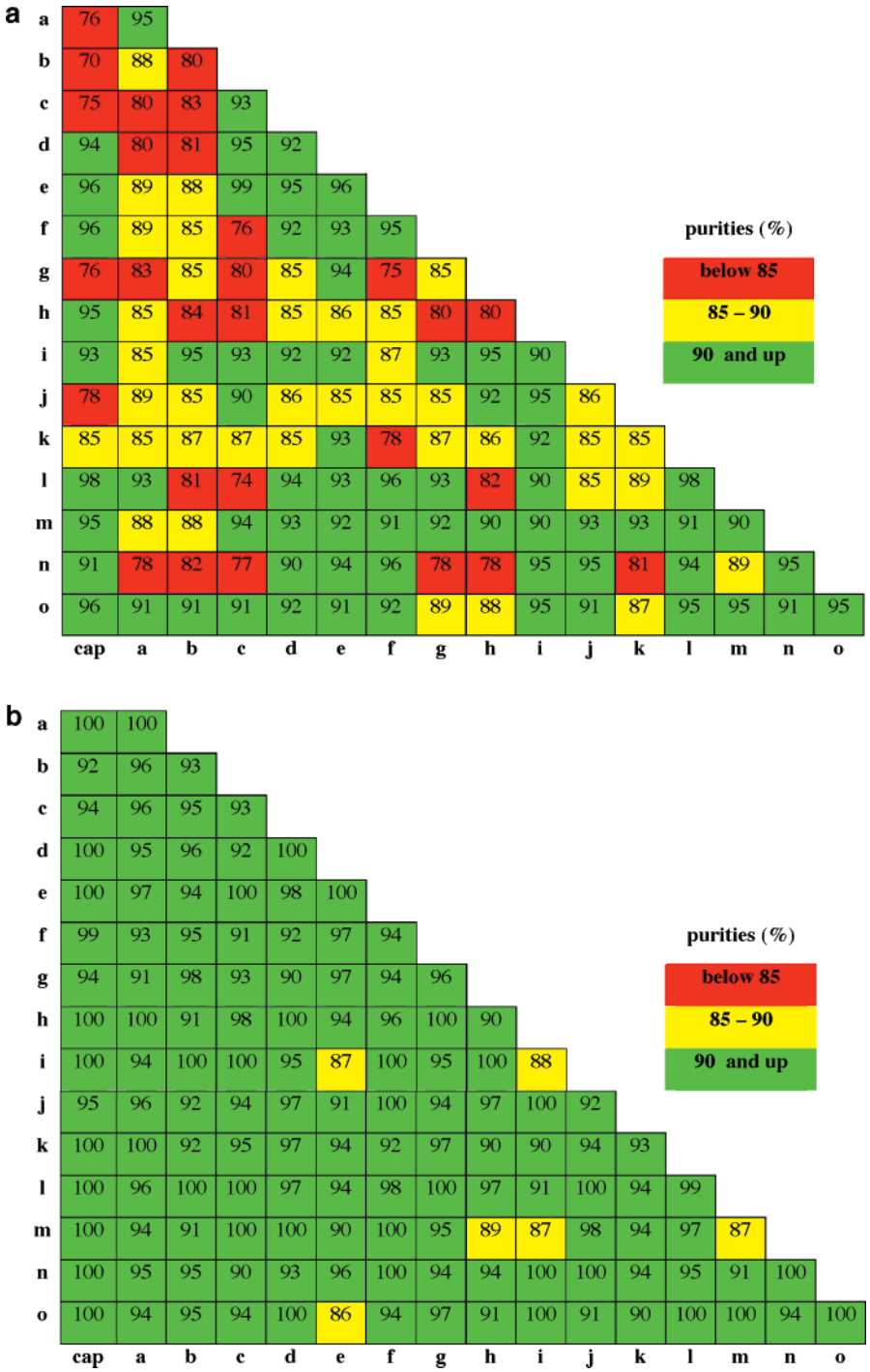



***Figure 5.*** Purities of the library of compounds **3** where the detection method was (a) UV set at 254 nm, and (b) SEDEX detection. The term "cap" is used for morpholine.

further exploration of hits identified in such assays. Consequently, we searched for a nonfluorescent tagging-group that would not make the compounds much more lipophilic, but which could be easily used to add a fluorescent label. An oligoethylene glycol-linked terminal alkyne was selected for this purpose. The oligoethylene glycol fragment was intended to separate the alkyne from the presumed pharmocophores in the peptidomimetics and to maintain some water solubility. Copper-mediated azide-alkyne cycloadditions[36,37] could then be used to add azide-functionalized labels[38] when the time was right.

The reactions used to prepare compounds **3**, Scheme 3, largely parallel those shown in Scheme 2 for the library of fluorescein-labeled molecules **2**. Substitution of a fluorescein label with the alkyne tag **8** is conceptually trivial but has some important practical consequences. First, both coupling reactions to the triazine were slower than in the DTAF-containing compounds so longer reaction time was used (8 rather than 4 h for the first coupling and 7 d instead of 3 d for the second one). The triethylene glycol-containing tag also changes the solubility characteristics of the products; in all cases the DMSO was

(36) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2001**, *40*, 2004−2021.
(37) Angell, Y.; Burgess, K. *Chem. Soc. Rev.* **2007**, *36*, 1674−1689.
(38) Seo, T. S.; Li, Z.; Ruparel, H.; Ju, J. *J. Org. Chem.* **2003**, *68*, 609−612.

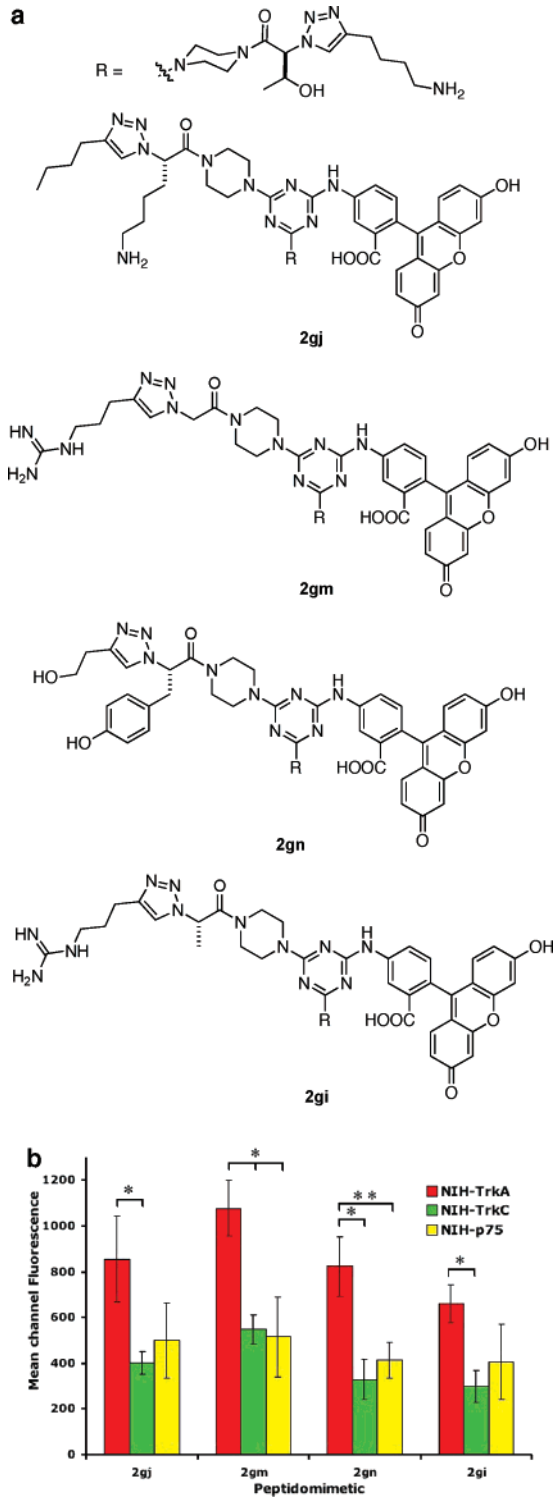



***Figure 6.*** Direct binding FACScan assays with FITC-peptidomimetics: (a) structures of compounds **2gm**, **2gn**, **2gi**, and **2gj**; (b) plot of mean channel fluorescence of direct-binding FACS assay (significant differences, (∗) $p < 0.005$ and (∗∗) $p < 0.05$). The background MCFs of NIH-IGF-1R were subtracted to analyze the specific MCF binding to test cells. Depending on the peptidomimetic, the MCFs subtracted ranged from 50 to 200 fluorescent units and was <30% of the calculated specific signal. As positive controls, antireceptor mAbs were used for each cell line: anti-TrkA mAb 5C3 for NIH-TrkA (raw MCF ≈ 550), anti-TrkC mAb 2B7 for NIH-TrkC (raw MCF ≈ 300), anti-p75 mAb MC192 for NIH-p75 (raw MCF ≈ 700), and aIR3 for NIH-IGF-1R (raw MCF ≈ 450) (data not shown).[44,45] These positive control mAbs did not bind to cells not expressing its cognate target, and the MCF of negative control mouse IgG-FITC ranged from 8 to 20 for all the cell lines.

removed, the residue was acidified with 5% HCl, and then the products were extracted into methanol with decantation away from the precipitated salts. The methanol was removed, and the product was extracted into methanol again, followed by another decantation. Analyses via HPLC with SEDEX detections indicated that more than 90% of the inorganic salts were removed via this procedure.

Figure 5 shows the purities of the crude products obtained after aqueous workup of the library of compounds **3**. This library was actually designed for the NIH repository for which the threshold was >90% purity via either UV or SEDEX detection. All the compounds in the library met this criterion.

**Evaluation of the Libraries in a Model Assay for Trk Selectivities.** Many of the dipeptide sequences chosen in the design of the monovalent molecules **5** designed for this library were based on the turn regions of nerve growth factor (NGF). We have a longstanding interest in evaluation of NGF mimics as artificial ligands for the TrkA receptor.[39,40] Fluorescently labeled compounds, as in the library of compounds **2**, are ideal for a first-pass fluorescence-activated cell sorting (FACS) assay that involves binding to transfectant cells expressing the TrkA receptor. The FACS output gives a measure of direct binding to the cells and provides an ideal way to access the selectivity of the binding to the Trk receptors. Competition with labeled forms of the natural ligand (NGF) is *not* required; this is highly advantageous because NGF has such a high affinity for its receptor ($K_d$ = ca. $10^{-11}$ M),[41] early stage small molecule leads are unlikely to able to compete with it, and because labeled NGF is prohibitively expensive.

Direct FACS assays were performed as previously described.[42] Briefly, the FITC-peptidomimetics **2** were tested via quantitative FACS for direct binding to NIH-3T3 cells transfected to express a targeted receptor or a control receptor: NIH-TrkA cells, NIH-TrkC cells, NIH-p75 cells. NIH-3T3 cells overexpressing the transfected insulin-like growth factor-1 receptor (NIH-IGF-1R cells) were used as negative control, because a neurotrophin mimetic should not bind to their surface. As positive binding controls mAbs 5C3 (anti-TrkA), 2B7 (anti-TrkC), MC192 (anti-p75), and αIR3 (anti-IGF-1R) were used, and nonbinding mouse IgG-FITC was used as negative control. All the FACS assays were repeated independently at least four times.

Many of the peptidomimetics bound strongly to the Trk-expressing cells; they gave even higher staining than monoclonal antibodies that bind the Trk receptors with affinities in the $10^{-9}$ M range (see Supporting Information). However, the goal of this study was to ascertain if *selectivity* was possible, rather than to determine *absolute affinity*. The reason for this is that it is relatively easy to find molecules that stick to proteins, especially if the molecules contain fluorescein that tends to be a promiscuous binder.[43] However, if a fluorescein-containing

(39) Pattawarawarapan, M.; Burgess, K. *J. Med. Chem.* **2003**, *46*, 5277−5291.
(40) Burgess, K. *Acc. Chem. Res.* **2001**, *34*, 826−835.
(41) Lee, F. S.; Kim, A. H.; Khursigara, G.; Chao, M. V. *Curr. Opin. Neurobiol.* **2001**, *11*, 281−286.
(42) Zaccaro, M. C.; Lee, H. B.; Pattarawarapan, M.; Xia, Z.; Caron, A.; Burgess, K.; Saragovi, H. U. *Chem. & Biol.* **2005**, *12*, 1015−1028.
(43) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. *J. Med. Chem.* **2002**, *45*, 1712−1722.
(44) Zaccaro, M. C.; Lee, B. H.; Pattarawarapan, M.; Xia, Z.; Caron, A.; L'Heureux, P.-J.; Bengio, Y.; Burgess, K.; Saragovi, H. U. *Chem. Biol.* **2005**, *12*, 1015−1028.
(45) Ivanisevic, L.; Zheng, W.; Woo, S. B.; Neet, K. E.; Saragovi, H. U. *J. Biol. Chem.* **2007**, *282*, 16754−16763.

molecule binds to a Trk receptor selectivity, whereas other fluorescein-containing molecules do not, then that constitutes a lead that can then be optimized without fluorescein labels. That process involves different assays, and extensive SAR that again is beyond the scope of this paper.

In preliminary assays, four peptidomimetics, **2gj**, **2gm**, **2gn**, and **2gi** exhibited preferential binding to TrkA-expressing cells when compared versus IGF-1R-expressing cells. These four compounds were resynthesized, purified (>95%) and retested for binding to a panel of NIH3T3 cells expressing either TrkA, TrkC, p75, or IGF-1R (Figure 6). Analyses on the data for each compound show that they all bind significantly better to TrkA than to TrkC. Both **2gm** and **2gn** bind significantly better to TrkA than to p75, with **2gm** being better than **2gn**. Compounds **2gm** (TK-GR) and **2gn** (TK-YS) are the better ligands for TrkA; **2gj** (TK-KI) and **2gi** (TK-AR) bind TrkA relatively poorly. In addition, the four peptidomimetics bind equally and relatively poorly to p75. The differences in binding between **2gm** and **2gi** suggest that the alanine side chain may cause steric hindrance. Functional group differences observed in these compounds may reflect the fact that NGF has *four* different turns (with different side chains) that have been implicated in binding the TrkA receptor.

## Conclusion

Monovalent molecules **1** were shown to be readily accessible on gram scales. This is important because the logistics of preparing large libraries of bivalent materials require that appreciable quantities of monovalent starting materials are available. The methodologies outlined here facilitated construction of bivalent libraries without use of protecting groups or coupling agents. A positive feature of the methodology is that probes, sites for further functionalization, or groups to modulate physiochemical properties can be added to the triazine backbone. Further studies are required to determine if the mimics **1** can be *functional* analogues of $\beta$-turns, but the FACS data described here indicate they can contribute to binding. This has revealed several possibilities for a selective TrkA probe.

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**Supporting Information Available:** Procedures and characterization data for preparation of the monovalent compounds and the two bivalent libraries, methods for the FACS assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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