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ARTICLE *in* ORGANIC LETTERS · APRIL 2005

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ORGANIC LETTERS

2005
Vol. 7, No. 7
1203−1206

Bridged Peptide Macrocycles as Ligands for PDZ Domain Proteins

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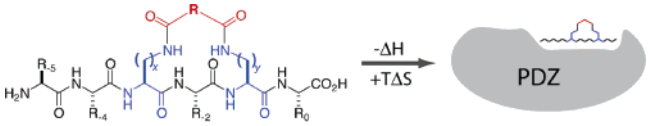
Received November 22, 2004 *(Revised Manuscript Received February 18, 2005)*

ABSTRACT



Conformationally constrained side chain-bridged cyclic peptides were prepared using bis-carboxylic acid ring spacers. These macrocyles were designed to inhibit protein–protein interactions mediated by the third PDZ domain (PDZ3) of a mammalian neuronal protein, PSD-95. Isothermal titration calorimetry (ITC) experiments measured dissociation constants in the low micromolar range. For each compound, the change in entropy (*TΔS*) of binding either is comparable in magnitude to the enthalpy change (*ΔH*) or is the predominant driving force for association.

Cyclic peptides are an enticing molecular class for the development of protein-binding ligands for a variety of reasons: the prospect of high affinity and selectivity; cellular stability from proteolytic resistance; and relative ease of assembly compared to nonpeptide organic macrocycles.[1] These were all motivating factors when we initiated a program to design cellular probes for the PDZ domain protein family, which led to the development of a nontraditional form of macrocyclic peptide for the third PDZ domain (PDZ3) of the neuronal postsynaptic density-95 protein (PSD-95).[2] Several compounds from this first generation exhibited good in vitro affinity, and a selected member demonstrated efficacy in vivo.[3] We now report further progress in this direction, presenting the design, synthesis, and thermodynamic binding analysis for a new series of PDZ domain-targeting macrocycles employing a modified ring assembly strategy.

As a large class of autonomously folding and binding substructure found within larger proteins, PDZ domains are active participants in mediating protein−protein interactions in numerous mammalian proteins,[4] most notably those found in neuronal cells.[5] The development of stable, selective, high-affinity chemical probes for PDZ domains will aid cellular studies investigating endogenous activity of proteins containing these binding modules.

The three PDZ domains of PSD-95 are of particular neurobiological interest, given the functional roles exhibited by the parent protein at the synaptic membrane. From a pharmacological perspective, targeting PSD-95 may prove to be useful for therapeutic intervention in conditions such as stroke-associated ischemic brain damage[6] and psychostimulant addiction.[7]

Coupled to our interest in developing tools for PDZ cell biology was the desire to address a fundamental biophysical question: what are the thermodynamic consequences of attempting to improve the binding affinity of a linear ligand though cyclization? With their structural stability and penchant for recognizing relatively small epitopes, many PDZ domains may serve as "nonartificial model systems" for investigating protein−protein thermodynamic binding properties and serve as useful platforms for the development and testing of new constrained ligand designs.

In this study, we expand upon a strategy developed for generating cyclic peptides in which α-, β-, and γ-amino acids

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10.1021/ol0475966 CCC: $30.25
Published on Web 03/08/2005

were used as *bridging elements* to tether amino- and carboxy-functionalized residue side chains (Figure 1, route A).[2] This

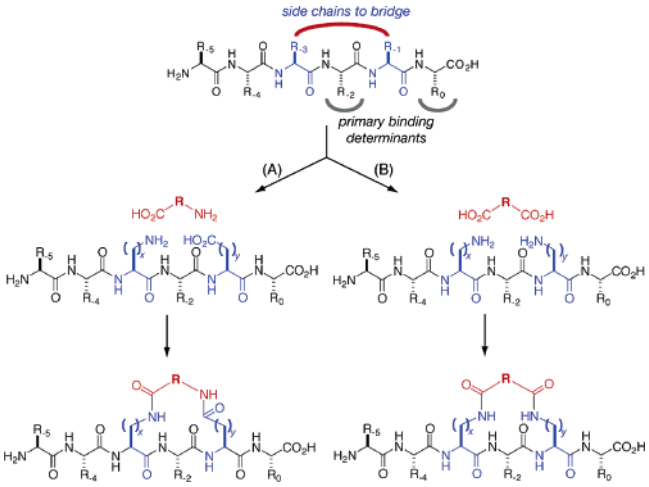


Figure 1. Design strategies for side chain-bridged ligands.

modular approach allows for the rapid and convenient manipulation of functionality as well as bridge length and ring size. The present design employs an organic *bis-carboxylic acid* as a bridging constraint, in which both carboxylates form amide bonds with the two amine-presenting side chains within the peptide backbone (Figure 1, route B). As in keeping with a design criterion from our prior generation of ligands, the critical residues at the "0" and "−2" positions remain exposed and available for binding to the PDZ domain.

Seven macrocyclic peptides (**1**−**7**) were prepared using bisacid bridges to connect the side chains of lysine residues occupying the "−1" and "−3" positions in the hexapeptide Tyr-Lys-*Lys*-Thr-*Lys*-Val (Figure 2). This peptide is based upon a known PDZ3-binding sequence, the C-terminus (Tyr-

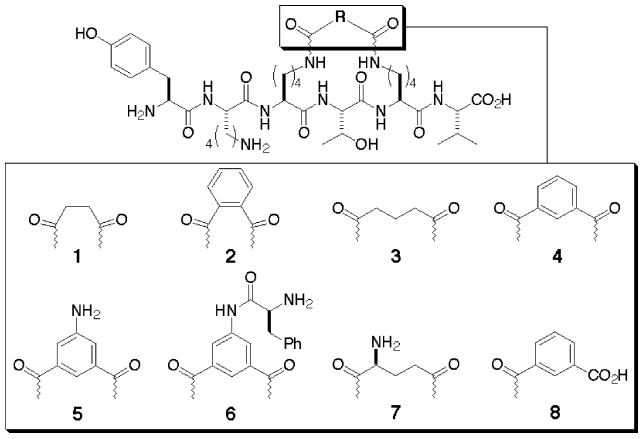
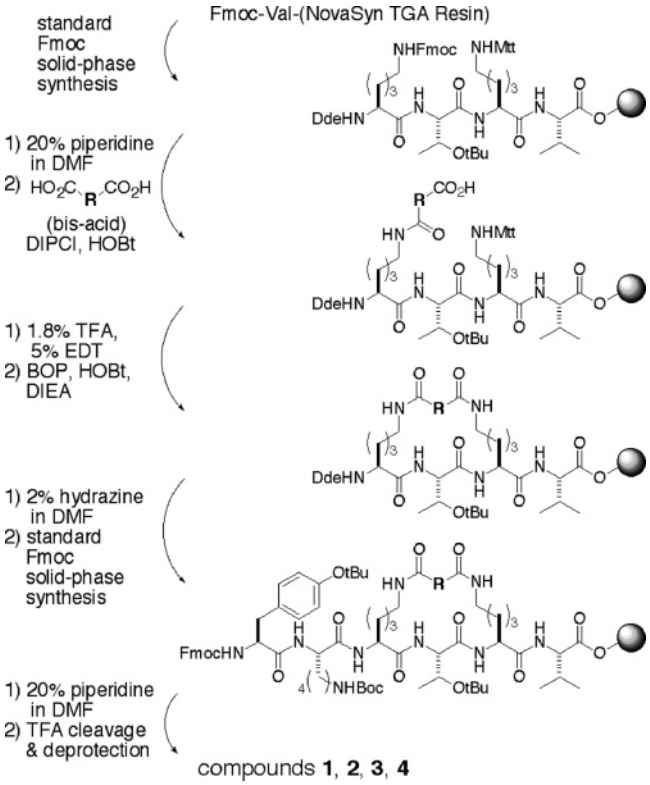


Figure 2. Peptide scaffold and bisacid bridging components for ligands **1**−**8**.

Lys-Gln-Thr-Ser-Val) of the CRIPT protein.[8] The bisacids were chosen on the basis of their propensities to impart varying degrees of length and rigidity to the resulting macrocyclic rings. Although there are nonproteinogenic amino acids that possess amine-bearing side chains of different lengths that can be used to further attenuate the ring size,[2] for this initial investigation these were kept fixed with lysine.

Two synthetic routes were devised to achieve the required protecting group orthogonality, depending on whether the bisacid tether was purely carbogenic (**1**−**4**, Scheme 1) or

Scheme 1. Synthetic Route for Ligands with Nonfunctionalized Bisacid Bridging Elements (**1**−**4**).



whether an amine functionality was present (**5**−**7**, Scheme 2). Bisacids **5** and **7** bore Fmoc amino protection prior to coupling to the Lys side chain; the former was prepared by adapting a known procedure for protecting a structurally related aniline.[9] A linear control, **8**, was prepared using Scheme 2, except that the second coupling step (amidation of the "−1" Lys) was not performed.

PDZ3−ligand interactions were analyzed using isothermal titration calorimetry (ITC), a solution technique that provides the full panel of thermodynamic binding parameters; these include the changes in Gibbs free energy (ΔG), enthalpy

(8) Niethammer, M.; Valtschanoff, J. G.; Kapoor, T. M.; Allison, D. W.; Weinberg, T. M.; Craig, A. M.; Sheng, M. *Neuron* **1998**, *20*, 693−707.

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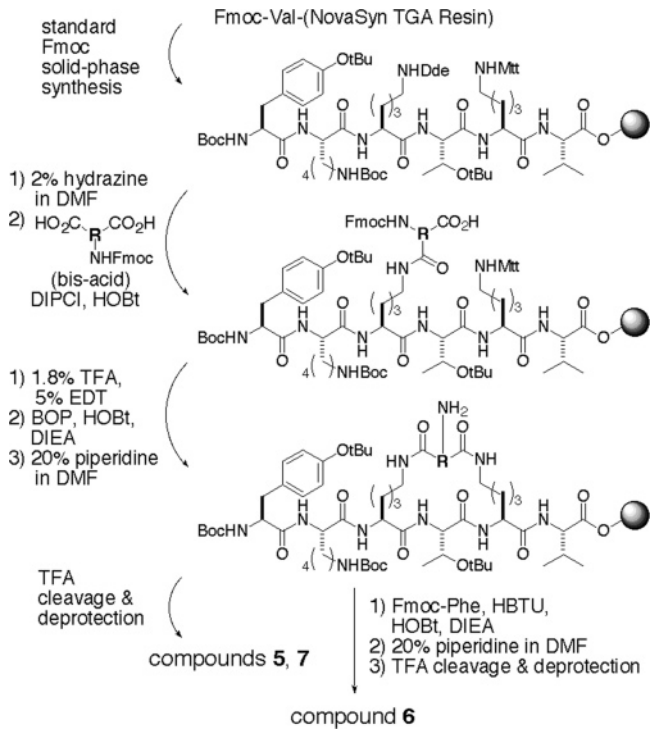
Scheme 2. Synthetic Route for Ligands with Amine-Functionalized Bisacid Bridging Elements (**5**−**7**)[a]



[a] For **7**, the bisacid bridge (Glu) is protected as a phenylisopropyl ester (Pip) at its side chain carboxylate.

(Δ*H*), and entropy (*T*Δ*S*), as well as the stoichiometry (*n*).[10] While the affinity (K_d or Δ*G*) is of primary interest when assessing and ranking the efficacy of ligands, changes in enthalpy and entropy, reflecting specific molecular interactions, ultimately determine the observed binding constants. Since thermodynamic values can often be rationalized with different molecular explanations, in the absence of explicit structural characterization the interpretations are tentative.

Data from the separate ITC experiments, in which the solubilized ligands were individually titrated into a solution of PDZ3, are shown in Table 1 and Figure 3. The structural modification of **1**→**2**, as well as that of **3**→**4**, is expected to increase both the rigidity and the hydrophobic surface area of the ligand. Either preorganization into the bound conformation or desolvation of nonpolar regions of a molecule would lead to a gain in the observed change in entropy, if not the actual affinity. But should *T*Δ*S* increase as desired, the confounding and often unpredictable effect of *entropy*−*enthalpy compensation* frequently dampens the anticipated improvement in Δ*G* by somehow arranging molecule interrelationships, including those with solvent, to reduce the net favorable contribution of enthalpy.[11] So while the slight 2-fold improvement in K_d of the 1,2-phenyl-linked **2** over the flexible ethyl chain of **1** is due to a gain in −Δ*H* at the expense of *T*Δ*S*, the favorable *T*ΔΔ*S* (+2.16 kcal/mol)

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(11) Sharp, K. *Protein Sci.* **2001**, *10*, 661−667.

Table 1. Thermodynamic Values for the Calorimetric Titration of Bisacid-Bridged Cyclic Peptides into PDZ3 from PSD-95[a]

compd	K_d (μM)	Δ*G* (kcal/mol)	Δ*H* (kcal/mol)	*T*Δ*S* (kcal/mol)
1	15.4 (±4.1)	−6.59 (±0.15)	−1.85 (±0.02)	4.74 (±0.18)
2	7.17 (±0.55)	−7.02 (±0.05)	−3.44 (±0.30)	3.58 (±0.25)
3	4.87 (±0.56)	−7.25 (±0.07)	−3.88 (±0.21)	3.37 (±0.14)
4	3.78 (±0.02)	−7.41 (±0.02)	−1.87 (±0.16)	5.53 (±0.14)
5	3.76 (±0.31)	−7.40 (±0.05)	−1.85 (±0.05)	5.55 (±0.10)
6	6.34 (±0.17)	−7.09 (±0.01)	−3.49 (±0.01)	3.60 (±0.03)
7	15.0 (±0.3)	−6.58 (±0.01)	−2.66 (±0.02)	3.92 (±0.03)
8	14.9 (±3.6)	−6.60 (±0.15)	−3.32 (±0.00)	3.28 (±0.15)

[a] Values are the mean of at least two independent experiments (error shown below reflects the range). Binding stoichiometry values (*n*) ranged from 0.93 to 1.11.

experienced by the 1,3-phenyl-linked **4** over less-constrained **3** is compensated by a nearly matched change in ΔΔ*H* (+2.01 kcal/mol).

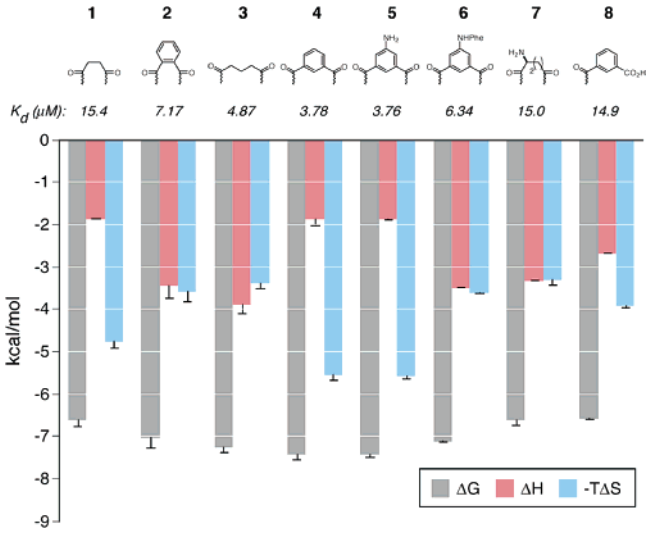

Figure 3. Bar graph of thermodynamic parameters for ligands **1**−**8**. Note that *T*Δ*S* is plotted as the negative value.

It is possible to analyze compounds **1**−**4** from a different perspective, as ring expansion (**1**→**3**) and ring isomerization (**2**→**4**) perturbations. The addition of a methylene equivalent to **1** adds a partially rotatable bond and leads to an expected unfavorable decrease in binding entropy. Yet the affinity improves 3-fold, due to a surge in the Δ*H* contribution. This may result from a ring that is able to adopt a conformation

improving specific protein−ligand interactions, whereas the presumably more constrained **1** cannot access such a state without some energetic cost. The strategy of methylene unit augmentation (as in **1**→**3**) was a key feature of our earlier PDZ-targeting macrocycles[2] but has also been applied in diverse ways to ligand design for the SH2 domain,[12] in peptide mimics of the arginine-rich motif of the HIV-1 Tat protein,[13] in agonists for receptors,[14] and for enzyme inhibitors.[15]

The comparison between ligands **2** and **4** attempts to diminish the contribution of desolvation differences that might be due to nonequivalent hydrophobic surface areas, since the pair are isomers. While **4** possesses a slightly elongated internal ring size, it is tied with **5** for largest entropy change of the series, with a $T\Delta\Delta S$ of almost 2 kcal/mol over **2**. In the absence of further structural or conformational characterization, however, it cannot be concluded that **4** is necessarily more rigid or constrained than **2**, since there may be additional global restraints present in the former.

The next step in development was to functionalize the bridge to permit the rapid formation of derivatives. This specific design strategy was implemented with the idea of preparing ligands that might exhibit selectivity between various PDZ domains by allowing for unique interactions with regions distal from the canonical binding site. NMR structural studies of an earlier developed amino acid-bridged macrocyle with a PDZ domain showed that contacts can occur between the protein surface and the bridging unit.[3]

Placement of an amine on the bridge (**5**) maintains the same affinity as the unmodified parent, allowing for potential "expansion" of the ligand binding surface through various chemical transformations. As an example, a simple amidation reaction was performed in which Fmoc-phenylalanine was coupled to the amine in a manner analogous to peptide synthesis (**6**; Scheme 2). With an enthalpic enhancement of $\Delta\Delta H \approx 1.6$ kcal/mol, this suggests that additional binding interactions may be formed with **6** that are not possible with **5**. Even if this speculation is correct, the energetic enhancement is effectively abrogated by a more than compensating decrease in entropy, with a $T\Delta\Delta S$ of approximately −2 kcal/mol. This might be the price of restricting the additional rotors introduced with the Phe residue.

Along similar lines, the amino analogue of **3** was also prepared (**7**), although in this case the affinity decreased. This ligand demonstrates that our design strategy is not restricted to symmetrical bisacids, although the use of asymmetrical bridge components does require the protection of one of the carboxylates to ensure that assembly yields a single isomer.

A control ligand (**8**) was also synthesized, which represents a linear analogue of **4**. Compound **8** can be thought of as a bond-cleavage product of **4** if the latter were hydrolyzed at the "−1" lysine side chain. The macrocycle **4** does, in fact, exhibit a favorable change in entropy over the linear **8** of $T\Delta\Delta S \approx 2.3$ kcal/mol, which is the source for a 3-fold improvement in affinity. Although **8** is linear, in fact two changes have been wrought: ring rupture *and* formation of ionizable amine and carboxylic acid functional groups. Ideally, an appropriate control would entail the cleavage of an aliphatic C−C bond, so as to minimize complicating effects due to potential changes in bonding interactions.

In summary, the presented modular ligand design enables the synthetic addition or subtraction of single-carbon units, thus allowing for incremental expansion or contraction of ring size, which can impact affinity and, perhaps eventually, selectivity. Combining the results from this report with those of our previous study,[2] we have demonstrated that such modulation can significantly influence the binding strength of PDZ domain−ligand interactions. Further, the PDZ macrocycles prepared provide useful information about the nature of constrained protein-binding molecules and can potentially serve as compounds for the design of cellular probes.

Finally, this macrocyclic scaffold may also prove to be of use for ligand development outside the realm of PDZ domains, since the design is in keeping with the notion of creating a broader, wider binding surface area that may be necessary to inhibit association with certain proteins. Many protein−protein interaction interfaces have larger surface areas that may not succumb to disruption in the presence of a competing linear inhibitor; this report provides one possible solution by more densely functionalizing a compound without simply resorting to elongation.

Acknowledgment. Funding for this research was provided by the National Institutes of Health (GM63021).

Supporting Information Available: Experimental procedures for preparation and characterization of peptide ligands. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0475966

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