

# Rigidified Clicked Dimeric Ligands for Studying the Dynamics of the PDZ1-2 Supramodule of PSD-95

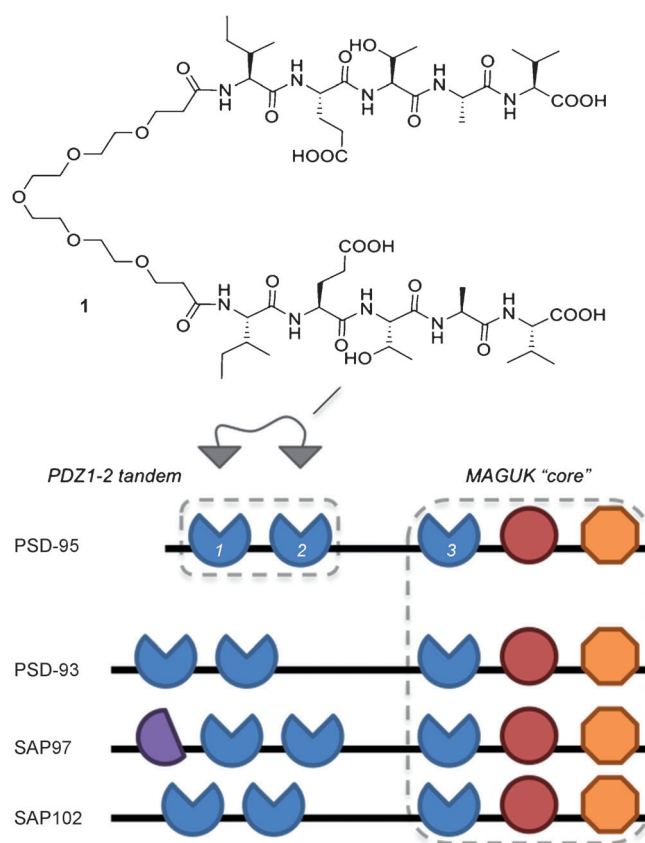
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PSD-95 is a scaffolding protein of the MAGUK protein family, and engages in several vital protein–protein interactions in the brain with its PDZ domains. It has been suggested that PSD-95 is composed of two supramodules, one of which is the PDZ1-2 tandem domain. Here we have developed rigidified high-affinity dimeric ligands that target the PDZ1-2 supramodule, and established the biophysical parameters of the dynamic PDZ1-2/ligand interactions. By employing ITC, protein NMR, and stopped-flow kinetics this study provides a detailed insight into the overall conformational energetics of the interaction between dimeric ligands and tandem PDZ domains. Our findings expand our understanding of the dynamics of PSD-95 with potential relevance to its biological role in interacting with multivalent receptor complexes and development of novel drugs.

Protein–protein interactions (PPIs) are vital for cellular and biochemical processes, and have attracted particular attention because of their potential as new promising drug targets for the treatment of many diseases.<sup>[1,2]</sup> The postsynaptic density protein-95 (PSD-95)/discs large/zona occludens 1 (PDZ) protein domain family is one of the most widespread in the human genome and is involved in several crucial PPIs.<sup>[3,4]</sup> PDZ domains are important for intracellular communication networks downstream of receptor activation and are often found in multi-domain scaffold and anchoring proteins involved in trafficking and assembling intracellular enzymes and membrane receptors into signaling-transduction complexes.<sup>[5,6]</sup> They are typically composed of 90 amino acids, and the different domains are structurally very similar, with a binding pocket accommodating C-terminal peptide ligands.<sup>[7,8]</sup>

PSD-95, a typical PDZ-containing protein,<sup>[9,10]</sup> is emerging as an attractive drug target for a number of diseases in the brain, most importantly cerebral ischemia.<sup>[12,13]</sup> PSD-95 and a number

of structurally related proteins are members of the membrane-associated guanylate kinase (MAGUK) protein family, which plays important roles in membrane formation and function.<sup>[14,15]</sup> MAGUK proteins are generally composed of three consecutive PDZ domains (PDZ1–3), followed by a Src homology 3 (SH3) and a guanylate kinase (GK) domain (Figure 1).<sup>[15,16]</sup> The role of the PDZ domains of MAGUK proteins as C-terminal recognition modules is well-established,<sup>[4,6,17]</sup> whereas the roles of the SH3 and in particular the GK domain have only recently been elucidated, with the latter being a phosphate-binding module.<sup>[17,18]</sup> Interestingly, recent studies have shown that PSD-95 (as likely also other MAGUK proteins) functionally and struc-



**Figure 1.** Dimeric ligand targeting the PDZ1-2 tandem domain and domain organization of selected members of MAGUK proteins. In compound 1, two pentapeptide ligands (IETAV) are linked by amidation at the N termini with PEG4 dicarboxylic acid. The two supramodules (PDZ1-2 tandem, and PDZ3-SH3-GK domains (MAGUK "core")), are highlighted; dimeric peptide (1) targeting the PDZ1-2 tandem is shown. PDZ: blue, SH3: red, GK: orange, L27: purple.

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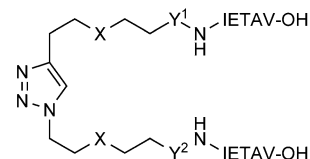
turally folds into two “supramodules”: one comprises the PDZ1-2 tandem domain, and the other contains the PDZ3, GK, and SH3 domains; together these form the supertertiary structure of PSD-95.<sup>[19–22]</sup> The two supramodules are highly flexible, and this allows considerable interdomain movement within each supramodule, as proposed for the PDZ1-2 tandem.<sup>[19–21]</sup> In the PDZ1-2 supramodule, the two PDZ domains change from a fixed to a more flexible conformation upon binding to peptide ligands, thus potentially providing increased conformational entropy for the whole system and improving the binding affinity to the PDZ domains.<sup>[23,24]</sup>

We have previously shown that dimeric ligands created by cross-linking two pentapeptides (e.g., **1**, Figure 1) bind PDZ1 and PDZ2 of PSD-95 simultaneously; the affinity was 145-fold higher than for the corresponding monomeric pentapeptide.<sup>[25]</sup> Biophysical characterization of these flexible dimeric ligands suggested that interdomain motion of the PDZ1-2 domain takes place upon binding,<sup>[24–26]</sup> however it was not clear to what extent this flexibility in PDZ1-2 was important for affinity.

Here, we examined the effects of rigidifying the linker in **1**. Firstly, we wanted to examine if reducing the entropic penalty for binding of the flexible dimeric ligands to PDZ1-2 would result in increased affinity of the rigidified ligands. Secondly, rigidified ligands could elucidate the role of interdomain flexibility of the PDZ1-2 supramodule and its importance in binding to dimeric ligands. Hence, we designed and synthesized a series of rigid dimeric ligands and evaluated these in biophysical experiments.

To reduce the flexibility of the poly(ethylene glycol) (PEG) linker, we introduced triazole moieties,<sup>[27,28]</sup> and generated the dimeric ligands by copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reactions.<sup>[29,30]</sup> The introduction of triazoles could also allow a more versatile synthetic procedure for generating dimeric ligands. First, we designed three essentially different dimeric ligands (**2–4**, Table 1 and Scheme S1 in the Supporting Information), all of which contained a rigidifying triazole and the same ligand-binding pentapeptide motif (IETAV) but differed in the nature of the linker (PEG or aliphatic) and linker-ligand junction (N-alkylation or amidation). The three ligands were generated by preparing six appropriate linker building blocks (**5–10**, Scheme S1) with a terminal azide

**Table 1.** Binding affinities of rigid dimeric ligands **2–4**.

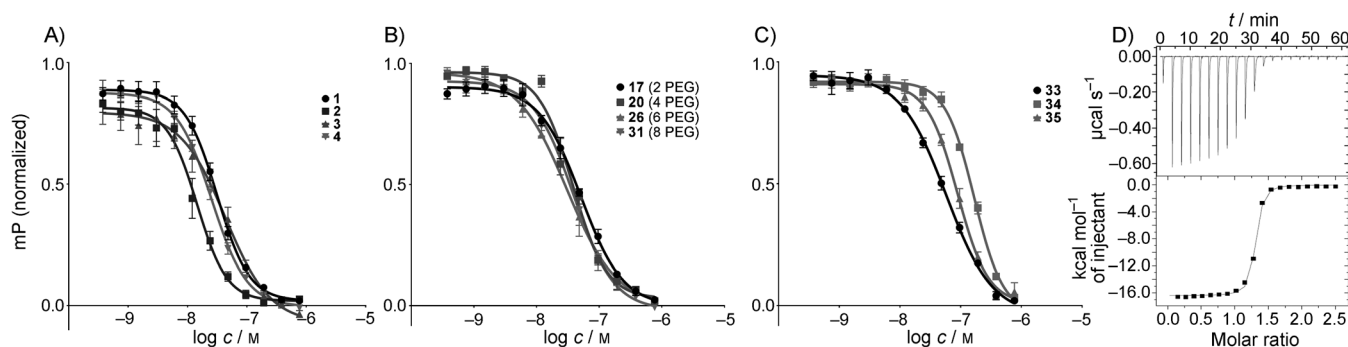
|  |                 |                                  |                                     |                                    |
|------------------------------------------------------------------------------------|-----------------|----------------------------------|-------------------------------------|------------------------------------|
| Compound                                                                           | X               | Y <sub>1</sub>                   | Y <sub>2</sub>                      | K <sub>i</sub> [nM] <sup>[a]</sup> |
| <b>1</b>                                                                           | –               | –                                | –                                   | 13 ± 1                             |
| <b>2</b>                                                                           | CH <sub>2</sub> | CH <sub>2</sub>                  | CH <sub>2</sub>                     | 4.7 ± 0.9                          |
| <b>3</b>                                                                           | O               | OCH <sub>2</sub> CH <sub>2</sub> | OCH <sub>2</sub> CH <sub>2</sub>    | 20 ± 3                             |
| <b>4</b>                                                                           | O               | OCH <sub>2</sub> CO              | OCH <sub>2</sub> CH <sub>2</sub> CO | 11 ± 1                             |

[a] K<sub>i</sub> values are mean ± SEM (n > 4).

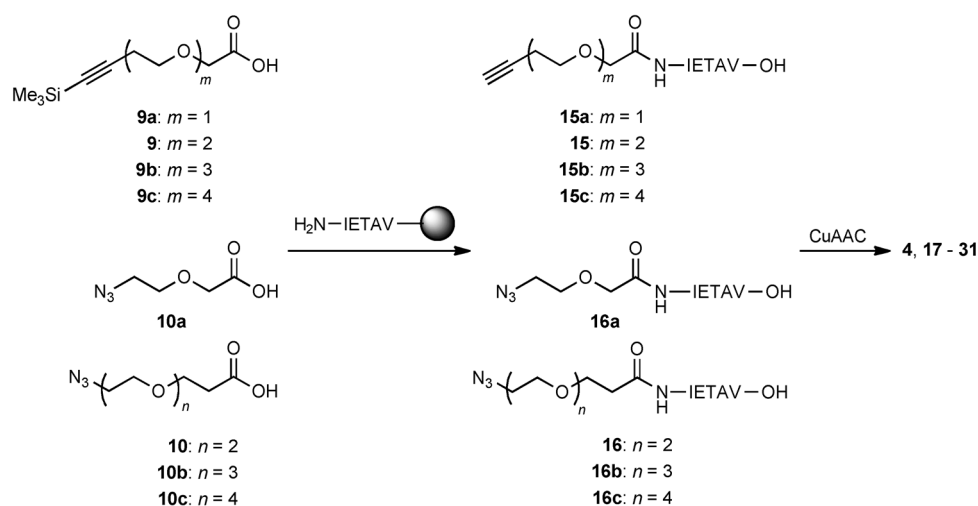
or alkyne.<sup>[31–38]</sup> The linker building blocks were subsequently attached to the ligand-binding peptide by N-terminal derivatization of the resin-bound pentapeptide with N-alkylation by Fukuyama–Mitsunobu chemistry or amidation, followed by cleavage from the resin to yield the six peptide-linker building blocks (**11–16**, Scheme S1). The three target compounds, **2–4**, were obtained by pairwise dimerization of the appropriate alkyne and azide peptide-linker building blocks. The three rigidified dimeric ligands were evaluated in a fluorescence polarization (FP) assay,<sup>[24]</sup> and gratifyingly all three ligands displayed affinities towards PDZ1-2 in the low nanomolar range (Table 1 and Figure 2A). Thus, introduction of triazoles into the linker region is clearly well tolerated, and the nature of the linker (aliphatic or PEG) and linker–ligand attachment is less important for affinity.

Next, we investigated the effect of modifying the linker for the rigidified dimeric ligands. We used dimeric ligand **4** because of its high affinity for PDZ1-2 and its synthetic feasibility. We designed a systematic set of building blocks with four different alkyne linkers (**9**, **9a–c** (1–4 PEG units); Scheme 1) and four different azide linkers (**10**, **10a–c** (1–4 PEG units); Scheme 1).

CuAAC combination of the alkyne and azide peptide-linker building blocks provided 16 different rigid dimeric ligands (**4**, **17–31**). These ligands differed in both linker length and rela-



**Figure 2.** Binding affinities determined by FP and ITC. A)–C) Dose-inhibition curves for the flexible dimeric ligand **1**, rigidified dimeric ligands **2–4**, mono-triazole dimeric ligands with different linker lengths, **17**, **20**, **26**, and **31** (2, 4, 6, and 8 PEG unit spacers, respectively), and extra-rigid analogues **33–35**; D) ITC data measured for **4** binding to PDZ1-2: K<sub>d</sub> = 41 ± 4 nM.

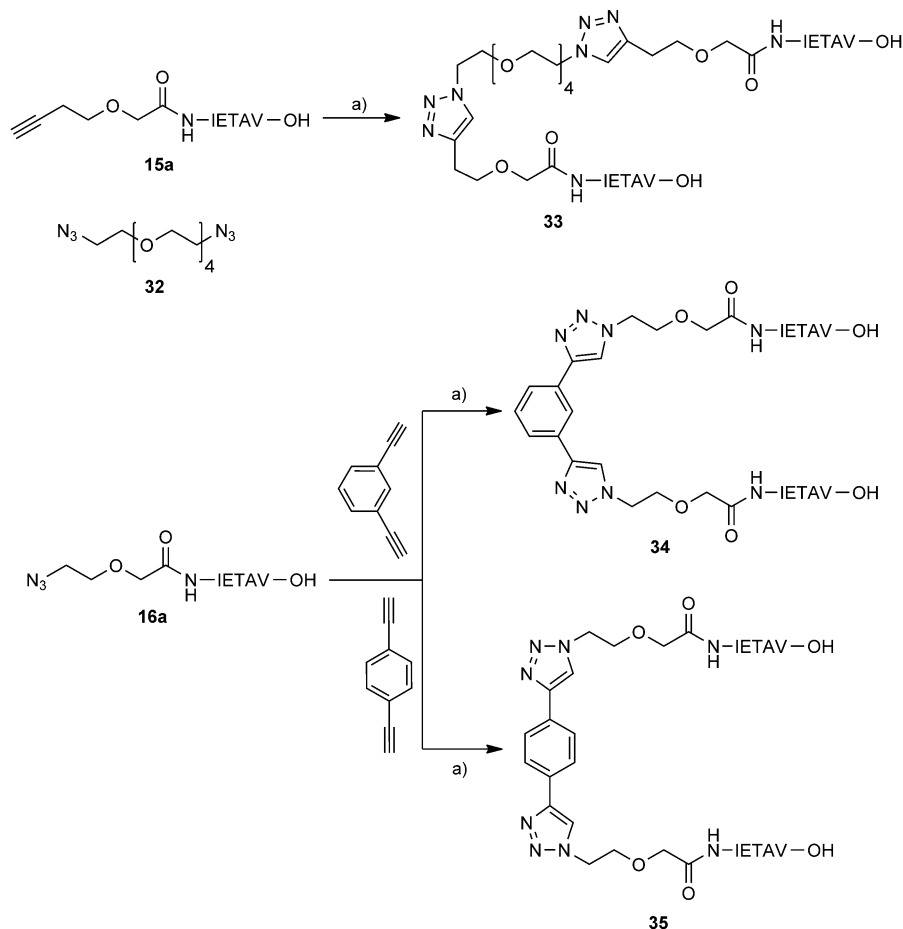


**Scheme 1.** Synthesis of compounds **4** and **17–31**.

tive position of the triazole, and were examined in an FP assay (Figure 2B and Table S1). Interestingly, all rigid dimeric ligands displayed affinities in the low nanomolar range (11–33 nM), regardless of linker length. This is in stark contrast to our previous results for dimeric ligands with flexible linkers (both increasing and in particular decreasing linker length had a major impact on affinity).<sup>[25]</sup> To explore how increased rigidity of the linker would affect affinity and other biophysical parameters, we designed and synthesized three compounds containing additional rigidifying elements, that is, either two triazole moieties (**33**) or a combination of a triazole and a phenyl ring (**34** and **35**; Scheme 2). The affinity of **33** ( $K_i = 27 \pm 2$  nM) was similar to those of **17–31**, whereas **34** and **35** displayed slightly lower affinities ( $K_i = 80 \pm 3$  and  $46 \pm 2$  nM, respectively; Figure 2C). So, rigid linkers bearing one triazole group generally provide dimeric ligands with affinities in the same range as that of **1**, but allow greater variation in linker length compared to flexible PEG-based linkers. However, with the introduction of further constraints into the linker, for example, three aromatic moieties (**34** and **35**), affinities started to decrease.

Next, we employed ITC to elucidate the thermodynamic prop-

erties of selected rigidified dimeric ligands (**4**, **17**, **22**, **25**, **30**, **31**, and **33–35**). First, we confirmed that all ligands bind PDZ1-2 with 1:1 binding stoichiometry ( $N \sim 1$ ; Figures 2D and S1, Table S2), as had been observed for flexible dimeric ligands such as **1**, and which is indicative of a true dimeric binding mode.<sup>[24,25]</sup> The  $K_d$  values determined by ITC generally showed good correlation with the  $K_i$  values from the FP assay. In general, the change in free energy ( $\Delta G$ ) relative to **1** was up to  $1.2 \text{ kcal mol}^{-1}$ , with the largest changes observed for the more rigid dimeric ligands (**33–35**; Table S2 and Figure S1), as anticipated from the FP data. In contrast, no obvious correlation was observed between linker rigidity and observed changes in enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) for the ligands; this had also been difficult to predict for



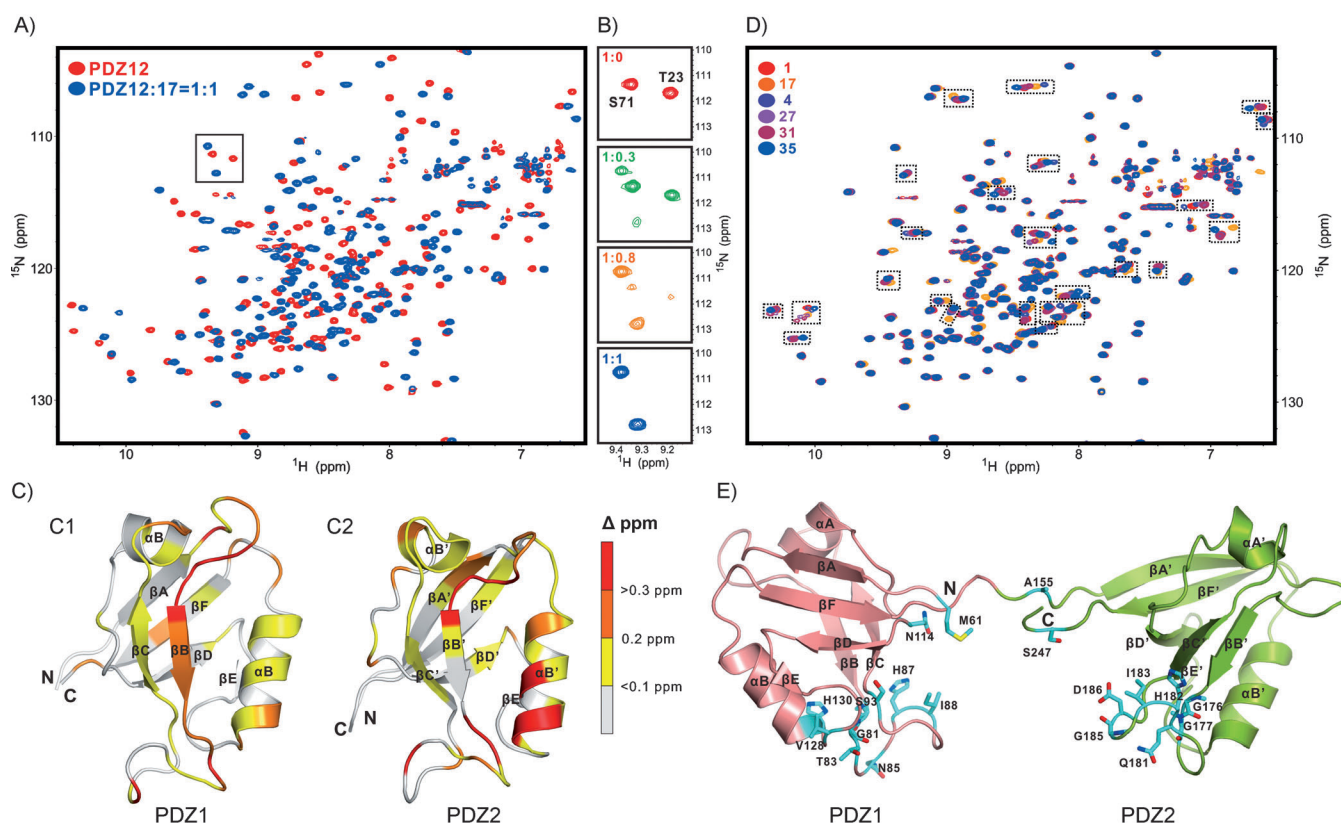
**Scheme 2.** Synthesis of extra-rigid dimeric ligands **33–35**. Reaction conditions: A) 20% copper(II) sulfate, 100% sodium ascorbate, DMF/H<sub>2</sub>O (5:1).

interactions between other polyvalent ligands and their targets.<sup>[39,44]</sup> Noticeable, the dimeric ligands **33** and **35** (two and three aromatic moieties in the linker, respectively) showed improved enthalpy ( $\sim 1.5$  kcal mol<sup>-1</sup>), whereas increased entropic penalty (2.0–2.5 kcal mol<sup>-1</sup>) was observed compared to **1** (Table S2). This is counterintuitive, as a linker with increased rigidity would be expected to incur reduced entropic penalty upon binding. However, as ITC measures the energy for the entire system including contributions from protein flexibility, we speculate that a putative reduction in the entropic penalty of the ligand can be more than compensated for by increased restriction of interdomain mobility of the PDZ1-2 tandem upon ligand binding, thereby leading to the observed increase in entropic penalty and, despite improved enthalpy, unfavorable free energy. However, the observed changes could also at least in part be explained by changes in hydrophobic interactions, hydrogen bond strengths, or other factors.

To investigate the interactions with PDZ1-2 at the structural level, we selected **1** and a range of rigidified dimeric ligands (**4**, **17**, **27**, **31**, **33**, and **35**) for NMR titration experiments (Figure 3). First, we confirmed that the ligands bind to PDZ1-2 with high affinity (Figure 3A and B) and in the expected bind-

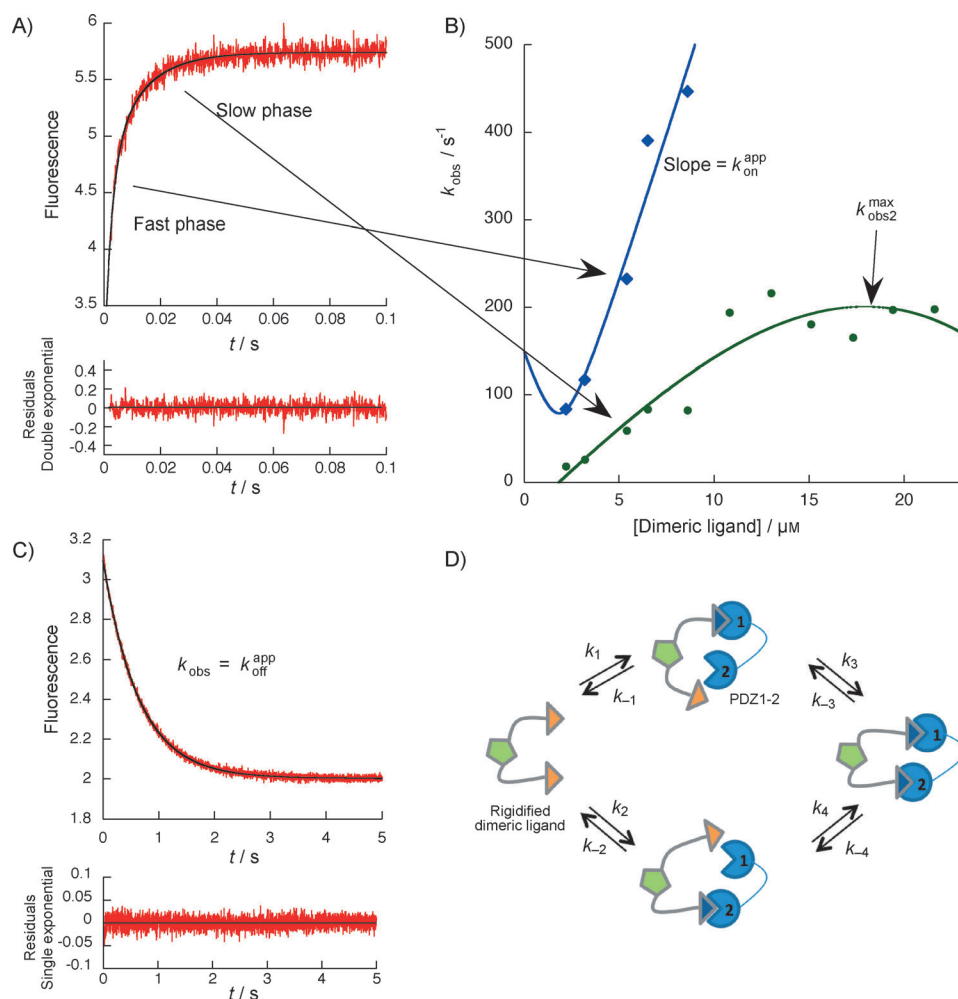
ing mode, as residues in the C-terminal ligand-binding site of PDZ1 and PDZ2 displayed changes in chemical shifts during titrations (Figure 3C). In addition, chemical shift changes were observed in the dynamic  $\beta$ B– $\beta$ C loop as well as the PDZ1-2 linker region, and, interestingly, the rigid dimeric ligands induced different chemical shifts particularly in these two regions (Figure 3D–E and Figure S2).

Finally, to elucidate the kinetic ligand-binding mechanism, we investigated the selected rigid dimeric ligands (**4**, **17**, **22**, **25**, **30**, and **31**) in more detail by stopped-flow kinetics (Figure 4 and Table S3). The  $K_d$  values from these experiments correlated well with the ITC  $K_d$  values (Table S2), thus validating the assumptions and approximations in our analysis (Figure S3). The binding scheme of a dimeric ligand to tandem PDZ domains is complex, but can be approximated with a “double square” (a single square is shown in Figure 4D). First, one peptide of the dimeric ligand binds to either PDZ1 or PDZ2 in the tandem domain (intermolecular interaction) followed by binding of the other peptide ligand to the second PDZ domain (intramolecular interaction; Figure 4D).<sup>[24,25]</sup> The intramolecular second binding event is the basis for the increased affinity of dimeric ligands relative to monomeric



**Figure 3.** Binding of dimeric ligands to PDZ1-2 characterized by NMR-based titrations of PDZ1-2 with increasing concentrations of **17**. A) 2D NMR of PDZ1-2 with and without **17**. B) Detail at different concentrations of **17** showing chemical shift changes for two PDZ1 residues. The slow exchange between the ligand-bound and ligand-free PDZ1-2 in the middle of the titration is consistent with the high affinity of **17**. C) Mapping of binding-induced chemical shift changes to the structure of PDZ1-2 by **17** to illustrate the ligand binding sites on PDZ1-2: **17** binds to the conserved  $\beta$ B– $\alpha$ B PDZ binding groove in both PDZ1-2. For clarity, the two PDZ domains are presented separately so that their respective  $\beta$ B– $\alpha$ B grooves can be recognized. D) Overlay of  $^1\text{H}$ ,  $^{15}\text{N}$  HSQC spectra of PDZ1-2 in complex with different dimeric ligands: **1** (red), **17** (orange), **4** (purple), **27** (pink), **31** (magenta), **35** (blue). The PDZ1-2 peaks showing obvious chemical shift differences upon binding to these compounds (dashed rectangles). E) Structure of PDZ1-2; residues identified in boxes (above) are rendered as stick models. A comparison of the data from A) and D) indicates that the overall binding properties of these dimeric compounds are similar; subtle differences are found in the  $\beta$ B– $\beta$ C loop and the PDZ1-2 linker.





**Figure 4.** Binding kinetics of the PDZ1-2 tandem with dimeric ligands. A) PDZ1-2 (2  $\mu\text{M}$ ) was mixed with **4** (6  $\mu\text{M}$ ) and the fluorescence of an engineered Trp was followed over time. The kinetic trace displayed a slow and a fast phase and was fitted to a double exponential equation to obtain two observed rate constants  $k_{\text{obs}1}$  and  $k_{\text{obs}2}$ . B) The observed rate constants were plotted against concentration of dimeric ligand. The slope of  $k_{\text{obs}1}$  ( $\blacklozenge$ ) at high concentration of **4** is the apparent association rate constant  $k_{\text{on}}^{\text{app}}$ . The maximum value of  $k_{\text{obs}2}$  ( $\bullet$ ) was estimated by using a polynomial function. C) The overall or apparent dissociation rate constant  $k_{\text{off}}^{\text{app}}$  was determined in a displacement reaction. At high concentration of the displacing ligand (dansyl-SIESDV),  $k_{\text{obs}}$  is approximately equal to  $k_{\text{off}}^{\text{app}}$ . D) This square scheme illustrates the binding of our dimeric inhibitors to the PDZ1-2 tandem and defines microscopic rate constants used in the text. Note that each of the peptides of the inhibitor can initially bind to either PDZ1 or PDZ2. Therefore, a “double square” is the most realistic model for binding, and this was taken into account when the  $K_{\text{d}}$  values in Table S3 were estimated from measured rate constants with the equation in Figure S3.

ones,<sup>[25]</sup> because of the vicinity of the second ligand to the PDZ domain and the resulting high effective concentration.<sup>[25]</sup> These experiments demonstrated that the rate constants of the intramolecular steps ( $k_{\text{obs} \text{ max}} \sim k_3$ ) were largely unaffected by changing the length of the linker (Table S3). Likewise, the overall dissociation rate constant  $k_{\text{off}}^{\text{app}}$  changed very little with linker length. Thus, neither the binding mechanism nor the magnitude of rate constant is affected significantly by the introduction or position of a triazole or linker length (Table S3).

In conclusion, we have designed and synthesized a set of rigidified dimeric peptide ligands targeting the PDZ1-2 supramodule of PSD-95. These dimeric ligands contain triazoles as a rigidifying element in the linker region, and the ligands vary

in both type of linker, linker attachment, linker length, and relative position of the triazole moiety. The versatile synthetic approach for these dimeric ligands allows a highly systematic exploration of analogues, and provides an expedient and general way for obtaining dimeric ligands with peptide binding motifs. Overall, we observed a marked tolerance for introduction of triazole moieties into the linker region, as most ligands showed very high affinity to PDZ1-2. Surprisingly, we observed very limited effect on affinity from drastic changes in linker length. Thus, the introduction of a triazole moiety into the linker allows greater tractability with respect to linker length in the design of dimeric ligands; given the therapeutic potential of such dimeric ligands,<sup>[24]</sup> this could have important implications in the future development of drug candidates and in vivo active compounds.

Biophysical studies using ITC, NMR, and stopped-flow kinetics revealed an intriguing compensation between rigidity of the dimeric ligands and changes in enthalpy and entropy of the dimeric ligand/PDZ1-2 interaction. Interestingly, the most rigid dimeric ligands showed improved enthalpy contributions, thus indicating that these ligands engage in very favorable interactions with PDZ1-2, but at the cost of an increased entropic

penalty likely from constricting interdomain motion of the protein. These structure–activity relationship studies are consistent with recent NMR and small-angle X-ray scattering (SAXS) studies of PDZ1-2 flexibility upon binding to monomeric and dimeric ligands.<sup>[23,24]</sup> Therefore, the rigidified dimeric ligands developed here will be useful tools in unraveling the complex dynamics of binding to the PDZ1-2 supramodule of PSD-95 and could provide important insights for further development of such ligands as medically important compounds.

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