ORGANIC LETTERS

2012
Vol. 14, No. 5
1194–1197

Synthesis of Water-Soluble Scaffolds for Peptide Cyclization, Labeling, and Ligation

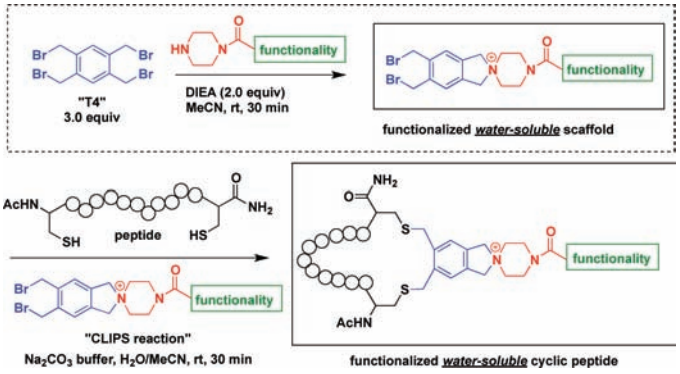
Linde E. J. Smeenk,[†] Nicolas Dailly,[‡] Henk Hiemstra,[†] Jan H. van Maarseveen,*[,†] and Peter Timmerman*[,‡]

Van't Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands, and Pepscan Therapeutics, Zuidersluisweg 2, 8243 RC Lelystad, The Netherlands

J.H.vanmaarseveen@uva.nl; p.timmerman@pepscan.com

Received December 7, 2011

ABSTRACT ret]



The synthesis and applications of water-soluble scaffolds that conformationally constrain side chain unprotected linear peptides containing two cysteines are described. These scaffolds contain a functionality with orthogonal reactivity to be used for labeling and ligation. This is illustrated by the chemical ligation of two dissimilar constrained peptides via oxime ligation or strain-promoted azide–alkyne cycloaddition in aqueous media.

The mimicry of protein function by using small peptide-based 'mini-proteins' remains an enormous challenge in the field of biomimetic chemistry.[1] The biological activity of proteins is mostly confined in small specific parts of their three-dimensional structure.[2] Examples of active protein mimics based on small linear peptides have been reported[3] but generally lack appreciable functionality due to improper folding. It has been verified that mimics of structurally more complex binding sites (conformational or discontinuous)

[†] University of Amsterdam.
[‡] Pepscan Therapeutics.

(1) (a) Altmann, K. H.; Mutter, M. *Int. J. Biochem.* **1990**, *22*, 947–956. (b) Degrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622–628. (c) Hecht, M. H.; Richardson, J. S.; Richardson, D. C.; Ogden, R. C. *Science* **1990**, *249*, 884–891.

(2) Fairlie, D. P.; West, M. L.; Wong, A. K. *Curr. Med. Chem.* **1998**, *5*, 29–62.

(3) (a) Bittle, J. L.; Houghten, R. A.; Alexander, H.; Shinnick, T. M.; Sutcliffe, J. G.; Lerner, R. A.; Rowlands, D. J.; Brown, F. *Nature* **1982**, *298*, 30–33. (b) Langeveld, J. P. M.; Casal, J. I.; Osterhaus, A.; Cortes, E.; Deswart, R.; Vela, C.; Dalsgaard, K.; Puijk, W. C.; Schaaper, W. M. M.; Meloen, R. H. *J. Virol.* **1994**, *68*, 4506–4513. (c) Dimarchi, R.; Brooke, G.; Gale, C.; Cracknell, V.; Doel, T.; Mowat, N. *Science* **1986**, *232*, 639–641.

(4) (a) Kuwabara, I.; Maruyama, H.; Kamisue, S.; Shima, M.; Yoshioka, A.; Maruyama, I. N. *J. Immunol. Methods* **1999**, *224*, 89–99. (b) Cui, X. L.; Nagesha, H. S.; Holmes, I. H. *J. Virol. Methods* **2003**, *114*, 109–112. (c) Villen, J.; Borras, E.; Schaaper, W. M. M.; Meloen, R. H.; Davila, M.; Domingo, E.; Giralt, E.; Andreu, D. *ChemBioChem* **2002**, *3*, 175–182. (d) McGaughey, G. B.; Citron, M.; Danzeisen, R. C.; Freidinger, R. M.; Garsky, V. M.; Hurni, W. M.; Joyce, J. G.; Liang, X.; Miller, M.; Shiver, J.; Bogusky, M. J. *Biochemistry* **2003**, *42*, 3214–3223. (e) Dakappagari, N. K.; Lute, K. D.; Rawale, S.; Steele, J. T.; Allen, S. D.; Phillips, G.; Reilly, R. T.; Kaumaya, P. T. P. *J. Biol. Chem.* **2005**, *280*, 54–63.

(5) (a) Singh, Y.; Dolphin, G. T.; Razkin, J.; Dumy, P. *ChemBioChem* **2006**, *7*, 1298–1314. (b) Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. *Science* **2004**, *305*, 1466–1470. (c) Reichwein, J. F.; Wels, B.; Kruijtzer, J. A. W.; Versluis, C.; Liskamp, R. M. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 3684–3687. (d) Angell, Y. L.; Burgess, K. *Chem. Soc. Rev.* **2007**, *36*, 1674–1689. (e) Schneider, J. P.; Kelly, J. W. *Chem. Rev.* **1995**, *95*, 2169–2187.

10.1021/ol203259a
Published on Web 02/14/2012

require adopting the protein's native secondary and/or tertiary structure for decent activity.[4] A common way to achieve this is by making use of synthetic scaffolds.[5]

However, to reach the next level of complexity, multiple (cyclic) peptide fragments of a (discontinuous) binding site need to be linked together. Therefore, a generally applicable and straightforward synthetic approach toward scaffolds that (*i*) conformationally constrain linear peptides and (*ii*) can be ligated together in order to mimic discontinuous binding sites is desired. Here we present the synthesis of novel water-soluble scaffolds that fulfill these requirements.

Our method builds upon CLIPS technology,[6] a powerful method to constrain and cyclize side chain unprotected dithiol-containing peptides. This chemoselective cyclization reaction utilizes the exquisite reactivity of dibromoxylene scaffolds, such as **1** (Figure 1) toward free cysteine residues in aqueous conditions, and provides a cyclized peptide containing two robust thioether bonds.[7]

Recently, the set of available scaffolds was extended by Hartman et al., who described the functionalization of 1,3,5-tribromomesitylene via a monocarboxylation reaction (**2**, Figure 1).[8]

We envisioned a different way to generate functionalized dibromoxylene scaffolds, namely by reacting functionalized primary amines with excess of 1,2,4,5-tetrabromodurene (**T4**). We found that only 'crowded' primary amines (e.g., amino acids with bulky side chains) gave stable products in this reaction (**3**, Figure 1). The products from sterically unhindered primary amines, like propargyl amine, were highly unstable and polymerized as a result of overalkylation at the nucleophilic tertiary nitrogen that is formed. In order to overcome this problem, we reacted **T4** with a variety of secondary amines. The reaction products were chemically very stable and turned out to be highly water-soluble as a result of the quaternary ammonium ion present (scaffolds **4**, Figure 1). However, the presence of this tetrahedral nitrogen center could also cause the formation of diastereomeric product mixtures after peptide coupling, particularly when asymmetric amines are used (R_1R_2NH).

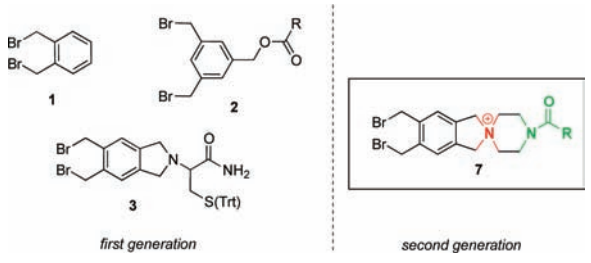
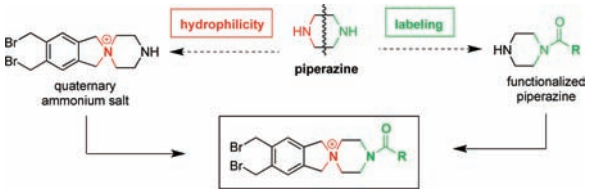


Figure 1. First and second generation of CLIPS scaffolds.

In order to circumvent this problem, we used 1,4-piperazine as the 'connector' unit between the functionalities and the dibromoxylene unit (Scheme 1). 1,4-Piperazine turned out to be the molecule of choice here, because (*i*) it precludes any chirality issue as mentioned, (*ii*) it is commercially available in various monoprotected forms (Boc, Fmoc, Cbz, etc.), and (*iii*) the free amine group is readily amenable for further functionalization via amide bond formation using a wide variety of carboxylic acids.

Scheme 1. Design of Second Generation Functionalized and Water-Soluble CLIPS Scaffolds



For convenience, the synthesis of scaffolds **4** was conducted by coupling of functionalized carboxylic acids **5** to the mono-*N*-protected 1,4-piperazines using standard amide coupling conditions (HBTU, DIEA). The alternative approach, i.e. coupling of the mono-*N*-protected 1,4-piperazine to **T4** prior to amide coupling, failed because of the instability of the benzyl bromide functionalities under conditions for Boc-, Fmoc-, or Cbz-deprotection. Table 1 shows the different carboxylic acids (**5a**–**g**) that we used for coupling. These include precursor functionalities for a strain-promoted azide–alkyne cycloaddition (entries 1–2), oxime ligation (entries 3–4), thiol–ene ligation (entries 5–6), or decoration with a biotin label (entry 7).

Each functionalized carboxylic acid was reacted with a suitable mono-*N*-protected piperazine (Boc-, Cbz-, or Fmoc-), depending on the acid or base sensitivity of the functional groups in the acids. For example, Boc-piperazine was used in the case of the azide **5a**, cyclooctyne **5b**, alkene **5e**, and biotin **5g**, which are known to be stable under the acidic conditions for Boc-removal, while the acid-sensitive diethyl acetal **5c** and the Boc-protected hydroxylamine **5d** were preferably synthesized using Cbz-protected piperazine. For coupling of the Cys(Trt)-containing carboxylic acid **5f** we used the Fmoc-protected piperazine, since Cbz removal failed due to poisoning of the Pd catalyst by the presence of the thioether moiety.

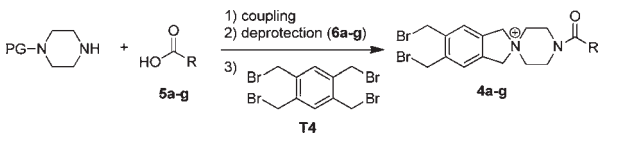
After amide-coupling and subsequent Boc-, Fmoc-, or Cbz-removal, the resulting amines **6a**–**g** were reacted with **T4** to form scaffolds **4**. It was found that the use of relatively low amine concentrations (typically 10 mM) and an excess of **T4** (typically 3 equiv) were sufficient to completely suppress further reaction of scaffolds **4** with a

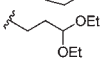
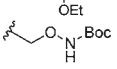
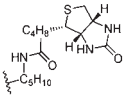
(6) Timmerman, P.; Beld, J.; Puijk, W. C.; Meloen, R. H. *ChemBioChem* **2005**, *6*, 821–824.

(7) (a) Neri, D.; Brandli, A. W. *Nat. Chem. Biol.* **2009**, *5*, 452–453. (b) Heinis, C.; Rutherford, T.; Freund, S.; Winter, G. *Nat. Chem. Biol.* **2009**, *5*, 502–507.

(8) Dewkar, G. K.; Carneiro, P. B.; Hartman, M. C. T. *Org. Lett.* **2009**, *11*, 4708–4711.

Table 1. Yields for the Coupling Reaction[a] of Mono-*N*-protected 1,4-Piperazine with Carboxylic Acids, Followed by Boc[b], Fmoc,[c] or Cbz[d] Removal and Reaction with **T4**[e]



entry	PG		R	6 yield (%)[f]	4 yield (%)
1	Boc	**5a**		**6a** (97)	**4a** (93)
2	Boc	**5b**		**6b** (65)	**4b** (67)
3	Cbz	**5c**		**6c** (94)	**4c** (71)
4	Cbz	**5d**		**6d** (73)	**4d** (97)
5	Boc	**5e**[g]		**6e** (99)[g]	**4e** (68)
6	Fmoc	**5f**		**6f** (77)	**4f** (92)
7	Boc	**5g**		**6g** (56)[g]	**4g** (61)[g]

[a] Reaction conditions: acid **5** (1.2 equiv), HBTU (1.2 equiv), amine (1.0 equiv), and DIEA (2.5 equiv), THF, rt, 1 h. [b] 1:1 DCM/TFA, rt, 1 h. [c] 1:1 THF/DIEA, rt, 1 h. [d] 1:1 EtOAc/*i*PrOH, Pd/C, H_2, overnight. [e] **T4** (3.0 equiv), amine **6a**–**g** (1.0 equiv), DIEA (2.0 equiv), MeCN, rt, 30 min. [f] Isolated yield over two steps. [g] Different synthetic route toward **6e**, **6g**, and **4g**; see Supporting Information.

second equivalent of the amine. Moreover, the choice of solvent and base determined to a large extent the outcome of the reaction. Some of the bases tested slowed down the reaction considerably (K_2CO_3, 2,6-lutidine), while others (triethylamine, pyridine) interfered with the process by reacting with the benzylic bromides. DIEA was the only base capable of driving the reaction cleanly toward completion in ~30 min.

We also searched for the optimal solvent. Product formation was not at all observed in toluene and only at a low rate in $CHCl_3$, THF, and DCM, while formation of numerous side products was observed in DMSO and DMF. By using MeCN, a fast reaction (< 30 min) was observed without any byproduct formation. The product could be collected conveniently by precipitation after adding cold Et_2O.

Piperazines **6a**–**g** were all reacted with **T4** using the optimized procedure (**T4** (3 equiv), DIEA (2 equiv) in MeCN, 30 min at room temperature) to give scaffolds **4a**–**g** in moderate to excellent yields (56–99%) (Table 1). The products were contaminated with a maximum of 0.7 equiv of the DIEA•HBr salt that could not be removed by washing with ether. Purification by HPLC using acid-free conditions did effectively remove the DIEA•HBr salt but significantly lowered the yield due to hydrolysis of the benzyl bromide functionalities in **4**. Therefore, scaffolds **4a**–**g** were used without further purification, since the DIEA•HBr salt did not harm the subsequent CLIPS reactions to any extent.

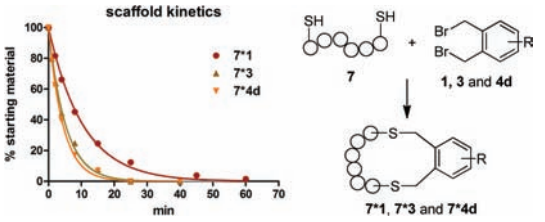


Figure 2. Kinetic studies of CLIPS reactions: peptide **7** (0.14 mM), scaffold **1**, **3**, or **4d** (1.25 equiv), H_2O/MeCN (1:1), 200 mM $NaHCO_3/Na_2CO_3$, pH 8.8.

Subsequently, we compared the reactivity of ammonium-containing scaffolds **4** in a peptide cyclization reaction to that of the first generation (water-insoluble) neutral scaffolds **1** and **3**, using the super hydrophobic peptide Ac-CSIAFRAARFAISC-NH_2 (**7**) as a model (Figure 2). The CLIPS macrocyclization reaction of peptide **7** with scaffolds **3** and **4d** ($t_{1/2}$ = 4 min) proceeded twice as fast as the reaction starting from **1** ($t_{1/2}$ = 8 min). The reason for this is not entirely understood. Electronic effects of the neutral and positively charged aminomethyl group(s) are unlikely, as they are expected to be very different in nature for **3** and **4d**. Presumably, the annulated 5–6 ring structure present in both **3** and **4d** might result in some stabilization of the transition state in the S_N2-type attack of the bromide. This assumption is currently under investigation.

A characteristic feature of scaffolds **4** is their ability to increase the polarity and, consequently, water-solubility of any cyclized peptide prepared from **4**. UPLC analysis indeed showed increased polarity of cyclic peptide **7*4d** (retention volume 30% MeCN, Figure 3) compared to the corresponding linear peptide **7** (45% MeCN in Figure 3), while the cyclic peptide **7*1** is even more hydrophobic than **7** itself and elutes at 60% MeCN.

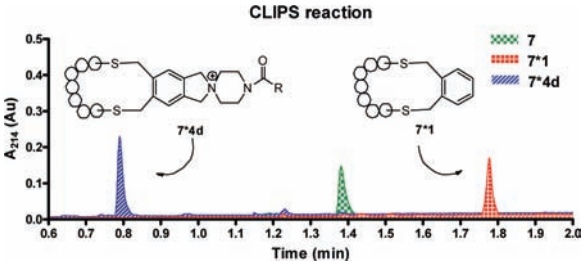


Figure 3. UPLC spectrum (5–95% MeCN in 3 min; gradient 30% MeCN/min) showing the UV-absorbance (215 nm) of cyclic peptides **7*1** and **7*4d** after the CLIPS reaction.

In view of our final goal to make water-soluble discontinuous protein binding site mimics, we optimized the chemical ligation of two different scaffold-constrained peptides by connection via the remaining mutually reactive moieties at these scaffolds. This ligation should ideally

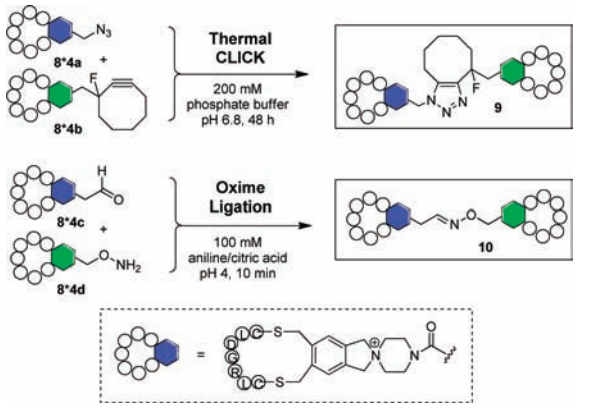
meet the following requirements: (*i*) proceed at low concentrations (typically 1 mM or lower) and at room temperature in <24 h, (*ii*) no cross-reactivity of the reactive groups for ligation with any (unprotected) side chain peptide functionality in the peptides, and (*iii*) the covalent linkage formed upon ligation should be chemically stable. To achieve these goals, we investigated the following three different ligation methods: (*i*) the strain-promoted azide–alkyne cycloaddition[9] (i.e., with **8*4a** and **8*4b**, Scheme 2), (*ii*) the aniline-catalyzed oxime formation[10] (i.e., with **8*4c** and **8*4d**, Scheme 2), and (*iii*) the photoinduced thiol–ene reaction[11] (i.e., with **8*4e** and **8*4f**). The side chain unprotected RGD-peptide Ac-CLRGDLC-NH_2 (**8**) was used as a model compound and was cyclized via reaction with all seven available scaffolds (**4a**–**g**). After cyclization, some of the cyclic peptides needed deprotection of the reactive functionalities, followed by HPLC purification prior to ligation. Hydrolysis of the diethyl acetal liberating the aldehyde toward **8*4c** was achieved by adding a 1% TFA solution in H_2O to the mixture for 1 h. Boc-removal toward **8*4d** was completed by stirring the peptide for 1 h in a TFA/H_2O 1:1 solution. Finally, Trt-group removal for **8*4f** was accomplished by adding a mixture of 5% TFA, 3% TES, and DCM for 1 h.

The strain-promoted azide–alkyne cycloaddition reaction between **8*4a** and **8*4b** was performed at 1 mM in a phosphate buffered solution at pH 6.8. Even though product formation proceeded slowly (48 h to completion) double-cyclic peptide **9** was formed in 51% yield (Scheme 2).

The best results were obtained via the aniline-catalyzed oxime ligation reaction. Full conversion of peptides **8*4c** and **8*4d** at 1 mM in a 100 mM aniline/citric acid buffer at pH 4 in H_2O/MeCN 3:1 was observed in 10 min at room temperature giving the oxime-ligated double-cyclic peptide **10** in 76% yield (Scheme 2).

Unfortunately, ligation of cyclic peptides **8*4e** and **8*4f** via the UV-induced thiol–ene reaction was not successful, using any of the reported literature procedures. The lack of reactivity is likely due to the low equimolar concentrations used (1 mM). However, for our purposes, the use of large excesses of either peptide (**8*4e** or **8*4f**) to speed up the reaction was not an option.

Scheme 2. Synthesis of Double-CLIPS Peptides **9** and **10** via Chemical Ligation of Functionalized CLIPS-Peptides **8*4a**–**d**



We finally showed that peptides can be simultaneously cyclized and biotin-labeled using the biotin-functionalized scaffold **4g**. As an alternative, functionalized scaffolds **4a**–**f** may be used for indirect labeling, i.e. by coupling of an appropriately functionalized FLAG, biotin, fluorescein, rhodamine, or luciferin tag to a cyclized peptide carrying the compatible functional group at the scaffold.

The scaffold synthesis described here provides a general and easy applicable route toward complex water-soluble double-loop mimics of discontinuous protein binding sites and can also be used to solubilize or label cyclic peptides. The method opens new perspectives in the field of protein mimicry. Further use of this methodology is currently under investigation.

Acknowledgment. This work was financially supported by The Netherlands Organization for Scientific Research (NWO) ECHO 700.57.017.

Supporting Information Available. Experimental procedures, characterization, UPLC analysis, tables and copies of ^1H and ^{13}C spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(9) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
(10) Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581–7584.
(11) Dondoni, A.; Massi, A.; Nanni, P.; Roda, A. *Chem.—Eur. J.* **2009**, *15*, 11444–11449.