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Serendipitous discovery of an efficient method for the synthesis of dimeric-RGD analogues using DMAP-photoirradiation†

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We describe a novel disulfide reaction *via* UV/*N*,*N*-dimethylaminopyridine (DMAP) methodology for efficient construction of alkyl and aryl symmetrical disulfides. Compared with other chemical strategies, our methodology is distinguished in that the dimerization reaction can proceed efficiently without metal catalysts, expensive reagents or forcing conditions. This methodology was successfully applied to the preparation of complex dimeric biological peptide-based molecules, and the dimeric RGD peptides produced by this methodology had better binding affinity than the commercially available E-[RGDfK]$_2$. Our methodology will greatly extend the method to the construction of a complex disulfide bond under mild conditions.

The conversion of thiols to the corresponding disulfides has gained significant applications both in chemical industry[1] and biological science.[2] Disulfides are valuable intermediates for the production of sulfenyls and sulfinyls in organic chemistry.[3] Furthermore, disulfide bonds are the principal entities responsible for stabilizing the secondary or tertiary structure of proteins.[4] Procedures involving the use of halogen derivatives,[5] transition metal salts,[6] peroxides,[7] molecular oxygen,[8] nitric oxide,[9] 2,6-dicarboxypyridinium chlorochromate[10] and cerium salts[11] have been introduced for oxidative coupling of thiols to disulfides. Despite these promising examples, there are still several drawbacks including the need for expensive reagents used in excess, long reaction times, and forcing reaction conditions. Additionally, these methods are mainly focused on producing a simple disulfide bond based on small molecules, and it is uncertain whether these protocols are amenable to the synthesis of complex biomolecules.

Dimerization of cysteine residues has become a vital method for maintaining biologically active conformations of physiologically important peptides such as somatostatin and vasopressin.[12] The development of new and efficient protocols for the preparation of complex disulfide biomolecules under mild reaction conditions is an important challenge for medicinal chemistry.

Multivalent interactions are known to play a critical role in many biological processes.[13] The synthesis of multivalent peptides has further enhanced the interaction of individual ligands with their receptors. For example, the RGD (arginine–glycine–aspartic acid) tripeptide motif plays an essential role in the molecular recognition of integrin $\alpha_v\beta_3$, which is overexpressed in various types of tumors.[14] Monomeric RGD-based probes have been successfully prepared and exhibit selectivity for integrin $\alpha_v\beta_3$ *in vitro* and *in vivo*.[15] However, monomeric RGD-based probes exhibited low cellular uptake *in vitro*.[16] To overcome this issue, multimeric RGD ligands have been developed, and they demonstrate higher receptor binding affinity *in vitro* and better tumor retention *in vivo* due to their multivalent composition.[17] Therefore, there still remains a need for mild reaction conditions capable of preparing multimeric RGD peptides with high flexibility, efficiency, and chemoselectivity.

Thiol–yne click chemistry has become an important tool for the construction of both multivalent molecules of biological interest and assorted materials (Scheme 1a).[18] More recently, Sun and co-workers have successfully applied thiol–yne click chemistry to the construction of multivalent peptide-based imaging probes.[19] It is worth noting that UV-induced thiol–yne reaction inevitably produces some amount of disulfide as the main byproduct.[20] Therefore, we envisioned that the UV-irradiation of thiol–yne reaction provides a possible method to make disulfide bonds under mild conditions. More recently, good yields have been reported for disulfide reaction catalyzed by bases such as tetramethylguanidine and Et_3N.[21] Inspired by both facts, herein we developed a photoirradiation methodology for the efficient preparation of dimeric RGD analogue products *via* substituting

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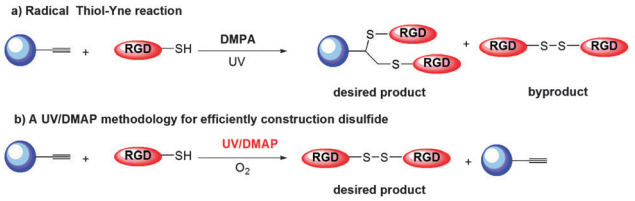
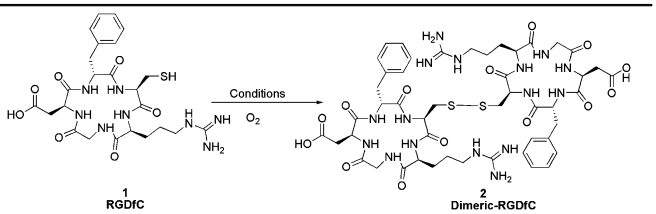


Scheme 1 (a) UV-induced thiol–yne click chemistry; (b) UV/DMAP method for dimerization of RGD.

the typical thiol–yne click reaction catalyst 2,2-dimethoxy-2-phenylacetophenone (DMPA) for *N*,*N*-dimethylaminopyridine (DMAP) (Scheme 1b).

Initially, we chose RGDfC (**1**) as a model reaction to establish the best conditions for dimerization of the RGD analogue (**2**) under various conditions (Table 1). The desired product was obtained in 65% yield after reaction in water for 3 h under UV irradiation (4 W, 365 nm) in the presence of DMAP (entry 1). The results of MALDI-TOF-MS (ESI,† Fig. S1, m/z = 1156.223) and ESI-MS (Fig. S2, m/z = 1155.5, ESI†) confirmed the dimer-RGDfC as the desired product. A better yield was obtained using DMF as solvent (yield 75%, entry 2), while the optimal result was achieved using DMSO (yield 78%, entry 3). The role of polar aprotic solvents having high dielectric constants, such as DMF and DMSO are known to increase the oxidation rate of thiol with oxygen.[21b] Therefore, the better yields obtained from organic solvents were consistent with the literature. It was also found that the DMAP loading presents a critical determinant of the reaction efficiency in this reaction; decreasing the amount of DMAP from 1 equiv. to 0.1 equiv. can extend the reaction time from 5 to 12 h and reducing yields from 78% to 35% (entries 3–5). Finally, we investigated whether DMAP or UV could catalyze this dimerization reaction exclusively. It is noteworthy that DMAP alone could catalyze the reaction to furnish moderate yields (46%) of the dimer, albeit with extended reaction time (entry 6), while only a little desired product was observed under UV irradiation in the absence of a catalyst after a long time of 12 h (entry 7, 8%). It is well known that DMAP is a good example of a modern low-molecular organic base catalyst with a powerful effect on many organic reactions.[22] Hence, we speculate a possible base-catalyzed mechanism *via* DMAP in our methodology.

Table 1 Optimization for dimerization of RGDfC



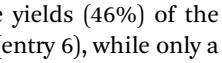
Entry	Catalyst[a]	Solvents	Time (h)	Yield[b] (%)
1	DMAP/UV (1 equiv.)	H_2O	3	65
2	DMAP/UV (1 equiv.)	DMF	3	75
3	DMAP/UV (1 equiv.)	DMSO	3	78
4	DMAP/UV (0.5 equiv.)	DMSO	5	74
5	DMAP/UV (0.1 equiv.)	DMSO	12	35
6	DMAP (1 equiv.)	DMSO	12	48
7	UV	DMSO	12	8

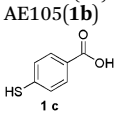
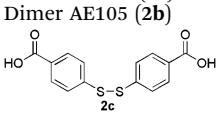
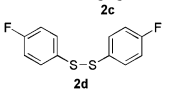
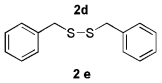
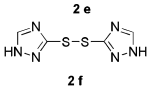
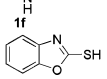
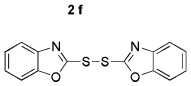
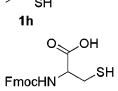
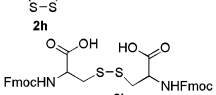
[a] Reaction conditions: [RGDfC] = 0.01 M in solvent, UV source: UVGL-55 Handheld UV Lamp, 4 W, 365 nm. [b] Isolated yield.

The scope of the dimerization reaction was investigated using a range of substrates and the optimized UV/DMAP conditions (Table 2). A similar yield was obtained with the RGD analogue RADfC (entry 1, 75%), and the dimeric RADfC was confirmed by MALDI-TOF-MS (Fig. S3, m/z = 1184.3, ESI†) and ESI-MS (Fig. S4, m/z = 1183.6, ESI†). Then a more complex dimeric-peptide (AE105, 11-mer peptide antagonist) was also successfully prepared *via* our methodology (entry 2, 54%), and the dimeric peptide was confirmed by MALDI-TOF-MS (Fig. S5, m/z = 2792.3, ESI†). In light of these results, we then turned our attention to the suitability of our method for preparing small disulfides. To our delight, every thiol substrate tested including aryl and alkyl thiols produced the corresponding disulfides in good to excellent yields (entries 3–8, 78–88%), with Fmoc-cysteine giving only moderate yield (entry 9, 42%). It is possible that the low yield of dimeric Fmoc-cysteine is attributable to DMAP-mediated Fmoc cleavage during the reaction. The structure of every disulfide product was confirmed by ESI-MS, ^{1}HNMR and ^{13}CNMR (ESI†). Based on the above results, our methodology mainly has the following advantages. Firstly, our research efforts have established that the bulky macrocycles of RGD analogues or small molecule thiols could be effectively converted into the corresponding disulfides in the presence of simple and cheap DMAP/UV. Secondly, this disulfide reaction can occur under mild reaction conditions and can tolerate a wide range of functional groups such as –OH and –COOH.

To evaluate whether dimeric RGD analogues prepared *via* our methodology maintained binding affinity and specificity for

Table 2 Synthesis of disulfides under UV/DMAP



Entry	Thiols (**1**)	Product (**2**)	Time (h)	Yield[a] (%)
1	RADfC (**1a**)	Dimer RADfC (**2a**)	3	75
2	AE105(**1b**)	Dimer AE105 (**2b**)	5	54
3	 **1 c**	 **2c**	0.5	80
4	 **1d**	 **2d**	0.5	82
5	 **1e**	 **2 e**	0.5	87
6	 **1f**	 **2 f**	0.5	88
7	 **1 g**	 **2 g**	0.5	82
8	 **1h**	 **2h**	0.5	78
9	 **1i**	 **2i**	0.5	42

[a] Isolated yields.

Table 3 IC_{50} values for mono and dimer RGD analogues

Entry	Peptides	IC_{50}/nM
1	Monomeric-RGDfC	68.0 ± 7.8 nM
2	Dimeric-RGDfC	25.0 ± 5.1 nM
3	E-$[RGDfK]_2$	46.0 ± 6.7 nM
4	Monomeric-RADfC	>800 nM
5	Dimeric-RADfC	>800 nM

integrin $\alpha_v\beta_3$, competitive cell binding assay using ^{125}I-echistatin as the integrin $\alpha_v\beta_3$ specific radioligand was performed on U87MG human glioblastoma cells.[23] The mono- and dimeric RGD and RAD analogues, and the obtained IC_{50} values are summarized in Table 3. As expected, the dimeric RGDfC peptide showed higher binding affinity (IC_{50} = 25.0 ± 5.1 nM) compared to the monomeric-RGDfC (IC_{50} = 68.0 ± 7.8 nM). The dimeric RGD peptide constructed by our method exhibited better binding affinity than the commercially available dimeric RGD (E-$[RGDfK]_2$, cyclic RGD, IC_{50} = 46.0 ± 6.7 nM). It is possible that the presence of a mini-PEG linker in E-$[RGDfK]_2$ decreases the binding avidity of RGD in this dimer. Finally, mono- and dimeric RAD showed non-specific binding to the integrin $\alpha_v\beta_3$, consistent with the *in vitro* study by Garanger *et al.*[24]

In conclusion, we have reported a novel and efficient method for the construction of disulfides from thiols using UV irradiation in the presence of DMAP. This method has been successfully applied to the construction of a library of dimeric RGD analogues. Moreover, the dimeric RGD analogues exhibited higher binding affinity than commercially available dimers. Finally, this methodology is amenable to the synthesis of other dimeric peptide-based small molecules or biomolecules. Their great versatility and flexibility are very important for future applications.

Experimental

Synthesis of AE105

The peptide AE105 (Cys-Gly-Asp-Cha-Phe-(D)Ser-(D)Arg-Tyr-Leu-Trp-Ser-NH_2) was synthesized on Tentagel S RAM resin using traditional Fmoc solid-phase peptide chemistry. After deprotection and cleavage from the resin using 93% TFA, 5% TIPS, and 2% H_2O for 2 h, the peptide was precipitated in cold Et_2O and washed with Et_2O three times. The dried peptide was purified by prep-HPLC and checked by MALDI-MS: *m*/*z* 1397.0.

Cell binding assay

U87MG cells (1×10^5) were suspended in 500 μL of DMEM seeded in 12-well tissue culture plates and incubated at 37 °C overnight. The plate was incubated with ^{125}I-echistatin in the presence of increasing concentrations of different RGD and RAD peptide analogues (0–1000 nM). After the cells were incubated for 2 h, the supernatant was removed and washed with binding buffer. Radioactivity was determined using a gamma counter. The best-fit 50% inhibitory concentration (IC_{50}) values for the U87MG cells were calculated by fitting the data with non-linear regression using Graph-Pad Prism (GraphPad Software, Int.).

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