ORGANIC LETTERS

2002
Vol. 4, No. 23
4041−4044

Peptide–Small Molecule Hybrids via Orthogonal Deprotection–Chemoselective Conjugation to Cysteine-Anchored Scaffolds. A Model Study

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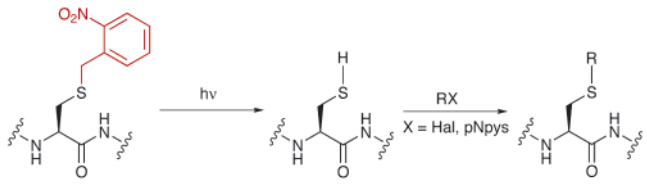
Received August 14, 2002

ABSTRACT



The feasibility of an orthogonal deprotection–conjugation protocol, holding the promise of libraries of functionally diverse chemical probes attached to cysteine-anchored peptide scaffolds, has been explored with a model system. The necessary tools for assembly of the hybrid libraries have been prepared and the tandem procedure optimized. S-Alkylation and S-sulfenylation are featured as the chemoselective ligation reactions.

In an attempt to enhance the potency of HIV entry inhibitors (i.e., CD4 mimetics), built on miniprotein (scorpion toxin) scaffolds,[1] we recently initiated a program targeting the *chemical* modification of the peptide leads, identified through *genetically encoded* diversity screening.[1c] Specifically, we wish to generate synthetic hybrids of the miniproteins, employing libraries of small (MW ≤ 250 Da) molecular probes of the functional space, thereby uniting the biological potential of amino acid-based scaffolds with the unlimited potential of the functional diversity of small molecules. The initial goal of this approach is to discover site-directed recognition motifs that are inaccessible with the 20 DNA-encoded building blocks. Since the miniprotein leads are expected to provide basal recognition in a structurally conserved mode, a significant advantage of the hybrid design would be the compilation of the structure/activity relationships (SARs) for what are otherwise weak binders outside of the context of the peptide scaffold. The long-term goal is the conversion of these conjugates into drug-like small-molecule ligands.

To rapidly map the SAR of the hybrid library, we envisioned late-stage chemoselective ligation (Figure 1) as a viable and, importantly, expeditious alternative to parallel synthesis of individual analogues. In the former case, peptide assembly, side chain deprotection, folding, and other processing steps can be optimized for a common, structurally characterized peptide precursor. Such *scaffolds* can then be split into separate reaction vessels for spatially addressable

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10.1021/ol026736d CCC: $22.00
Published on Web 10/12/2002

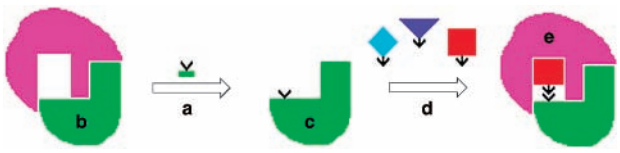


Figure 1. Schematic illustration of the post-assembly chemoselective ligation strategy: site-specific installation of orthogonal functionality (a) on the established peptide lead (b) provides a scaffold (c), from which uniquely reactive small-molecules (d) can be displayed (ligated) in a structurally conserved mode and then screened against the target (e) in a high-throughput fashion.

modification step(s), coupled directly to high-throughput screening.

A key requirement for the successful generation of such a library would be strict chemoselectivity during the conjugation. Several chemical transformations have been generally recognized as being fully orthogonal to the richly functionalized biopolymers while involving *mutually* and *uniquely* reactive functionalities.[2] The long-term stability of the resulting conjugates in the assay media, their unambiguous stereostructure, and the availability of the required diversity elements were also considered as critical criteria for the choice of the ligation reaction.

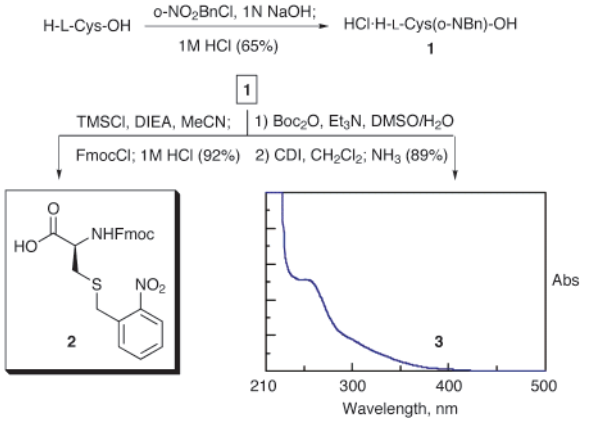
The natural amino acid cysteine is recognized as a unique chemical handle, enabling site-specific modifications to be conducted on peptides and proteins at the post-assembly stage.[3] Both chemoselective alkylations and sulfenylations can be used for library generation from an unpaired cysteine due to the enhanced reactivity of both the thiol and thiolate anion, toward respective electrophiles in the presence of potentially competing nucleophiles.

At the post-assembly stage, chemical manipulation of the highly reactive and sensitive cysteine residue requires that the unpaired thiol group remain uniquely masked by an appropriate protecting group, being unaffected by the conditions necessary for scaffold assembly. The subsequent release of the thiol handle must also be accomplished with no chemical or structural damage to the attendant functionalities. With these strict criteria in mind, we turned to photolysis-mediated unmasking, as a mild, reagent-free, and chemically orthogonal deprotection strategy. [4]

o-Nitrobenzyl (oNBn) heteroatom derivatives represent a class of UV-excitable chromophores capable of undergoing *intramolecular* hydrogen abstraction (Norrish-type II reaction) to afford hydrolysis-prone *o*-nitroso-benzaldehyde derivatives.[5] Both postsynthetic *caging* of a cysteine-containing peptide[6] and Boc peptide synthesis, inserting an oNBn-protected cysteine into a peptide,[7] have been reported. Therefore, we expected an analogous *S*-*o*-nitrobenzyl-protected cysteine congener to be compatible with Fmoc solid-phase peptide synthesis. In addition to chemical caging, an in vitro translation technique, utilizing a nonsense anticodon tRNA, has been successfully applied to generate a cysteine-caged analogue of a functional protein.[8] To the best of our knowledge, however, further chemical manipulations of the photoliberated cysteine have not been described. To optimize the proposed tandem deprotection−conjugation sequence and to prepare all the necessary tools for hybrid library generation, we initiated the model study that follows.

The requisite Fmoc *S*-*o*-nitrobenzyl cysteine **2** for solid-phase peptide synthesis was prepared from *o*-nitrobenzyl-protected amino acid hydrochloride **1**[9] by first masking the carboxylate group transiently as a trimethylsilyl (TMS) ester[10] prior to reaction with fluorenylmethylchloroformate (Scheme 1).[11] Model system **3** was also synthesized from the same precursor by blocking both the amine and carboxylate termini with the Boc and amido groups, respectively.

Scheme 1. Synthesis of the Caged Cysteine Congeners for Solid-Phase Peptide Synthesis (**2**) and for Photolysis Model Study (**3**)



The UV spectrum of the fully protected cysteine congener **3** (Scheme 1) features a λ_{max} at ca. 260 nm with an extended shoulder, potentially rendering the chromophore photosensitive up to 370−390 nm. Under such an irradiation regime, even the photosensitive amino acid tryptophan should remain intact. Deprotection of **3** was investigated via irradiation in a photoreactor at 366 nm with six 300 μW/cm^2 UV tubes

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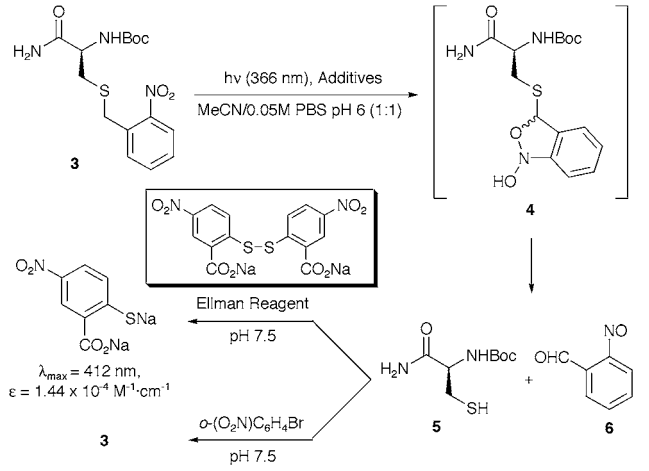
(11) The caged cysteine **2** is fully compatible with Fmoc solid-phase peptide synthesis: Manjappara, U.; Chaiken, I. M.; Savinov S. N.; Smith, A. B., III. Unpublished results.

placed at a distance of about 5 cm from the Pyrex reaction flask. A deoxygenated mixed solvent system (1:1 MeCN/0.05M PBS, pH 6) was employed to bring all substrates and reagents into one phase and to promote hydrolysis of the photochemically derived mixed *S,O*-acetal (Scheme 2).

Scheme 2. Effect of Additives on Photodeprotection



entry	Semicarbazide (5 mol. eq.)	L-(–)-Ascorbic acid (2 mol. eq.)	yield of **5**	yield of **3**
1	–	–	0	–
2	+	–	44	29
3	+	+	quant.	90

The extent of photolytic consumption of **3** proved to be highly dependent on substrate concentration. At concentrations of 20 mM or higher, the photoprocess stalled before reaching completion, hence the irradiations were carried out at 10 mM substrate concentration or lower. A kinetic analysis was performed by following the disappearance of the sulfide **3** (10 mM) via ^{1}H NMR (1:1 MeCN-d_3/0.05 M PBS, pH 6).[12] A complex kinetic pattern was observed, with the initial first-order reaction ($k_{1'} \approx 0.028$ min^{-1}) being substantially retarded after $t_{1/2}$ (ca. 25 min), presumably due to accumulation of competing byproduct chromophores. Hence, for reliable thiol photorelease, deprotection of the *o*-nitrobenzyl peptides must be conducted at low concentrations ($\leq$1 mM) so that the first-order kinetics persists throughout the reaction.

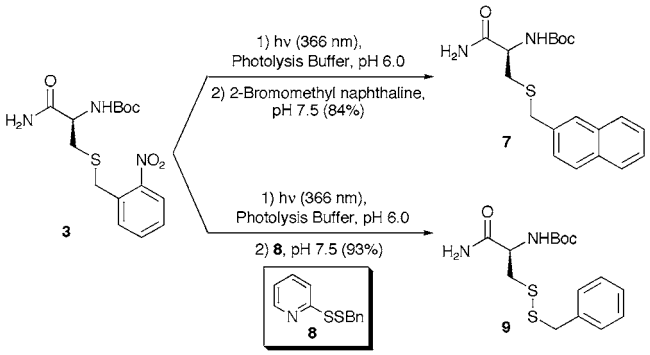
The initial photolysis experiment produced no thiol, according to the chromogenic sulfhydryl-selective Ellman reagent,[13] although near complete consumption of the photosubstrate could be demonstrated by both TLC and ^{1}H NMR. Side reactions, caused by the oxidative sensitivity of the desired thiol and the electrophilic nature of the released *o*-nitroso-benzaldehyde, were suspected to compromise the desired product recovery, via generation of oxidized byproducts (cystine, cysteic acid, etc.) and by the promiscuous reactivity of the aldehyde.

To preserve the chemical integrity of the thiol, inclusion of scavengers and traps was explored. However, it was not clear whether the presence of additional reagents would permit the subsequent ligation step. The critical effect of additives is illustrated in Scheme 2. Semicarbazide hydrochloride, a carbonyl scavenger,[5] led to significant thiol recovery (44%), as judged by the Ellman's assay. Simultaneous inclusion of semicarbazide and L-(−)-ascorbic acid, the latter a generic antioxidant,[14] further improved the photodeprotection yield to near quantitative.[15]

The free thiol was next subjected to alkylation with *o*-nitrobenzyl bromide (1.5 mol equiv), upon adjusting the pH of the reaction mixture to 7.5, effectively regenerating the protected cysteine in situ (Scheme 2). This ligation proved to be both efficient (90% yield) and rapid (ca. 10 min), as again judged by Ellman's assay. Importantly, both semicarbazide and L-(−)-ascorbic acid appeared to be fully compatible with the ligation step, even when present in significant excess.

Other representative electrophiles proved to be viable conjugation candidates. For example, electron-rich 2-naphthyl bromide was shown to react with the liberated thiol with an efficiency comparable to the electron-deficient *o*-nitrobenzyl bromide (Scheme 3). An alternative conjugation scenario,

Scheme 3. Photodeprotection Followed by in Situ Reaction of Liberated Cysteine with Representative Electrophiles



complementary from the structural perspective, was envisioned to involve disulfide formation.[16] Thus, 2-thiopyridine-activated[17] benzyl sulfide **8** was employed to obtain disulfide **9**.

Despite the promising results with the selected electrophiles, their use would be restricted in a high-throughput format, due to various factors. For example, the dependence of the alkylation rate on the π-orbital character[18] of the electrophile was recognized as a potential liability in expand-

(12) See Supporting Information.

(13) (a) Boyne, A. F.; Ellman, G. L. *Anal. Biochem.* **1972**, *46*, 639. (b) For a review, see: Tawfik, D. S. *Protein Protocols Handbook*, 2nd ed.; Walker, J. M., Ed; Humana Press, Inc.; Totowa, NJ, 2002; pp 483−484.

(14) Bendich, A.; Machlin, L. J.; Scandurra, O.; Burton, G. W.; Wayner, D. D. M. *Adv. Free Radical Biol. Med.* **1986**, *2*, 419 and references therein.

(15) Inclusion of L-(−)-ascorbic acid permitted the photodeprotection to be carried out without rigorous oxygen exclusion, in 96-well plates with minimal, if any, thiol photooxidation (see Supporting Information).

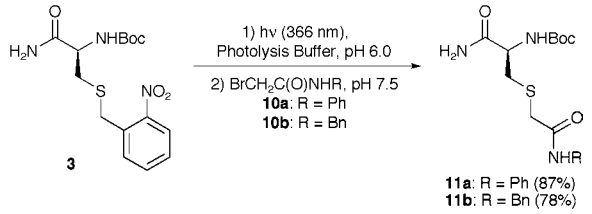
(16) For a review, see: Andreu, D.; Albericio, F.; Sole, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. *Methods in Molecular Biology: Peptide Synthesis Protocols*; Pennington, M. W.; Dunn, B. M., Eds.; Humana Press, Inc.; Totowa, NJ, 1994; Vol. 35, pp 91−169.

(17) Castell J. V.; Tun-Kyi, A. *Helv. Chim. Acta* **1979**, *62*, 2507.

ing the diversity of the arylmethyl halides. With 2-thiopyridine derivatives such as **8**, only a modest level of activation is accessible,[19] requiring use of a mildly basic buffer to effect disulfide exchange, a chemical feature likely to be detrimental to the integrity of existing disulfide bridges.

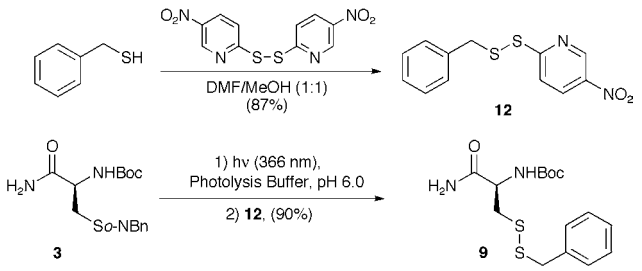
We therefore turned our attention to 2-bromo-acetamides and 2-bromo-acetanilides, a well-precedented class of protein modification reagents.[20] A generally higher[18] and, importantly, uniform level of reactivity can be expected from members of this alkylation library, essentially independent of the electronic nature of precursor amines and anilines. Two representative members **10a** and **10b** were readily prepared from aniline and benzylamine.[21] The deprotection−alkylation sequence was then conducted as before to provide conjugates **11a** and **11b** (Scheme 4).

Scheme 4. Conjugation of 2-Bromo-acetanilide **10a** and 2-Bromo-acetamide **10b**



The reactivity of the sulfenylation reagents can be predictably increased by lowering the pK_a of the thiol leaving group, without compromising the chemoselectivity of the process.[22] For example, stable and highly reactive 5-nitro-2-thiopyridine (pNpys)-activated sulfides are known to participate effectively in sulfenyl transfer at acidic pH.[23] The pNpys group could be conveniently introduced by treatment of a thiol with 2,2′-dithiobis(5-nitropyridine) in 1:1 DMF/MeOH, as shown in Scheme 5 for benzyl mercaptan. Unlike 2-pyridinedisulfide

Scheme 5. Preparation and Conjugation of Sulfenyl Transfer Reagent (pNpysBn)**12**



7, the pNpys reagent **12** reacted readily with sulfhydryl group without adjusting the pH of the photolysis buffer.[24]

In summary, an efficient orthogonal deprotection−chemoselective conjugation protocol has been demonstrated with a model cysteine. 2-Bromo-acetanilides, 2-bromo-acetamides, and pNpys-activated thiols proved to be the reagents of choice.[25] Studies to prepare and evaluate mini-protein scaffolds, incorporating orthogonally protected cysteine, are underway and will be reported in due course.

Acknowledgment. Support was provided by the National Institutes of Health through grant GM-056550 for financial support.

Supporting Information Available: Experimental details for the synthesis and characterization of compounds **1**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) For convenient, flash chromatography-free isolation of 2-bromo-acetamide products, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was utilized instead of 1,3-dicyclohexyl-carbodiimide (DCC) in a procedure otherwise identical to: Amblard, M.; Rodríguez, M.; Martinez, J. *Tetrahedron* **1988**, *44*, 5101. A small amount of 2-chloro-acetamides (<5%) was isolated with 2-bromo-acetamides as the result.

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(23) Rabanal, F.; DeGrado, W. F.; Dutton, L. *Tetrahedron Lett.* **1996**, *37*, 1347.

(24) The extent of the disulfide exchange can be conveniently monitored from the intense yellow color of the reaction mixture due to the release of 5-nitro-2-pyridinethiol.

(25) Pilot sublibraries of these crystalline, stable, and uniformly reactive electrophiles have been prepared (Savinov S. N.; Smith, A. B., III. Unpublished results) and will be described in the context of a hybrid library assembly.