ORGANIC LETTERS

2000
Vol. 2, No. 1
23−26

N^{α}-2-Mercaptobenzylamine-Assisted Chemical Ligation

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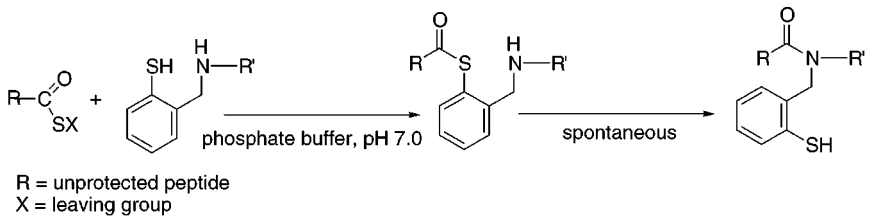
Received October 25, 1999

ABSTRACT

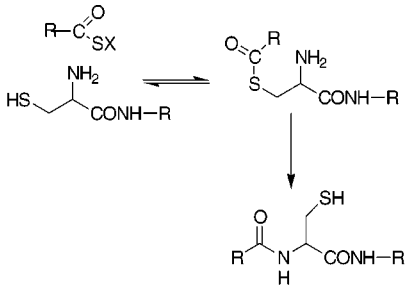


The selective formation of an amide bond in the presence of unprotected functional groups is a challenging problem of peptide chemistry. A 2-mercaptobenzyl group tethered at the N-terminus of model peptides was observed to facilitate amide bond formation when a peptide thioester was added under mild aqueous conditions.

The highly chemoselective reaction between an unprotected peptide thioester of either synthetically or biologically expressed origin and an unprotected N-terminal cysteine peptide to give a natural amide-bond linkage has empowered the field of synthetic protein engineering.[1] However, the requirement for an N-terminal cysteine residue is not always compatible with the synthetic target and additional ligation sites would relieve this constraint.[2] Ligation of a thioester peptide to even a single additional amino acid, such as glycine, could effectively double the number of ligation sites available in a protein sequence and allow for greater flexibility in the synthetic design of a protein.

Cysteine-mediated "native chemical ligation" occurs by a two-step mechanism. First, a reversible transthioesterification of the cysteine side chain by a C^{α}-thioester peptide results in an acylated N-terminal cysteine side chain. This thioester intermediate rapidly undergoes an acyl rearrangement, through a favorable five-membered ring, giving an amide bond at the ligation site (Scheme 1). The reaction between 1 equiv of each peptide proceeds at low peptide concentration (<10 mM) in neutral aqueous solution below 37 °C.

Scheme 1. Native Chemical Ligation[a]



[a] R = unprotected peptide; X = leaving group (either MPAL or benzyl).

In principle, reversible attachment of the functional equivalent of a cysteine side chain onto the N-terminus of a peptide would provide a universal ligation chemistry. This approach has been attempted using a cleavable aminooxy-

(1) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779. (b) Tam, J. P.; Lu, Y. A.; Chuan-Fa, L.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489. (c) Muir, T. W.; Sandhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710.

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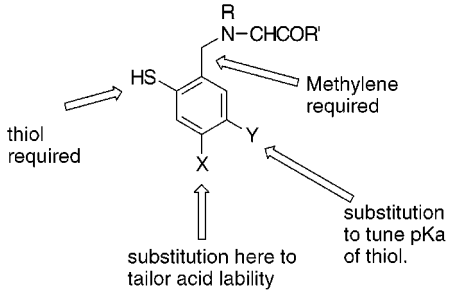
10.1021/ol991184t CCC: $19.00
Published on Web 12/14/1999

linked ethanethiol side chain.[3] However, because of slow rearrangement of the thioester intermediate through a less favorable six-membered ring onto an aminooxy group this system has been limited in application. Nevertheless, this study demonstrated the potential of N-linked thiol tethers for peptide ligation.

Acyl rearrangements using substituted 2-hydroxybenzylamine groups were explored by Kemp[4] and have been utilized extensively as the 2-hydroxy-4-methoxybenzyl (Hmb) group in backbone protection strategies for solid-phase peptide synthesis.[5] Hmb makes use of an O to N acyl shift via a constrained six-membered ring from the phenol to a secondary amine. However, acylation of the phenol group requires highly activated, protected amino acids, conditions incompatible with the ligation of unprotected peptides. In contrast, it has been shown that thiophenol can be easily acylated by peptide thioesters in the course of ligation reactions.[6] It is also well-known that thioesters undergo aminolysis much more easily at neutral pH than the corresponding oxygen esters.[7] These observations strongly suggest that substitution of the hydroxy group with a thiol would facilitate rapid acylation and maintain the rearrangement properties of the constrained six-membered ring. Consequently, an N-linked 2-mercaptobenzyl group could form the basis of a new peptide ligation chemistry (Scheme 2).

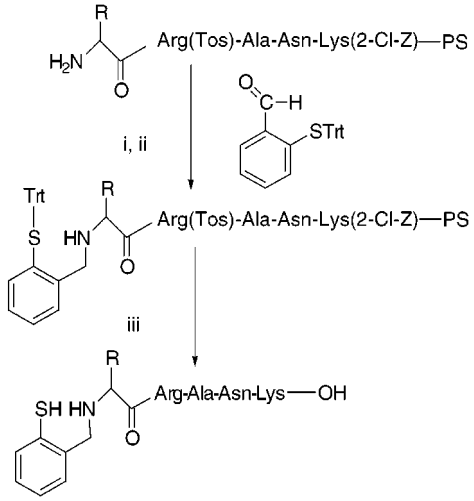
Scheme 2. Design of a 2-Mercaptobenzylamine Tether, Illustrating the Potential for Extended Chemical Ligation[a]



[a] In this work we have studied systems in which X = H, Y = H and X = H, Y = NO_2. An important design feature is an inherent safety catch: when R = H (benzylamine), the tether is acid-stable independent of X, and when R = acyl (benzylamide), it becomes acid-labile when X is a suitable substitution.[5]

The Hmb system has been intensively characterized and demonstrated to be compatible with peptide chemistry. Additionally it has several desirable practical features. (1) It can be simply prepared by either stepwise synthesis or reductive amination directly to the resin.[8] (2) The N-terminal benzylamine is stable to the strong acid conditions used for peptide cleavage/deprotection.[5c] (3) The benzylamide formed after a successful ligation is labile to strong acids. (4) The aromatic ring can be substituted to change its properties: for example, acid lability and nucleophilicity of the thiol functionality[5c,d] (Scheme 3).

Scheme 3. Preparation of a N^{α}-2-Mercaptobenzylamine-Substituted Peptide[a]



[a] (i) DMF/CH_3CO_2H (99:1), 30 min; (ii) $NaCNBH_3$, 1 h; (iii) HF, *p*-cresol (30:1), 0 °C; 1 h.

A characteristic feature of this intramolecular rearrangement is the large variation in acyl migration time observed across the range of 2-hydroxybenzylamine-substituted amino acids. The migration is arrested with increased bulk of the side chain. In anticipation of this effect, the ligation sites glycine, alanine, and valine were chosen to sample the different steric environments. The mercaptobenzyl group was incorporated into the sequences GRANK, ARANK, and VRANK by reductive amination of 2-tritylthiobenzaldehyde[9] onto the N-terminus of a resin-bound, side chain protected peptide (Scheme 3).

Previous studies from this laboratory demonstrated that ligation of the peptides LYRAX (where X is any amino acid thioester) onto CRANK, which have a representative range of side chain functionalities, can accurately model the ligation

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(4) (a) Kemp, D. S.; Vellaccio, F. *J. Org. Chem.* **1975**, *40*, 3464−3465. (b) Kemp, D. S.; Kerkman, D. J.; Leung, S.; Hanson, G. *J. Org. Chem.* **1981**, *46*, 490−498. (c) Kemp, D. S. *Biopolymers* **1981**, *20*, 1793−1804.

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(7) Jenks, W. P.; Cordes, S.; Carriuolo, J. *J. Biol. Chem.* **1960**, *235*, 3608−3614.

(8) (a) Ede, N. J.; Ang, K. H.; James, I. W.; Bray, A. M. *Tetrahedron Lett.* **1996**, *37*, 9097−9100.

(9) 2-Thiobenzaldehyde was prepared from 2-thiobenzyl alcohol (Lancaster) by the method of: Kasmai, H. S.; Mischke, S. G. *Synthesis* **1989**, 763−764. The trityl protection was added as described in: Annis, I.; Chen, L.; Barany, G. *J. Am. Chem. Soc*. **1998**, *120*, 7226−7238. The reductive amination of the peptide was initially problematic, especially for glycine, because of side reactions of thiosalicaldehydes with primary amines. Corrigan, M. F.; Rae, I. D.; West, B. O. *Aust. J. Chem*. **1978**, *31*, 587−594. Very low yields of the desired monoalkylated material were obtained; therefore, the thiol was protected with trityl to give, after reductive amination onto the resin-bound peptide, monoalkylated product in reasonable yield (50% after preparative HPLC).

rates of larger peptides.[2] Cysteine-mediated chemical ligation proceeds efficiently, with thioester peptides incorporating all the amino acids, excluding β-branched amino acids and proline. The results for LYRAA and LYRAF thioesters (Table 1) strongly suggest that mercaptobenzyl-tethered glycine peptides are compatible with a similar set of thioester peptides. Similarly, it is reasonable to predict from these results that glycine thioester peptides should ligate efficiently with mercaptobenzyl-tethered peptides containing unhindered N-terminal amino acids.

Table 1. Approximate Half-Lives for Ligations between Mercaptopropionic Acid Leucine (MPAL) Thioesters and N^{α}-2-Mercaptobenzylamine-Modified Peptides[a]

	$t_{1/2}$, h		
	GRANK	ARANK	VRANK
LYRAG	0.5	3.0	24 (<5%)
LYRAA	4.0	48.0	
LYRAF	5.0		

[a] Values were determined by inspection of analytical HPLC traces for time courses of each ligation, identified by E/S MS.[10] Ligation conditions: aqueous pH 7.0 buffered with phosphate (100 mM); 37 °C; individual peptide concentration of approximately 6 mM in the presence of tris(2-carboxyethyl)phosphine hydrochloride (20 mM).

Reducing conditions were necessary to prevent the formation of disulfides, and so the water-soluble reducing agent tris(2-carboxyethyl)phosphine hydrochloride was added. Phosphate buffer (100 mM) was used to maintain a neutral pH; below pH 6 the ligation slowed considerably. The ligation product was followed by analytical HPLC over a time course and identified by electrospray mass spectrometry.[10] However, this analysis does not differentiate between rearranged and unrearranged product, and unrearranged material was a serious problem in earlier investigations.[3] The identity of the product was confirmed by treating with hydroxylamine, which left the material unaltered (thioesters are labile), and also the product was observed to undergo complete aerial oxidation to a material with the correct mass profile for the disulfide (the unrearranged material could not form disulfide).

This matrix of ligation reactions suggests that reactions involving glycine at either the C or N position of the ligation junction, but excluding β-branched amino acids or proline, go to completion in under 24 h in neutral aqueous buffer at ~6 mM peptide concentration and are therefore of practical use in synthesis (Table 1). The low concentration of the peptides was chosen to model reasonable conditions for the ligation of larger peptide segments; peptide ligation reactions at concentrations as high as 50 mM have been reported.[11]

The thioester peptides used in this study were prepared as mercaptopropionic acid leucine (MPAL) esters.[2,12] These esters are not strongly activated to acylation. Therefore, thioester exchange with benzyl mercaptan was carried out prior to ligation. A marginal improvement in ligation time of the benzyl thioester peptide was observed (Figure 1).

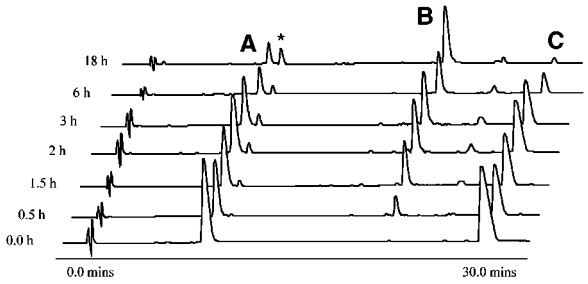

Figure 1. Analytical HPLC traces for the time course of the ligation reaction for LYRAG benzyl thioester (**C**) onto N^{α}-2-mercaptobenzylamine-ARANK (**A**) to give product (**B**). The asterisk denotes the putative thiol leaving group. Ligation conditions: see Table 1. Analytical HPLC conditions: Vydac C4 column (5 μm, 0.46 × 15 cm), 0−30% B in A, linear gradient for 30 min (1 cm^3 min^{-1} 215 nm UV monitor detection) where buffer A is 0.1% aqueous TFA and buffer B is 90% acetonitrile−10% buffer A.

However, more activated thioesters can be prepared, such as the 3-carboxy-4-nitrophenyl thioester.[1a,3]

Modification of the benzyl ring can alter the reactivity of the thiol (Scheme 2). Substitution of a nitro or similar group into the 2-hydroxybenzyl skeleton has been shown to dramatically accelerate rearrangement.[4b,c,5d] However, peptide ligations with the analogous 2-mercapto-4-nitrobenzylamine[13] series under conditions identical with those for the 2-mercaptobenzylamine series were unsuccessful, suggesting that the concomitant loss of nucleophilicity of the thiol group suppressed the initial transthioesterification. Further exploration of avenues to increase the generality of this ligation will be guided by previous investigations of the 2-hydroxybenzylamine system.[4,5]

The quantitative, specific coupling of single equivalents of unprotected peptides at a position of choice has long been a stated goal of peptide chemists.[4,14,15] Although peptide ligation using mercaptobenzylamine anchored to an amine group on an unprotected peptide has been demonstrated, subsequent detachment from the product has not. Future studies will involve tailoring the mercaptobenzyl skeleton

(10) Electrospray ionization MS data for products shown in Table 1: LYRAG(**Mba**)GRANK M, obsd 1227 (calcd 1227.5); LYRAG(**Mba**)-ARANK M, obsd 1242.5 (calcd 1242.5); LYRAF(**Mba**)GRANK M, obsd: 1319 (calcd 1318.6); LYRAG(**Mba**)VRANK M, obsd 1268.5 (calcd 1269.5).

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(12) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111−117.

(13) 5-Nitro-2-thiobenzaldehyde was synthesized from 2-chloro-5-nitrobenzaldehyde (Acros) by the published method: Korobov, M. S.; Minkin, V. I.; Nivorozhkin, L. E. *J. Org. Chem. USSR (Engl. Transl.)* **1975**, *11*, 826−831. It was unnecessary to protect the thiol group during reductive amination.

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for lability (Scheme 2). A reversible group for assisting chemoselective ligation would deliver highly flexible routes for protein synthesis. This concept would also be generally applicable to chemoselective amide bond formation between any two moieties to generate a variety of natural products or bioconjugates.[16]

Acknowledgment. We thank The Skaggs Insitute for Chemical Biology and the NIH (Grant No. R01GM59380) for financial support.

OL991184T

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