Peptide ligation *via* side-chain auxiliary† ‡

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A new peptide ligation strategy based on a side-chain auxiliary was developed; the auxiliary is fairly simple and can be removed, without product isolation, under basic conditions.

Intramolecular acyl transfer to form an amide bond is the basis of several peptide ligation strategies. This step is markedly accelerated by a high local concentration of the reactive groups *i.e.* a proximity effect. Wieland and Brenner[1] were the first to show the importance of an entropic activation/proximity effect to peptide bond formation by intramolecular acyl transfer. The pioneering work of the Kemp group on the intramolecular O → N acyl transfer in peptide segment coupling using the dibenzofuran auxiliary brought these concepts into practice for the synthesis of large peptides.[2] Unarguably, in large polypeptide synthesis, native chemical ligation (NCL) and expressed protein ligation (EPL), employing S → N acyl transfer, have been the most effective ligation methods.[3]

Mimicking the cysteine function in NCL/EPL with a removable auxiliary is a promising approach in assisting S → N acyl transfer at various amino acid junctions. In this regard, peptides with N-linked auxiliaries such as 1-phenylethanethiol and 2-mercaptobenzyl have been investigated and successfully applied to the synthesis of large peptides that correspond to folded proteins.[4] In these elegant examples the attachment of the auxiliary to the N-terminal peptide generates a secondary amine, which is engaged in the S → N acyl transfer. On the other hand, this leads to an increased steric hindrance at the transition state, rendering efficient ligation mainly at the Gly–Gly junction.[5]

Recently, we introduced a new glycopeptide ligation approach named sugar-assisted ligation (SAL).[6] In SAL, instead of anchoring an auxiliary to the N-terminal peptide, we take advantage of the existing sugar by slightly modifying its acetamido group on the C2 position to facilitate the ligation.[6] This in turn allows the primary amine to be engaged in the acyl transfer in contrast to previous methods where a secondary amine is employed. Following SAL, the sugar moiety can be cleaved enzymatically using PNGase A, affording the peptide structure.[6b] However, in our previous studies, the enzymatic removal of the sugar was not efficient, providing the desired peptide in a very low yield (10–15%). Moreover, using SAL in which the sugar moiety serves as a removable auxiliary for the synthesis of peptides rather than glycopeptides would be an expensive and tedious method.

In principle, a removable auxiliary with similar characteristics to the sugar moiety can be attached to one of the amino acid side-chains in order to assist peptide ligation. Here we report that the removable thiol auxiliary based on cyclohexane and cyclopentane molecules can indeed mimic the sugar role in SAL and facilitate S → N acyl transfer for peptide ligation.

Our design principle of utilizing a side-chain auxiliary to assist peptide ligation is described in Scheme 1. A thiol-modified cyclohexane is attached through an ester bond with the carboxylate side-chain of an aspartic acid (Asp) or glutamic acid (Glu) residue.

The modified cyclohexane is meant to play two crucial roles. First, to allow capture of the thioester peptide through the transthioesterification step. Second, it will serve to position the N-terminal amine and the acyl group in proximity so rapid and chemoselective S → N acyl transfer can occur, hence acting as a rigid template for facilitating acyl transfer. As in SAL, the peptide is extended with an extra amino acid, next to the residue bearing the auxiliary, to allow efficient ligation. Finally, the auxiliary can be removed through a saponification step to generate the unmodified peptide.

To test our strategy, we first synthesized building block **1**, starting from the racemic mixture of *trans*-amino cyclohexanol, in order to include it close to the N-terminal peptide (ESI†). Peptide synthesis was carried out on Fmoc-Rink amide resin employing routine HBTU–DIEA coupling conditions (Scheme 2A). Upon completion of coupling building block **1**, the resin was split into four portions in which different amino acids (Gly, Ala, His, Asp) were coupled to the free N-terminal peptide to generate peptides **3a–3d** as diastereomeric mixtures in 55–65% yield (ESI†).

To test the ligation reaction, peptides were dissolved at a final concentration of 5 mM in 6 M guanidine·HCl, pH 7.5–8 (1 : 1.2 molar ratio of peptide to thioester). The ligation reactions were performed at 37 °C and the progress of each reaction was monitored using analytical HPLC and MALDI-TOF. Upon completion of the ligation reaction, the pH was increased to 10 by addition of 1 M NaOH. The reaction was left at this pH for 5 min, at room temperature, during which a full hydrolysis of the auxiliary was achieved (Fig. 1B). Notably, the auxiliary removal is done *in situ* in contrast to other studies, including SAL, where the ligation product is isolated first and then subjected to auxiliary removal.[4]

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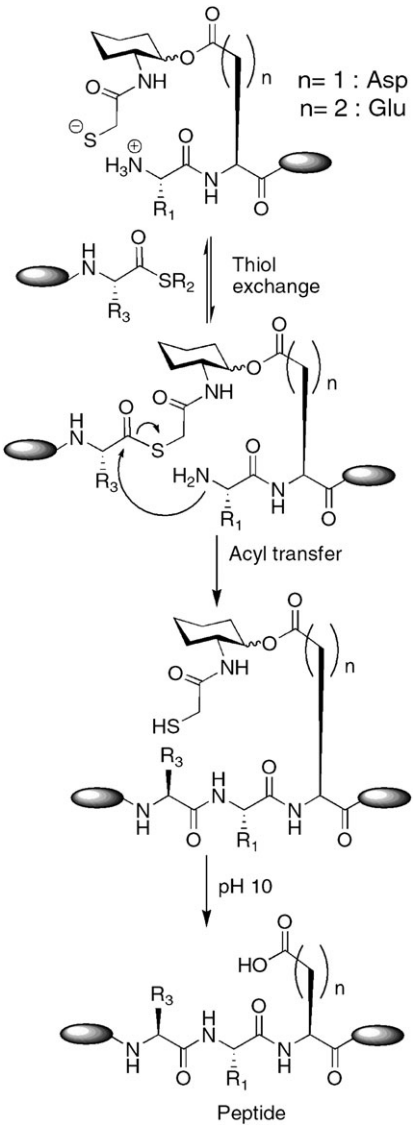
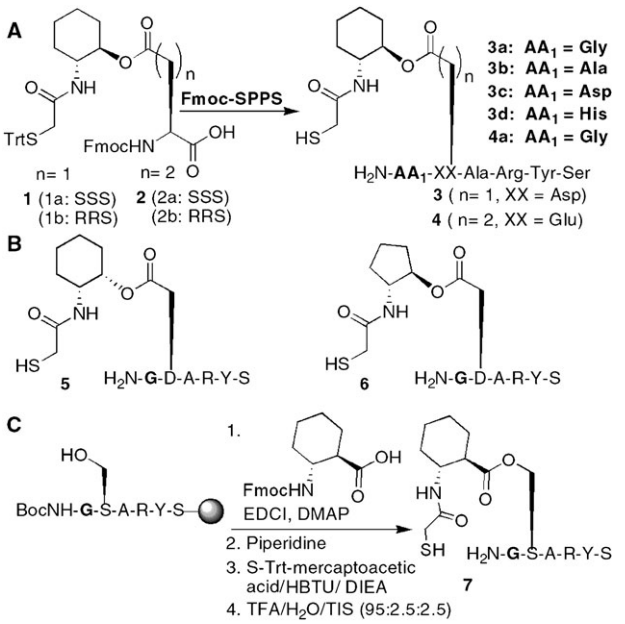
‡ Dedicated to Professor Ehud Keinan on the occasion of his 60th birthday.



Scheme 1 Proposed mechanism for ligation *via* side-chain auxiliary. R_1, R_3 are variations of amino side-chains, R_2 = $-CH_2CH_2CONH_2$.

The rates reported in Table 1 provided the following insights when compared to SAL. (1) Similar to SAL and in contrast to NCL,[7] the ligation reaction proceeds through S → N acyl transfer as the rate determining step (ESI†). (2) The ligation reaction tolerates different amino acids at the N-terminal of peptide-(auxiliary) with preference for amino acids that bear side-chains serving as a general base in the ligation pathway (Entries 2 and 3, Table 1) and those that are sterically less hindered (Entry 1, Table 1). (3) The observed ligation rate is affected by the C-terminal amino acid of the peptide thioester (Entries 8, 9 and 10, Table 1). (4) The ligation is chemoselective and compatible with the presence of amine and hydoxyl groups. In this regard, similar ligation results were obtained when the thioester peptide included Lys residues (ESI†). (5) Despite the changes in the ligation junctions, the desired product was observed as the major peak by HPLC analysis. Preparative HPLC of the ligation reactions gave the desired peptides, after auxiliary removal, in 60–70% overall yield (Table 1).



Scheme 2 Peptides bearing side-chain auxiliaries. (A) Aspartic acid and glutamic acid bearing the *trans*-cyclohexane-based auxiliary and its incorporation into model peptides. (B) Peptides containing *cis*-cyclohexane- and cyclopentane-based auxiliaries. (C) Synthesis of peptide with the auxiliary attached to a serine side-chain.

Since the connectivity between the auxiliary and side-chain of aspartic acid is an ester, aspartamide formation is a competing reaction.[8] Careful analysis of the ligation reaction (Entries 2 and 9, Table 1) revealed that after 24 h reaction time, ~10% of the ligation product undergoes aspartamide formation (peak c, Fig. 1A). However, this side product, which generates isoaspartyl residue, increased during ester hydrolysis to 20% of the final ligation product, as we found after careful

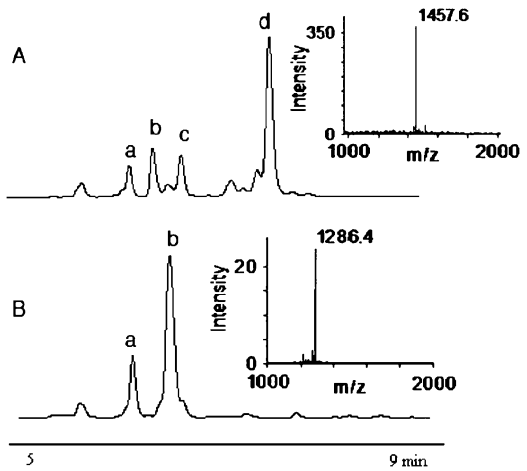
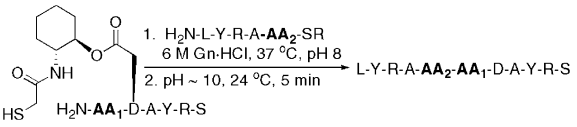


Fig. 1 Representative analytical HPLC traces/(MALDI-TOF/MS) of ligation reactions (Entry 2, Table 1): peak a, thioester hydrolysis with the expected mass of 579.27 ± 0.2 (1.2 equiv. of peptide thioester was used in the ligation reaction); peak b, unreacting thioester with the expected mass of 666.32 ± 0.2; peak c, byproduct generated from aspartamide formation with the expected mass of 1268.6 ± 0.2; peak d, ligation product with the expected mass of 1457.6 ± 0.2 Da. (B) Ligation reaction after auxiliary removal (pH 10, 5 min): peak a, hydrolyzed thioester; peak b, ligation product with the expected mass of 1286.4 ± 0.2 Da.

Table 1 Scope of peptide ligation *via* side-chain auxiliary



Entry	-AA_2-	-AA_1-	-AA_2-AA_1-	t/h[a]	Obsd mass	Isolated yield (%)[b]
1	Gly	Gly	Gly-Gly	~8	1228.7 ± 0.2	68
2	Gly	Asp	Gly-Asp	~6	1286.4 ± 0.2	70
3	Gly	His	Gly-His	~6	1308.3 ± 0.2	68
4	Gly	Ala	Gly-Ala	~24	1242.6 ± 0.2	60
5	His	Gly	His-Gly	~12	1308.6 ± 0.2	60
6	His	His	His-His	~8	1388.4 ± 0.2	62
7	His	Ala	His-Ala	~24	1322.5 ± 0.2	59
8	Ala	Gly	Ala-Gly	~36	1242.5 ± 0.2	62
9	Ala	His	Ala-His	~30	1322.5 ± 0.2	62
10	Ala	Asp	Ala-Asp	~24	1300.5 ± 0.2	63

[a] t represents the time at which over 80% of the peptide-(auxiliary) was consumed with the major peak being the desired product. [b] Reported yield is for ligation and auxiliary removal steps. R = $-CH_2CH_2CONH_2$.

separation of the ligation product (ESI†). In a peptide sequence that contains -Asp-AA-, where AA is a sterically hindered amino acid, aspartamide formation should decrease substantially and should not be observed if AA is proline. In principle, the use of backbone protection or cleavage of the ligation product with HF would eliminate this side reaction, regardless of the nature of the aspartyl α-carboxy amide bond.[8]

In SAL, we found that regardless of the glycopeptide type (α-O-linked, β-O-linked and N-linked glycopeptide),[6] the ligation rates were similar. To evaluate the effect of the substituents' configuration on our new auxiliary, we compared the rates of ligation for peptides bearing the *trans*- and *cis*-cyclohexane-based auxiliaries. We also prepared a similar peptide sequence bearing the cyclopentane-based auxiliary **6**, to examine the effect of the ring type on the ligation rate (Scheme 2B). The three peptides with the Gly extension, **3a**, **5**, **6**, were reacted with the C-terminal glycine thioester under similar ligation conditions. Notably, our results showed no differences in the ligation rates between the three types of auxiliaries. This could be explained by the large ring that the reaction uses to undergo S → N acyl transfer, in which the changes in the configuration and ring type of the different auxiliaries can be tolerated.

The successes with the auxiliary anchored to the side-chain of aspartic acid prompted us to explore the attachment of our auxiliary to the glutamic acid side-chain. Similar synthetic schemes were adopted to prepare the necessary building block and peptide-(auxiliary). Peptide **4a** bearing the glycine extension was ligated to a C-terminal glycine thioester. Interestingly, the time of the reaction increased to 12 h compared to 8 h with peptide **3a**. The slower rate could be explained by the increase in the flexibility of the glutamic acid side-chain due to the extra methylene group, which leads to a less favorable S → N acyl transfer.

Ligation assisted by an auxiliary which is attached to the serine side-chain was also examined. The synthesis of the desired peptide-(auxiliary) was fully accomplished on the solid support (Scheme 2C). Peptide **7**, bearing the Gly extension, was ligated under similar conditions to a C-terminal glycine thioester. In this case, the ligation went to completion at a slightly faster rate (6 h) compared to ligation with peptide **3a** (8 h) (ESI†). Auxiliary removal was accomplished under similar conditions to give the product in 70% yield. A similar trend was observed when the N-terminal glycine in peptide **7** was substituted with alanine and compared with peptide **3b**. Here, the reaction was completed in 16 h compared to 24 h with peptide **3b**. Although in the serine-(auxiliary) and aspartic acid-(auxiliary) the reactions use a similar ring size for the acyl transfer, the ester bond in peptide **7** is reversed compared to the one in peptide **3**, which seems to favorably affect the S → N acyl transfer reaction. Interestingly, when mercaptoacetic acid was attached directly to the serine side-chain, without the cyclohexane moiety, only 15–20% of the ligation product was observed, despite extending the reaction time to 24 h. In this example, the reaction proceeds through an 11-membered ring transition state, which is similar to the ring size of the transition state in the dibenzofuran auxiliary.[2] While the ring size is an important factor, the rigidity of the template/auxiliary to position the nucleophilic amine to the acyl group is crucial.[9]

In summary, we have shown that attaching a removable auxiliary to the side-chain of an amino acid to allow for the primary amine to be involved in the ligation reaction results in higher flexibility at the ligation junction. Moreover, three amino acid side-chains successfully served to anchor the auxiliary, thereby expanding the scope of peptide ligation beyond the cysteine residue.

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