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Synthesis and Application of an Auxiliary Group for Chemical Ligation at the X-Gly Site

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Abstract—An efficient synthesis of an auxiliary group, the 2-mercapto-4,5-dimethoxybenzyl (Dmmb) moiety, to form a Gly-building block is presented. The building block was incorporated into peptides to study the reaction with thiobenzyl-activated derivatives. The target peptides have been obtained by standard chemical ligation reaction, followed by TMSBr-assisted cleavage to remove the auxiliary group. Prior to Dmmb removal, under acidic conditions an unexpected rearrangement was observed and evidence for a mechanism is provided. © 2002 Elsevier Science Ltd. All rights reserved.

During recent years, Native Chemical Ligation (NCL) has become one of the most powerful methods for protein synthesis.[1] The ability to synthesize longer polypeptides by the solid-phase methodology followed by assembly to proteins has a major impact when trying to assess functional aspects of interesting domains. However, for the classical approach, a crucial Cys residue at the N-terminal of one of the fragments is required to achieve ligation at an X-Cys site.

Recently, in several papers an extension of the NCL method has been reported to address the X-Gly site. The 2-mercaptoethoxy,[3] the phosphinobenzenethiol,[4] and the 2-mercaptobenzyl group[5] have been applied for chemical ligation. In addition, while our work was in progress, the 1-phenyl-2-mercaptoethyl[6–8] and the 2-mercapto-4,5-dimethoxybenzyl group[9] (Dmmb) have been described. In the latter contribution, the Gly building block has been obtained by reductive amination of an N^{α}-glyoxylyl peptide, produced by periodate oxidation of an N-terminal Ser, with 2-tritylsulfanyl-4,5-dimethoxybenzylamine (**2**). Ligation has been performed with peptides containing a C-terminal Gly-thioester to address the Gly-Gly ligation site and removal of the Dmmb group has been accomplished using 1 M TFMSA in TFA.

Herein, we wish to report more detailed investigations on the Dmmb group. Starting from commercially available 2-mercapto-4,5-dimethoxybenzylamine (**1**), we have synthesized the corresponding Gly-derivative **3** in three steps in an overall yield of 24% (see Fig. 1). 1.2–1.5 equivalents of compound **3** were activated with equimolar amounts of TATU and DIEA (2 equivalents with respect to TATU) at ambient temperature and the coupling was carried out for 4 h. In all cases, the Kaiser test[10] indicated quantitative acylation.

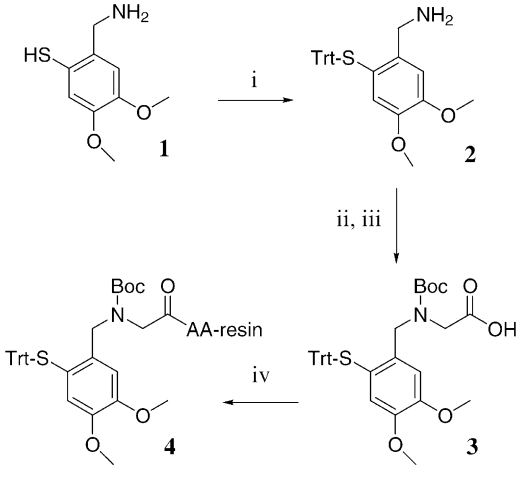

Figure 1. Synthesis of a Dmmb-modified peptide: (i) Trt-Cl, DMF, 87%; (ii) methyl bromoacetate, DIEA, DMF, 40%; (iii) 2 N NaOH, THF then Boc_2O in situ, 68%; (iv) H-peptidyl resin, TATU, DIEA, DMF.

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Alternatively, we have also used **2** to form the Gly derivative on the resin after reaction with the resin-bound bromoacetyl-peptide. However, we felt more confident to incorporate the building block after synthesis according to the Fmoc strategy, since standard coupling procedures can be applied.

In this study, two different peptide-thioesters, H-Tyr-Ser-Leu-SBzl **6** and Z-Lys-SBzl **7**, have been used to produce the target peptides listed in Table 1.

In the case of the Dmmb group, the reaction is thought to pass through a six-membered ring in the transition state, followed by a rearrangement to produce the desired amide bond. In the first step, we have examined the kinetics in comparison to the NCL method, in which ligation proceeds through a five-membered ring transition state.

The time course of the ligation reactions under standard conditions, that is 0.1 M phosphate buffer, 6 M guanidine, 40 mM TCEP, 3% thiophenol, pH 7.4, was followed by analytical HPLC. In the native situation **8**, the reaction was finished after 6 h (Fig. 2). In the case of the Dmmb group **9**, 25 h were required to achieve quantitative conversion to the ligated product.

We have anticipated that the Dmmb group would be cleaved under acidic conditions, for example >90% of TFA, as commonly applied in peptide synthesis. However, the removal of the Dmmb group using a variety of scavengers in TFA was not successful. Surprisingly, an earlier eluting peak as compared to the purified ligation product was observed when injecting directly the TFA cleavage solution onto an RP-HPLC column. Attempts to isolate this new, more polar compound failed. Both ligation products, **9** and **11**, displayed a similar behaviour upon treatment with TFA. LC–MS analysis revealed the identical mass for the early eluting peak and the ligation product, thus we assumed the existence of an equilibrium between the amide bond-containing ligation product and the intermediate thioester which is formed in the first step of the ligation (Fig. 3A).

Table 1. List of synthetic peptides

Compd	Peptide
8	Z-Lys-Cys-Pro-Trp-Trp-OH
9	Z-Lys-(Dmmb)Gly-Pro-Trp-Trp-OH
10	Z-Lys-Gly-Pro-Trp-Trp-OH
11	H-Tyr-Ser-Leu-(Dmmb)Gly-Ala-Tyr-OH
12	H-Tyr-Ser-Leu-Gly-Ala-Tyr-OH
13	H-Tyr-Ser-Leu-(S-methyl-Dmmb)Gly-Ala-Tyr-OH
14	(H-Tyr-Ser-Leu-(Dmmb)Gly-Ala-Tyr-OH)$_2$

The following results support this suggestion and are rationalized in Figure 3:

1. the earlier eluting peak completely disappears if the pH is raised to 8;
2. no earlier eluting peak is produced after selective methylation of the sulfur of the Dmmb group to obtain **13**[2] followed by TFA treatment;
3. no earlier eluting peak could be detected after oxidation to the disulfide containing dimer **14** followed by TFA treatment.

Interestingly, the modified Dmmb group attached to compound **13** could be cleaved upon prolonged treatment (20 h) in a TFA solution (93% TFA, 5% H_2O, 1% TIPS, 1% EDT). Under these conditions at ambient temperature, compound **11** was almost completely converted to the putative internal thioester.

Since postsynthetic methylation may be cumbersome, we were looking into further methods for Dmmb removal. For this reason, cocktails containing 1 M TMSBr, 1 M thioanisole, 1% EDT in TFA and 1 M TFMSA, 1 M thioanisole in TFA[9] have been tested. The cleavage reactions to obtain compounds **10** and **12** were carried out at ambient temperature for 1 and 20 h, respectively. The Dmmb group could be successfully cleaved and in the case of the TMSBr-assisted cleavage,

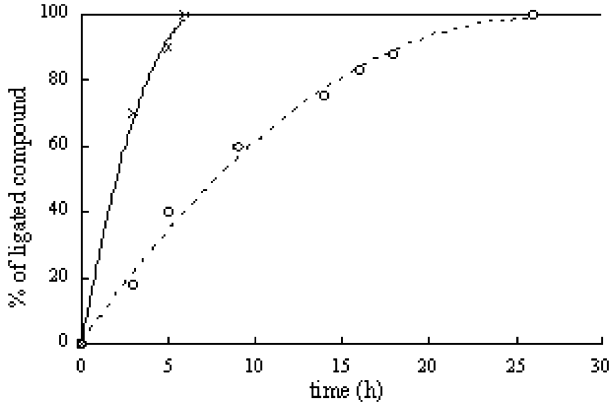

Figure 2. Plot of the ligation kinetics. Formation of **8** (solid line) and **9** (dotted line). Peptide concentrations were 6 mM and the reaction was carried out at ambient temperature. Relative peak areas in the HPLC chromatograms were used to calculate the percentage of conversion.

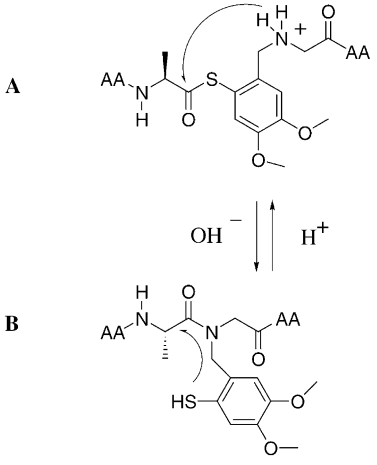

Figure 3. Rearrangement of the ligation product under acidic conditions: (**A**) internal thioester; (**B**) ligation product containing the amide bond.

the target peptides **10** and **12** were obtained in yields (Dmmb cleavage followed by HPLC purification and lyophilization) of 52 and 70%, respectively. Due to the superior quality of the crude target peptide obtained after TMSBr-assisted cleavage, we recommend to use 1 M TMSBr, 1 M thioanisole, 1% EDT in TFA for removal of the Dmmb group.

In conclusion, we have described a new method for the synthesis of a Gly-derivative containing the 2-mercapto-4,5-dimethoxybenzyl moiety (Dmmb). This approach is compatible with regular Fmoc chemistry since the building block is incorporated in a standard coupling reaction. The use of a TMSBr-assisted cleavage procedure, instead of the previously reported TFMSA treatment[9] to remove this auxiliary group, improved the quality of the crude product. Furthermore, we have elaborated on the interesting feature of a rearrangement from an amide bond to an internal thioester under acidic conditions.

In addition, we have demonstrated that the Dmmb group can be successfully applied for ligation sites other than Gly-Gly. The efficacy of the Dmmb group in the reaction involving Leu-Gly and Lys-Gly ligation sites has been demonstrated and the kinetics of the ligation reaction using the Dmmb auxiliary have been compared to the corresponding situation for native chemical ligation.[11]

References and Notes

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11. Abbreviations: AA: amino acid; DIEA: diisopropylethylamine; Dmmb: 2-mercapto-4,5-dimethoxybenzyl; EDT: 1,2-ethanedithiol; Fmoc: 9-fluorenylmethoxy-carbonyl; NCL: native chemical ligation; RP-HPLC: reversed-phase high performance liquid chromatography; TATU: *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TBTU: *N*-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methanaminium tetrafluoroborate *N*-oxide; TCEP: tris-(2-carboxyethyl)-phosphin hydrochloride; TFA: trifluoroacetic acid; TFMSA: trifluoro-methanesulfonic acid; TIPS: triisopropyl-silane; TMSBr: trimethylsilyl bromide; Trt-Cl: trityl chloride.