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Synthesis of Glyco(lipo)peptides by Liposome-Mediated Native Chemical Ligation

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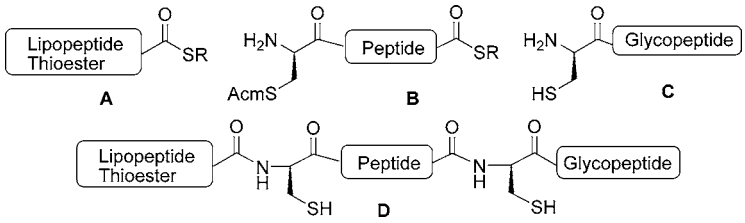
ABSTRACT



Although native chemical ligation (NCL) is emerging as a powerful method for the assembly of (glyco)peptide building blocks, its applicability is reduced when peptide segments are poorly soluble in aqueous buffer. We have found that incorporating reactants in liposomes allows NCL of lipophilic peptides and lipopeptides. Furthermore, the reaction rates of *liposome*-mediated NCL are higher than traditional reaction conditions resulting in improved yields.

Recently, we demonstrated[1] that the three-component vaccine candidate **1** (Figure 1) composed of the tumor-associated Tn

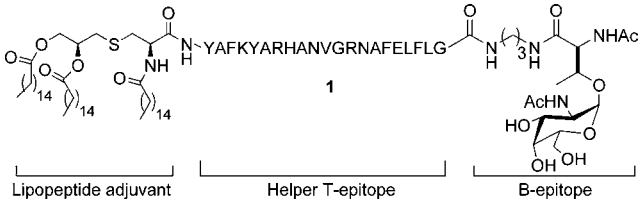


Figure 1. Fully synthetic vaccine candidate **1**.

antigen,[2−4] the peptide T-epitope YAFKYARHANVGRNAFELFL (YAF),[5] and the lipopeptide *S*-[(*R*)-2,3-dipalmitoyloxy-propyl]-*N*-palmitoyl-(*R*)-cysteine (Pam_3Cys)[6,7] can elicit IgG antibody responses.

This finding was significant because it had been difficult to elicit relevant immune responses against tumor-associated carbohydrates.[8,9]

To optimize the immunological properties of a three-component vaccine, a synthetic methodology was required, which would allow a convenient assembly of a number of B- and T-epitopes and lipopeptide adjuvants into a range of vaccine candidates. During our investigation, we discovered that *liposome*-mediated native chemical ligation (NCL) is a

(1) Buskas, T.; Ingale, S.; Boons, G. J. *Angew. Chem., Int. Ed.* **2005**, *44*, 5985−5988.
(2) Springer, G. F. *Science* **1984**, *224*, 1198−1206.
(3) Kagan, E.; Ragupathi, G.; Yi, S. S.; Reis, C. A.; Gildersleeve, J.; Kahne, D.; Clausen, H.; Danishefsky, S. J.; Livingston, P. O. *Cancer Immunol. Immunother.* **2005**, *54*, 424−430.
(4) Toyokuni, T.; Dean, B.; Cai, S. P.; Boivin, D.; Hakomori, S.; Singhal, A. K. *J. Am. Chem. Soc.* **1994**, *116*, 395−396.
(5) Wiertz, E.; van Gaans-van den Brink, J. A. M.; Gausepohl, H.; Prochnickachalufour, A.; Hoogerhout, P.; Poolman, J. T. *J. Exp. Med.* **1992**, *176*, 79−88.
(6) Spohn, R.; Buwitt-Beckmann, U.; Brock, R.; Jung, G.; Ulmer, A. J.; Wiesmuller, K. H. *Vaccine* **2004**, *22*, 2494−2499.
(7) Metzger, J.; Jung, G.; Bessler, W. G.; Hoffmann, P.; Strecker, M.; Lieberknecht, A.; Schmidt, U. *J. Med. Chem.* **1991**, *34*, 1969−1974.
(8) Kuduk, S. D.; Schwarz, J. B.; Chen, X.-T.; Glunz, P. W.; Sames, D.; Raghupathi, G.; Livigston, P. O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 12474−12485.
(9) Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836−863.

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useful approach that greatly increases the reaction rates and yields of ligations of sparingly soluble peptide reactants. Importantly, for the first time, the new approach makes it possible to employ lipidated peptides in NCL. The methodology is also attractive for NCL of lipophilic peptides, which usually give low yields of products under classical reaction conditions.

NCL is a chemoselective reaction that occurs at physiological pH between an N-terminal cysteine residue and a C-terminal peptide thioester.[10−12] In the first step of ligation, a reversible trans-thioesterification takes place between the C-terminal thioester and the sulfhydryl group from the N-terminal cysteine residue. The ligated peptide thioester then undergoes a rapid, irreversible, and spontaneous intramolecular S→N shift, generating the thermodynamically favored native amide bond at the ligation junction. NCL occurs uniquely at an N-terminal cysteine residue regardless of the presence of any additional internal cysteine residues and, as this ligation method is compatible with both carbohydrates and peptides, provides access to glycopeptides. NCL is emerging as a powerful tool to assemble highly complex (glyco)peptides and small proteins.

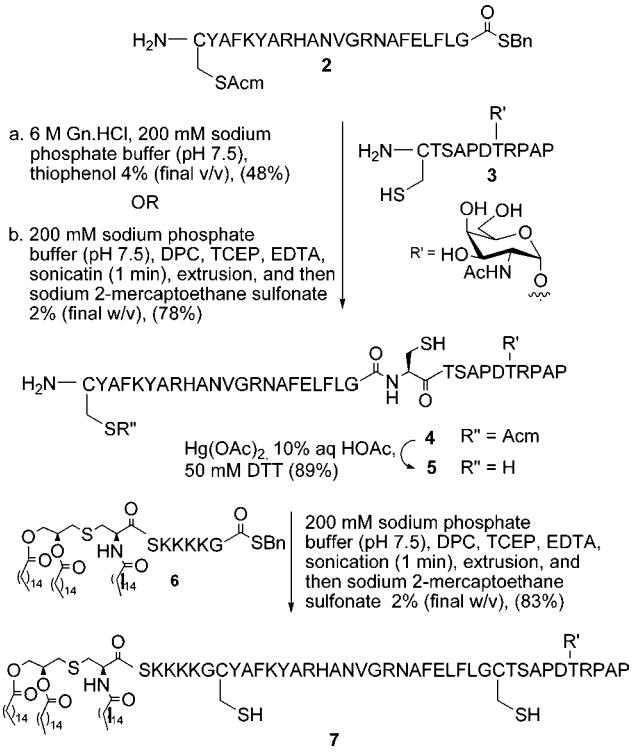
Compound **7**, which is composed of the tumor-associated glycopeptide derived from MUC-1,[13] the well-documented T-cell epitope YAFKYARHANVGRNAFELFL (YAF),[5] and the lipopeptide Pam_3CysSK_4,[6,7] was selected as a synthetic target. It was envisaged that this compound could be prepared from building blocks **2**, **3**, and **6** by sequential NCL. Thus, NCL between the cysteine moiety of **3** and the thioester of **2** should link the B- and T-epitopes. Next, removal of the *S*-acetamidomethyl (Acm) protecting group[14] of the N-terminal cysteine of the ligation product should reveal a free cysteine thiol, which can then be ligated with the thioester of **6** to give required adduct **7**.

MUC-1 epitope **3** was assembled by automated solid-phase peptide synthesis (SPPS) using Fmoc-protected amino acids and N^{α}FmocThr(α-AcO_3-D-GalNAc)OH (Tn antigen[15]) on Rink amide resin. After the assembly, the glycopeptide was cleaved from the solid support by treatment with TFA (94.0%), water (2.5%), ethanedithiol (2.5%), and TIS (1%). Next, the acetyl esters of the saccharide moiety were cleaved by treatment of 5% aqueous hydrazine in the presence of DTT to give glycopeptide **3**.

Peptide thioester **2** was synthesized on a sulfonamide "safety-catch" linker.[16−18] Cleavage of the fully assembled peptide from the resin was accomplished by a two-step procedure entailing alkylation of the sulfonamide with iodoacetonitrile followed by treatment with benzyl mercaptan to give a protected peptide having a C-terminal thioester. The acid-sensitive protecting groups of the peptide were removed by treatment with reagent B (TFA, phenol, water, and TIS; 88:5:5:2) to give **2**. This compound is equipped with an N-terminal cysteine residue carrying the orthogonal Acm thiol protecting group, which is stable under conventional side-chain deprotection with TFA but can be cleaved using Hg(II) or Ag(I) or oxidatively by using I_2.[14] Finally, Pam_3CysSK_4 α-thioester **6** was synthesized similar to the preparation of compound **2**.

Having building blocks **2**, **3**, and **6** at hand, we focused our attention on the preparation of glycolipopeptide **7** by sequential NCL (Scheme 1). The ligation of **2** with **3** was performed under standard conditions using a phosphate buffer (pH 7.5) containing 6 M guanidinium-hydrochloride.[17] The ligation was catalyzed by the addition of 4% thiophenol (v/v),[19] and the progress of the reaction was monitored by LC/MS. The reaction was rather sluggish, and after a reaction time of 18 h, partial conversion of **2** and **3** into **4** and some hydrolysis of the thioester were observed. Purification by semipreparative RP-HPLC gave **4** in a yield of 48%. Next, the Acm group of **4** was removed using mercury(II) acetate to give glycopeptide **5**, containing a free sulfhydryl moiety.

Scheme 1. Synthesis of Three-Component Vaccine by NCL



(10) Dawson, P. E.; Muir, T. W.; Clark-lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(11) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960.
(12) Yeo, D. S. Y.; Srinivasan, R.; Chen, G. Y. J.; Yao, S. Q. *Chem.−Eur. J.* **2004**, *10*, 4664−4672.
(13) Snijdewint, F. G. M.; von Mensdorff-Pouilly, S.; Karuntu-Wanamarta, A. H.; Verstraeten, A. A.; Livingston, P. O.; Hilgers, J.; Kenemans, P. *Int. J. Cancer* **2001**, *93*, 97−106.
(14) Veber, D. F.; Varga, S. L.; Hirschman, R.; Milkowski, J.; Denkewalter, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456−5461.
(15) Cato, D.; Buskas, T.; Boons, G. J. *J. Carb. Chem.* **2005**, *24*, 503−516.
(16) Kenner, G. W.; McDermot, Jr.; Sheppard, R. C. *J. Chem. Soc. D, Chem. Commun.* **1971**, 636.
(17) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684−11689.
(18) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11374.
(19) Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325−4329.

Unfortunately, a second NCL of compound **5** with the thioester **6** in a phosphate buffer containing 6 M guanidinium-hydrochloride and thiophenol did not provide target compound **7**. The failure of this reaction is probably due to the poor solubility of **6**. Addition of detergents such as SDS[20] and DPC,[21] at ambient and elevated reaction temperatures (40−50 °C), did not improve the ligation. Furthermore, the use of alternative catalysts such as a mixture of sodium thiophenate and thiophenol or sodium 2-mercaptoethane sulfonate did not lead to product formation. Attempts to perform the ligation in a phosphate buffer containing 8 M urea and use of trifluoroethanol as a reaction solvent also led to failure.

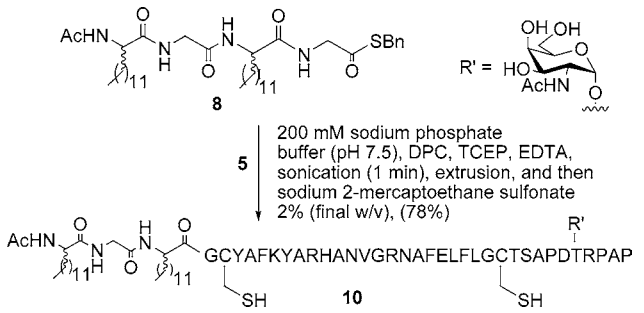
We envisaged that the incorporation of compounds **5** and **6** into liposomes would facilitate solubilization[22,23] and hence increase the rate of ligation. Thus, a film of dodecylphosphocholine, thiol **5**, and thioester **6** was hydrated by incubation at 37 °C for 4 h in a phosphate buffer (pH 7.5) in the presence of carboxyethyl phosphine and EDTA. The latter two reagents were added to suppress disulfide formation. The mixture was ultrasonicated for 1 min, and the resulting vesicles were sized to 1 μm by passing through a polycarbonate membrane filter. The ligation was catalyzed by the addition of sodium 2-mercaptoethane sulfonate,[24] and surprisingly, after a reaction time of 2 h, LC-MS showed completion of the reaction. After purification by RP-HPLC over a C-4 column, compound **7** was obtained in a high yield of 83%. The use of thiophenol as a catalyst resulted in a significantly slower reaction rate, and after 4 h, the reaction had proceeded to only ~60% completion. After a reaction time of 16 h, LC-MS revealed significant hydrolysis of palmitoyl esters.

Encouraged by the successful preparation of **7**, we focused our attention again on the synthesis of glycopeptide **4**, this time using the new methodology. The preparation of this compound by traditional NCL was relatively low yielding due to the poor solubility of **2** in a phosphate buffer containing 6 M guanidinium-hydrochloride. It was envisaged that incorporation of **2** and **3** into liposomes would increase the solubility, and hence a higher yield of product may be expected. Thus, a liposomal preparation of peptide **2** and glycopeptide **3** was prepared using the conditions employed for the preparation of **7**. The ligation was catalyzed by the addition of sodium 2-mercaptoethane sulfonate, and after a reaction time of 2 h, the product was purified by RP-HPLC to give **4** in an excellent yield of 78%.

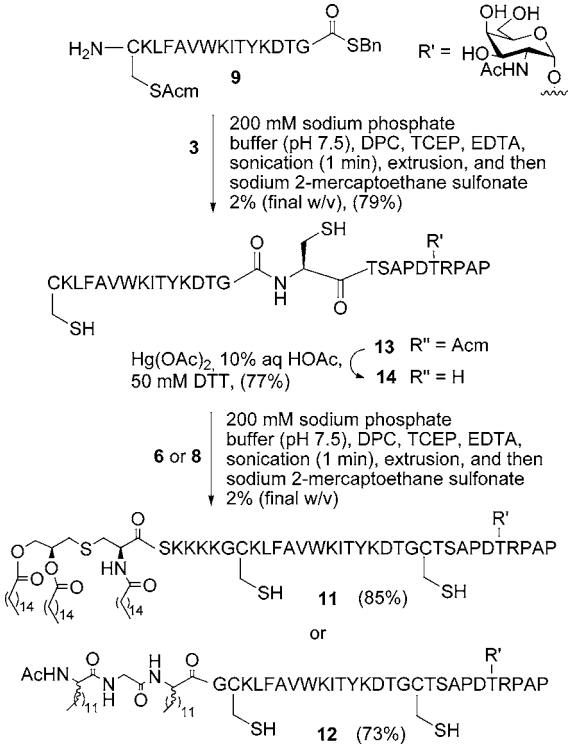
Interestingly, no product formation was observed when a solution of **3** was added to a liposomal preparation of **2** using sodium 2-mercaptoethane sulfonate as the promoter (compound **3** has reasonable solubility in phosphate buffer). The results of these experiments indicate that NCL takes place within the lipid environment of the liposome and not at the water−liposome interface.

To examine the utility of the approach, compounds **10** (Scheme 2), **11**, and **12** (Scheme 3), which differ in (glyco)-peptide and lipid composition, were prepared by sequential liposome-mediated NCL starting from building blocks **2**, **3**, **6**, **8**, and **9**. Thus, glycolipopeptide **10** could easily be obtained by ligation of **5**, which was prepared from compounds **2** and **3** with thioester **8**. Derivatives **11** and **12** were prepared by ligation of **3** with **9** to give glycopeptide **13**, which after removal of the Acm group (→**14**) was ligated with thioesters **6** or **8**, respectively. In each liposome-mediated NCL, the thioester was consumed within 2 h as determined by LC-MS, and after purification by semi-

Scheme 2. Preparation of **10** from **5** and **8**



Scheme 3. Preparation of **11** and **12** from **3**, **6**, **8**, and **9**



(20) Valiyaveetil, F. I.; MacKinnon, R.; Muir, T. W. *J. Am. Chem. Soc.* **2002**, *124*, 9113−9120.
(21) Clayton, D.; Shapovalov, G.; Maurer, J. A.; Dougherty, D. A.; Lester, H. A.; Kochendoerfer, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4764−4769.
(22) Hunter, C. L.; Kochendoerfer, G. G. *Bioconjugate Chem.* **2004**, *15*, 437−440.
(23) Otaka, A.; Ueda, S.; Tomita, K.; Yano, Y.; Tamamura, H.; Matsuzaki, K.; Fujii, N. *Chem. Commun.* **2004**, 1722−1723.
(24) Grogan, M. J.; Kaizuka, Y.; Conrad, R. M.; Groves, J. T.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2005**, *127*, 14383−14387.

preparative RP-HPLC, the glycopeptides or glycolipopeptides were obtained in high yield.

Previously, Kochendoerfer and co-workers[22] performed a NCL between a synthetic hydrophobic polypeptide incorporated into a cubic lipidic phase and a tetrapeptide, which was added to the membrane preparation. This mode of ligation is different from the approach described here because only one of the two reactants is incorporated into the membrane. Furthermore, Otaka and co-workers[23] reported that lipid bilayer assisted NCL between a thioester and an N-terminal cysteine peptide can successfully be used for the synthesis of membrane protein segments possessing two transmembrane regions and one extracellular domain. In this approach, peptides were embedded in a palmitoyloleoyl phosphatidylcholine membrane and the reaction was catalyzed by the addition of thiophenol.

The results of our study demonstrate that incorporation of a lipophilic (lipo)peptide thioester and an N-terminal cysteine glycopeptide into DPC-liposomes facilitates NCL to afford a range of glycopeptides and glycolipopeptides. Surprisingly, the new approach is not limited to peptides that have a trans- and an extracellular domain. Furthermore, it was found that 2-mercaptoethane sulfonate is a more effective catalyst compared to thiophenol. In this respect, it was observed that the liposome-mediated NCLs were completed within 2 h, which is remarkably fast for the type of substrates employed. The high reaction rate can probably be attributed to a concentration effect in the liposomes.

In conclusion, we have developed a novel approach for native chemical ligation by the entrapment of reactants in liposomes. The new methodology is particularly suited for the synthesis of lipophilic (glyco)peptides of biological importance.[25−30] For example, it allows the synthesis of a range of three-component vaccine candidates by a modular approach using an array of B- and T-epitopes and lipopeptide adjuvants. A modular approach is attractive because it provides greater synthetic flexibility than linear synthesis. In this respect, each building block can be used for the preparation of several different target compounds. Furthermore, compared to conventional linear SPPS, a block synthetic approach will minimize byproduct buildup in the growing peptide chain. In this respect, the DT sequence of the MUC-1 glycopeptide is prone to aspartimide formation,[31] which can occur at each coupling step. In a convergent block synthesis, the individual building blocks can be purified by RP-HPLC and characterized by NMR and MS prior to assembly, providing a sound basis for highly pure final products.

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Supporting Information Available: General experimental methods, analytical data, and spectra of the corresponding compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(25) Dziadek, S.; Kowalczyk, D.; Kunz, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 7624−7630.
(26) Guo, Z.; Shao, N. *Med. Res. Rev.* **2005**, *25*, 655−678.
(27) Buskas, T.; Ingale, S.; Boons, G. J. *Glycobiology* **2006**, *16*, 113R−136R.
(28) Dube, D. H.; Bertozzi, C. R. *Nat. Rev. Drug Discovery* **2005**, *4*, 477−488.
(29) Doores, K. J.; Gamblin, D. P.; Davis, B. G. *Chem.−Eur. J.* **2006**, *12*, 656−665.
(30) Macmillan, D.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 1355−1359.
(31) Mergler, M.; Dick, F.; Sax, B.; Stahelin, C.; Vorherr, T. *J. Pept. Sci.* **2003**, *9*, 518−526.