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Synthesis And Liposome-Formation Of A Thermostable Lipid Bearing Cell Adhesion Peptide Sequence

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Abstract: A peptide containing the cell adhesion sequence, Arg-Gly-Asp-Ser, was attached to 2,3-diphytanoxypropaneamine (NH_2-DPhy), an archaebacterial lipid model. The peptide moiety contained a thioester group at its *C*-terminus and was synthesized by the solid-phase method. The thioester group was activated with silver ions to yield a peptide bond between the peptide and the archaebacterial lipid. The resulting peptide-lipid conjugate formed stable liposomes upon sonication.

An essential requirement for the use of liposomes as drug carriers is the ability to target specific cells. One of the successful approaches has been to use monoclonal antibodies as the targeting mechanism.[1] Alternatively, the antibodies could be replaced with the peptide ligands of various receptors. Generally, these ligands are small compared to antibodies, and hence their design and synthesis could be easily accomplished by chemical means.

We have previously shown that the model lipid of the archaebacterial halophiles, DPhyPC,[2] forms stable vesicles upon sonication.[3] The liposomes retain Na^+ and Cl^- ions as well as CF at temperatures as high as 70°C. The stability of the liposomes is mainly derived from the phytanyl groups that make up DPhyPC.

In order to examine the possibility of using thermostable lipids as drug carriers, we designed a method to immobilize peptide ligands onto a lipid. In the present study, a peptide containing the cell adhesion sequence of fibronectin, Arg-Gly-Asp-Ser, was anchored to NH_2-DPhy, which retains phytanyl groups as DPhyPC (Fig. 1) and the formation of liposomes was tested. In practice, the peptide ligands would promote the association of the drug-carrying liposomes to fibronectin's receptors. These receptors, the integrins, are highly expressed on the surface of lymphocytes.

As conjugate **1** is composed of both peptide and lipid components, each of which has a different stability toward chemical reagents, it was therefore necessary to prepare each component separately prior to the condensation. NH_2-DPhy was prepared according to the method shown in Fig. 2. 1,2-diphytanylglycerol, prepared as previously described,[3] was tosylated with tosyl chloride in the presence of Et_3N. The product was

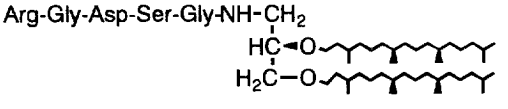

Fig. 1. Structure of peptide-lipid conjugate **1**.

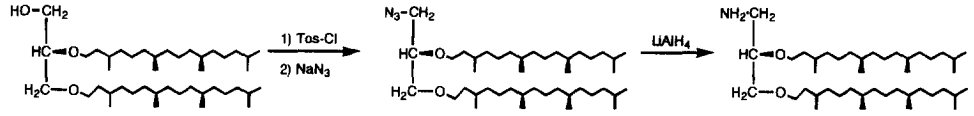

Fig. 2. Pathway for synthesis of NH_2-DPhy.

treated with sodium azide in DMF, followed by reduction using $LiAlH_4$ in ether to obtain NH_2-DPhy. The yield was 28% based on 1,2-diphytanylglycerol.

To determine the most efficient way to produce conjugate **1**, two different methods were used for the synthesis of protected peptide and subsequent coupling to NH_2-DPhy. Preparation of the peptide was carried out by the solid-phase method.

Method A: In this method, WSCI was used for coupling between peptide and NH_2-DPhy. Thus, maximally protected peptide was prepared in order to avoid side reactions during condensation. Starting from Fmoc-Gly-$OCH_2C_6H_4OCH_2C_6H_4$-resin (1 mmol of Gly), the peptide chain was elongated using Fmoc-amino acids with TFA-stable side chain protecting groups. Protection of the side chain carboxyl group of Asp was achieved using O-2-Ad, which is reported to suppress aspartimide formation.[4] In a preliminary experiment, benzyl ester was used for this purpose, but the yield of protected peptide was 5% due to cyclic imide formation at the Asp-Ser sequence. After completion of the chain assembly, the protected peptide was detached from the resin using 5% ethanedithiol-TFA. The crude product was purified using RPHPLC to obtain Arg(Mts)-Gly-Asp(O-2-Ad)-Ser(Bzl)-Gly. The yield was 54% based on Gly on the initial resin. The amino group of the peptide was protected with a Boc group using Boc-OSu (4x amino group) in DMSO to yield quantitative amounts of Boc-Arg(Mts)-Gly-Asp(O-2-Ad)-Ser(Bzl)-Gly (peptide **2**).

Peptide **2** was condensed with NH_2-DPhy (1x peptide **2**) in CH_2Cl_2 by WSCI (1.2x peptide **2**). The progress of the reaction was monitored by TLC. The reaction was almost complete within 3h without any serious side reactions. Deprotection was carried out using 1 M CH_3SO_3H in TFA. Under the above conditions, all protecting groups except O-2-Ad group were removed. The compound was further treated with 1M CF_3SO_3H in TFA to remove the O-2-Ad group. Although the O-2-Ad group had been removed, FAB-MS analysis confirmed that one of the phytanyl groups had also been cleaved during deprotection.[5] Thus, the yield of the peptide-lipid conjugate was negligible using this procedure.

Method B: In order to avoid the loss of a phytanyl group during deprotection, the condensation between the peptide and NH_2-DPhy was carried out without aspartyl side chain protection. Instead, a partially protected peptide thioester was used to achieve coupling. As the thioester group can be selectively activated during condensation by silver ions,[6] protection of side chain carboxyl groups was not necessary. This peptide thioester has been used for protein synthesis.[7] Preparation of the peptide was carried out as shown in Fig. 3. Boc-Nle and Boc-Gly-SCH_2CH_2COOH were successively introduced on to the MBHA-resin (0.5 mmol) by the DCC-HOBt method. Peptide chain elongation was carried out on this resin using Boc-amino acids. The Arg residue was incorporated using Fmoc-Arg(Mts) to avoid removal of the terminal amino protecting group during HF treatment. The resin was then treated with anhydrous HF containing 10% anisole at 0 °C for 1.5 h. The resulting crude product was purified by RPHPLC to yield Fmoc-Arg-Gly-Asp-Ser-Gly-SCH_2CH_2CO-Nle-NH_2 (peptide **3**).[8] The yield was 24% based on the concentration of the amino group on the initial resin.

Condensation of peptide **3** with NH_2-DPhy was carried out as previously described.[7] Peptide **3** and

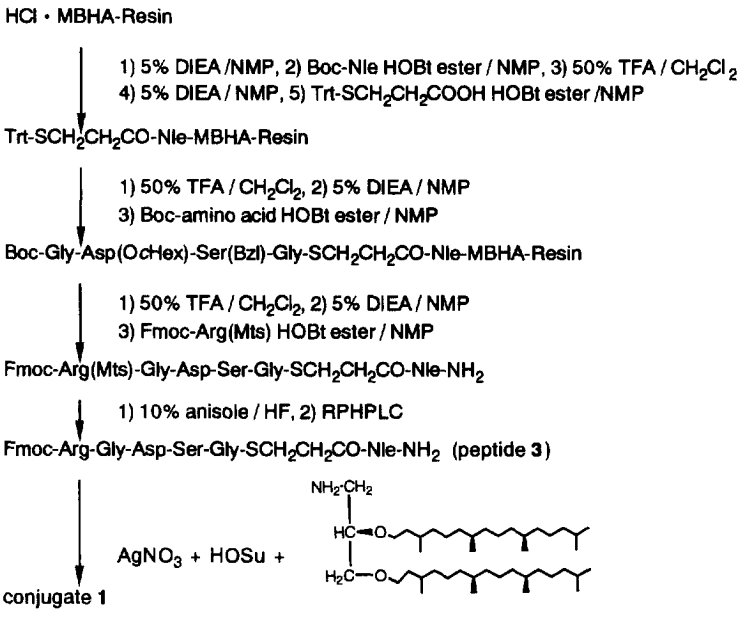

Fig. 3. Pathway for synthesis of conjugate **1**.

NH_2-DPhy (0.7x peptide **3**) were dissolved in DMSO containing HOSu (10x peptide **3**) and DIEA (2.4x peptide **3**). In order to initiate the coupling reaction, silver ions (4x peptide **3**) were added to the solution. TLC monitoring showed that the reaction was completed within 1 h without any serious side reactions. Piperidine was then added to the reaction mixture to remove the Fmoc group. Next, DMSO and piperidine were extracted with ether and the residual oil washed with distilled water to remove excess peptide. The product was extracted with 40% aqueous acetonitrile containing 0.1% TFA. Analysis by TLC and FAB mass spectrometry showed that highly pure peptide-lipid conjugate (conjugate **1**) was obtained using this procedure.[9] The yield was 42% based on NH_2-DPhy.

Conjugate **1** or mixtures of conjugate **1** and DPPC (1:10, 1:1) were dissolved in distilled water and sonicated at 50 °C for 25 min. The suspension was centrifuged at 2000 xg for 15 min, and then the supernatant was analyzed by transmission electron microscopy. Conjugate **1** itself or mixtures of conjugate **1** with DPPC formed uni- and multilamellar vesicles of 200 to 1000 Å in diameter. Typical examples are shown in Fig. 4. The liposomes composed of conjugate **1** and DPhyPC (1:10) retained high thermal stablity as DPhyPC liposomes, judging from the leakage rate of CF from the liposomes (the data are not shown).

Using method B, the peptide component was easily prepared by the solid-phase method. Due to the selective activation of the thioester group, condensation between the peptide and lipid components was accomplished without requiring side chain protecting groups. As a result, the final deprotection conditions were sufficiently mild for both the peptide and the lipid, despite their differing chemical stabilities. Thus, method B provides a simple and general route for the preparation of the peptide-lipid conjugate. Cell adhesion activity of the liposomes prepared from conjugate **1** is under study.

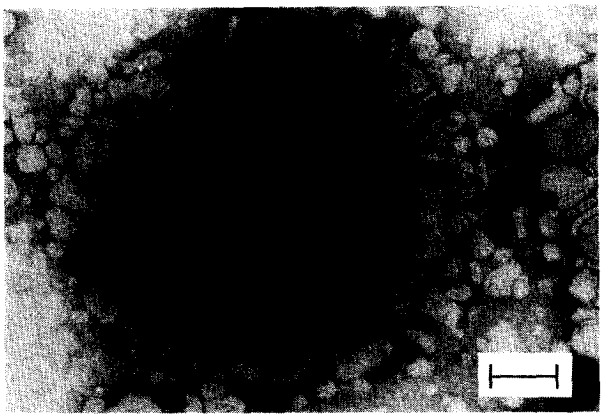

Fig.4. Transmission electron micrograph of liposomes composed of conjugate **1**; negative staining with phosphotungustic acid / sodium hydroxide (pH 7). The bar corresponds to 1000 Å.

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References and notes:

1. F. Martin and D. Papahadjopoulos, *J. Biol. Chem.*, **257**, 286-288 (1982).
2. Abbreviations: Ad, adamantyl; CF, 5(6)-carboxyfluorescein; *c*Hex, cyclohexyl; DIEA, diisopropylethylamine; DPhyPC, 1,2-diphytanylglycero-3-phosphocholine; DPPC, 1,2-dipalmitoylglycero-3-phosphocholine, MBHA, 4-methylbenzhydrylamine; Mts, mesitylenesulfonyl; NMP, 1-methyl-2-pyrrolidinone; TFA, trifluoroacetic acid; WSCI, water-soluble carbodiimide.
3. K. Yamauchi, K. Doi, M. Kinoshita, F. Kii, and H. Fukuda, *Biochim. Biophys. Acta*, **1110**, 171-177 (1992).
4. Y. Okada, S. Iguchi, and K. Kawasaki, *J. Chem. Soc. Chem. Commun.*, 1532-1534 (1987).
5. Found: m/z 844.5 $(M+H)^+$. Calculated for the product, in which one of the phytanyl group was replaced with hydrogen atom: m/z 844.6 $(M+H)^+$.
6. The reaction proceeds as follows:
   $R\text{-}CO\text{-}SR' + HOSu \xrightarrow{Ag^+} R\text{-}CO\text{-}OSu + Ag\text{-}SR' \xrightarrow{NH_2\text{-}R''} R\text{-}CO\text{-}NH\text{-}R''$, where R-CO-SR' is a partially protected peptide thioester, -SR' is $-SCH_2CH_2CO\text{-}Nle\text{-}NH_2$, and NH_2-R" is an amino component peptide.
7. H. Hojo, S. Yoshimura, M. Go, and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **68**, 330-336 (1995).
8. Found: m/z 913.4 $(M+H)^+$. Calcd: m/z 913.4 $(M+H)^+$.
9. Found: m/z 1125.3 $(M+H)^+$. Calcd: m/z 1124.9 $(M+H)^+$.

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