

Synthesis of VIP-Lipopeptide Using a New Linker to Modify Liposomes: Towards the Development of a Drug Delivery System for Active Targeting

Toru Masaka,^{a,b} Takuya Matsuda,^a Yingpeng Li,^a Yuki Koide,^a Akira Takami,^a Kenji Yano,^a Ryosuke Imai,^a Risa Ichihara,^a Nobuhiro Yagi,^a Hideharu Suzuki,^a Hidemasa Hikawa,^a Katsuhide Terada,^a and Yuusaku Yokoyama*^a

^aSchool of Pharmaceutical Sciences, Toho University; 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan; and

^bSakura Medical Center, Toho University; 564-1 Shimoshizu, Sakura, Chiba 285-8741, Japan.

Received June 27, 2013; accepted August 9, 2013; advance publication released online August 23, 2013

A new component for the solid phase peptide synthesis of lipopeptide, 2-[(4*R*,5*R*)-5-((9*H*-fluoren-9-yl)-methoxy)carbonylaminoethyl]-2,2-dimethyl-1,3-dioxolan-4-yl)methoxy]acetic acid (2**), was designed and synthesized from (–)-2,3-*O*-isopropylidene-*D*-threitol (**3**) in 4 steps. The key step was the selective alkylation of **3** with benzyl bromoacetate in the presence of Cs₂CO₃. Vasoactive intestinal peptide (VIP)-lipopeptide (**1**) incorporating this linker was synthesized by solid phase peptide synthesis.**

Key words drug delivery system; linker; isopropylidene-*D*-threitol; lipopeptide; peptide synthesizer

Surface modification of liposomes is essential for the development of drug delivery systems (DDS),^{1,2)} especially for the active targeting of tumor cells.^{3,4)} Peptides are generally used to modify liposomes in order to recognize the target receptor on the cell membrane.^{5–9)} It is easiest to modify the liposome surface when the functional groups of a pre-formed liposome are reacted with peptides in an aqueous environment.¹⁰⁾ However, such methods have several limitations^{5,6)}: 1) steric hindrance of the liposome leads to low yield, 2) purification of the liposomes is very difficult due to by-products and unreacted reagents, and 3) the amino acid sequence of the peptide can hinder reactivity. Although a promising approach to solving this problem is to produce a lipid-protein/peptide conjugate molecule by total synthesis and use it to make liposomes, the modification of the phospholipid requires many protection and deprotection steps of the phosphate group, resulting in poor yields due to hydrolysis in aqueous media.^{7,8)}

We therefore designed^{11,12)} a novel lipid analog mimicking the structural features of phospholipids that is easily synthesized using a peptide synthesizer (Fig. 1). The lipid moiety was composed of the following four domains: the anchor domain (anchoring the molecule into the liposome), a glycerol

mimic domain (structurally equivalent to the glycerol in phospholipids), a hydrophilic domain (structurally equivalent to the phosphate group in phospholipids), and a linker domain (to prevent steric hindrance in the liposome).

We intend to explore this technology for generating active targeted DDS for tumor cells. It has been reported^{1,13)} that the receptor for vasoactive intestinal peptide (VIP-R) is overexpressed in some tumor cells. We believed that a peptide-lipid analog conjugate (VIP-lipopeptide) could easily be synthesized and modified on the liposome surface. Further, encapsulation of an anti-tumor drug, doxorubicin (DOX), in the liposome could prove effective as an active targeted anti-tumor DDS.

The purification of this lipopeptide and its modification of liposomes proved difficult due to the unexpected amphiphilic characteristics of the compound. These characteristics arise from the combination of a lipophilic alkyl chain and the hydrophilic peptide. A new, more hydrophilic linker domain was therefore designed and incorporated into the lipopeptide, providing better orientation of the peptide on the aqueous surface of the lipid bilayer. The new linker domain contained two hydroxyl groups (lipopeptide **1**, Fig. 2). Incorporation of this linker into the lipopeptide using a peptide synthesizer re-

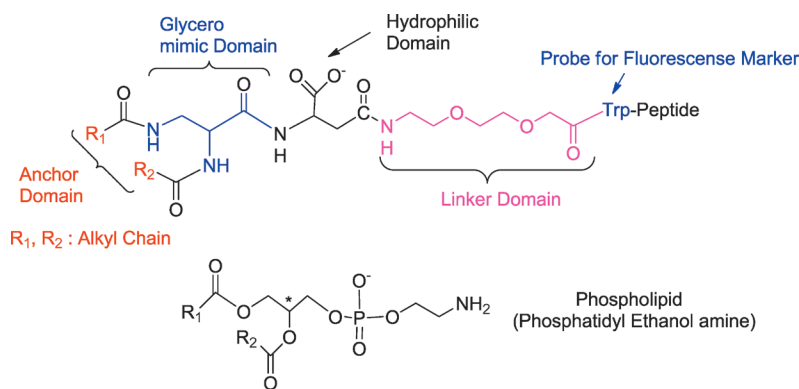


Fig. 1. General Structure of the Novel Lipid Analogue (Lipopeptide)

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: yokoyama@phar.toho-u.ac.jp

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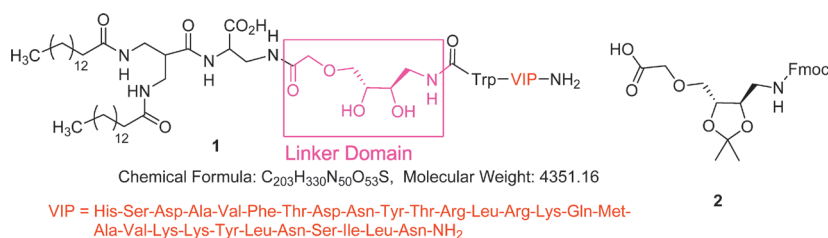


Fig. 2. Structure of VIP-Lipopeptide (1) with the New Linker Domain

Table 1. Results of Alkylation

Run	Base (eq)	Solvent	BrCH ₂ COOBn (eq)	Yield of 4 (%)
1	NaH (1.8)	THF	2	—
2	NaOH (1.5)	Toluene	1.5	—
3	K ₂ CO ₃ (1.1)	DMF	1.5	11
4	Cs ₂ CO ₃ (1.8)	DMF	1.6	34

quired a 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acid with an acetonide-protected hydroxyl group (2). The synthesis of linker 2 and lipopeptide 1 using a solid phase peptide synthesizer is described below.

(-)-2,3-*O*-Isopropylidene-D-threitol (3) was selected as the starting material. Due to our experience developing unprotected synthesis strategies for highly polar compounds such as free amino acids,^{14–17} we attempted the direct introduction of benzyl acetate moiety into unprotected diol 3.

The reaction of benzyl bromoacetate with diol 3 provided a complex mixture in non-polar aprotic solvent [tetrahydrofuran (THF), toluene] with strong base (NaH, NaOH) (Chart 1, Table 1, runs 1, 2). The desired product 4 was isolated in low yield (11%) when a weaker base, K₂CO₃ in dimethylformamide (DMF), was used, and no residual starting material was detected (run 3). By changing the base, reaction temperature, and molar ratio, the yield was improved to 34% using Cs₂CO₃ as a base (run 4). The low yield could have been due to the simultaneous formation of the carbanion of benzyl

bromoacetate and the hydroxyl anion of 3, resulting in side reactions that provided a complex product mixture. Indeed, selective mono-alkylation of more acidic poly-phenolic compounds with benzyl bromoacetate smoothly proceeded to give the desired product in high yield under similar conditions.^{18,19}

Although the yield was not satisfactory, we carried out the next step. Tosylation of 4 smoothly proceeded to give 5 (73% yield), which was reacted with azide anion to give the desired azide 6 in 84% yield (Chart 2).

Non-selective reduction of 6 to reduce the azide group and remove the benzyl group by H₂/Pd-C provided amino acid 7, which was reacted with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) without purification to give the final product 2 in 77% yield (Chart 3).

VIP-Lipopeptide (1) with new linker 2 was synthesized on TGS-RAM resin by Fmoc solid-phase synthesis using an automated peptide synthesizer (Chart 4). Fmoc-protected amino acids were coupled sequentially to synthesize VIP using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*-hydroxybenzotriazole (HOBT) as condensation reagents (8→9). Fmoc-tryptophan (probe for fluorescence marker), Fmoc-Asp(OH)-OtBu and Fmoc-Dap(Fmoc)-OH were coupled sequentially on the synthesizer (9→10→11→12). Since the last condensation reaction, with palmitic acid (anchor domain), did not proceed satisfactorily due to its insolubility in DMF, palmitic acid was

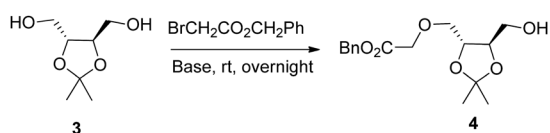


Chart 1. Alkylation with Benzyl Bromoacetate

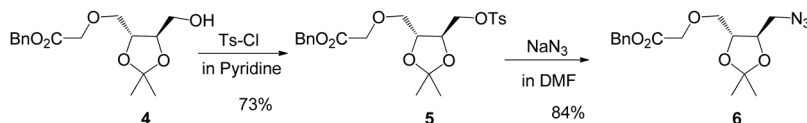


Chart 2. Conversion from Alcohol 4 to Azide 6

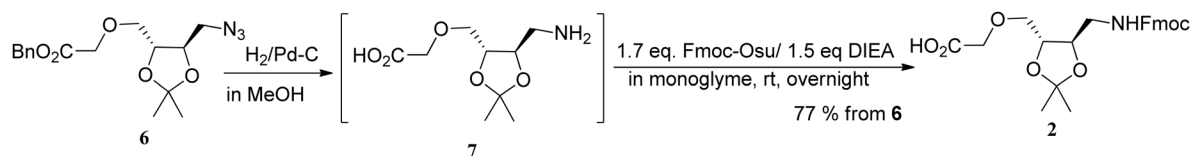


Chart 3. One-Pot Synthesis of Linker Component 2

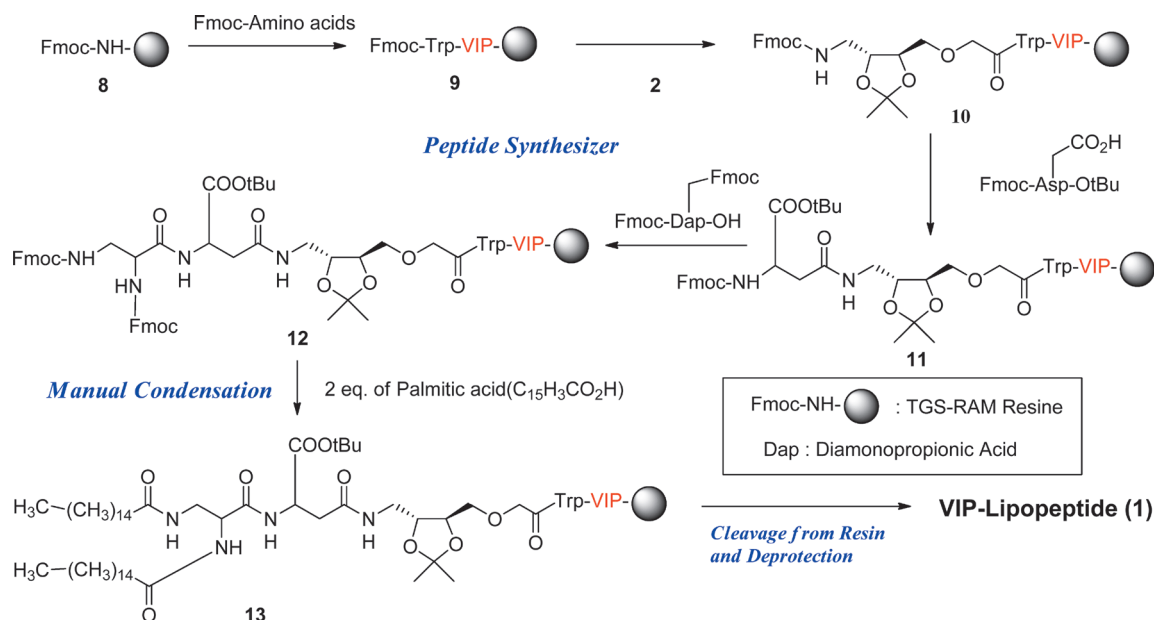


Chart 4. Solid Phase Synthesis of VIP-Lipopeptide (1) with the New Linker

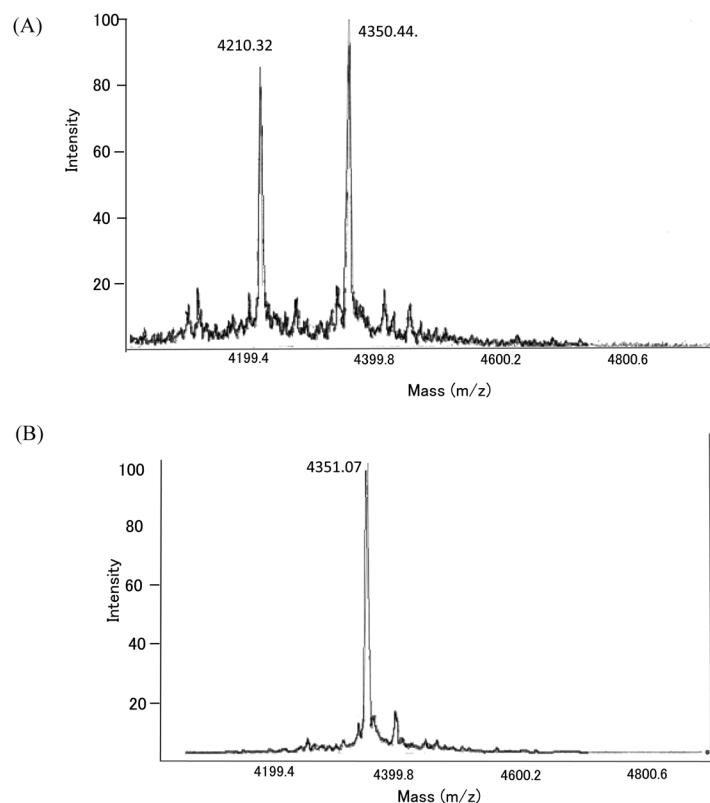


Fig. 3. TOF-MS Spectrum of 1: (A) before Purification, (B) after Purification

Molecular weight was 4351.16 ($C_{203}H_{330}N_{50}O_{53}S$).

manually reacted with *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) in the presence of HOBt and *N*-methylmorpholine (NMM) (12→13). After de-protection and cleavage from the resin, crude VIP-lipopeptide (1) was obtained as a yellow solid, which was purified by preparative HPLC to give pure 1 in 28% yield. The purity and structure of 1 were confirmed by time-of-flight mass (TOF-MS) spectra and analytical HPLC (Figs. 3, 4).

We synthesized Fmoc-protected amino acid 2 in 4 steps in 16% overall yield. Compound 2 can be used as a component of the linker domain during the synthesis of VIP-lipopeptide (1) using a peptide synthesizer. The key aspects of this synthetic route are selective alkylation of diol 3 by benzyl bromoacetate, as shown in Chart 2, and one-pot reaction of 6 (reduction and protection of Fmoc group) to provide the final product 2, as shown in Chart 3. The synthesis and straight-

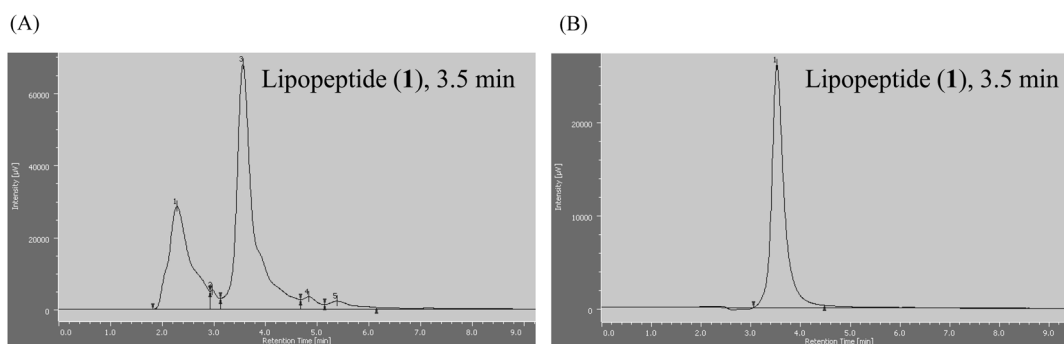


Fig. 4. HPLC Profile of **1**: (A) before Purification, (B) after Purification

HPLC column [Capcell pak Phenyl UG120 (Shiseido) 5 μ m, 4.6 mm ϕ ×250 mm]; detector, UV (280 nm); flow rate, 1.0 mL/min; gradient, H₂O (0.1% TFA)–CH₃CN (0.1% TFA): 40:60.

forward purification of VIP-lipopeptide (**1**) was accomplished using solid phase peptide synthesis and preparative HPLC. Modification of liposomes with **1** and their evaluation as active targeting DDS for cancer therapy will be published in the near future.

Experimental

General chemistry methods, synthesis procedures, spectral data, and bioassay methods are given in Supplemental Information.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research [(C)-No. 13672237] from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We are also grateful to Toho University for supporting the Toho University Joint Research Fund.

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