Toward Semisynthetic Lipoproteins by Convergent Strategies Based on Click and Ligation Chemistry

Hans-Jürgen Musiol,[a] Shouliang Dong,[a] Markus Kaiser,[a] Ralf Bausinger,[b] Andreas Zumbusch,[b] Uwe Bertsch,[c] and Luis Moroder*[a]

Cell-membrane proteins are anchored to the lipid bilayer by single or multiple insertion of transmembrane helices or by regioselective single or dual lipidation in co- and post-translational enzymatic processes, including acylation with fatty acids, prenylation, and rather commonly C-terminal amidation with glycosylphosphatidylinositols (GPI).[1] Procedures for native and neolipidation of peptides have been comprehensively reviewed,[2] and, more recently, even the synthesis of complex GPIs has been reported.[3] However, lipidation of proteins at defined sites and particularly grafting of GPIs or related mimetic structures to the C termini of proteins still represent formidable long-term goals of chemistry and molecular biology. So far, lipoproteins have been obtained by chemical ligation of synthetic lipopeptides with recombinant protein fragments by the highly selective and efficient maleinimide/thiol addition reaction, as shown for the RAS protein, for example,[4] or by total synthesis through orthogonal protection schemes for regioselective lipidation of side-chain amino groups, for example, with 1,2-dipalmitoyl-glycero-3-succinate.[5] In view of the recently developed efficient procedures for the semisynthesis of proteins by native-chemical [6] and expressed-protein ligation,[7] we have performed model studies toward C-terminal lipidation of proteins by exploiting the copper(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition of terminal alkynes to azides to form a stable triazole product[8] and the transthioesterification between peptide thioesters and *N*-cysteinyl-lipopeptides followed by intramolecular S→N acyl shift as the synthetic strategy set forth in Scheme 1. Aside from validating the methodology of a combined click[9] and ligation chemistry,[6] incubation of HeLa cells with the micellar solution of the lipopeptide confirmed its fast uptake, as visualized by confocal fluorescence microscopy.

It is well established that dual vicinal lipid chains, as present in the di-fattyacyl glycerol moiety of natural GPI anchors are required for an almost irreversible capture of peptides and proteins by lipid bilayers.[10] Correspondingly, to properly mimic the GPI anchor, phosphatidylethanolamine was converted into the corresponding azide **1** by $CuSO_4$-catalyzed diazotransfer with triflyl azide[11] to produce the key intermediate for subsequent application of the click chemistry. The crystalline azide **1** was then used for the 1,3-cycloaddition reaction with the S-protected model peptide **2**, which contained a C-terminal propargylglycine (Pra) residue as suitable reaction partner (Scheme 1). The azide–alkyne cycloaddition was performed with CuI as catalyst in organic solvent, and the lipopeptide derivative **3** was isolated by silica gel chromatography in yields of 70–75%.[12] Upon removal of the acid-labile *S*-trityl and N^{α}-Boc groups from **3** with TFA, the subsequent native chemical ligation of the cysteinyl-lipopeptide with *N*-dansyl- or *N*-rhodamine B-labeled Gly-Pro-Gly-Gly-SPh ester **4** was performed in micellar solutions of 2% octyl-β-D-glucopyranoside.[13] Ligation was found to proceed smoothly in the presence of tris(2-carboxyethyl)phosphine (TCEP) if excess of the thioesters was carefully avoided to prevent bisacylation as a side reaction.[14] HPLC served to isolate the fluorescence-labeled lipopeptides **5a,b** in yields of 60–70% as analytically well-characterized compounds, as shown in Figure 1 for compound **5b**. The lipopeptide **6** was obtained in practically quantitative yield by treatment of the C-terminal propargylglycine residue with azide **1** in aqueous–organic media and in the presence of

[a] *H.-J. Musiol, Dr. S. Dong, Dr. M. Kaiser, Prof. Dr. L. Moroder*
Max-Planck-Institut für Biochemie
Am Klopferspitz 18, 82152 Martinsried (Germany)
Fax.: (+49) 89-8578-2847
E-mail: moroder@biochem.mpg.de

[b] *Dipl.-Phys. R. Bausinger, Dr. A. Zumbusch*
Institut für physikalische Chemie
Butenandtstraße 5–13, 81377 München (Germany)

[c] *Dr. U. Bertsch*
Zentrum für Neuropathologie und Prionforschung, LMU München
Feodor Lynen Straße 23, 81377 München (Germany)

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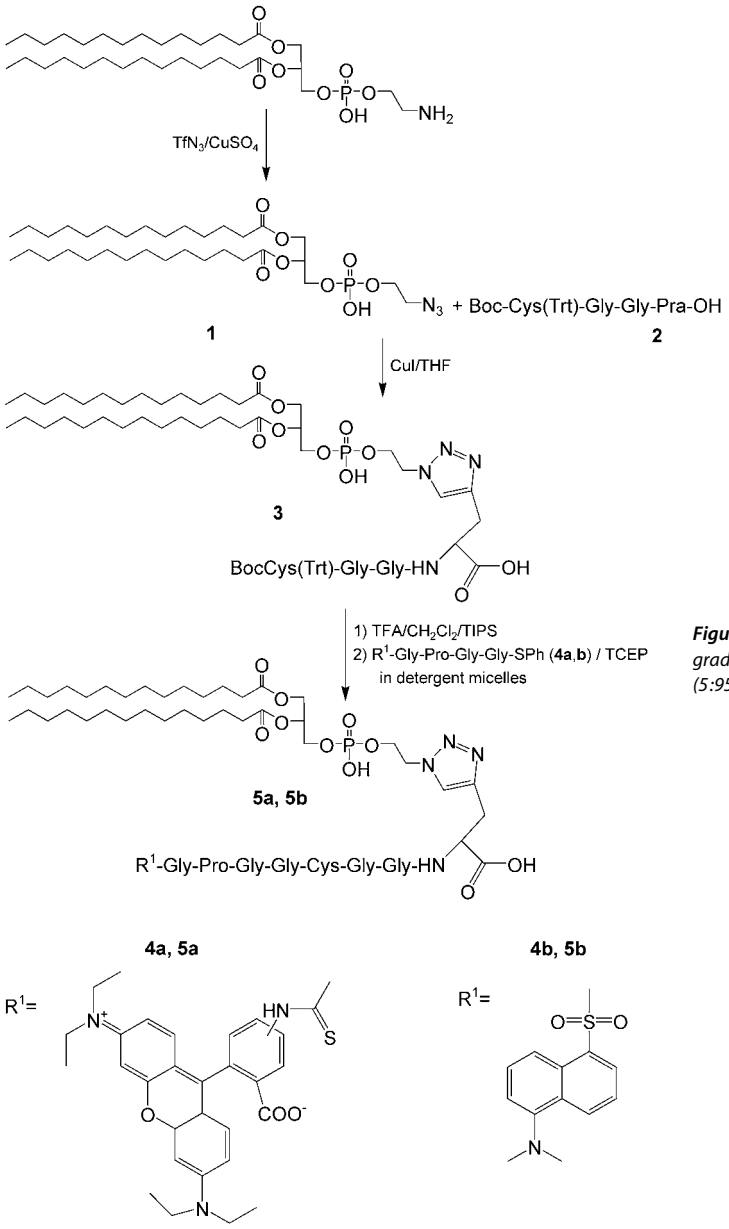


Scheme 1. *Synthesis of a C-terminally lipidated and N-terminally fluorescently labeled model peptide by the use of the click chemistry and native chemical-ligation procedures.*

$CuSO_4$/sodium ascorbate.[15] Upon reductive cleavage of the *S-tert*-butylthio group with tributylphosphine, ligation of the lipopeptide **6** with the dansylpeptide thioester **4b** was performed in micellar solution of 1% sodium dodecylsulfate (SDS; Scheme 2), and the fluorescent compound **7** was isolated by preparative HPLC and characterized analytically.[16] This reaction could also be carried out directly, since in the reductive-ligation reaction media cleavage of the thiol protecting group occurs concurrently with the transesterification in the presence of TCEP[17] or mercaptans.[18] By performing the ligation step with an expressed C-terminal thioester-activated protein fragment in micellar solution, as required for solubilization of the lipo fragment, oxidative refolding of the semisynthetic lipoprotein should even be favored,[19] and direct transfer onto vesicles or cell membranes should be straightforward.

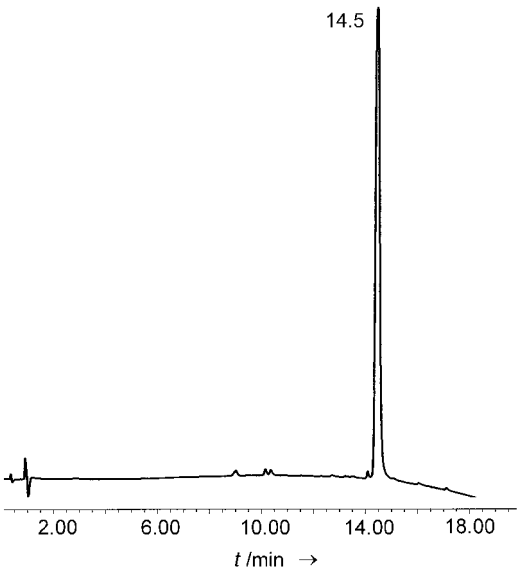
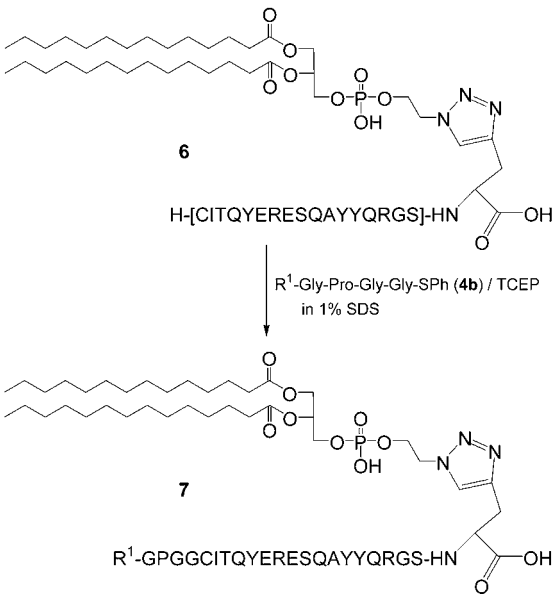


Figure 1. *HPLC profile of compound **5b** [EC 125/4 Nucleosil 300–5 C-4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.1% TFA in H_2O/CH_3CN (5:95)].*



Scheme 2. *The C-terminally lipidated fragment 214–231 of human prion protein, a key intermediate for the semisynthesis of this lipoprotein, was ligated in a model reaction with the dansyl-peptide **4b** in SDS micellar solution.*

Incubation of the HeLa cells with the lipopeptide **5a** revealed a fast uptake within 30 min. Rather surprising was the similarly fast intracellular distribution of the fluorescence dye with staining of the membrane-containing organelles as known to occur with rhodamine (Figure 2).[20,21] Internalization of the fluorescence dye could result from fast flip-flop motions of the lipopeptide followed by proteolytic cleavage of the peptide moiety and thus cytoplasmatic release of the dye. However it must also occur by endocytosis, since directed cellular

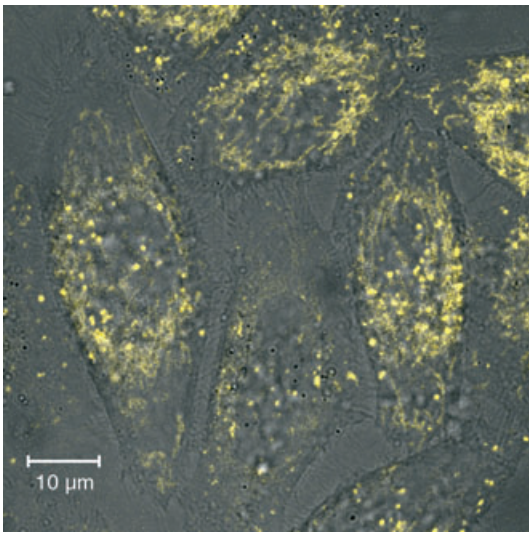


Figure 2. *Confocal microscopy of HeLa cells stained with the lipopeptide* ***5a****.*

trafficking of **5a**-labelled vesicles was detectable until the end of the experiment (7 h) without affecting the viability of the cells.

In summary, the goal outlined in Scheme 1 has been met with model peptides, and thus the semisynthesis of lipoproteins seems amenable by this convergent strategy. The lipopeptide **6** corresponds to the C-terminal fragment 214–231 of the human prion protein (PrP) with a propargylglycine at position 231 for the site-specific lipidation. It represents the key intermediate for assembly of this ubiquitous GPI membrane-anchored protein, whose conversion from the native cellular PrP^{c} to the pathogenic PrP^{sc} scrapie form has been associated with a group of unusual neurodegenerative disorders in humans as well as with scrapie in sheep and BSE in cattle.[22] Since this conversion probably occurs in calveolae or in cholesterol-rich membrane rafts,[23] the accessibility of PrP^{c} lipidated with a GPI mimic and possibly labeled with suitable fluorescent probes presently represents a challenge of greatest priority.

Experimental Section

1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanol-azide: Trifluoromethanesulfonic anhydride (0.39 mL, 2.36 mmol) was added dropwise to an ice-cooled solution of NaN_3 (0.61 g, 9.43 mmol) in H_2O (2.5 mL) and CH_2Cl_2 (4.5 mL) under vigorous stirring. After 2 h, the organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2 (3×2.5 mL). The combined organic layers containing the triflyl azide were washed with sat. $NaHCO_3$ and used directly in the next reaction step.

Triethylamine (0.33 mL, 2.36 mmol) and $CuSO_4{\cdot}5H_2O$ (19.7 mg, 0.08 mmol) were added to a suspension of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol-amine (0.50 g, 0.79 mmol) in MeOH/H_2O (8:2, 10 mL). This was followed by addition of the triflyl azide solution in CH_2Cl_2 (ca. 12 mL). The mixture was stirred overnight at RT, and the clear solution was concentrated to an oily residue. This was distributed between EtOAc (60 mL) and H_2O (10 mL). The organic layer was washed with 5% $KHSO_4$ (5 mL) and H_2O (2×5 mL), and then dried ($MgSO_4$). The solution was evaporated to a semi-solid residue, which was separated by chromatography on silica gel by elution with $CHCl_3$/MeOH/17% ammonia (40:10:1). Fractions containing homogeneous product were pooled, evaporated, and recrystallized from $CHCl_3/CH_3CN$. Yield: 0.48 g (90% as ammonium salt); m.p. 66–68 °C; TLC ($CHCl_3$/MeOH/17% ammonia, 40:10:1): $R_f = 0.35$, TLC ($CHCl_3$/MeCN/80% AcOH, 50:20:10): $R_f = 0.40$; HPLC [EC 125/4 Nucleosil 300–5 C-4, linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.1% TFA in H_2O/CH_3CN (5:95)]: $t_R =$ 16.15 min; ESI-MS: $m/z = 662.6$ $[M+H]^+$, calcd for $C_{33}H_{64}N_3O_8P$: 661.4; ^{1}H NMR (400.13 MHz, $CDCl_3$, 27 °C): 7.5 (br, 4H; NH_4^+), 5.2 (m, 1H; OC*H*-$(CH_2)_2$-), 4.65, 4.17 (dd, 2H; CH-CH_2-O-P), 4.00 (dd, 2H; P-O-CH_2-CH_2), 3.95 (t, 2H; COO-CH_2-CH), 3.45 (t, 2H; CH_2-N_3), 2.28 (br, 4H; 2CH_2-CO), 1.60 (dd, 4H; 2CH_2-CH_3), 1.27 (br, 40H; 20-CH_2-CH_2-CH_2), 0.85 (t, 6H; 2CH_3).

CAUTION: Azide ions form explosive compounds with dichloromethane.[24]

Staining of HeLa cells with lipopeptide 5a: HeLa cells were grown on glass cover slips in CO_2-independent growth medium (D-MEM with 20 mM HEPES buffer). Prior to microscopy, they were incubated for 15 min with a solution of **5a** in PBS buffer (1 µM), washed with pure PBS buffer, and covered with fresh growth medium. Confocal microscopy was performed at 37 °C by using a Zeiss LSM510 with an excitation wavelength of 543 nm and long-pass-filtered fluorescence detection above 560 nm.

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Keywords: alkynes · azides · cycloadditions · lipopeptides · native chemical ligation

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[12] Peptide **2** was treated in peroxide-free THF with azide **1** in equimolar mixture by addition of diisopropylethylamine (4 equiv) and CuI (0.2 equiv). Washing in EtOAc with 3% $KHSO_4$ and H_2O, followed by silica gel chromatography ($CHCl_3$/MeOH/AcOH/H_2O, 60:18:2:3) and crystallization from CH_2Cl_2/CH_3CN gave **3** in 78% yield that was homogeneous on TLC. HPLC [EC 125/4 Nucleosil 300–5 C-4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.1% TFA in H_2O/CH_3CN (5:95)]: t_R = 15.8 min; ESI-MS: m/z = 1335.0 $[M+H]^+$; calcd for $C_{69}H_{124}N_7O_{15}PS$: 1334.7.

[13] Upon acidolytic deprotection of the lipopeptide **3**, evaporation from $CHCl_3$/EtOH (2:1) produced a film, which was solubilized in 2% octyl-β-D-glucopyranoside. Its lyophilizate was dissolved in phosphate buffer (100 mM, pH 7.5) by ultrasonication, and TCEP·HCl (4 equiv) was added followed by **4a** (1 equiv) in CH_3CN/H_2O (3:1). After 12 h, **5a** was isolated by preparative HPLC [VP 250/21 Nucleosil C-4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.1% TFA in H_2O/CH_3CN (5:95)] in 50% yield. Because of the isomeric mixture of rhodamine B isothiocyanate used in the synthesis of **4a**, compound **5a** gives a double peak in HPLC [EC 125/4 Nucleosil 300–5 C-4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.1% TFA in H_2O/CH_3CN (5:95)]: t_R = 15.0 and 15.4 min; ESI-MS: m/z = 1761.4 $[M+H]^+$; calcd for $C_{85}H_{127}N_{14}O_{20}PS_2$: 1760.1. Compound **5b** was obtained by the identical procedure in 67% yield; homogeneous on HPLC (see Figure 1); ESI-MS: m/z = 1494.0 $[M+H]^+$; calcd for $C_{68}H_{109}N_{12}O_{19}PS_2$: 1493.8.

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[15] The reaction was performed in borate buffer (50 mM, pH 8.2)/*tert*-butanol (2:1) by using 4 equiv of azide and a higher amount of Cu^{2+}/sodium ascorbate (1:5, 0.1 equiv) than in ref. [8b]. Upon centrifugation and washing with water, the lipopeptide **6** was obtained in almost quantitative yield. HPLC [EC 125/4 Nucleosil 300–5 C4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.08% TFA in H_2O/CH_3CN (5:95)]: t_R = 14.1 min (>90%); ESI-MS: m/z = 1420.2 $[M+2H]^{2+}$, 947.2 $[M+3H]^{3+}$; calcd for $C_{126}H_{199}N_{30}O_{40}P$: 2839.2.

[16] Compound **7** was isolated by preparative HPLC [EC 250/21 Nucleosil 300–5 C4; linear gradient from 0.1% TFA in H_2O/CH_3CN (80:20) to 0.08% TFA in H_2O/CH_3CN (15:85) over 80 min]; HPLC (conditions as reported for **5a**[13]): t_R = 13.2 min; ESI-MS: m/z = 1671.4 $[M+2H]^{2+}$, 1114.8 $[M+3H]^{3+}$, 836.6 $[M+4H]^{4+}$; calcd. for $C_{149}H_{228}N_{35}O_{46}PS_2$: 3340.79.

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