

Tritium labeling of a cholesterol amphiphile designed for cell membrane anchoring of proteins

Balázs Schäfer¹, Erika Orbán^{2,#}, Zoltán Kele³, Csaba Tömböly^{1*}

¹Laboratory of Chemical Biology, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, Temesvári krt. 62., 6726 Szeged, Hungary

²Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Pázmány Péter sétány 1/a, 1117 Budapest, Hungary

³Department of Medicinal Chemistry, Szeged University, Dóm tér 8., 6720 Szeged, Hungary

[#]Current address: Institute of Biophysical Chemistry, Goethe University Frankfurt, Max-von-Laue str. 9, 60438 Frankfurt, Germany

Abstract

Cell membrane association of proteins can be achieved by the addition of lipid moieties to the polypeptide chain, and such lipid-modified proteins have important biological functions. A class of cell surface proteins contains a complex glycosylphosphatidylinositol (GPI) glycolipid at the C-terminus, and they are accumulated in cholesterol-rich membrane microdomains, i.e. lipid rafts. Semisynthetic lipoproteins prepared from recombinant proteins and designed lipids are valuable probes and model systems of the membrane-associated proteins. Because GPI-anchored proteins can be reinserted into the cell membrane with the retention of the biological function, they are appropriate candidates for preparing models via reduction of the structural complexity. A synthetic headgroup was added to the 3 β -hydroxyl group of cholesterol, an essential lipid component of rafts, and the resulting cholesterol derivative was used as a simplified GPI mimetic. In order to quantitate the membrane integrated GPI mimetic after the exogenous addition to live cells, a tritium labeled cholesterol anchor was prepared. The radioactive label was introduced into the headgroup, and the radiolabeled GPI mimetic anchor was obtained with a specific activity of 1.37 TBq/mmol. The headgroup labeled cholesterol derivative was applied to demonstrate the sensitive detection of the cell membrane association of the anchor under in vivo conditions.

Introduction

A unique class of cell membrane-associated proteins contains a C-terminal glycosylphosphatidylinositol (GPI) moiety, and these lipoproteins associate with the outer leaflet of the membrane bilayer via the lipid chains of the GPI molecule. GPI-anchored proteins are functionally diverse including enzymes, cell adhesion molecules, receptors, complement regulatory proteins, immunoproteins and further proteins (e.g. prion protein) with unknown functions.^[1] It was shown that their biological functions were retained after exogenous addition to membranes,^[2-5] therefore semisynthetic lipoproteins containing diverse reporters in the headgroup of the lipid moiety can be valuable probes for investigating GPI-anchored proteins.^[6-13] The GPI moiety directs the attached protein towards the extracellular space and locates the attached protein in special membrane microdomains, i.e. lipid rafts. Because cholesterol maintains the dynamic lipid-lipoprotein associates of GPI-anchored proteins, sphingolipids and cholesterol,^[14;15] it is hypothesized that cholesteryl lipoproteins may also be accumulated in lipid rafts.^[16] Based on the finding that cholesteryl poly(ethylene glycol) ethers accumulate in cholesterol rich membranes and their cytotoxicity is low,^[17] we have recently prepared a cholesterol derivative containing both a maleimide and a fluorescent reporter in the headgroup for protein conjugation and imaging, respectively. It was shown that after protein conjugation the semisynthetic cholesteryl lipoprotein could be purified via a β -cyclodextrin inclusion complex that made the cholesterol moiety soluble in polar solvents. Then the lipid excess- and detergent-free lipoprotein could be introduced into the cell membrane of live cells with minimal disruption of the membrane, and the fluorescence of the headgroup could be unambiguously assigned to the attached protein.^[18] In order to further study the membrane associated cholesteryl lipoproteins it is important to quantitate the lipoprotein incorporated into the cell membrane. It can be achieved by determining the radioactivity of cell membrane preparations or intact cells treated with a tritium labeled analogue of the anchor molecule. Herein we report our results on the preparation and biological application of a tritium labeled cholesterol derivative designed for that purpose.

Experimental

The purity of all reagents and solvents were analytical or the highest commercially available grade. Protected propargylglycine (Pra) and 5-amiofluorescein were purchased from Bachem Feinchemicalen AG (Bubendorf, Switzerland), coupling reagents and substituted di(ethylene glycols) were obtained from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Other chemicals were from Sigma Ltd. (St. Louis, MO, USA). Tritium labeling was carried out in a self-designed vacuum manifold,^[19;20] radioactivity was measured with a Packard Tri-Carb 2100 TR liquid scintillation analyser using Hionic-Fluor scintillation cocktail of PerkinElmer. Analytical thin layer chromatography (TLC) was performed on 5×10 cm glass plates precoated with silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany), spots were visualized with UV light, ninhydrin or phosphomolybdic acid, radioactivity was detected with a Berthold LB511 linear TLC analyzer. Flash chromatography was carried out on silica gel 60 (Sigma Ltd., St. Louis, MO, USA) using the indicated solvents. Analytical and semipreparative HPLC separations were performed with a Merck-Hitachi LaChrom system under the indicated conditions. Radio-HPLC was performed on a Jasco HPLC system equipped with a Packard Radiomatic 505 TR Flow Scintillation Analyser. NMR spectra were recorded on a Bruker Spectra DRX 500 MHz spectrometer in the indicated solvent, chemical shifts (δ) are reported in ppm after calibration to the solvent signals. Molecular weight of the compounds were determined by MS analysis on a Finnigan TSQ 7000 spectrometer equipped with an ESI source, or on a Bruker reflex III MALDI-TOF spectrometer. In the case of MALDI-MS, 2,5-dihydroxybenzoic acid matrix was applied. Mass spectrometric measurements of [²H]piperidine were performed on a Micromass Q-TOF Premier mass spectrometer (Waters MSTechnologies, Manchester, UK) equipped with a nanoelectrospray ion source. The instrument was scanned in the mass range of 85-90. Peak intensities were calculated for $M+H]^+$, $M(^2H_1)+H]^+$, $M(^2H_2)+H]^+$, $M(^2H_3)+H]^+$ molecule ions by the Masslynx software. The measured peak intensities of mono-, di- and tri-deuterated ions were corrected by the calculated intensities of overlapping $M(^{13}C)+H]^+$, $M(^2H_1, ^{13}C)+H]^+$, and $M(^2H_2, ^{13}C)+H]^+$ peaks, respectively. The effect of $M(^{13}C_2)+H]^+$ and $M(^2H_1, ^{13}C_2)+H]^+$ and ¹⁵N containing ions were neglected. Live cells treated with the

fluorescent anchor were visualized with an Olympus IX81 confocal laser scanning microscope and images were prepared by the FluoView 500 software.

[²H]piperidine 1

An amount of 5 mg of 4-bromopiperidine hydrobromide (20 μmol) was dissolved in 400 μL of 10 M NaOH and it was extracted with 1 mL of EtOAc. The organic phase was washed with brine, dried over Na₂SO₄ and evaporated at atmospheric pressure. The resulting amine was dissolved in 500 μL of EtOAc and it was reduced under 0.4 bar deuterium gas in the presence of 11 mg of Pd/C (10% Pd) catalyst. After 3 h, the catalyst was filtered off and the EtOAc solution was extracted with 1 M HCl. [²H]piperidine hydrochloride **1** was obtained after repeated evaporations from the acidic aqueous solution (2 mg, 82%). R_f 0.38 (*n*-BuOH–AcOH–H₂O 2:1:1); ESI-MS calcd for C₅H₁₀DN 86.10, found 87.09 [M+H]⁺.

[³H]piperidine

Tritium labeling was performed with 2.1 mg of 4-bromopiperidine base (12.9 μmol) dissolved in 1 mL of EtOAc in the presence of 5 mg of Pd/C (10% Pd) catalyst. The reaction mixture was degassed prior to tritium reduction by a freeze-thaw cycle, and then it was stirred under 0.4 bar of tritium gas for 3 h, followed by the filtration of the catalyst. The EtOAc solution was extracted with 1 M HCl, and labile tritium was removed by repeated evaporations from 1 M HCl solution. Finally 20.5 GBq of [³H]piperidine hydrochloride was isolated as a white solid (s.a. 1.58 TBq/mmol), that was immediately used for the next step.

[³H]N-nitrosopiperidine

The solid [³H]piperidine hydrochloride (20.5 GBq) was dissolved in 100 μL of 1 M HCl and the solution was cooled into ice. Then 250 mg of KNO₂ was added and the reaction mixture was stirred at 0 °C for 2 h followed by extraction with Et₂O. The ethereal solution was evaporated at atmospheric

pressure yielding 17.8 GBq (87%) of crude [³H]N-nitrosopiperidine. It was dissolved in 100 μL THF and immediately used in the next step without further purification. R_f 0.28 (petroleum ether–acetone 9:1), 93% radiochemical purity by radio-TLC.

[³H]N-aminopiperidine 2

The solution of 17.8 GBq of [³H]N-nitrosopiperidine in 100 μL of THF was transferred to a reaction vessel equipped with a condenser. A suspension of 4.5 mg of LiAlH₄ in 600 μL of THF was added and the reaction mixture was stirred under Ar at 60 °C for 3.5 h. After cooling, 100 μL of 10 M NaOH was added and it was stirred for further 20 min. The resulting slurry was extracted with Et₂O, and the ethereal solution was extracted with 3 M HCl. The HCl solution was evaporated and labile tritium was removed by repeated evaporations from water : ethanol (1:1) resulting in 8.4 GBq (47%) of solid [³H]N-aminopiperidine hydrochloride (0.8 mg), which was used for the preparation of **8** without further purification. R_f 0.38 (*n*-BuOH–AcOH–H₂O 2:1:1), 73% radiochemical purity by radio-TLC, s.a. 1.33 TBq/mmol.

Cholesteryl (S)-1-(4-(2-(tert-butoxycarbonylamino)-2-carboxyethyl)-1H-1,2,3-triazol-1-yl)-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate 3

Boc-Pra-OH (60 mg, 281.4 μmol) was dissolved in 1 ml of DMF, and it was added to the solution of cholesteryl 1-azido-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate^[18] (230 mg, 335.0 μmol) in CH₂Cl₂ (4 ml). Then a solution of L(+)-ascorbic acid sodium salt (33.4 mg, 168.8 μmol) and CuSO₄·5H₂O (21.1 mg, 84.4 μmol) in water (4 ml) was added. The reaction mixture was stirred for 8 h at room temperature and then it was evaporated in vacuo. The crude product was purified by column chromatography (CHCl₃–MeOH–AcOH 90:9:1) to give 230.4 mg (91%) of pure **3** as a white solid. R_f 0.30 (CHCl₃–MeOH–AcOH 90:9.5:0.5); k' = 8.61 (t_R = 12.5 min; Vydac 214TP5410 C4 column (100 × 4.6 mm, 5 μm), gradient elution with 1.8 %/min ACN (0.08% (v/v) TFA) in H₂O (0.1%(v/v) TFA) starting from 50% ACN, flow rate: 1 mL/min, λ = 216 nm); ¹H NMR (500 MHz, CDCl₃) δ 7.63 (s, 1H),

6.63 (s, 1H), 5.61 (s, 1H), 5.34 (d, 1H, $J= 3.7$ Hz), 4.58 (s, 2H), 4.53 (m, 1H), 4.47 (s, 1H), 3.86 (s, 2H), 3.60 (m, 10H), 3.42 (q, 2H, $J= 4.6$ Hz), 3.28 (m, 2H), 2.62 (t, 2H, $J= 6.7$ Hz), 2.47 (t, 2H, $J= 6.7$ Hz), 2.29 (d, 2H, $J= 7.6$ Hz), 1.98 (m, 1H), 1.93 (m, 1H), 1.83 (m, 3H), [1.62-1.00 (m, 18H): 1.57 (3H), 1.52 (1H), 1.48 (2H), 1.40 (1H), 1.32 (1H), 1.30 (2H), 1.20 (1H), 1.14 (1H), 1.11 (4H), 1.09 (2H)], 1.42 (s, 9H), 1.00 (s, 3H), [0.99-0.86 (m, 3H): 0.94 (2H), 0.88 (1H)], 0.90 (d, 3H, $J= 6.4$ Hz), [0.86 (d, 3H, $J= 1.8$ Hz), 0.84 (d, 3H, $J= 1.8$ Hz)], 0.66 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 172.7, 172.1, 155.7, 142.7, 139.7, 124.1, 122.7, 80.0, 74.5, 70.7, 70.5, 70.3, 70.1, 69.9, 69.5, 56.8, 56.2, 53.3, 50.5, 50.1, 42.4, 39.8, 39.6, 39.3, 38.2, 37.1, 36.7, 36.3, 35.9, 32.0 (2C), 31.0, 30.0, 28.5, 28.3, 28.12, 28.07, 27.8, 24.4, 23.9, 22.9, 22.7, 21.1, 19.4, 18.8, 12.0; MS(MALDI) calcd for $\text{C}_{49}\text{H}_{81}\text{N}_5\text{O}_{10}$ 899.60, found 900.44 $[\text{M}+\text{H}]^+$.

Cholesteryl (S)-1-(4-(2-amino-2-carboxyethyl)-1H-1,2,3-triazol-1-yl)-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate 4

Compound **3** (200 mg, 222.3 μmol) was dissolved in 2 ml of TFA- CH_2Cl_2 1:1 and it was stirred for 30 min. The solution was evaporated in vacuo to give 197.0 mg (97%) of pure **4** trifluoroacetate as a pale yellow solid. R_f 0.12 (CHCl_3 -MeOH-AcOH 90:9.5:0.5); k' = 4.15 (t_R = 6.7 min; Vydac 214TP5410 C4 column (100 \times 4.6 mm, 5 μm), gradient elution with 1.8 %/min ACN (0.08% (v/v) TFA) in H_2O (0.1%(v/v) TFA) starting from 50% ACN, flow rate: 1 mL/min, λ = 216 nm); MS(MALDI) calcd for $\text{C}_{44}\text{H}_{73}\text{N}_5\text{O}_8$ 799.55, found 800.48 $[\text{M}+\text{H}]^+$.

Cholesteryl (S)-1-(4-(2-carboxy-2-(3-(maleimido)propanamido)ethyl)-1H-1,2,3-triazol-1-yl)-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate 5

The amino acid derivative **4** (140 mg, 153.2 μmol) and DIEA (61 μL , 350.4 μmol) were dissolved in MeOH (4 ml) and 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (56.0 mg, 210.4 μmol) was added. The mixture was stirred for 3 h at room temperature and then it was evaporated in vacuo. The crude product was purified by column chromatography (CHCl_3 -MeOH-AcOH 85:14:1) to give 118

mg (81%) of pure **5** as a white solid. R_f 0.43 (CHCl₃-MeOH-AcOH 80:19:1); $k' = 6.53$ ($t_R = 9.8$ min; Vydac 214TP5410 C4 column (100 × 4.6 mm, 5 μm), gradient elution with 1.8 %/min ACN (0.08% (v/v) TFA) in H₂O (0.1%(v/v) TFA) starting from 50% ACN, flow rate: 1 mL/min, $\lambda = 216$ nm); ¹H NMR (500 MHz, CDCl₃) δ 7.70 (s, 1H), 7.02 (brs, 1H), 6.68 (s, 2H), 6.63 (brs, 1H), 5.34 (d, 1H, $J = 3.7$ Hz), 4.77 (brs, 1H), 4.57 (m, 2H), 4.49 (s, 1H), 3.82 (m, 4H), 3.61 (m, 10H), 3.42 (q, 2H, $J = 4.6$ Hz), 3.24 (m, 2H), 2.62 (t, 2H, $J = 6.7$ Hz), 2.58 (t, 2H, $J = 6.3$ Hz), 2.47 (t, 2H, $J = 6.7$ Hz), 2.30 (d, 2H, $J = 7.6$ Hz), 2.01 (m, 1H), 1.94 (m, 1H), 1.83 (m, 3H), [1.62-1.03 (m, 18H): 1.57 (3H), 1.52 (1H), 1.48 (2H), 1.40 (1H), 1.32 (1H), 1.30 (2H), 1.20 (1H), 1.14 (1H), 1.11 (4H), 1.09 (2H)], 1.00 (s, 3H), [0.99-0.86 (m, 3H): 0.94 (2H), 0.88 (1H)], 0.90 (d, 3H, $J = 6.4$ Hz), [0.86 (d, 3H, $J = 1.8$ Hz), 0.84 (d, 3H, $J = 1.8$ Hz)], 0.66 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 172.80, 172.7, 172.2, 170.7, 169.9, 142.7, 139.8, 134.3, 124.3, 122.8, 74.5, 70.6, 70.5, 70.3, 70.0, 69.9, 69.4, 56.8, 56.3, 53.5, 50.5, 50.1, 42.4, 39.8, 39.6, 39.4, 38.2, 37.1, 36.7, 36.3, 35.9, 34.6, 34.3, 32.0 (2C), 31.0, 30.0, 28.4, 28.1 (2C), 27.9, 24.4, 24.0, 23.0, 22.7, 21.2, 19.4, 18.8, 12.0; MS(MALDI) calcd for C₅₁H₇₈N₆O₁₁ 950.57, found 951.45 [M+H]⁺.

Fluorescent cholesterol anchor 6

The maleimide derivative **5** (50 mg, 52.6 μmol) and HATU (20.0 mg, 52.6 μmol) were dissolved in THF (5 ml) and DIEA (9.1 μL, 52.6 μmol) was added to the solution. It was stirred for 5 min followed by the addition of fluorescein amine (54.8 mg, 157.8 μmol) in THF (2 mL). The reaction mixture was stirred for 16 h at room temperature, then it was evaporated in vacuo and the crude product was purified by column chromatography (CHCl₃-MeOH-AcOH 90:9:1) to give 37.0 mg (55%) of pure **6** as an orange solid. R_f 0.41 (CHCl₃-MeOH-AcOH 90:9:1); $k' = 8.53$ ($t_R = 12.4$ min; Vydac 214TP5410 C4 column (100 × 4.6 mm, 5 μm), gradient elution with 1.8 %/min ACN (0.08% (v/v) TFA) in H₂O (0.1%(v/v) TFA) starting from 50% ACN, flow rate: 1 mL/min, $\lambda = 216$ nm); ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.30 (s, 1H), 10.21 (brs, 2H), 8.43 (s, 1H), 8.32 (d, 1H, $J = 5.7$ Hz), 7.89 (t, 1H, $J = 5.4$ Hz), 7.79 (s, 1H), 7.74 (d, 1H, $J = 8.3$ Hz), 7.15 (d, 1H, $J = 8.3$ Hz), 6.97 (s, 2H), 6.90 (s, 2H), 6.87 (dd,

2H, $J= 8.6, 2.6$ Hz), 6.73 (dd, 2H, $J= 8.6, 1.6$ Hz), 5.31 (d, 1H, $J= 3.2$ Hz), 5.09 (q, 1H, $J= 5.8$ Hz), 4.47 (t, 2H, $J= 5.1$ Hz), 4.42 (m, 1H), 3.92 (m, 2H), 3.77 (t, 2H, $J= 5.1$ Hz), 3.45 (brs, 10H), 3.38 (q, 2H, $J= 5.7$ Hz), 3.12 (m, 1H), 3.05 (dd, 1H, $J= 14.9, 7.1$ Hz), 2.71 (m, 2H), 2.43 (t, 2H, $J= 6.7$ Hz), 2.34 (t, 2H, $J= 6.7$ Hz), 2.22 (d, 2H, $J= 7.6$ Hz), 1.92 (m, 1H), 1.85 (m, 1H), 1.76 (m, 3H), [1.60-0.92 (m, 21H): 1.48 (2H), 1.52 (1H), 1.47 (1H), 1.44 (2H), 1.39 (1H), 1.28 (3H), 1.17 (1H), 1.13 (1H), 1.11 (4H), 1.02 (1H), 1.00 (1H), 0.97 (1H), 0.96 (1H), 0.91 (1H)], 0.95 (s, 3H), 0.87 (d, 3H, $J= 6.4$ Hz), 0.84 (d, 3H, $J= 2.0$ Hz), 0.81 (d, 3H, $J= 2.0$ Hz), 0.65 (s, 3H, 18-H); ^{13}C NMR (125 MHz, $(\text{CD}_3)_2\text{SO}$) δ 171.5, 171.2, 171.0, 170.6, 169.3, 168.5, 159.2, 152.7, 146.5, 142.8, 142.1, 139.2, 134.1, 129.6, 125.9, 125.5, 125.3, 123.1, 121.9, 116.9, 114.0, 109.1, 101.3, 83.8, 72.8, 69.8, 69.7 (2C), 69.6, 69.3, 69.2, 56.1, 55.9, 55.4, 49.5, 49.4, 42.1, 39.1, 39.0, 38.8, 37.7, 36.6, 36.1, 35.7, 35.0, 34.9, 34.5, 31.8, 31.1, 29.7, 29.2, 27.9, 27.7, 27.4, 27.2, 23.9, 23.3, 22.6, 22.4, 20.9, 19.0, 18.6, 11.7; MS(MALDI) calcd for $\text{C}_{71}\text{H}_{89}\text{N}_7\text{O}_{15}$ 1279.64, found 1280.56 $[\text{M}+\text{H}]^+$.

Cholesterol anchor 7

The maleimide derivative **5** (25 mg, 26.3 μmol), HOBt (3.6 mg, 26.3 μmol) and DIC (4 μL , 26.3 μmol) were dissolved in DMF (2 mL), then *N*-aminopiperidine (3.1 μL , 29 μmol) was added and the solution was stirred for 16 h at room temperature. It was evaporated in vacuo and the crude product was purified by HPLC on a Discovery Bio Wide Pore C_5 column to give 16 mg (59%) of pure **7** as a white solid. R_f 0.45 (CHCl_3 -MeOH-TEA 90:9.9:0.1); $k' = 4.31$ ($t_R = 6.9$ min; Vydac 214TP5410 C_4 column (100 \times 4.6 mm, 5 μm), gradient elution with 1.5 %/min ACN (0.08% (v/v) TFA) in H_2O (0.1%(v/v) TFA) starting from 60% ACN, flow rate: 1 mL/min, $\lambda = 216$ nm); ^1H NMR (500 MHz, CDCl_3) δ 7.63 (s, 1H), 7.08 (s, 1H), 6.68 (s, 2H), 6.60 (s, 1H), 6.48 (s, 1H), 5.34 (d, 1H, $J= 3.7$ Hz), 4.87 (brs, 1H), 4.55 (m, 2H), 4.46 (s, 1H), 3.83 (s, 4H), 3.61 (brs, 10H), 3.42 (q, 2H, $J= 4.6$ Hz), 3.20 (m, 2H), 2.73 (m, 4H), 2.62 (t, 2H, $J= 6.7$ Hz), 2.58 (t, 2H, $J= 6.7$ Hz), 2.47 (t, 2H, $J= 7.6$ Hz), 2.29 (d, 2H), 1.99 (m, 1H), 1.96 (m, 1H), 1.83 (m, 3H), 1.71 (m, 4H), [1.58-1.01 (m, 20H): 1.55 (3H), 1.50 (1H), 1.47 (2H), 1.43 (m, 3H), 1.32 (1H), 1.30 (2H), 1.23 (1H), 1.14 (1H), 1.11 (4H), 1.09 (1H), 1.05 (1H)], 1.00 (s, 3H), [0.96-

0.83 (m, 3H): 0.96 (1H), 0.92 (1H), 0.90 (1H)], 0.88 (d, 3H, $J= 6.4$ Hz), [0.86 (d, 3H, $J= 1.8$ Hz), 0.84 (d, 3H, $J= 1.8$ Hz)], 0.66 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 172.7, 172.2, 170.7, 169.9, 156.8, 142.7, 139.8, 134.3, 124.3, 122.8, 74.5, 70.6, 70.5, 70.3, 70.0, 69.9, 69.4, 63.4, 56.8, 56.3, 53.6, 50.5, 50.1, 42.4, 39.8, 39.6, 39.4, 38.2, 37.1, 36.7, 36.3, 35.9, 34.6, 34.3, 32.0 (2C), 31.0, 30.0, 28.4, 28.14, 28.12, 27.9, 27.2, 24.4, 24.0, 23.5, 23.0, 22.7, 21.2, 19.4, 18.8, 12.0; MS(MALDI) calcd for $\text{C}_{56}\text{H}_{88}\text{N}_8\text{O}_{10}$ 1032.66, found 1033.58 $[\text{M}+\text{H}]^+$.

*[^3H]Cholesterol anchor **8***

The maleimide derivative **5** (1 mg, 1 μmol) was dissolved in DMF (300 μL) containing HOBt (0.13 mg, 1 μmol), DIC (0.14 μL , 1 μmol) and DIEA (0.09 μL , 0.5 μmol). Then [^3H]N-aminopiperidine (100 MBq) dissolved in 24 μL of DMF was added and the solution was stirred for 16 h at room temperature. It was evaporated in vacuo and the crude product was dissolved in HFIP and it was purified by HPLC on a Vydac 214TP5410 column to give 17.4 MBq of pure **8**. $k' = 4.31$ ($t_{\text{R}} = 6.9$ min; Vydac 214TP5410 C4 column (100 \times 4.6 mm, 5 μm), gradient elution with 1.5 %/min ACN (0.08% (v/v) TFA) in H_2O (0.1%(v/v) TFA) starting from 60% ACN, flow rate: 1 mL/min, $\lambda = 216$ nm), s.a. 1.37 TBq/mmol.

Cell culture

SH-SY5Y (ATCC: CRL-2266) human neuroblastoma cells were used to test the membrane association potency of **6** and **8**. Cells were cultured in DMEM (Sigma Ltd., St. Louis, MO, USA) medium containing 10% FCS, L-glutamine (2 mM), gentamycin (160 $\mu\text{g}/\text{mL}$), 1 mM pyruvate and non-essential amino acids (Sigma Ltd., St. Louis, MO, USA). The cell culture was maintained at 37°C in a humidified atmosphere with 5% CO_2 .

Live cell imaging with confocal laser scanning microscopy

20000 cells per well were plated on an 8-well Lab-Tek II Chambered coverglass. After 48 h incubation at 37°C, cells were treated with 1 μM of **6** dissolved in serum-free medium containing 10 μM β -cyclodextrin for 30 min. Cells treated with serum-free medium for 30 min were used as a negative

control. After treatment and incubation, cells were washed with serum-free medium and the nuclei of the cells were stained for 5 min with DRAQ5. Sequential excitation was applied for fluorescein, and DRAQ5. Fluorescein was excited with an Ar ion laser (488 nm) and emitted photons were collected through a BA 505-525 nm filter; DRAQ5 was excited with a He-Ne laser at 633 nm and emitted photons were collected through a BA 660 nm filter.

*Membrane incorporation of [³H]cholesterol anchor **8***

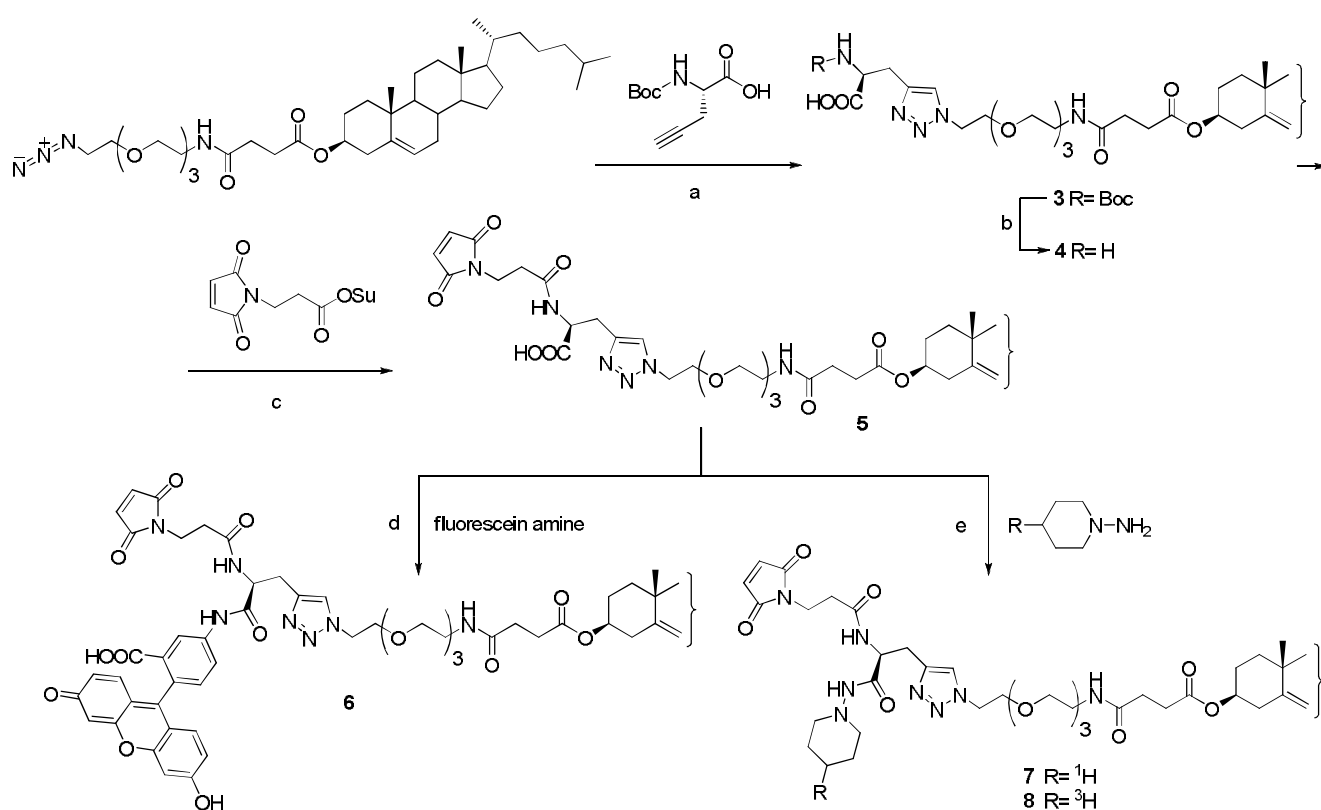
10⁶ cells per well were plated on a 24-well plate, and after 12 h incubation at 37°C cells were treated with 1 – 100 nM of **2** or **8** dissolved in serum-free medium containing 10 μM β-cyclodextrin for 10 and 30 min. Then cells were washed with serum-free medium and trypsinized. The resulting cell suspensions were filtered on a GF/B filter (Whatman) using a 24-well cell harvester (Brandel) and washed with PBS buffer pH7.4. Filter discs containing the cells were immersed into Ultima Gold XR scintillation cocktail and they were counted in glass vials with a Packard Tri-Carb 2100 TR LSA. Cells treated with serum-free medium for 30 min were used for background counting.

Results and Discussion

Our earlier studies revealed that a maleimide functionalized fluorescent cholesterol derivative is capable of anchoring proteins to the plasma membrane of live cells.^[18] The headgroup of the anchor molecule contains a diethylene glycol residue which increases the polarity of the molecule and exposes the attached protein toward the extracellular space. When applied in a form of β-cyclodextrin inclusion complex, the headgroup fluorescence of the cholesterol anchor can be used for the unambiguous visualization of the attached protein after cell membrane delivery. In order to determine the membrane concentration of the cholesterol anchor, a tritium labeled analogue (**8**) was designed. Using this compound, the quantitation of the membrane incorporated protein anchor can be achieved by measuring the radioactivity of cell membranes preparations or intact cells. For this purpose a defined position of the radioactive label is crucial. Although [1,2-³H]cholesterol complexed with β-cyclodextrin

was reported to use for the fast enrichment of cell membranes with labeled cholesterol,^[21] and it was also used in studying the cyclodextrin-mediated cholesterol transfer between different lipid layers,^[22] cholesterol labeling is not appropriate for our purposes. The headgroup of the cholesterol anchor can be enzymatically removed via the hydrolysis of the cholesteryl ester, and as a result the labeled cholesterol moiety remains in the cell membrane and the unlabeled headgroup is released to the cell culture medium. In this case the membrane radioactivity is not affected by the presence or lack of the headgroup, and the process cannot be detected resulting in overestimation of the amount of the membrane associated cholesterol anchor. In contrast, if the tritium label is incorporated into the headgroup of the anchor molecule, enzymatic removal of the headgroup will result in decreasing radioactivity of the cell membrane preparation, and also emerging the labeled headgroup in the cell culture medium.

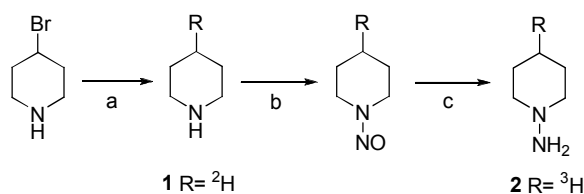
In order to introduce the radioactive label into the cholesterol anchor headgroup, a convergent synthetic strategy was applied. The tritium label was incorporated into a small molecule that was coupled to an anchor fragment bearing both the cholesterol residue and the bulk of the headgroup. The advantage of this approach is that tritium incorporation either into the cholesterol moiety or into other parts of the headgroup is excluded. The cholesterol amphiphile **4** is an appropriate intermediate, because a primary amine and a carboxylic acid functions in the headgroup are available for the introduction of a radiolabeled or a fluorescent tag, and a protein conjugation function as well. It was prepared from *N*^α-*t*Boc-protected propargylglycine and cholesteryl 1-azido-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oate via a dipolar cycloaddition in the presence of CuSO₄ and sodium ascorbate (Scheme 1). The *N*^α-*t*Boc-protecting group was removed by TFA in DCM and the amine **4** was liberated in situ with DIEA during the next step. Then **4** was used for the preparation of two constitutionally related, tritium or fluorescein labeled anchors. First the thiol reactive maleimide group was coupled to the amine **4**, because this way the co-existence of a Michael acceptor and a primary amine in an intermediate compound is excluded that otherwise could lead to self-addition under the basic conditions of the introduction of the labeling tag. It was found that the amino function of **4** reacts



Scheme 1 Preparation of the fluorescein and tritium labeled cholesterol anchors: a) NaAsc, CuSO₄·5H₂O, DMF–CH₂Cl₂–H₂O (1:4:4), RT, 8h; b) TFA–CH₂Cl₂ (1:1), RT, 30 min; c) 2 eq DIEA, MeOH, RT, 3h; d) HATU, DIEA, THF, RT, 16h; e) HOBT, DIC, DMF, RT, 16h.

faster with an *N*-hydroxysuccinimide ester than with a maleimide, and accordingly the *N*-acylation of **4** with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester in DMF resulted in **5**. This maleimide serves as a possible protein conjugation function in biological applications. In order to avoid the addition of nucleophiles to the maleimide of **5** an aniline and an alkyl-hydrazine – both are less basic than primary amines – were used to introduce the fluorescein and the tritium label, respectively. In a preliminary study it turned out, that the aniline moiety of 5-aminofluorescein did not react with the Michael acceptor of *N*-(2-maleimidoethyl)-2-aminopent-4-ynamide under the coupling condition applied for the preparation of **6**. Then the fluorescent anchor **6** was obtained by coupling of the carboxyl group of **5** with 5-aminofluorescein in the presence of the coupling agent HATU. The NMR structure investigation revealed that 5-aminofluorescein was conjugated to the carboxylic function of **5**.

Based on earlier observations on isotopomer radioligands,^[20;23] the tritium label was introduced via



Scheme 2 Preparation of [^3H]N-aminopiperidine: a) Pd/C (10% Pd), $^2\text{H}_2$ (**1**) or $^3\text{H}_2$ (**2**), EtOAc, RT, 3h; b) KNO_2 , 1 M HCl, 0 °C, 2h; c) LiAlH_4 , THF, Ar atm., 60 °C, 3.5h.

an aliphatic compound instead of aniline to increase the radiolytic stability of the labeled anchor molecule. *N*-Aminopiperidine was chosen for that purpose, because it is less basic than cyclohexylamine,^[24;25] and after *N*-acylation it results in stable hydrazides. Coupling of tritium labeled *N*-aminopiperidine to the carboxylic acid function of **5** results in an anchor molecule possessing a headgroup homologous to that of **6**, and this way the nonspecific tritium incorporation into the cholesterol moiety or into the headgroup is not feasible. Before preparing the tritium labeled anchor by *N*-acylation of [^3H]N-aminopiperidine with **5**, the unlabeled isotopologue **7** was prepared in order to optimize the reaction conditions and carry out NMR measurements. For the preparation of the tritium labeled *N*-aminopiperidine, 4-bromopiperidine was considered as a precursor in a deuterium labelling experiment (Scheme 2). The 4-bromopiperidine base was liberated from the hydrobromide salt and it was reduced with deuterium gas in the presence of Pd/C (10% Pd) catalyst. The resulting [^2H]piperidine hydrochloride was analyzed by TLC and ESI-MS. The separation of 4-bromopiperidine and piperidine was achieved on silica gel 60 F₂₅₄ glass plates in *n*-BuOH–AcOH–H₂O 2:1:1, as their R_f values were found to be 0.48 and 0.38, respectively. The TLC analysis of the crude [^2H]piperidine hydrochloride revealed that the conversion of 4-bromopiperidine was complete. Furthermore, the deuterium incorporation level was estimated by an ESI-MS analysis. It was found that 0.9 deuterium atoms/piperidine molecules were incorporated on average, but isotopologous species containing two or three deuterium atoms were also identified (Figure 1). These results suggested that this precursor is satisfactory for achieving an approximate molar activity of 1 TBq/mmol. In the next step [^3H]N-aminopiperidine was prepared from 4-bromopiperidine (Scheme 2). Reduction of the brominated piperidine

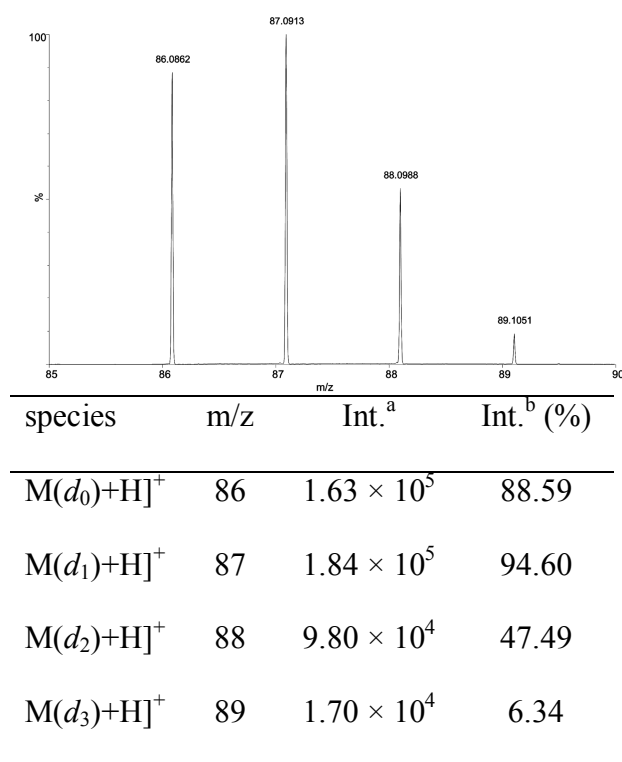


Figure 1 ESI mass spectrum of [²H]piperidine and the abundance of the isotopologues; ^a measured intensity, ^b relative intensity corrected with the natural abundance of ²H, ¹³C.

with tritium gas in the presence of Pd/C (10% Pd) catalyst resulted in tritium labeled piperidine that was nitrosated. The resulting [³H]*N*-nitrosopiperidine was then reduced with LiAlH₄, and [³H]*N*-aminopiperidine was isolated as a hydrochloride salt. The carboxylic acid **5** was activated with HOBt in the presence of DIC, and the resulting active ester was used *in situ* for the *N*-acylation of [³H]*N*-aminopiperidine to give the tritium labeled anchor **8**. HPLC analysis revealed that a significant amount of tritium labeled *N*-aminopiperidine was coupled to **5** (Figure 2). The purification of the crude cholesterol derivative was achieved by RP-HPLC resulting in 17.4 MBq of pure **8** with an isolated radiochemical yield of 17%. The sterical hindrance of the carboxylic acid function of **5** and the more diluted reaction mixture as compared to that of **6** and **7** resulted in the low yield of **8**. Quantitative analysis of the concentration and radioactivity of the labeled anchor **8** was performed by RP-HPLC via UV and radioactivity detection using a calibration curve made by **7**. The specific activity of the tritiated cholesterol anchor **8** was found to be 1.37 TBq/mmol that is higher than the theoretical value. Beyond

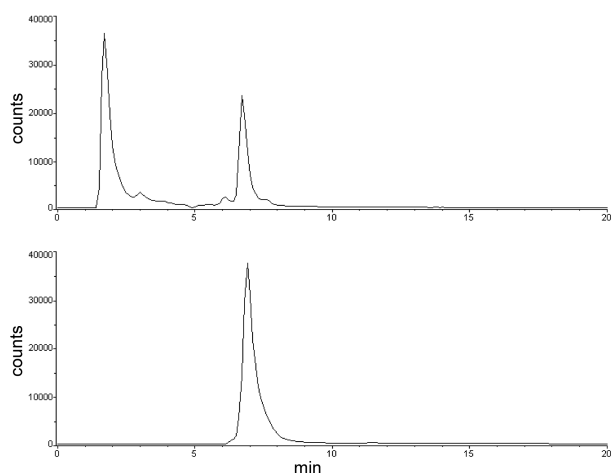


Figure 2 Radio-HPLC chromatogram of (A) the crude and (B) the purified tritium labeled cholesterol anchor **8**. [^3H]N-aminopiperidine was eluted without retention, **8** was eluted at $t_{\text{R}} = 6.90$ min.

tritium substitution of Br in 4-bromopiperidine, catalytic $^1\text{H}/^3\text{H}$ exchange reactions also took place presumably at the positions 2 and 6 of the piperidine ring. When 2-3 h of reaction time is applied for catalytic dehalotritiation, the catalytic exchange becomes significant and increases the specific activity.^[23] The ESI-MS analysis of the deuterium labeled piperidine also indicated catalytic exchange reactions. It is important to note that the convergent synthetic strategy ensures that all the tritium atoms are localized in the piperidine ring of the headgroup, because after the tritium gas reduction of 4-bromopiperidine further transformations do not affect the tritium distribution.

The control compound **6**, a fluorescent GPI anchor mimetic was used to assess the membrane association property of the designed [^3H]anchor. The solubility of **6** was investigated in an aqueous medium before the imaging application. It was found that the amphiphile **6** partially dissolved in a serum free medium as micellar associates and the remaining portion formed precipitates. The resulting heterogeneous mixture was incubated with 10 eq β -cyclodextrin that forms water-soluble inclusion complexes with cholesterol and 3 β -hydroxyl-modified cholesterol derivatives.^[26] This way 0.5 mM aqueous solution of the cholesterol amphiphile was prepared. Then SH-SY5Y cells were treated with 1 μM of the inclusion complex of **6** for 30 min. After delivering to live SH-SY5Y cells, an intensive membrane staining was observed, i.e. **6** incorporated into the cell membrane similarly to our original anchor^[18] (Figure 3). It indicates that a slight modification in the α -amino acid function of the head-

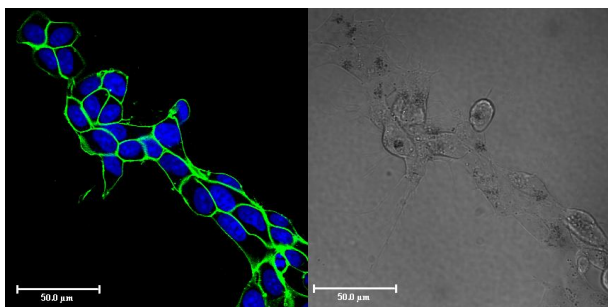


Figure 3 Confocal laser scanning (left) and differential interference contrast (right) microscopy of SH-SY5Y cells after 30 min incubation with 1 μ M cholesterol anchor **6** followed by a treatment with the nuclear dye DRAQ5 for 5 min at 37°C. On the image fluorescein is green and nuclei are blue.

group is tolerated with the retention of the membrane association properties. The tritium labeled anchor **8** could also be solubilized in cell culture medium with this method, and it was applied for studying the cell membrane incorporation of **8**. SH-SY5Y cells were incubated with 1 – 100 nM of **8** or **2** at 37°C for 10 and 30 min, and then cells were washed and trypsinized. The membrane incorporated radioactivity was determined by liquid scintillation counting of the filtered cells. It was found that **2** was not incorporated into the cell membranes, as the radioactivity of these cell population was slightly above the background (Figure 4). However, when cells were treated with **8**, concentration- and time-

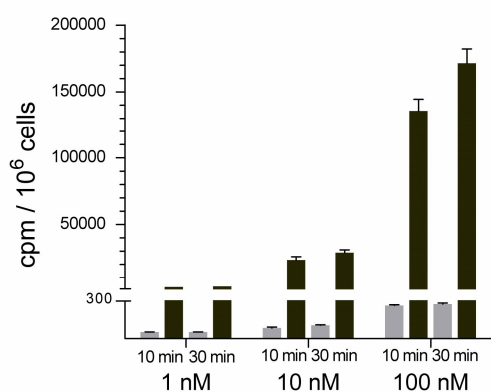


Figure 4 Incorporation of the [³H]cholesterol anchor **8** to the membrane of SH-SY5Y cells. Cells were incubated with different concentration of **2** (grey bars) or **8** (black bars) in the presence of 10 μ M β -cyclodextrin followed by liquid scintillation counting of the washed and trypsinized cells. The error bars represent SEM values of three parallel measurements.

dependent incorporation of the radioactive cholesterol anchor was observed. The radioactivity of the cell cultures was ca. 20% higher after 30 min incubation than after 10 min incubation indicating fast plasma membrane incorporation of **8**.

Conclusions

Semisynthetic lipoproteins prepared from recombinant proteins and designed lipid derivatives are valuable probes of the membrane-associated lipoproteins. In this article we reported the synthesis of a novel tritium labeled cholesterol anchor that contains the radioactive label in the headgroup. It was achieved in a convergent synthetic approach by the acylation of [³H]N-aminopiperidine with the 1-hydroxybenzotriazole ester of the carboxylic acid derivative **5**. This structure of the headgroup features the introduction of the tritium label into the same position where a fluorophore was introduced in **6**. Thus the fluorescein and tritium labeled anchors are constitutionally related, and the fluorescence microscopic images are relevant to the [³H]cholesterol incorporation data. Each synthetic step was optimized by using unlabeled standard compounds and by tracer experiments, and finally the target cholesterol amphiphile **8** was obtained with a radiochemical purity of 98%, and with a chemical purity of 99%. It was demonstrated that the membrane incorporation of the resulting headgroup labeled [³H]cholesterol anchor is concentration- and time-dependent, and that it can be investigated at low concentrations, below the working concentration of fluorescent analogues. It is advantageous because under these conditions the exogenous introduction of cholesterol derivative **8** results in low membrane concentration and thus, low level of membrane perturbation, cellular stress.

Acknowledgements

Financial support from the Hungarian Scientific Research Fund (K77783), from the Hungarian National Development Agency (TÁMOP 4.2.2.A-11/1/KONV-2012-0052), the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (Cs.T.) and technical help of Balázs Csibrány in cell culturing are acknowledged.

References

- [1.] T. Kinoshita, M. Fujita, in *The Enzymes. Volume 26: Glycosylphosphatidylinositol (GPI) anchoring of proteins*, (Eds.: A. K. Menon, T. Kinoshita, P. Orlean, F. Tamanoi) Academic Press, New York, **2009**, pp. 1-30.
- [2.] M. E. Medof, T. Kinoshita, V. Nussenzweig, *J. Exp. Med.* **1984**; *160*, 1558.
- [3.] C. W. van den Berg, T. Cinek, M. B. Hallett, V. Horejsi, B. P. Morgan, *J. Cell Biol.* **1995**; *131*, 669.
- [4.] P. E. Milhiet, M. C. Giocondi, O. Baghdadi, F. Ronzon, B. Roux, C. Le Grimellec, *EMBO Rep.* **2002**; *3*, 485.
- [5.] C. Metzner, M. M. Mostegl, W. H. Gunzburg, B. Salmons, J. A. Dangerfield, *FASEB J.* **2008**; *22*, 2734.
- [6.] M. E. Medof, S. Nagarajan, M. L. Tykocinski, *FASEB J.* **1996**; *10*, 574.
- [7.] B. Bader, K. Kuhn, D. J. Owen, H. Waldmann, A. Wittinghofer, J. Kuhlmann, *Nature* **2000**; *403*, 223.
- [8.] D. R. Premkumar, Y. Fukuoka, D. Seveler, E. Brunschwig, T. L. Rosenberry, M. L. Tykocinski, M. E. Medof, *J. Cell Biochem.* **2001**; *82*, 234.
- [9.] F. R. Taylor, D. Wen, E. A. Garber, A. N. Carmillo, D. P. Baker, R. M. Arduini, K. P. Williams, P. H. Weinreb, P. Rayhorn, X. Hronowski, A. Whitty, E. S. Day, A. Boriack-Sjodin, R. I. Shapiro, A. Galdes, R. B. Pepinsky, *Biochemistry* **2001**; *40*, 4359.
- [10.] C. Peters, A. Wolf, M. Wagner, J. Kuhlmann, H. Waldmann, *Proc. Natl. Acad. Sci. U. S. A.* **2004**; *101*, 8531.
- [11.] M. J. Grogan, Y. Kaizuka, R. M. Conrad, J. T. Groves, C. R. Bertozzi, *J. Am. Chem. Soc.* **2005**; *127*, 14383.
- [12.] M. G. Paulick, M. B. Forstner, J. T. Groves, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.* **2007**; *104*, 20332.
- [13.] K. Teruya, K. Nishizawa, K. Doh-ura, *Protein J.* **2010**; *29*, 493.

- [14.] K. Simons, M. J. Gerl, *Nat. Rev. Mol. Cell Biol.* **2010**; *11*, 688.
- [15.] D. Lingwood, K. Simons, *Science* **2010**; *327*, 46.
- [16.] S. Agarwal, C. Schroeder, G. Schlechtingen, T. Braxmeier, G. Jennings, H. J. Knolker, *Bioorg. Med. Chem. Lett.* **2013**; *23*, 5165.
- [17.] S. B. Sato, K. Ishii, A. Makino, K. Iwabuchi, A. Yamaji-Hasegawa, Y. Senoh, I. Nagaoka, H. Sakuraba, T. Kobayashi, *J. Biol. Chem.* **2004**; *279*, 23790.
- [18.] B. Schäfer, E. Orbán, A. Borics, K. Huszár, A. Nyeste, E. Welker, Cs. Tömböly, *Bioconj. Chem.* **2013**; *24*, 1684.
- [19.] G. Tóth, S. Lovas, F. Ötvös, in *Methods in Molecular Biology: Neuropeptide Protocols*, (Eds.: G. B. Irvine, C. H. Williams), Humana Press, Totowa, NJ, **1997**, pp. 219-230.
- [20.] Cs. Tömböly, A. Péter, G. Tóth, *Peptides* **2002**; *23*, 1573.
- [21.] A. E. Christian, M. P. Haynes, M. C. Phillips, G. H. Rothblat, *J. Lipid Res.* **1997**; *38*, 2264.
- [22.] R. Leventis, J. R. Silvius, *Biophys. J.* **2001**; *81*, 2257.
- [23.] Cs. Tömböly, R. Dixit, I. Lengyel, A. Borsodi, G. Tóth, *J. Label. Compd. Radiopharm.* **2001**; *44*, 355.
- [24.] R. Hinman, *J. Org. Chem.* **1958**; *23*, 1587-1588.
- [25.] H. K. Hall Jr., *J. Am. Chem. Soc.* **1957**; *79*, 5441.
- [26.] R. Ravichandran, S. Divakar, *J. Inclusion Phenom. Mol. Recognit. Chem.* **1998**; *30*, 253.