11.7 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 4.90 – 4.84 (m, 2H, H-1, H-4′), 4.69 (dd, 1H, J = 6.2, 10.9 Hz, OCH_2Fmoc), 4.51 (dd, 1H, J = 5.9, 10.9 Hz, OCH_2Fmoc), 4.10 (dd, 1H, J = 5.3, 12.3 Hz, H-9′b), 3.84 (dd, 1H, $J_{5,6a}$ = 7.5 Hz, $J_{6a,6b'}$ = 10.3 Hz, H-6a), 3.15 (dd, 1H, $J_{5,6b}$ = 4.4 Hz, $J_{6a,6b}$ = 10.3 Hz, H-6b), 2.69 (dd, 1H, $J_{3'e,3'a}$ = 12.6, $J_{3'e,4'}$ = 4.4 Hz, H-3′e), 1.22 (d, 3H, J = 6.5 Hz, T^{γ}); ^{13}CNMR (DEPT) (100.6 MHz, CD_3OD): δ = 100.77 (C-1), 99.93 (C-2′), 38.83 (C-3′), 19.32 (T^{γ}).

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[20] **19**: $[\alpha]_D^{22} = -20.8$ (c = 1.0, CH_3CN). FAB-MS (nitrobenzyl alcohol (nba), positive ion): m/z (%): 3754.4 (62.3) [$M(2\times{}^{13}C)-4\times$(*tert*-butyl)+2 × Na^+], calcd: 3753.74, 3755.1 (100) [$M(3\times{}^{13}C)-3\times$(*tert*-butyl)+2 × Na^+], 3755.8 (82.7) [$M(4\times{}^{13}C)-4\times$(*tert*-butyl)+2 × Na^+], 3757.2 (49.1) [$M(5\times{}^{13}C)-4\times$(*tert*-butyl) + 2 × Na^+]; ^{1}HNMR (400 MHz, [D$_6$]DMSO, TMS): δ = 8.48 – 7.41 (m, 18H, NH), 7.38 (m_c 5H, H_{Ar}-Bn), 7.28 – 7.16 (m, 5H, H_{Ar}-F), 7.11 (d, 2H, J = 7.9 Hz), 7.03 (d, 2H, J = 7.6 Hz, 2 × (H2-Y, H6-Y)), 6.82 – 6.76 (m, 4H, 2 × (H3-Y, H5-Y)), 6.70, 6.55 (S_{br}, $R^{NH(\varepsilon,\xi,\eta)}$), 5.22 – 5.14 (m, 5H, H-4, H-7′, H-8′, CH_2Bn), 4.92 – 4.85 (m, 2H, H-1, H-3); 4.78 – 469 (m, H-4′), 4.65 – 3.24 (m, 58H, F^{α}, 2 × Y^{α}, 2 × V^{α}, 3 × S^{α}, 3 × S^{β}, $CH_2CH_2(OCH_2CH_2CH_2)_2$= CH_2CH_2, R^{α}, D^{α}, 2 × A^{α}, 4 × P^{α}, 2 × T^{α}, G^{α}, V^{α}, H-2, H-5, H-6′, H-9′, 2 × T^{β}, 4 × P^{δ}, H-6a, H-5′), 3.22 – 2.60 (m, 10H, F^{β}, 2 × Y^{β}, H-6b, R^{δ}, H-3′e), 2.57 – 2.38 (m, 4H, D^{β}, CH_2CH_2Pmc), 2.38 – 2.35 (m, 2H, CH_2CONH-G-V), 2.49, 2.46 (s, 6H, *o,o′*-CH_3Pmc), 2.18 – 1.58 (m, 18H, 4 × P^{β}, 4 × P^{γ}, CH_2CH_2Pmc), 2.03, 2.00, 1.99, 1.93, 1.91, 1.85, 1.82, 1.75, 1.71, 1.66 (10 × s, 30H, m-CH_3Pmc, 6 × OAc, 3 × NHAc), 1.53 – 1.02 (m, 9H, 2 × V^{β}, R^{γ}, R^{β}, H3′a, 2 × A^{β}), 1.35, 1.23, 1.20, 1.15, 1.11, 1.08, 1.06, 1.05, (s, 69H, 7 × C(CH_3)$_3$, C(CH_3)$_2$Pmc), 0.98 – 0.90 (m, 3H, T_1^{γ}), 0.89 – 0.83 (m, 3H, T_2^{γ}), 0.82 – 0.78 (m, 12H, V^{γ}); ^{13}CNMR (DEPT) (100.6 MHz, [D$_6$]DMSO): δ = 98.40 (C-1), 98.01 (C-2′), 41.18, 40.97, 38.40, 36.95, 35.87 (2 × Y^{β}, F^{β}, C-3′, D^{β}, R^{δ}, CH_2CONH), 32.12 (CH_2CH_2Pmc), 19.05, 18.98, 18.75, 18.75, 18.16, 18.07, 17.95 (2 × V^{γ}, T_1^{γ}, *o,o′*-CH_3Pmc), 16.77 16.72, 16.00 (T_2^{γ}, 2 × A^{β}), 11.82 (m-CH_3Pmc).

[21] **20**: $[\alpha]_D^{22} = -76.9$ (c = 1.0, H_2O); FAB-MS (nba + LiBr, positive ion): m/z (%): 2770.7 (0.62) [M+Li^+], calcd: 2770.30; ^{1}HNMR (^{1}H-^{1}H COSY) (400 MHz, D_2O): δ = 7.37 – 7.24 (m, 3H), 7.23, 7.16 (m, 2H, H_{Ar}-F), 7.08 – 6.97 (m, 4H, 2 × (H2-Y, H6-Y)), 6.81 – 6.72 (m, 4H, 2 × (H3-Y, H5-Y)), 4.90 (m_c, 1H, H-1), 4.72 – 4.66 (m, 1H, D^{α}), 4.64 – 4.59 (m, 2H, F^{α}, R^{α}), 4.57 – 4.50 (m, 1H, A_1^{α}), 4.49 – 4.42 (m, 6H, A_2^{α}, 2 × S^{α}, 2 × Y^{α}), 4.41 – 4.33 (m, 6H, 4 × P^{α}, S_3^{α}, T_1^{α}), 4.32 – 4.29 (m, 2H, V_1^{α}, T_2^{α}), 4.28 – 4.23 (m, 1H, T_1^{β}), 4.22 – 4.17 (m, 1H, T_2^{β}), 4.12 – 4.03 (m, 3H, H-2, V_2^{α}, S_1^{β}), 4.01 – 3.27 (m, 40H, H-3, H-4, H-5, H-6, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′, NH(CH_2CH_2O)$_3CH_2CH_2$CONH, 2 × S^{β}, G^{α}, 4 × P^{δ}), 3.17 (m_c, 2H, R^{δ}), 3.10 – 2.99 (m, 1H, $F^{\beta a}$), 2.90 – 2.77 (m, 7H, 2 × Y^{β}, D^{β}, $F^{\beta b}$), 2.67 (dd, 1H, H-3′e), 2.60 – 2.54 (m, 2H, CH_2CONH-G-V-), 2.33 – 2.18 (m, 4H 4 × $P^{\beta a}$), 2.11 – 1.77 (m, 15H, 2 × V^{β}, 4 × $P^{\beta b}$, 4 × P^{ψ}, $R^{\beta a}$), 2.03, 2.01, 1.93 (s, 9H, 3 × NHAc), 1.76 – 1.59 (m, 4H, H-3′a, $R^{\beta b}$, R^{γ}), 1.34 (m, 6H, J = 6.5 Hz, 2 × A^{β}), 1.30 – 1.21 (m, 3H, T_1^{γ}), 1.15 (d, 3H, J = 6.2 Hz, T_2^{γ}), 0.97 – 0.85 (m, 12H, 2 × V^{γ}); ^{13}CNMR (DEPT) (100.6 Mhz, D_2O): δ = 99.62 (C-1), 99.20 (C-2′), 22.14, 21.94, 21.49, (3 × NHAc), 18.73, 18.39, 18.22, 17.54 (2 × T^{γ}, 2 × V^{γ}), 15.11, 14.92 (2 × A^{β}).

[22] For the importance of this key property, see Section 2 in ref. [2a], p. 885.

Synthesis and Membrane-Binding Properties of a Characteristic Lipopeptide from the Membrane-Anchoring Domain of Influenza Virus A Hemagglutinin**

Frank Eisele, Jürgen Kuhlmann, and Herbert Waldmann*

Key events in the establishment and progression of viral infection are attachment and fusion of the virus with the cell and budding of new virus particles from the infected cell. This complex multistep process is decisively influenced and determined by posttranslationally modified proteins embedded in the viral lipid bilayer. For instance, hemagglutinin from influenza virus A is glycosylated in the extracellular domain[1, 2] and S-palmitoylated next to the transmembrane region (Figure 1).[3, 4] The glycoprotein part is responsible for initiation of viral infection through selective binding to sialic acid receptors on the surface of the host cell. The lipid residues are required for the interaction between the cell membrane and the free capsid during budding of viral offspring[5, 6] and are thought to mediate protein – protein and protein – lipid interactions in the viruses.[7] In addition, the lipidated regions may play an important role in fusion processes of the viral membrane with the endosome after entry of the viral particle into the cell.[8, 9] However, this proposal is controversial, since other investigations indicated that the lipidated cytoplasmic tail of the complex viral lipoglycoproteins is not essential for its membrane fusion activity.[10]

For the study of these and related processes in precise molecular detail, lipidated peptides which represent the characteristic linkage region between the protein backbone and the lipid groups and which additionally carry a marker by which they can be traced in biological systems may be employed as efficient molecular probes.[11] However, their synthesis is complicated by the pronounced base lability of the palmitic acid thioesters which hydrolyse spontaneously at pH > 7.[12] For the synthesis of such labile peptide conjugates enzymatic methods may open up viable alternatives to classical chemical techniques.[11] In this paper we report on the development of the *p*-phenylacetoxybenzyl (PAOB) ester, a new enzyme-labile protecting group for carboxyl function-

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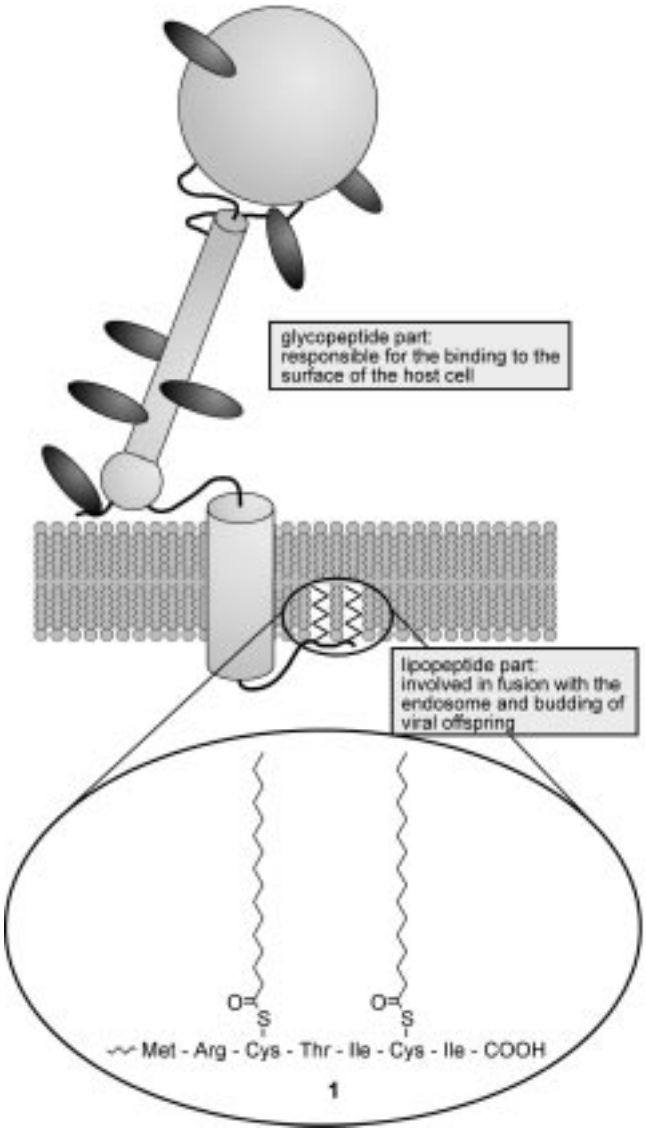
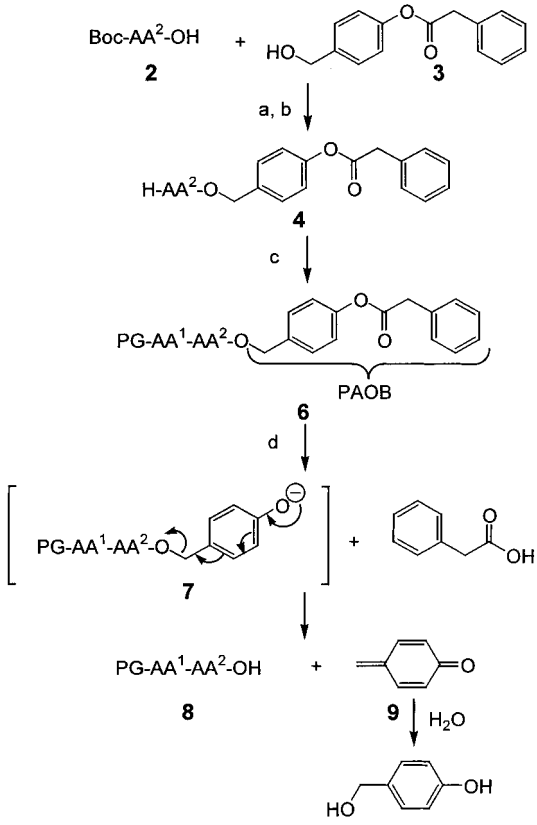


Figure 1. Schematic drawing of the glycosylated and lipidated influenza virus hemagglutinin A and the double-palmitoylated target peptide **1**.



8	PG	AA^1	AA^2	Yield (%)
8a	Aloc	Val	Ala	80
8b	Boc	Ser	Ile	80
8c	Boc	Thr	Phe	82
8d	Boc	Ala	Pro	82

Scheme 1. Synthesis and selective enzymatic deprotection of N-protected dipeptide PAOB ester **6**. a) DIC/DMAP (cat.), b) HCl/Et_2O, 63–90% (2 steps); c) PG-AA^1-OH **5**, EEDQ, 71–87%; d) phosphate buffer (pH 7), 10% methanol, penicillin G acylase. Boc = *tert*-butoxycarbonyl, PG = protecting group, Aloc = allyloxycarbonyl, DIC = *N*,*N*′-diisopropylcarbodiimide, DMAP: 4-dimethylaminopyridine, AA = amino acid, EEDQ = 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline.

alities, and its application in the construction of a fluorescent-labeled lipopeptide **1** from influenza virus A hemagglutinin.

In developing an enzyme-labile ester group that can be removed selectively under conditions that are mild enough for lipopeptide synthesis we resorted to the principle of fragmentation after cleavage of a suitable enzyme-labile bond. To this end, N-protected dipeptide PAOB esters **6** were synthesized (Scheme 1). The PAOB group embodies a phenylacetate which is specifically recognized and cleaved by the enzyme penicillin G acylase. Upon enzymatic hydrolysis a phenolate **7** is formed that fragments spontaneously to a quinone methide **9** and the desired carboxylic acids **8**. The quinone methide is trapped by water or added nucleophiles. This principle of deprotection by enzyme-induced fragmentation has been used for the development of enzyme-labile urethanes before.[13, 14] We stress, however, that even in the case of the urethanes the success of the enzyme-mediated fragmentation at pH 6–8 was rather surprising. Nonenzymatic induction of the fragmentation reaction requires the use of strong bases like ammonia[15] whereas in the presence of the biocatalysts the unmasking even occurs at neutral pH. In addition, in the case of the urethane protecting groups the entire unmasking is driven by the liberation of CO_2, thus shifting the equilibrium to the product side. This driving force is no longer operative in the case of an ester blocking function. Thus it was highly questionable whether this principle could be transferred successfully from an amino to a carboxy protecting group.

PAOB esters **6** were readily and efficiently synthesized by esterification of Boc-protected amino acids **2** with *p*-phenylacetoxybenzyl alcohol **3**, subsequent acid-mediated cleavage of the Boc group, and coupling of the resulting amino acid PAOB esters **4** with differently masked amino acids **5** (Scheme 1). Upon treatment of dipeptide PAOB esters **6** with penicillin G acylase at pH 7 and room temperature, to our delight, the desired selective C-terminal deprotection occurred smoothly. The enzyme saponifies the phenylacetate and, even at pH 7 and without the additional driving force of CO_2 liberation, the intermediately formed phenolates **7** undergo

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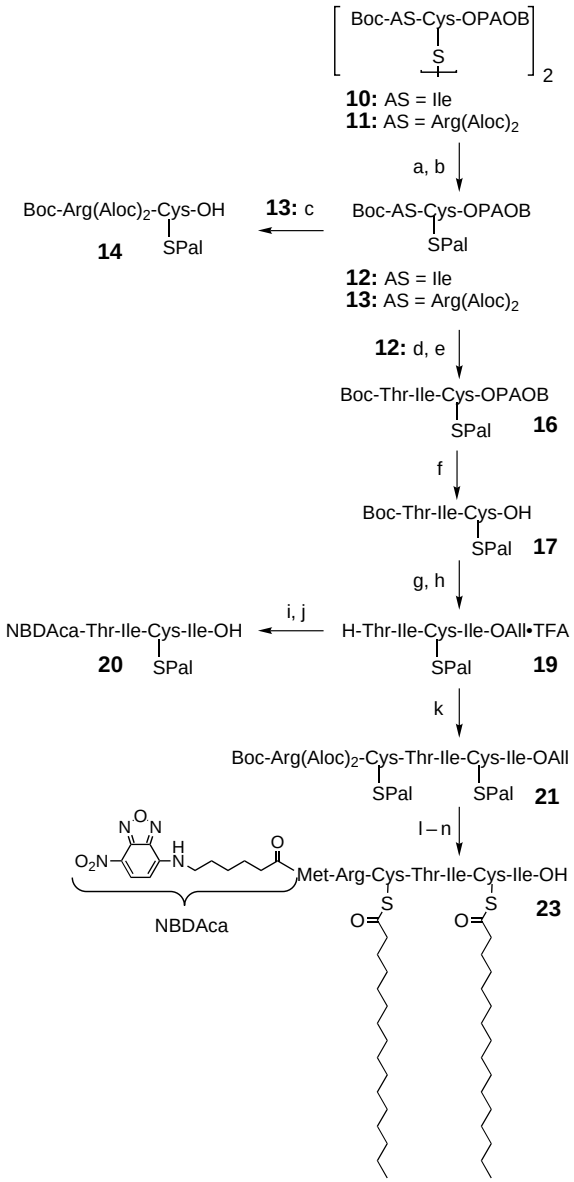
fragmentation to the desired unmasked dipeptides **8**. Quinone methide **9** is efficiently trapped by water, addition of stronger nucleophiles is not necessary. Penicillin G acylase is a readily available (immobilized, native, and as cross-linked enzyme crystals (CLECs)) and very stable enzyme with a broad substrate tolerance which does not attack peptide bonds or urethanes. In addition, in the PAOB group the site of enzyme attack is remote from the differing amino acids of the substrates. Thus the efficiency of the enzyme-induced deprotection is nearly independent of steric bulk and structure (that is, D- or L-amino acids, acyclic or cyclic amino acids, see Scheme 1). The desired peptides **8a**–**8d** were obtained in high yield.

On the other hand the Boc and Aloc groups can be removed selectively from dipeptide esters **6** without harm to the PAOB esters, that is, the protecting groups are orthogonally stable to each other (data not shown, see also below).

The full capacity of the enzyme-labile PAOB ester became evident in the synthesis of fluorescent-labeled derivatives of virus hemagglutinin peptide **1** (Scheme 2). This synthesis is complicated by the base lability of the thioesters and the need to additionally protect and deprotect the basic arginine side-chain functionality. Thus, a set of three orthogonally stable protecting groups is required, whereby the use of base-labile and hydrogenolytically removable blocking groups is not permitted. This problem was overcome by using the acid-labile Boc group for the N terminus, the enzymatically removable PAOB ester for the C terminus, and the Pd^0-sensitive Aloc group for the arginine guanidino side-chain function.

The synthetic sequence commenced with the construction of two S-palmitoylated dipeptide PAOB ester building blocks. Thus, cystinyl dipeptides **10** and **11** were synthesized in high yield as described above for other dipeptides. The disulfide bonds were then reduced by treatment with DTT and the liberated cysteine thiols were immediately converted into the corresponding palmitic acid thioesters (Scheme 2). S-Acylated dipeptides **12** and **13** were obtained in high yields. Upon treatment of arginyl peptide PAOB ester **13** with penicillin G acylase at pH 7 and 25 °C the C-terminal protecting group was hydrolyzed smoothly and without any undesired side reaction. The mildness of the reaction conditions and the substrate specificity of the biocatalyst guarantee that neither the thioester nor the bis-acylated guanidino group are attacked, and the selectively unmasked S-palmitoylated dipeptide **14** was isolated in high yield. The Boc group was removed selectively from isoleucyl peptide PAOB ester **12** and the peptide chain then was elongated with Boc-threonine to yield fully protected lipotripeptide **16**. Once again treatment of S-palmitoylated tripeptide PAOB ester **16** with the enzyme penicillin G acylase at pH 7 and 25 °C resulted in a smooth and completely selective removal of the C-terminal enzyme-labile blocking function. Selectively deprotected tripeptide thioester **17** was isolated in high yield. The enzyme-catalyzed transformations proceed only if dimethyl-β-cyclodextrin is added to the reaction mixture. In the absence of this cyclic hexasaccharide PAOB ester **16** was not hydrolyzed at all. The cyclodextrin has a hydrophobic core with which it can slip over the hydrophobic palmitic acid group. Thereby it solubilizes the peptide and renders it accessible to the biocatalyst. In addition, the formation of this inclusion complex may prevent the thioester from being hydrolyzed.



Scheme 2. Chemoenzymatic synthesis of **23**. a) DTT, NEt_3; b) 2 Pal-Cl, NEt_3, **12**: 83 %, **13**: 81 % (2 steps); c) penicillin G acylase, dimethyl-β-cyclodextrin, 0.05 M phosphate buffer (pH 7), 25 °C, 77 %; d) TFA; e) Boc-Thr-OH (**15**), EEDQ, NEt_3, 74 % (2 steps); f) penicillin G acylase, dimethyl-β-cyclodextrin, 0.05 M phosphate buffer (pH 7), 25 °C, 81 %; g) H-Ile-OAll · TosOH (**18**), EEDQ, NEt_3; h) TFA, 71 % (2 steps); i) NBDAca-OH, EDC, HOBt, NEt_3; j) $Pd(PPh_3)_4$, DMB, 69 % (2 steps); k) **14**, EEDQ, NEt_3, 46 %; l) TFA; m) NBDAca-Met-OH (**22**), EDC, HOBt, NEt_3; n) $Pd(PPh_3)_4$, DMB, 69 % (3 steps). Pal = palmitoyl, All = allyl, NBDAca = depicted in the scheme, DTT = dithiothreitol, TFA = trifluoroacetic acid, Tos = 4-toluenesulfonyl, EDC = *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, DMB = dimethylbarbituric acid.

Next S-palmitoylated tripeptide carboxylic acid **17** was condensed with isoleucine allyl ester **18** and the Boc group was removed to deliver lipotetrapeptide **19** in high overall yield. Compound **19** was blocked as allyl ester at the C terminus to allow for simultaneous Pd^0-catalyzed deprotection of both the arginine side-chain function and the

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C-terminal carboxylic acid towards the end of the synthesis. C-terminal deprotected and S-palmitoylated dipeptide building block **14** and N-terminally unmasked lipotetrapeptide **19** were then condensed to give double-palmitoylated lipohexapeptide **21**. The N-terminal Boc group was removed from **21** and the fluorescent 7-nitrobenz-2-oxo-1,3-diazole (NBD) label was introduced. Finally, the three allyl-type blocking functions were cleaved by Pd^0-catalyzed allyl transfer with *N*,*N*′-dimethylbarbituric acid as the accepting C nucleophile.[16] Similarly, introduction of the NBD label followed by selective removal of the allyl ester yielded lipopeptide **20**. The desired fluorescent and palmitoylated influenza virus hemagglutinin lipopeptides **20** and **23** were obtained in high overall yield.

These compounds may, for instance, be applied advantageously to determine the membrane binding properties of the viral proteins. In order to demonstrate this possibility lipidated peptides **20** and **23** were subjected to an assay determining a kinetic value which characterized their binding to liposome model membranes.[17]

For this assay methanolic solutions of the fluorescent-labeled lipopeptides **20** and **23** were mixed with a hundredfold molar excess of lipid (here: palmitoyl oleoyl phosphatidylcholine, POPC) and a twofold excess of a nonexchangeable fluorescence quencher (*N*-(lissamine rhodamine sulfonyl)phosphatidylethanolamine, Rho-DHPE). Both, lipid and quencher were dissolved in methanol. Buffer was added to generate mixed vesicles corresponding to a POPC concentration of 1 mM. Vesicles are trimmed by freeze–thaw cycles and extruder treatment[18] to generate vesicles of a defined size distribution (approximately 100 nm∅). If the NBD fluorophor of the lipopeptide incorporated in such a vesicle is excited at 460 nm its fluorescence emission at 535 nm is directly absorbed by the rhodamine dye of the quencher when Rho-DHPE is close to the lipopeptide. Mixing those vesicles with an excess of pure POPC vesicles allows mobile lipopeptides to leave their original environment. They enter the quencher-free vesicles where their NBD fluorescence is not quenched any longer resulting in an increase in fluorescence emission at 535 nm (Figure 2a).

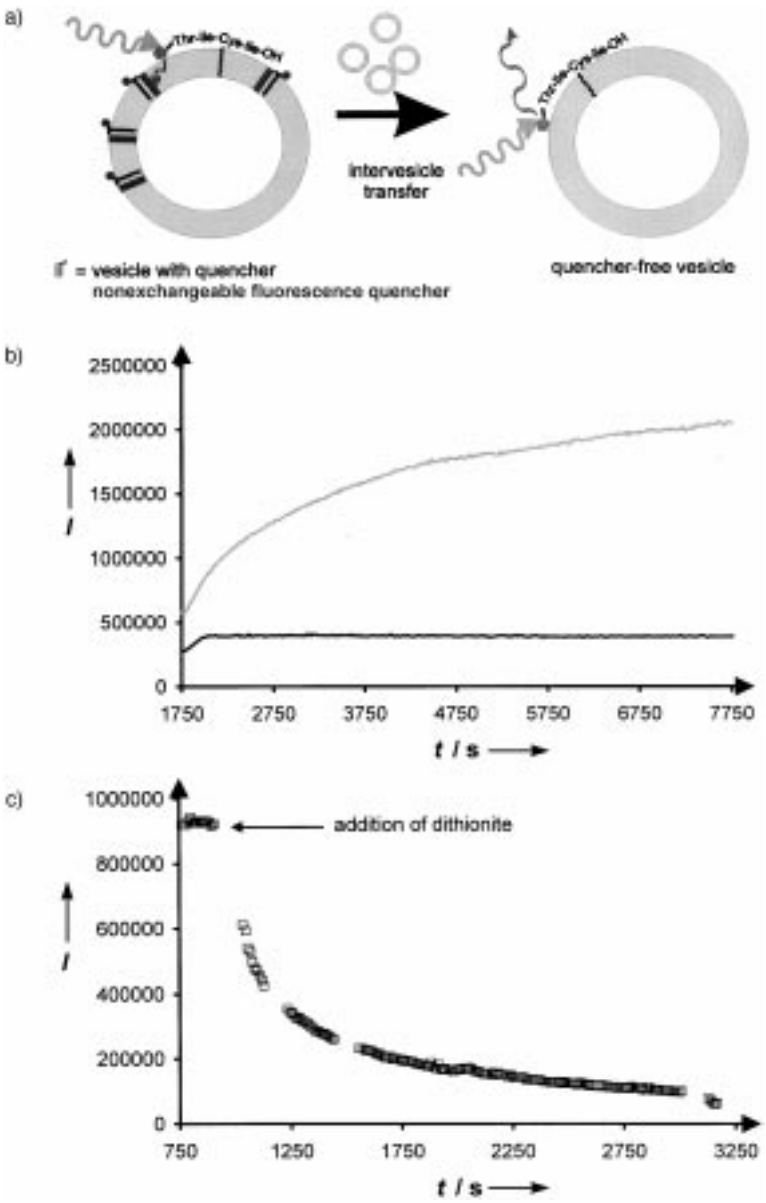

Figure 2. a) Principle of the intervesicle transfer of lipopeptides and the fluorometric detection employing a nonexchangeable fluorescence quencher; b) intervesicle transfer of the monopalmitoylated tetrapeptide **20** (gray line) and the double-palmitoylated heptapeptide **23** (black line); c) flip-flop diffusion of monopalmitoylated tetrapeptide **20**. I = relative fluorescence.

Mixed vesicles were generated containing 1 mol% of peptide **20** and **23** respectively and 2 mol% of Rho-DHPE. Only tetrapeptide **20** with a single palmitoyl-thioester showed an increase in fluorescence after mixing a solution of 5 μM POPC (mixed vesicles) with a 20-fold excess of pure POPC vesicles. This indicates the transfer of the lipopeptide to the vesicles free of Rho-DHPE, showing that one lipid modification is not sufficient for irreversible membrane insertion. In contrast the NBD emission of the heptapeptide **23** with two palmitoyl residues was not affected by the dilution with quencher-free vesicles (Figure 2b). This observation demonstrates that two C16 anchors are sufficient to fix the lipopeptide in its original environment.

The intervesicle transfer of lipopeptide **20** is composed of two separate processes. Peptides anchored at the outer face of the vesicle can directly migrate to the acceptor vesicle by diffusion. This step can be described as an irreversible first order mechanism if the acceptor vesicles are present in high excess. Depending on their distribution between inner and outer face of the vesicles the intravesicular lipopeptides have to perform a reversible flip-flop diffusion to appear on the outer face of the vesicles. In a first estimation we fitted the overall change in fluorescence for the tetrapeptide by a monoexponential function including a linear term for the flip-flop process. Best fits were obtained for a dissociation rate of $1.3 \times 10^{-3}\ s^{-1}$ and a linear drift of 1.8×10^{2} units of fluorescence s^{-1}. The drift corresponds to approximately 45% of the fitted amplitude.

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The separated analysis of the flip-flop exchange can be monitored by dithionite treatment of vesicles loaded with lipopeptide but without quencher. The addition of sodium dithionite resulted in fast reduction of all accessible NBD fluorophors at the outer face of the vesicles. This first loss of emission signal was followed by a slower decay, indicating the flip-flop of the intravesicular lipopeptides to the outside (Figure 2c).

The apparent rate constant for the intervesicle transfer of tetrapeptide **20** and heptapeptide **23** between POPC vesicles can be compared with data for lipopeptides with a single farnesyl modification or two hydrophobic residues (farnesyl thioether and palmitoyl thioester).[19] Here half-times of 21 s for a peptide with the sequence NBD-GCMGLPC(Far)-OMe and 155 h for the peptide NBD-GC(Pal)MGLPC(Far)-OMe were calculated for experiments at 37 °C, while tetrapeptide **20** has a half-time of about 9 min at 20 °C. Thus, in comparison to a farnesyl thioether, a single palmitoyl group confers a significantly enhanced stability of membrane insertion, however desorption from the model membrane still occurs at a relatively fast rate. Combination of a farnesyl thioether with a palmitic acid thioester results in a very slow but still detectable intervesicle transfer, but in the presence of two palmitoyl groups desorption of membrane-bound lipidated peptides cannot be detected at all. Thus, a bis-palmitoyl membrane anchor will lead to quasi irreversible membrane-anchoring of doubly palmitoylated proteins which can only be reversed by hydrolysis of the palmitic acid thioester bonds.

These values correspond well to data recorded for model peptides, which are derived from other lipidated proteins (such as Rho) and which have been used to predict the membrane binding properties of their parent proteins.[19, 20] Thus, determining a full set of data for several different hemagglutinin-derived lipopeptides in a detailed biophysical analysis should allow for an extrapolation to the membrane binding properties of this lipoglycoprotein as well.

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First Use of a Mineral Liquid Crystal for Measurement of Residual Dipolar Couplings of a Nonlabeled Biomolecule**

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Dedicated to Professor Fred Wudl on the occasion of his 60th birthday

The classic NMR strategy for determining the conformation of a biomolecule[1] involves exploiting the combination of the scalar coupling ^3J, to obtain dihedral angle information, and the ^1H–^1H dipolar cross-relaxation rate, which has a $(1/r^6)$ dependence where r is the internuclear distance. The limitation of this approach is that only short range structural information is obtained (a few bonds for the scalar coupling

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