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A New Lipophilic Fluorescent Probe for Interaction Studies of Bioactive Lipopeptides with Membrane Models

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Abstract—The new fluorescent lipophilic moiety 11-[(7-amino-4-methyl-2-oxo-2*H*-1-benzopyran-3-acetyl)amino]undecanoic acid (AMCA-ωAud-OH) was introduced by SPPS at the N-terminus of the immunodominant epitope GpMBP(74–85). FRET experiments using the new fluorescent lipopeptide demonstrate that the peptide interacts with much more affinity with the membrane compared to the lipid free analogue.

Peptide–biomembrane interaction is an essential step for various kinds of bioactive peptides, for example, hormones, antibiotics and antigens, to exhibit their functions. Therefore, many studies on peptide–lipid membrane interaction have been performed to clarify the structure–function relationship of natural peptides and to obtain fundamental information on their characteristics using model peptides. Investigations on the mechanism of interaction with artificial bilayer systems have shown that a variety of factors, such as the presence of lipophilic moieties, exert critical effects on the behavior of peptides in lipid membranes. There are examples on the literature demonstrating that conjugation of lipidic moieties to immunodominant peptides may affect T-cell responses.[1] Chemically defined modifications of peptides by the built-in immunoadjuvant lipopeptide Pam_3Cys-Ser was described as a reproducible way stimulating responses of cytotoxic T-lymphocytes (CTLs) in vivo, demonstrating that the new synthetic mitogens are highly suitable compounds for study in early events of the immune response.[2] In the past years, synthetic peptides modified also with simple lipophilic moieties, such as a palmitoyl group (Pam), have been shown to be efficient tools in inducing specialized CTLs. Their different bioactivity, compared to lipid free analogues, has been attributed to their anchoring to the cell membranes and to a subsequent facilitated interaction with membrane receptors.[3,4] Lipopeptides could, therefore, represent useful tools for the study of immunological responses.

We reported the immunoadjuvant effects of lipoconjugation of peptide antigens in an in vitro system by using CD4+ T cells.[5] The lipopeptides obtained by conjugating a palmitoyl moiety at the N^{α}-terminus of Gln^{74} or at the N^{ε} of Lys^{75} of GpMBP(74–85) (Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2) induced increased T-cell responsiveness compared to the native non-lipidated peptide. In particular, the lipopeptide Pam-GpMBP(74–85) increased the in vitro CD4+ T cell proliferative response in Lewis rats immunized with the immunodominant epitope GpMBP(74–85), compared to the lipid-free wild-type peptide. Lipoconjugation may, thus, favor the internalization of the peptide by APCs, as suggested by the increase of both CTL and CD4+ T cell response in the presence of lipopeptides. We also hypothesized that the moiety introduced at the N-terminus of the immunodominant epitope GpMBP(74–85) strongly contributes to the interaction of the peptide with cellular membranes so that the antigen can easily penetrate the bilayer. Antigens are then loaded on newly generated MHC II molecules, increasing the efficiency of their presentation.[5]

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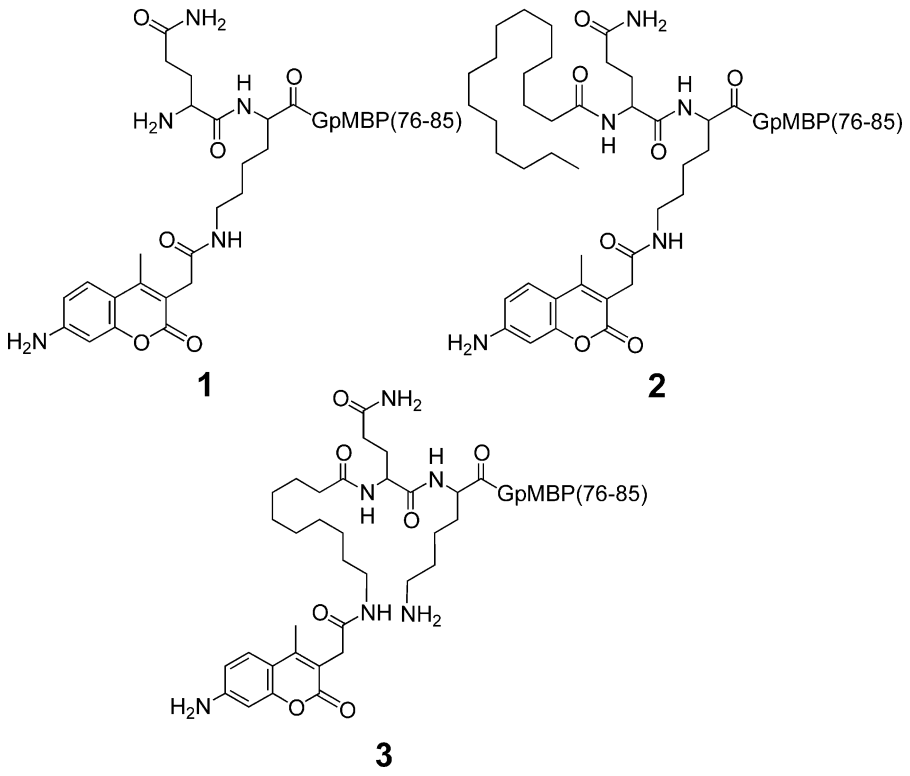


Figure 1. Fluorescent labeled peptides.

Table 1. Chemical data of the GpMBP(74–85) peptide derivatives

Peptides	Gradients at 1 mL min^{-1} for analytical HPLC	Yield (%)	ESI–MS $[M+H]^+$ Found (calcd)	R_t (min)	E% (R)
1	10–50% B in 10 min	90	1629.8 (1629.72)	7.64	24 (60 Å)
2	60–100% B in 10 min	95	1868.7 (1868.14)	5.83	63 (46 Å)
3	40–80% B in 20 min	75	1813.1 (1813.01)	11.5	76 (41 Å)

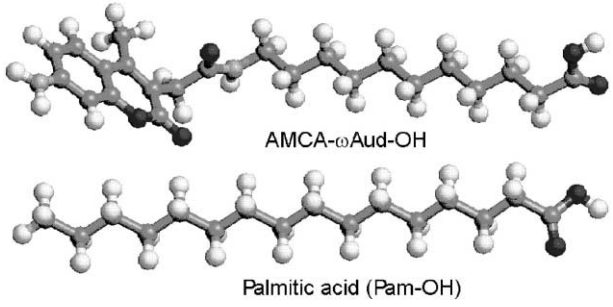


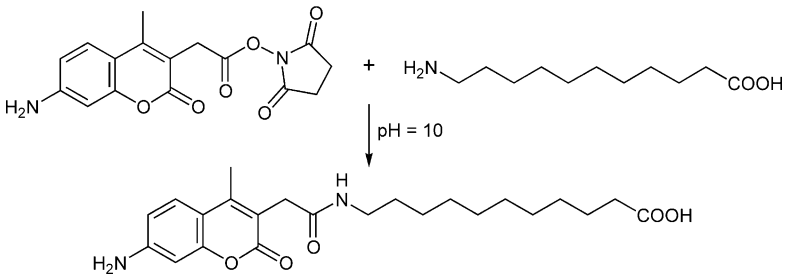
Figure 2. AMCA-ωAud-OH and Pam-OH.

In order to define the molecular mechanism and the role of the lipidic group, we used fluorescence resonance energy transfer (FRET) technique, with fluorescence-labeled peptides, to study the interaction between the peptides and phospholipid membranes. Coumarin derivatives have been widely used as fluorescent labels[6] for preparing fluorogenic substrates in immunohistochemistry. Among the coumarin compounds, 7-amino-4-methyl-2-oxo-2*H*-1-benzopyran-3-acetic acid [(7-amino-4-methylcumar-3-yl)acetic acid, AMCA] is extensively used for preparing blue fluorescent conjugates of protein and nucleic acids.[7] The dye can be optimally excited at 350 nm and emits near 435 nm. In this communication, we report the synthesis of a new lipophilic fluorescent probe 11-[(7-amino-4-methyl-2-oxo-2*H*-1-benzopyran-3-acetyl)amino]undecanoic acid (AMCA-ωAud-OH) and its application in interaction studies with model membranes.

Results and Discussion

In order to clarify the role of the lipidic moiety in Pam-GpMBP(74–85), we synthesized the fluorescent building block Fmoc-Lys(AMCA)-OH[8] to be introduced at position 75 of the lipopeptide Pam-GpMBP(74–85) and of the corresponding lipid-free wild type immunodominant peptide GpMBP(74–85).[9]

FRET was used to study the interaction between the labeled peptides and vesicles made up by phospholipids bearing a quencher on the polar head, that is, at the water–bilayer interface, or on the alkyl chain, inserted into the bilayer.[10] FRET technique, based on the interaction between AMCA as a fluorescence donor and BODIPY (Molecular Probes, Inc., OR, USA) as the corresponding acceptor, allows to evaluate the distance between the acceptor and the donor with $E = R_0^6/$



Scheme 1. Synthesis of AMCA-ωAud-OH.

$(R_0^6 + R^6)$ where E is the quenching efficiency, R_0 is Forster radius and R the donor-acceptor distance.[11]

[Lys^{75}(AMCA)]GpMBP(74–85) (**1**) and Pam-[Lys^{75}(AMCA)]GpMBP(74–85) (**2**) (Fig. 1 and Table 1) were found to interact only with BODIPY localized at the water–bilayer interface. In this case the Lys(AMCA) fluorophore apparently is too distant from the Pam moiety to provide more information on its localization in the bilayer. Therefore, by means of MacroModel (Version 6.5, Schroedinger Inc., 1999), the new fluorescent lipophilic moiety AMCA-ωAud-OH was designed able to expose the AMCA group at the end of the alkyl chain and plus mimicking, in terms of length, the palmitoyl moiety (Fig. 2). We synthesized the new fluorescent probe (Scheme 1)[12] which was introduced by SPPS at the N-terminus of the immunodominant epitope GpMBP(74–85), to obtain AMCA-ωAud-GpMBP(74–85) (**3**). FRET experiments using covesicles made up of DPPC/phospholipids bearing the BODIPY quencher on the alkyl chain/lipopeptide **3** (100:1:5) exhibited a very high quenching efficiency (E = 76%) corresponding to relatively small acceptor-donor distance (R≈40 Å) indicating that the AMCA moiety is localized close to the BODIPY quencher (Fig. 3). Control FRET experiments with vesicles containing BODIPY in the polar group region indicated negligible quenching by compound **3**. These results fully agree with insertion of the AMCA-labeled lipid moiety into phospholipid bilayer. This may lead to their permanence on the cell membrane and to a subsequent facilitated interaction with membrane receptors.[13]

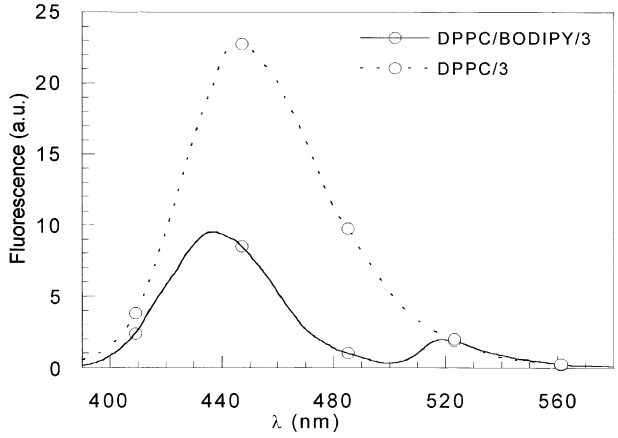


Figure 3. Emission spectra of covesicles made by DPPC, phospholipids bearing BODIPY on the alkyl chain and lipopeptide **3**.

In conclusion, we describe a synthetic approach to new lipophilic probes, containing alkyl chains of different length, to obtain labeled bioconjugates as useful tools in fluorescence techniques.

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9. All peptides were synthesized by the continuous flow solid-phase method on a semi-automatic apparatus (NovaSyn Gem Synthesizer) following the Fmoc/*t*Bu strategy. TentaGel S RAM (0.26 mmol/g) was purchased from Rapp Polymere (Germany) and the Fmoc-protected amino acids from Novabiochem (Switzerland). DMF was maintained over molecular sieves; piperidine was distilled from KOH. Fmoc-protected amino acids and Fmoc-Lys(AMCA)-OH, in 2.5-fold excess, were activated by TBTU, HOBt/NMM in DMF; AMCA-ωAud-OH, in 2.5-fold excess, was activated by HATU/NMM

in DMF. The palmitoyl moiety was introduced by using Pam-OPfp, in 2.5-fold excess, in DCM/DMF (2:1). Deprotection reactions were accomplished with 20% piperidine in DMF. Acylation end points were determined by checking the absorbance at 597 nm, due to the release of an anionic dye (acid Violet 17) from the cationic resin-bound amino groups. Deprotection reactions were followed by monitoring at 365 nm the dibenzofulvene–piperidine adduct. On completion of the synthesis, the resin was washed with DMF, DCM, and dried in vacuo. Peptides were cleaved and the side-chains deprotected at room temperature by TFA/phenol (95:5). Crude peptides were purified by semi-preparative HPLC on a Vydac column ODS 218TP1010 (250×10 mm). Analytical HPLC was performed on a Vydac ODS 218TP54 (250×4 mm), using gradients with the following eluants: A, 0.1% TFA in H_2O; B, 0.1% TFA in CH_3CN. HPLC-grade solvents were purchased from Carlo Erba (Italy). All other chemicals were commercially pure compounds and were used as received. Characterization of the products was performed using analytical HPLC, ESI–MS spectrometry (Micromass Model VG Quattro apparatus). Final HPLC purity of the peptides was always >98%.

10. Vesicles were prepared by dissolving dipalmitoyl-L-α-phosphatidylcholine (DPPC) in 3% MeOH and 7% $CHCl_3$ (2 mM). The solvent was removed under vacuum, for one night, leaving a thin film of lipid on the walls of the flask. The dried lipid was hydrated with an opportune volume of TRIS buffer (0.1 M, pH 7.4) and the suspension was vortexed to generate multilamellar vesicles (MLV). Unilamellar vesicles were prepared from MLV by extrusion at 65 °C with an extruder Lipex Biomembranes (Vancouver, Canada). We obtained 60±10 nm vesicles. Covesicles were made codissolving the peptide and DPPC and DPPC bearing BODIPY (DPPC/ BODIPY/peptide 100:1:5) in 3% MeOH and 7% $CHCl_3$ and then we used the procedure described previously.

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12. Synthesis of AMCA-ω-Aud-OH. AMCA-OSu (443 mg, 1.3 mmol) in DMF (6 mL) was added to a refluxing solution of 11-aminoundecanoic acid (241 mg, 1.2 mmol) in water (8 mL). The pH was adjusted to 10 with 10% Na_2CO_3. After 3 h, the solution was left to reach the room temperature and was stirred overnight. The white precipitate was recrystallized from an aqueous alkaline solution by acidification and washed with MeOH. Yield 70%; mp 198–199 °C; ^{1}H NMR (200 MHz, DMSO) δ 1.2–1.5 (16H, 8×CH_2), 2.17 (2H, t, Aud 2-H_2), 2.22 (3H, s, 4-CH_3), 2.99 (2H, *pseudo* q, Aud 11-H_2), 3.35 (2H, s, 3-CH_2), 5.99 (2H, s, NH_2), 6.38 (1H, d, J=2.2 Hz, 8-H), 6.57 (1H, dd, J=2.2 and 8.8 Hz, 6-H), 7.43 (1H, d, J=8.8 Hz, 5-H), 7.77 (1H, t, NH), 11.95 (1H, br s, COOH); ^{13}C NMR (50 MHz, DMSO) δ 14.86 (AMCA 4′-CH_3), 24.51, 26.35, 28.55–29.10, 30.68, 33.65 and 33.78 (Aud C-2–C-11, and AMCA 3′-CH_2), 98.37 (AMCA C-8′), 109.41, 111.21, and 113.50 (AMCA C-3′, C-4a′, and C-6′), 126.10 (AMCA C-5′), 149.59, 152.08, and 153.94 (AMCA C-4′, C-7′, and C-8a′), 161.46 (AMCA C-2′), 168.85 (CONH), 174.33 (COOH). Anal. calcd for $C_{23}H_{32}N_2O_5$·0.2H_2O: C, 65.76; H, 7.77; N, 6.67. Found: C, 65.52; H, 7.83; N, 6.95.

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