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A novel family of amphilic α-oxo aldehydes for the site-specific modification of peptides by two palmitoyl groups in solution or in liposome suspensions

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Abstract—Two amphiphilic α-oxo aldehydes were synthesized using solid-phase methodologies and evaluated for their ability to ligate with α-hydrazino acetyl peptides both in solution and when inserted into the lipidic bilayer of liposomes. © 2001 Elsevier Science Ltd. All rights reserved.

Many biological or formulation studies require the controlled modification of the physico-chemical or recognition properties of lipid bilayers. For example, an essential demand for the use of liposomes as drug carriers is the ability to target specific cells. This can be accomplished by insertion of adhesion motifs[1] or receptor ligands[2] into their lipid bilayers. The partition of biomolecules modified by long alkyl chains between membranes and solutions have been extensively studied[3] and show that a stable insertion requires at least two proximal alkyl chains. However, the lipidation of hydrophilic compounds is often troublesome due to the generally low solubility of the conjugates. To circumvent these difficulties, convergent strategies have been developed where the purified biomolecule is covalently bound to a functionalized lipidic anchor already inserted into the membrane through an amide bond,[4] a thioether bond,[5] or disulfide bridge.[6] However, these methodologies are not applicable to the site-specific immobilization of biomolecules bearing more than one amine or thiol functionalities. In a project aimed at anchoring complex polyfunctional molecules to lipid membranes, we have examined the utility of α-oxo hydrazone ligation of α-hydrazino acetyl peptides with an amphiphilic glyoxylic amide derivative of type **1** (Fig. 1).[7]

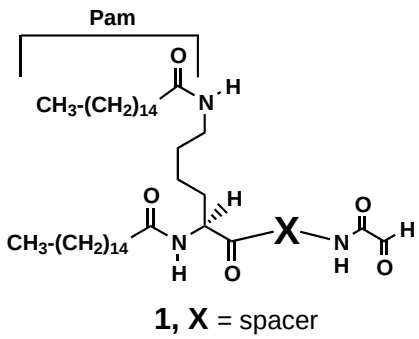

Figure 1. Amphiphilic α-oxo aldehyde.

The difficulty in preparing amphiphilic compounds such as **1** resides in the fine tuning of the physico-chemical properties to allow their solubilization, purification, characterization and the control of physisorption and non-specific binding. In addition, depending on the biomolecular interactions studied, the anchored peptide should be close to or distant from the membrane surface. Thus, to allow the spacer arm to be easily modulated, anchors **1** were elaborated on the solid phase using a (+)-dimethyl-2,3-*O*-isopropylidene-D-tartrate based linker as described in Scheme 1.[8] Spacer elongation and coupling of Fmoc-Lys(Fmoc)-OH was performed using standard Fmoc/*t*Bu solid phase peptide synthesis protocols (SPPS).[9] Following Fmoc removal with piperidine, palmitic acid was coupled twice using successively HBTU/HOBt/DIEA and cyanuric fluoride[10] activations. Using the acyl fluoride and long reaction times (3 h) was found to be necessary for the derivatization of all amino groups. This step was followed by extensive washings to remove any palmitic acid, which could have been trapped inside the solid

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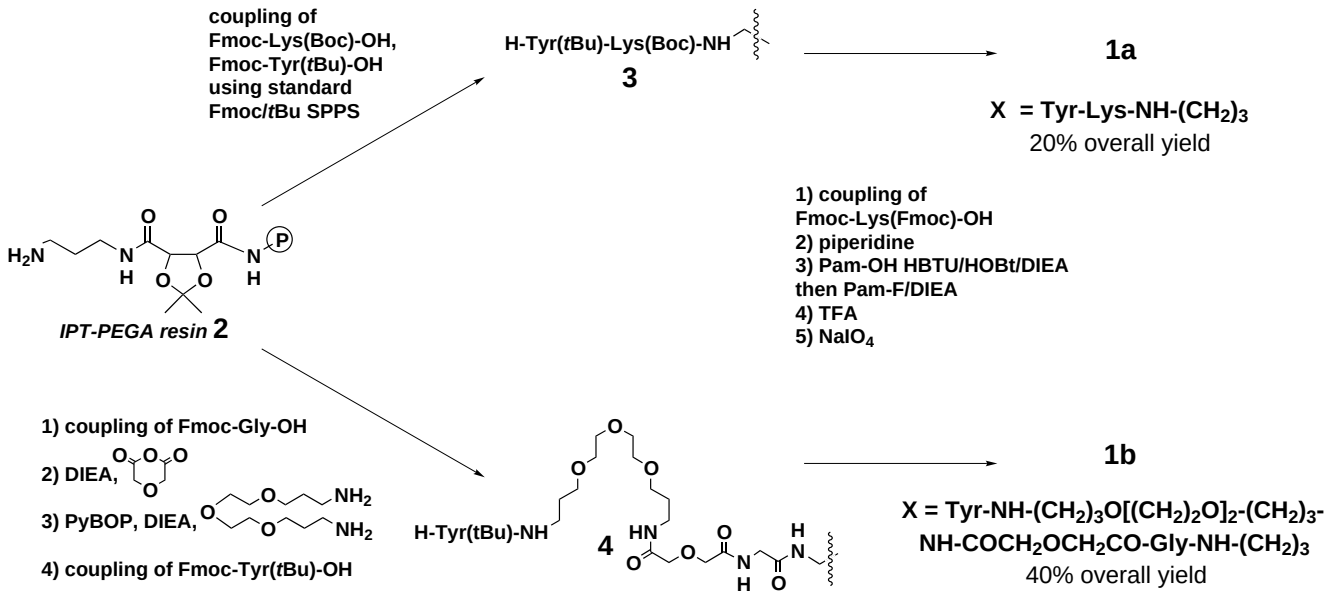

Scheme 1. Solid-phase synthesis of **1a**–**b**.

support. Treatment with concentrated TFA permitted the concomitant deprotection of the side chains and of the isopropylidene tartrate moiety, thus unmasking the vicinal diol of the linker. The final periodic oxidation required the presence of a lipophilic co-solvent to allow the swelling of the beads, whose interior was highly hydrophobic due to the presence of the palmitoyl groups. The best results were obtained using an acetic acid/water/methyl-2-propanol-2 1/2/3 (by vol.) mixture. Both compounds **1a** and **1b** were found to be insoluble in this solvent mixture, so that precipitation of the product occurred inside the beads, which were washed with water to remove the salts and ethanolamine used to quench the excess of periodate. The products were then extracted from the beads using methyl-2-propanol-2/MeOH and MeOH/CH_2Cl_2 at 50°C. Using this procedure, **1a** and **1b** were isolated in high purity and with 20 and 40% yield, respectively.[11]

The spacer of **1a** was designed to incorporate a UV chromophore and a positively charged group for RP-HPLC and mass spectrometry characterizations, respectively. Compound **1a** was found to be scarcely soluble in organic solvents usually employed for liposome preparation, but dissolved in the presence of DMPC, DMPG and cholesterol.[12] Hydrazone ligation with liposomes incorporating **1a** was studied using model α-hydrazino acetyl peptide H_2N–GRYL–NH_2 **5**.[13] Control experiments were performed with Ac–RYL–NH_2 **6** (**1a**/**5** or **1a**/**6**: 1 equiv./1 equiv.). Quantification of the amount of peptide associated with liposomes or remaining in solution was performed by amino acid analysis following total acid hydrolysis. 50% of peptide **5** was found to be associated with liposomes (20% for **6** probably due to ionic and/or hydrophobic interactions). The remaining of the peptide was found in solution, showing that the presence of phospholipids did not alter the amino acid analysis. The better association of peptide **5** with functional liposomes could be attributed to the formation of covalent hydrazone linkages. If one assumes a statistical distribution of **1a** into the liposome bilayer and that the membrane in not permeable to peptides **5** or **6**, the maximum yield for hydrazone ligation should be 50%, i.e. what was effectively obtained experimentally.

To solve the solubility problems associated with **1a**, a second spacer was designed based on the assembly of hydrophilic building blocks. Compound **1b** was effectively found to be more soluble in organic or partially organic solvents. Interestingly, **1b** unlike its parent **1a**, could be successfully engaged in hydrazone ligation in solution. Indeed, reaction of **1b** with α-hydrazino acetyl peptide **7** led to the formation of compound **8** with 41% yield following RP-HPLC purification (Fig. 2). Fig. 2 highlights the efficacy of the process, since the conversion was found to be as high as 60% after few seconds of reaction at 37°C. The same ligation proceeded successfully (60% yield) when **1b** was incorporated into liposomes which behaved as very efficient tools for the specific picking of proteins in a complex cytosolic medium.

In conclusion, a novel evolutionary family of amphiphilic α-oxo aldehydes for membrane immobilization through hydrazone ligation has been described. Studies devoted to the biological evaluation of the liposomes will be reported in due course.

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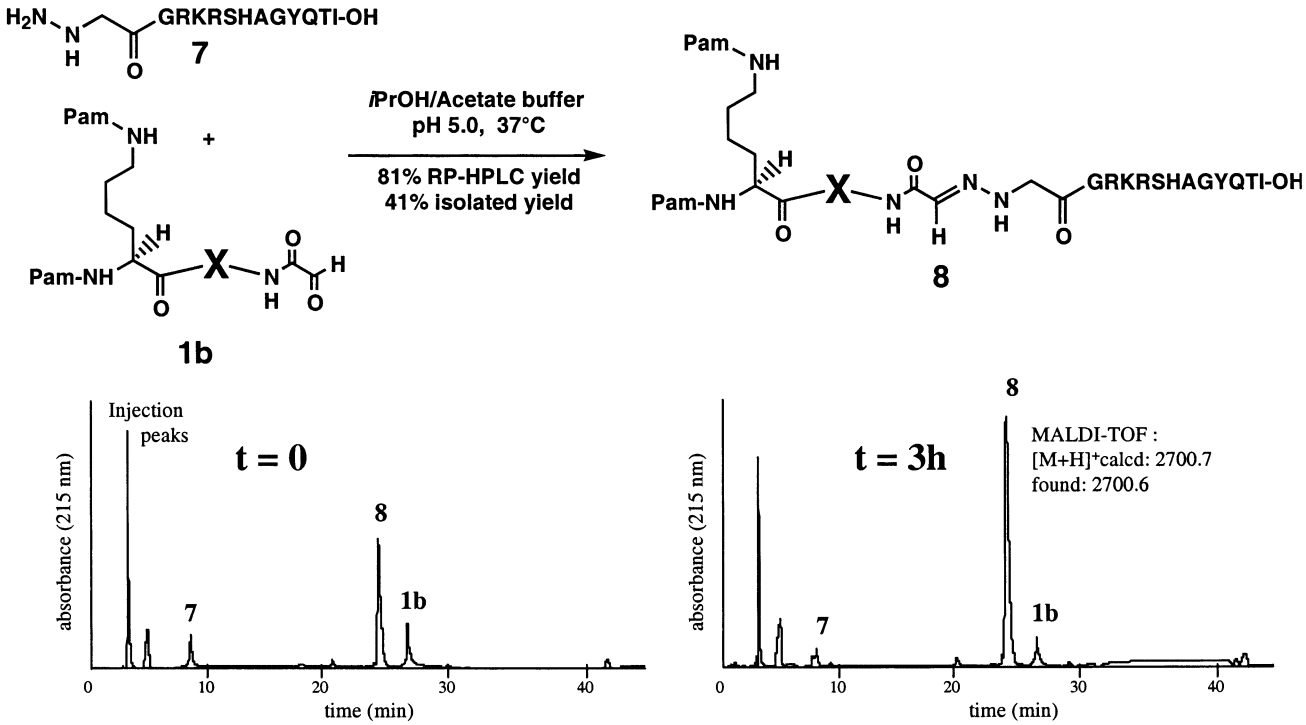

Figure 2. Ligation in solution of **1b** with peptide **7**. RP-HPLC monitoring of hydrazone ligation.

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11. **1a**: MALDI-TOF $[M+Na]^+$ calcd: 1048, found 1048. **1b**: MALDI-TOF $[M+Na]^+$ calcd: 1295.7, 1295.0, NMR ($CDCl_3/CD_3OD$ 1/1 by vol., 300 MHz) ^1H δ ppm 0.80 (t, $J=6.7$ Hz, 6H), 1.15 (m, 50H), 1.35 (m, 2H), 1.50 (m, 5H), 1.66 (m, 7H), 1.80 (m, 2H), 2.15 (m, 4H), 2.85 (m, 2H), 3.10 (m, 4H), 3.20–3.50 (m, 16H), 3.50 (m, 16H), 3.80 (s, 2H), 4.00 (s, 2H), 4.05 (s, 2H), 4.15 (t, $J=5.6$ Hz, 1H), 4.35 (m, 1H), 6.65 (d, $J=8.3$ Hz, 2H), 6.90 (d, $J=8.3$ Hz, 2H). ^{13}C δ ppm 13, 28, 36, 42, 48, 53, 60, 69, 95.
12. Large unilamellar vesicules: dimyristoyl phosphatidyl choline/dimyristoyl phosphatidyl glycerol/cholesterol/**1a** (54/42/6/10 by mol). Rehydratation by a citrate/phosphate buffer 50 mM, pH 6.
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