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A convenient route to conjugates of 1,2-diglycerides with functionalized oligoethylene glycol spacer arms

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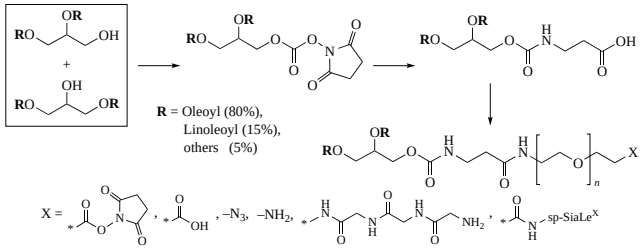
A controlled reaction of a mixture of structural isomers of diacylglycerol (DAG) with *N*,*N*-disuccinimidyl carbonate and then with β-alanine provided intermediates of 1,2-diacylglycerol (1,2-DAG) which were further modified with carboxy- and amino-terminated oligoethylene glycol spacer arms of different length. As an example of bioactive molecules intended for the incorporation in lipid bilayer of artificial or cell membranes, 1,2-DAGs bearing tetrasaccharide Sialyl Lewis X or triglycine at the terminus of polar spacer were synthesized. The synthetic scheme can be readily scaled-up by the use of DAGs from food industry.



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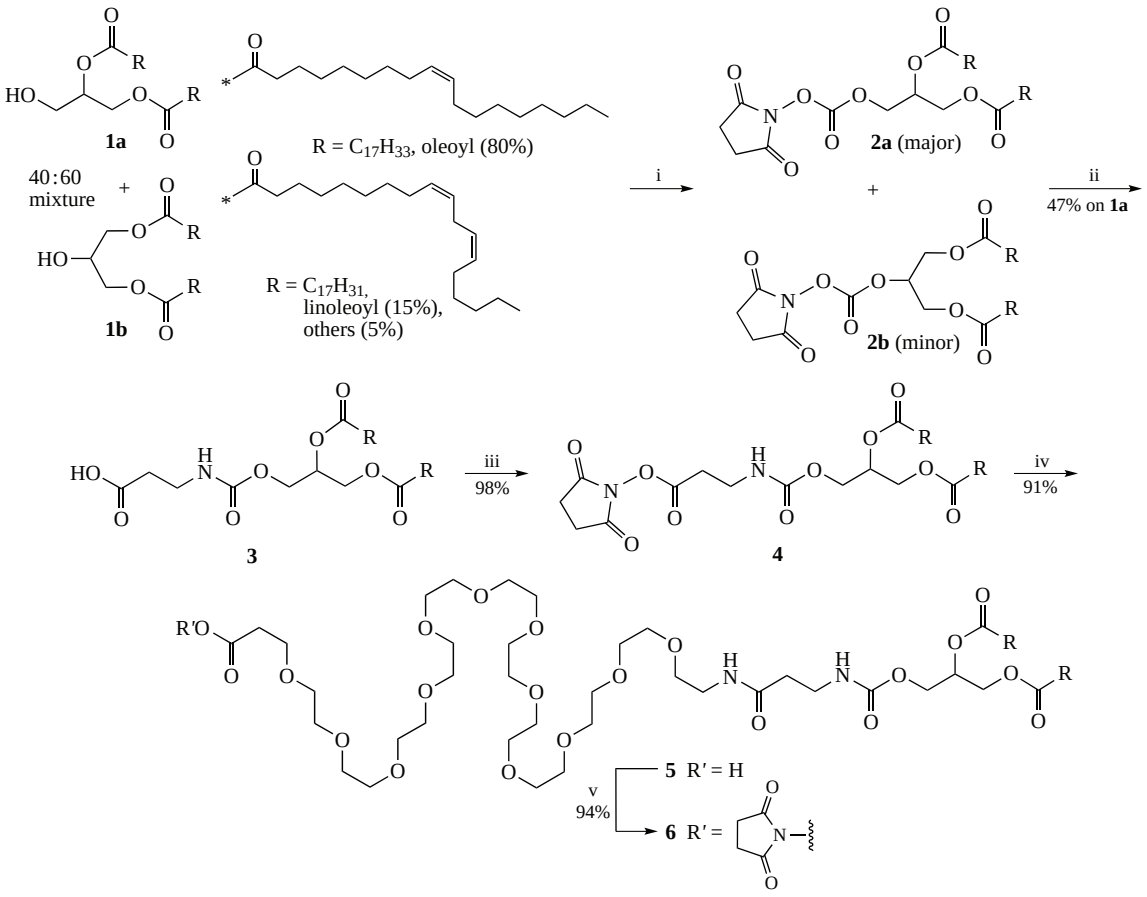
Lipophilic conjugates of biomolecules such as peptides, glycans, monoclonal antibodies or their fragments, and aptamers are in demand both in the studies of cell membranes and design/fabrication of drug delivery systems, such as liposomes, to ensure their targeting to specific cell receptors.[1,2] In the conjugate, the ligand is to be covalently bound to the polar head group of the lipid molecule capable of insertion and retention in the lipid bilayer. In dependence upon the research goal, a spacer of more or less length between membrane anchor and polar ligand is required to provide reliable presentation of the latter to its target macromolecule.[3] The spacer arm should bear functionality at its terminus distal from the lipid moiety for covalent linking with one or the other ligand. The most known commercially available compounds that meet all requirements are conjugates of phosphatidylethanolamine with functionalized polyethylene glycols (PEGs) of 2000 Da (mean polymerization numbers 45–48) or 5000 Da. The length of the spacer arms is designed as to avoid shielding of ligands at their termini,[4] since liposomes or solid lipid nanoparticles are usually coated with the corresponding PEGs for protection against immune cells.[5,6] However, stabilization of liposomes in the bloodstream can be also achieved by coating with glycolipids or phosphatidylinositol[7,8] when long spacers are not required. In addition, lipophilic conjugates for different research purposes require polar spacers of varied length and structure.[9,10]

Diglycerides, or diacylglycerols (DAGs), with C_{16}–C_{18} acyl groups can serve as reliable membrane anchors of amphiphilic molecules with prolonged polar moiety as well as phosphatidylethanolamine molecule can. The absence of a phosphate function facilitates synthetic transformations (for example, activation of carboxyl groups) and purifications. DAGs in the form of 1,2- and 1,3-diglycerides can be found in edible oils (though, at low concentrations, <10%) as the natural products of the hydrolytic activity of lipases during the maturation of oil fruits and seeds.[11,12] For their use in food industry, DAGs are produced chemically or enzymatically through direct esterification or glycerolysis.[11,13] Acyl migration occurs within DAG molecules and results in an equilibrium with 1,3-DAGs making up 60–70% of the total concentration since they are more thermodynamically stable.[11,12] Column chromatography on a silica gel provides more or less effective purification of the structural diglyceride isomers, however acyl migration renews already in the course of separation and continues upon storage (even without solvents, if not solidified). 1,2-DAGs are preferable as membrane anchors since they have a hairpin-shaped conformation of fatty acid chain arrangement which fits well in packing of lipid bilayer, in contrast to 1,3-DAGs forming V-shaped acyl chain arrangement.[12]

We propose to use a mixture of DAG structural isomers as a starting material for the synthesis of various lipophilic conjugates. The mixture can be isolated from a natural triglyceride oil (or partially hydrolyzed oil) by rough chromatography on silica gel, or retrieved from food industry, or synthesized using an individual fatty acid. Here, as model of composition of raw material from a natural source, we used a mixture of diglycerides containing 1,2- and 1,3-isomers in the ratio 2 : 3. The isomers were obtained by partial hydrolysis of olive oil followed by purification of products on silica gel; the acyl groups in the diglycerides were about 80% oleoyl and about 15% linoleoyl (for details, see Online Supplementary Materials). This is in good agreement with the literature data on the composition of olive oil.[14] The reaction of the 1,2- and 1,3-DAG **1a**,**b** mixture with *N*,*N*′-disuccinimidyl carbonate (DSC, 2 equiv.) in $MeCN/CH_2Cl_2$ in the presence of

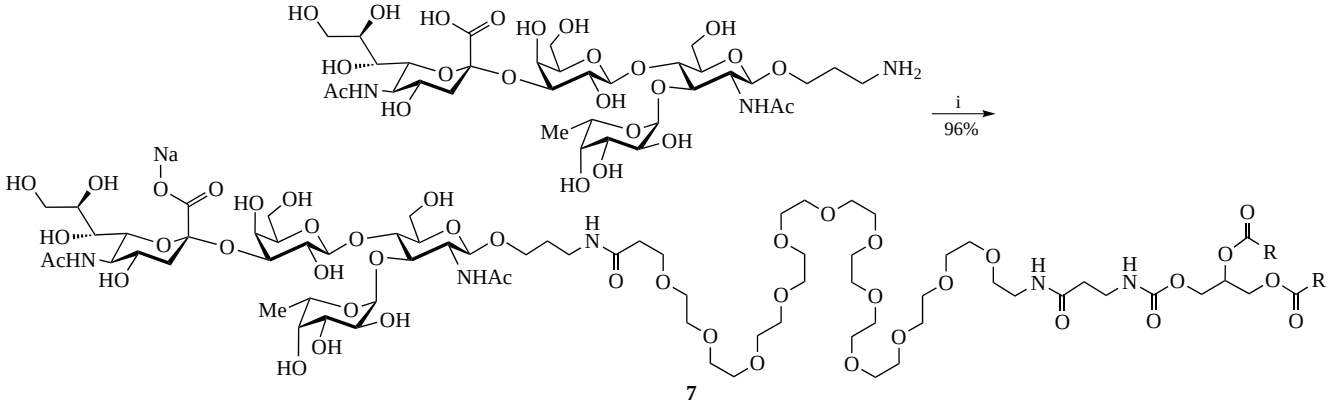


Scheme 1 *Reagents and conditions*: i, DSC (2 equiv.), Et_3N (1 equiv.), MeCN/CH_2Cl_2 (3:1), 20 °C, 1.7 h; ii, β-alanine (powder, excess), Et_3N, MeCN/CH_2Cl_2 (3:1), 20 °C, 1 h, separation on silica gel; iii, DSC (2 equiv.), Et_3N (1.5 equiv.), MeCN/CH_2Cl_2 (2:1), 20 °C, 40 min; iv, H_2N–PEG(12)–COOH (1 equiv.), Et_3N (1 equiv.), CH_2Cl_2, 20 °C, 2 h; v, DSC (3 equiv.), Et_3N (1.5 equiv.), MeCN/CH_2Cl_2 (2:1), 20 °C, 40 min.

triethylamine affords a mixture of *N*-succinimidyl carbonates of 1,2-diacyl- and 1,3-diacylglycerols **2a**,**b** (Scheme 1) in which the derivative of 1,2-diglyceride **2a** strongly prevails. The following one-pot reaction of these substances at the amino function of β-alanine leads to β-(1,2-diacylglyceryl-carbonylamino)propionic acid **3** and its 1,3-isomer in a ratio >10:1. These derivatives do not convert into each other unlike diglycerides do. Column chromatography on silica gel provided pure compound **3** in 47% yield calculated on 1,2-DAG **1a** in the starting mixture or ~27% based on unseparated mixture **1a**,**b** with its 1,3-isomer. A key step in the preparation of acid **3** from **1a**,**b** mixture is the selective activation of primary hydroxy group of 1,2-DAG **1a** resulting in *N*-succinimidyl carbonate **2a** as the main product. Under the chosen conditions, this reaction proceeds more than an order of magnitude faster than the reaction of the secondary hydroxy group of 1,3-DAG **2b**. It was possible to achieve an almost complete consumption of 1,2-DAG **1a** into *N*-succinimidyl carbonate **2a** with a low (<10%) conversion of 1,3-DAG to **2b**. An excess of DSC (before adding β-alanine, this excess is quenched with acetic acid) is necessary for the reaction to proceed quickly enough, however the conversion of diglycerides should be controlled. The prolongation of the reaction leads to the formation of noticeable amount of undesirable isomer **2b**.

At the next step, acid **3**, *rac*-1,2-diacyl-3-[*N*-(2-carboxyethyl)-carbamoyl]glycerol, was treated with DSC in MeCN/CH_2Cl_2 in the presence of triethylamine, followed by a gel exclusion chromatography on Sephadex LH-20 to yield *N*-succinimidyl ester **4** (~98%) with a purity >95% (see Scheme 1). Further transformations of ester **4** would depend upon the desirable length of the spacer arm and functionality required for conjugation with one or the other ligand. Reaction of ester **4** with an equivalent of oligoethylene glycol amino acid H_2N–PEG(12)–COOH yielded oligoethylene glycol acid diglyceride derivative **5** (91% after chromatography on a silica gel and Sephadex LH-20). Conversion of acid **5** into *N*-succinimidyl ester **6** (yield 94% with a purity >95% after gel exclusion chromatography on Sephadex LH-20) allows further modifications of the oligoethylene glycol derivative of diglyceride to be performed using compounds with an amino function.

The reaction of ester **6** with an equivalent of 3-aminopropyl glycoside of tetrasaccharide $SiaLe^X$, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ-sp-NH_2[15] in DMSO/dichloroethane, and a gel chromatography on Sephadex LH-20, afforded diglyceride glycoconjugate of $SiaLe^X$ **7**, yield 96% (Scheme 2). $SiaLe^X$ is a ligand of selectins (carbohydrate binding adhesion proteins), which are overexpressed on cell surface of activated leucocytes, endothelial cells, and platelets, and their targeting for drug delivery to tumors and inflammation foci is considered a promising strategy.[16] We have shown the specific anti-vascular effect of drug-loaded liposomes equipped with a diglyceride conjugate of $SiaLe^X$ in tumor bearing mice[17] and evidenced targeting of these liposomes to activated human endothelium *in vitro*.[18] $SiaLe^X$-conjugate used in the studies[17,18] also contained oligoethylene glycole spacer (n = 8–15), however the scheme for that synthesis was not optimal and did not provide scalability. We started from PEG bis(carboxymethyl) ether, therefore, PEG-containing module was linked to the rest modules in the molecule of lipophilic glycoconjugate *via* α-oxamethyl carboxamide bonds being less stable for long term storage (even at –20 °C) than β-oxaethyl carboxamide bonds as in the conjugate **7**; besides, diglyceride residue was linked *via* ester bond rather than through more stable carbamoyl bond.[19]



Scheme 2 *Reagents and conditions*: i, **6**, Et_3N (2 equiv.), DMSO/dichloroethane (1 : 1), 20 °C, 2 h; then NaOAc (aq., 20 equiv.), Sephadex LH-20, elution with Pr^iOH/water (3 : 5).

Alternatively, *N*-succinimidyl esters **4** and **6** were transformed into amino-terminated conjugates **8** (82%) and **10** (91%) with different lengths of polar spacers (Scheme 3) by the reaction with the excess of bis-amino derivative H_2N–PEG(3)–NH_2 (the latter was obtained by hydrogenation over Pd/C of azido amine derivative H_2N–PEG(3)–N_3 from Iris Biotech GmbH). The reaction of ester **6** with azido amine N_3–PEG(3)–NH_2 gave azido conjugate **9** in 92% yield (Scheme 3). This azido-terminated conjugate can be used for click reactions with alkyne-functionalized ligands. The Staudinger reduction of azide **9** with PPh_3 yielded stable under mild conditions iminophosphorane. Cleavage of the P=N bond in this compound at pH ~8 resulted in the desired amine **10**, however this process was accompanied by noticeable deacylation of the diglyceride.

Amino-terminated conjugates can be further functionalized, *e.g.*, with active esters of 3-maleimidopropionic acid (for thiol conjugation) and others, or peptides, *etc*. As an example, we have synthesized GlyGlyGly-terminated conjugate **11** by the reaction of amine **10** with *N*-succinimidyl ester of Fmoc derivative of triglycine in DMF followed by deprotection with piperidine with the yield of 81% after chromatography on silica gel and Sephadex LH-20. Diglyceride **11** can be further enzymatically conjugated to protein.

In conclusion, as far as we know, nobody previously used *N*-succinimidyl carbonates of 1,2-DAGs for the coupling with amines. To obtain carbamoyl bond with primary hydroxy group of 1,2-dialkylglycerol, activation with 4-nitrophenyl chloroformate was previously[20] reported. However, this reagent is more reactive towards alcohols than DSC, and therefore the selectivity of the formation of carbonate with primary hydroxyl in the mixture of 1,2- and 1,3-DAGs is expected to be lower. The scheme proposed herein allows one to access conjugates of 1,2-DAGs with polar functionalized arms starting from easily available mixtures of 1,2- and 1,3-diacylglycerols.

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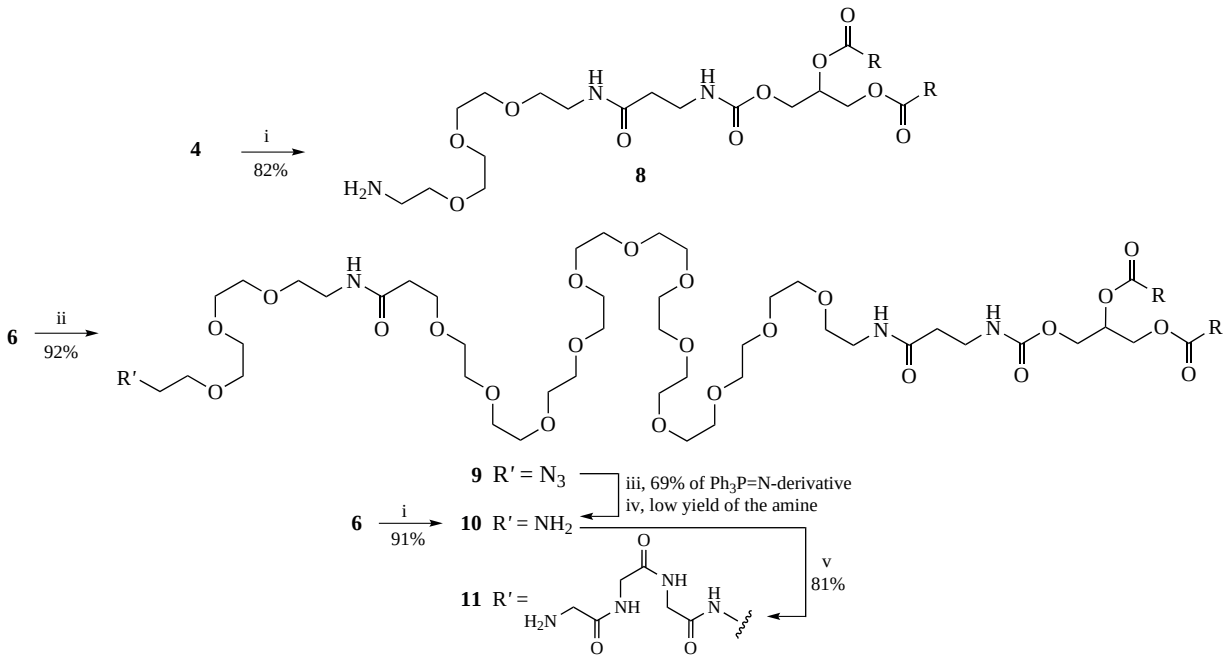

Scheme 3 *Reagents and conditions*: i, H_2N–PEG(3)–NH_2 diamine (8 equiv.), dichloroethane, 20 °C, 2 min, then AcOH; ii, N_3–PEG(3)–NH_2 amine (1.5 equiv.), $MeCN/CH_2Cl_2$/dichloroethane (2 : 2 : 2), 20 °C, 15 min; iii, PPh_3 (8 equiv.), 1,4-dioxane/water (4 : 1), 20 °C, 3 h; iv, 1,4-dioxane/water pH ~8, 20 °C, 15 h; v, Fmoc–Gly_3–ONSu (2 equiv.), DMF, 20 °C, 1 h, then piperidine (5% v/v), 20 °C, 15 min, then AcOH.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2021.07.034.

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