Synthesis and Evaluation as a Gene Transfer Agent of a 1,2-Dimyristoyl-*sn*-glycero-3-pentalysine Salt

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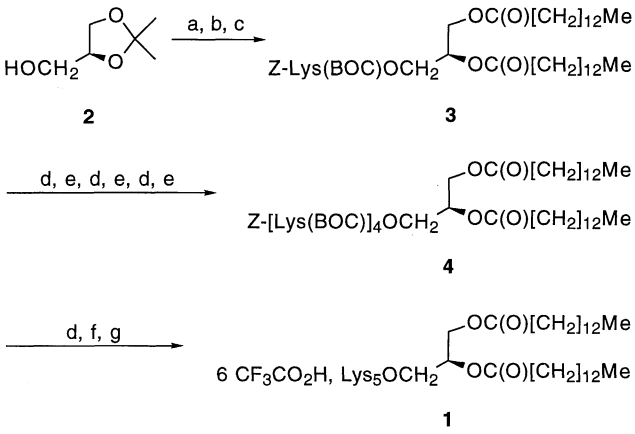
1,2-Dimyristoyl-*sn*-glycero-3-pentalysine hexakis(trifluoroacetate) **1** has been synthesized and its transfection efficiency has been compared with that of the commercially available lipid DOTAP (3T3 mouse fibroblast cells; CMV-Luc plasmid). When the positive (lipid) / negative (DNA) charge ratio was 6, the lipid **1** was 1.7 times more efficient than DOTAP.

Among the nonviral gene transfer techniques — *i. e.* nonviral techniques allowing the introduction, and subsequent expression, of exogenous DNA into living cells — the cationic lipid-mediated delivery has received an increasing attention in recent years[1] and several lipids consisting of a DNA-binding cationic moiety and of a self-aggregating lipidic moiety, are now commercially available. However, there is still a need for compounds with improved efficiency. In this Letter, we report the synthesis and evaluation of 1,2-dimyristoyl-*sn*-glycero-3-pentalysine hexakis(trifluoroacetate) **1** and its evaluation as a gene transfer agent in comparison with commercially available DOTAP.[2]

The design of lipid **1** was based – on the one hand – on the report that advantage has been taken of the DNA-binding ability of polylysines linked to proteins in targeted delivery of DNA to cells[3] and – on the other hand – on the fact that several amphiphilic diesters including the diacylglyceryl derivative DOTAP[4] have been described as effective vectors for transfection when incorporated into phospholipidic liposomes. Later, the successful transfection with lipopolyamine DOGS[2,5] alone showed that phospholipids were unnecessary. We would like to add that a polylysine conjugated to *N*-glutaryl-1,2-dioleoyl-phosphatidylethanolamine has been prepared and tested by others; however, with this compound, transfection was only observed when the cells were mechanically scraped after the incubation period or when a helper lipid was added to the preparation.[6]

The synthesis of the lipid **1** (Scheme 1) started with the acylation of 1,2-*O*-isopropylidene-*sn*-glycerol **2** with N^{α}-Z-N^{ε}-BOC-L-lysine, DCC[2] and DMAP[2]. After selective deprotection of the diol under mild acidic conditions and acylation with myristic acid using again DCC and DMAP, the α-amino group of lysine ester **3** was deprotected by catalytic hydrogenolysis. Three other N^{α}-Z-N^{ε}-BOC-L-lysines were then introduced using sequentially the DCC/HOBt[2] method for coupling and the catalytic hydrogenolysis for α-amino deprotection. Finally coupling with N^{α},N^{ε}-di-BOC-L-lysine and removal of all BOC[2] protecting groups with TFA[2] gave lipid **1**.[7]

The DNA / lipid **1** complex formation was investigated by adding various amounts of lipid **1** to a double stranded DNA and by examining the electrophoretic mobility of the complex on an agarose gel (1% w/v) stained with ethidium bromide. The DNA, which was obtained by treatment of the plasmid pUC12 with the restriction enzyme Hind III, appeared as a single band on the agarose gel. The tests were performed by mixing 0.5 μg of DNA



Scheme 1. a) Z-Lys-(BOC)-OH, DCC, DMAP, CH_2Cl_2, 6 h (91% yield); b) 0.01 N aqueous HCl, MeOH, 17 h (80%); c) $Me[CH_2]_{12}CO_2H$, DCC, DMAP, CH_2Cl_2, 6 h (65%); d) H_2, 10% Pd/C, EtOH, 2.5 h (88-98%); e) Z-Lys-(BOC)OH, DCC, HOBt, CH_2Cl_2 (81-89%); f) BOC-Lys-(BOC)-OH, DCC, HOBt, CH_2Cl_2 (85%); g) TFA/CH_2Cl_2 = 1/1, 0° C, 1 h (100%).

with 0, 0.02, 0.1, 0.5, 0.9, 1.8, 9.2, and 18.5 μg of lipid **1** so that the positive (lipid) / negative (DNA) charge ratio were 0, 0.04, 0.2, 1, 2, 4, 20, and 39 respectively.[8] Starting with test n° 5 (charge ratio = 2), no more migration of the DNA band occurred, indicating a rather large complex and / or an absence of negative charge.

The efficiency of lipid **1** in the transfection of 3T3 murine fibroblast cells with the CMV-Luc plasmid, which contains a reporter gene encoding firefly luciferase, was tested. Since this gene is normally absent from murine cells, the determination of luciferase activity in the cell extracts one day after transfection provides a measure of the transfection efficiency. The transfection experiments with lipid **1** were performed as follows.[8] The plasmid[9] (2 μg) and increasing amounts of ethanolic 2 mM lipid **1** were separately diluted with aqueous 150 mM NaCl (150 μL). After 10 min, the two solutions were mixed and after another 10 min, serum-free culture medium[10] (2.7 mL) was added. The so obtained transfection medium (1 mL/well) was added to the cells[10] after 10 min, and 2 h later, 10% FCS[2] (100 μL) was also added. The luciferase activity was measured after 24 h with Promega's luciferase assay system. To this end, the transfection medium was removed from the well and replaced by lysis buffer (100 μL; Promega); after 15 to 30 min, the cell extract was centrifuged and supernatant (20 μL) was mixed with luciferase assay reagent (100 μL). After a few seconds, the emitted light quantity was measured over 30 s in a luminometer (Biolumat LB

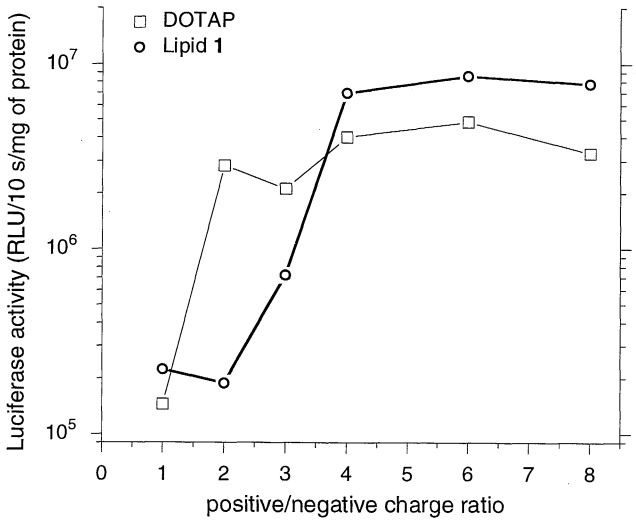


Figure 1. Efficiency of lipid **1** and of DOTAP as a function of the positive (lipid) / negative (DNA) charge ratio in the lipid-mediated transfection of 3T3 fibroblasts with CMV-Luc.

9500 Berthold). The luciferase background (150-200 RLU) was substracted from each measure and the luciferase activity was reported to the quantity of proteins present in the well (quantified with the BCA protein test from Pierce [enhanced protocol] in a Uvikon 930 Kontron Instruments spectrophotometer).

The Figure 1 shows that the transfection efficiencies of lipid **1** and of DOTAP[11] are highest when the positive / negative charge ratio is equal to 6, lipid **1** being then 1.7 times more efficient than DOTAP. Earlier reports by others had already mentioned that cationic lipid-mediated transfection reaches its maximum efficiency only when the nucleolipidic particles – formed from nucleic acid and lipid – bear a net positive charge. It is thought that they are thus able to bind to the negatively charged cell surface in a way that favors endocytosis.[1] However, when the charge ratio is higher than 8, less proteins are found in the wells; this may be attributable to some cytotoxicity of lipid **1**.

These encouraging preliminary results suggest that the influence of the length of the acyl chains and of the oligolysine on the transfection efficiency of **1** should be examined.

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References and Notes

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2 *Abbreviations:* BOC: *t*-butoxycarbonyl; CMV: cytomegalovirus; DCC: 1,3-dicyclohexylcarbodi-imide; DMAP: 4-dimethylaminopyridine; DMEM: Dulbecco's modified Eagle medium; DOGS: dioctadecylamidoglycylspermine; DOTAP: 1,2-dioleoyloxy-3-(trimethylammonio)propane; FCS: fœtal calf serum; HOBt: 1-hydroxybenzotriazole; RLU: relative light units; TFA: trifluoroacetic acid; Z: benzyloxycarbonyl.

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7 All intermediate compounds were purified by column chromatography and characterized by ^{1}H-NMR before use in the next step. The lipid **1** was sufficiently pure after deprotection with TFA to be used without purification. It appeared as a single spot (R_f = 0.29) on reversed phase TLC plates (Merck, HPTLC RP-18 WF_{254s}, 0.2mm; eluent: acetonitrile/10% aqueous TFA = 6/4). *^{1}H-NMR* (400 MHz, $CDCl_3$, CD_3OD, TFA) δ 0.72 (t, *J* 6.5 Hz, 2 x CH_3-), 1.10 (s, 2 x -$[CH_2]_{10}$-), 1.45 (m, 2 x -OC(O)CH_2**CH_2**-), 1.55 (m, 5 x -**CH_2**-CH_2-ND_3^+), 2.17 (td, *J* 7.5 Hz, 2.0 Hz, 2 x -OC(O)**CH_2**-), 2.80 (m, 5 x -**CH_2**-ND_3^+), 3.75-4.40 (m, -CO_2CH[**CH_2**OC(O)-]$_2$ and 5 x -C(O)**CH**[CH_2-]ND-), 5.11 (m, -CO_2**CH**[CH_2OC(O)-]$_2$). *FAB-MS* (*m*-nitrobenzyl alcohol) *m/z* 1175.7 (11%, $[M + Na]^+$), 1153.7 (100%, $[M + H]^+$), 1025.6 (4%, $[M - Lys + H_2]^+$), 897.5 (5%, $[M - Lys_2 + H_2]^+$), 769.5 (7%, $[M - Lys_3 + H_2]^+$), 641.4 (6%, $[M - Lys_4 + H_2]^+$), 495.3 (47%, $[M - Lys_5O]^+$), 285.2 (62%, $[M - Lys_5O$ – myristoyl$]^+$). *Electrospray MS* (aqueous acetonitrile, 1% formic acid; sampling cone voltage: 50 V) *m/z* 577.9 ($[M + 2 H]^{2+}$), 385.8 ($[M + 3 H]^{3+}$), 289.8 ($[M + 4 H]^{4+}$). In both mass spectra M stands for $C_{61}H_{120}N_{10}O_{10}$ = 1152.9.

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9 The CMV-Luc plasmid was a gift from Dr B. Demeneix (Museum National d'Histoire Naturelle, Paris).

10 The 3T3 murine fibroblast cells – a gift from Dr J.-P. Beck (Université Louis Pasteur, Illkirch) – were grown to confluency in DMEM[2] (Gibco) supplemented with 10% FCS[2] (Gibco), glutamine (0.286 mg/mL; Lancaster), glucose (2 mg/mL; Janssen), streptomycin (100 U/mL; Gibco), penicillin (100 IU/mL; Gibco), and kanamycin (0.100 mg/mL; Intermed).[8] They were transfered, 12 h before the transfection experiments, to Falcon 24-well dishes (45000-50000 cells/well in 2 mL culture medium). Just before transfection, the cells were rinsed with serum-free culture medium.

11 DOTAP is available from Boehringer-Mannheim and was used under the conditions recommended by the supplier.