

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

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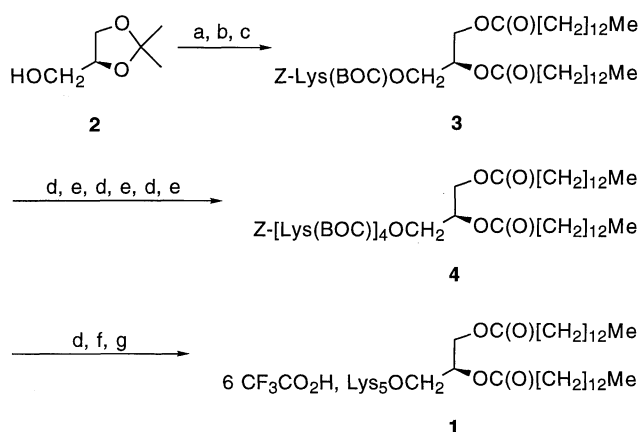
1,2-Dimyristoyl-*sn*-glycero-3-pentyllysine hexakis(trifluoroacetate) **1** has been synthesized and its transfection efficiency has been compared with that of the commercially available lipid DOTAP (3T3 mouse fibroblast cells; CMV-Luc plasmid). When the positive (lipid) / negative (DNA) charge ratio 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<sup>1</sup> 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-pentyllysine hexakis(trifluoroacetate) **1** and its evaluation as a gene transfer agent in comparison with commercially available DOTAP.<sup>2</sup>

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<sup>3</sup> and — on the other hand — on the fact that several amphiphilic diesters including the diacylglycerol derivative DOTAP<sup>4</sup> have been described as effective vectors for transfection when incorporated into phospholipidic liposomes. Later, the successful transfection with lipopolyamine DOGS<sup>2,5</sup> 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.<sup>6</sup>

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

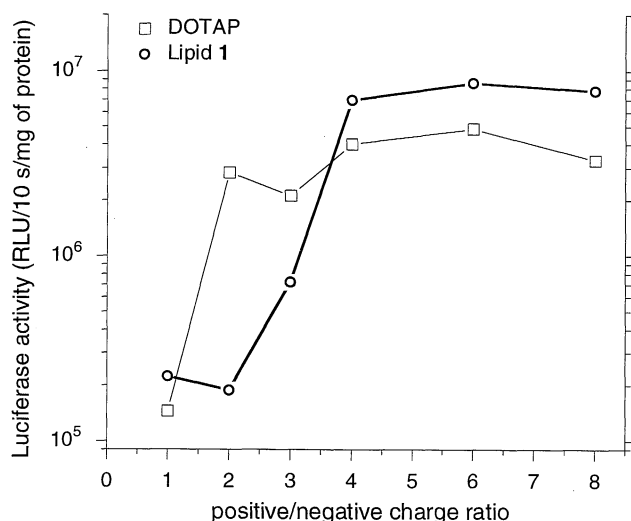
The DNA / lipid **1** complex formation was investigated by adding various amounts of lipid **1** to a double stranded DNA and by examining the electrophoretic mobility of the complex on an agarose gel (1% w/v) stained with ethidium bromide. The DNA, which was obtained by treatment of the plasmid pUC12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



**Scheme 1.** a) Z-Lys-(BOC)-OH, DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 6 h (91% yield); b) 0.01 N aqueous HCl, MeOH, 17 h (80%); c) Me[CH<sub>2</sub>]<sub>12</sub>CO<sub>2</sub>H, DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 6 h (65%); d) H<sub>2</sub>, 10% Pd/C, EtOH, 2.5 h (88-98%); e) Z-Lys-(BOC)OH, DCC, HOBt, CH<sub>2</sub>Cl<sub>2</sub> (81-89%); f) BOC-Lys-(BOC)-OH, DCC, HOBt, CH<sub>2</sub>Cl<sub>2</sub> (85%); g) TFA/CH<sub>2</sub>Cl<sub>2</sub> = 1/1, 0° C, 1 h (100%).

with 0, 0.02, 0.1, 0.5, 0.9, 1.8, 9.2, and 18.5  $\mu$ g 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.<sup>8</sup> 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.<sup>8</sup> The plasmid<sup>9</sup> (2  $\mu$ g) and increasing amounts of ethanolic 2 mM lipid **1** were separately diluted with aqueous 150 mM NaCl (150  $\mu$ L). After 10 min, the two solutions were mixed and after another 10 min, serum-free culture medium<sup>10</sup> (2.7 mL) was added. The so obtained transfection medium (1 mL/well) was added to the cells<sup>10</sup> after 10 min, and 2 h later, 10% FCS<sup>2</sup> (100  $\mu$ L) 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  $\mu$ L; Promega); after 15 to 30 min, the cell extract was centrifuged and supernatant (20  $\mu$ L) was mixed with luciferase assay reagent (100  $\mu$ L). 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 subtracted 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<sup>11</sup> 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.<sup>1</sup> 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

- 1 J.-P. Behr, *Acc. Chem. Res.*, **26**, 274 (1993).
- 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: foetal calf serum; HOBt: 1-hydroxybenzotriazole; RLU: relative light units; TFA: trifluoroacetic acid; Z: benzyloxycarbonyl.

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- 5 J.-P. Behr, B. Demeneix, J.-P. Loeffler, and J. Perez-Mutul, *Proc. Natl. Acad. Sci. USA*, **86**, 6982 (1989).
- 6 X. Zhou, A. L. Klibanov, and L. Huang, *Biochim. Biophys. Acta*, **1065**, 8 (1991); X. Zhou and L. Huang, *Biochim. Biophys. Acta*, **1189**, 195 (1994).
- 7 All intermediate compounds were purified by column chromatography and characterized by <sup>1</sup>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<sub>254s</sub>, 0.2mm; eluent: acetonitrile/10% aqueous TFA = 6/4). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>, CD<sub>3</sub>OD, TFA)  $\delta$  0.72 (t,  $J$  6.5 Hz, 2 x CH<sub>3</sub>-), 1.10 (s, 2 x -[CH<sub>2</sub>]<sub>10</sub>-), 1.45 (m, 2 x -OC(O)CH<sub>2</sub>CH<sub>2</sub>-), 1.55 (m, 5 x -CH<sub>2</sub>-CH<sub>2</sub>-ND<sub>3</sub><sup>+</sup>), 2.17 (td,  $J$  7.5 Hz, 2.0 Hz, 2 x -OC(O)CH<sub>2</sub>-), 2.80 (m, 5 x -CH<sub>2</sub>-ND<sub>3</sub><sup>+</sup>), 3.75-4.40 (m, -CO<sub>2</sub>CH[CH<sub>2</sub>OC(O)-]<sub>2</sub> and 5 x -C(O)CH[CH<sub>2</sub>-]ND-), 5.11 (m, -CO<sub>2</sub>CH[CH<sub>2</sub>OC(O)-]<sub>2</sub>). *FAB-MS* (*m*-nitrobenzyl alcohol)  $m/z$  1175.7 (11%, [M + Na]<sup>+</sup>), 1153.7 (100%, [M + H]<sup>+</sup>), 1025.6 (4%, [M - Lys + H<sub>2</sub>]<sup>+</sup>), 897.5 (5%, [M - Lys<sub>2</sub> + H<sub>2</sub>]<sup>+</sup>), 769.5 (7%, [M - Lys<sub>3</sub> + H<sub>2</sub>]<sup>+</sup>), 641.4 (6%, [M - Lys<sub>4</sub> + H<sub>2</sub>]<sup>+</sup>), 495.3 (47%, [M - Lys<sub>5</sub>O]<sup>+</sup>), 285.2 (62%, [M - Lys<sub>5</sub>O - myristoyl]<sup>+</sup>). *Electrospray MS* (aqueous acetonitrile, 1% formic acid; sampling cone voltage: 50 V)  $m/z$  577.9 ([M + 2 H]<sup>2+</sup>), 385.8 ([M + 3 H]<sup>3+</sup>), 289.8 ([M + 4 H]<sup>4+</sup>). In both mass spectra M stands for C<sub>61</sub>H<sub>120</sub>N<sub>10</sub>O<sub>10</sub> = 1152.9.
- 8 F. Barthel, J.-S. Remy, J.-P. Loeffler, and J.-P. Behr, *DNA and Cell Biol.*, **12**, 553 (1993).
- 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<sup>2</sup> (Gibco) supplemented with 10% FCS<sup>2</sup> (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).<sup>8</sup> They were transferred, 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.