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# New cationic lipids for gene transfer with high efficiency and low toxicity: T-shape cholesterol ester derivatives

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**Abstract**—New degradable cationic ester lipids with 'T-shape' configurations were synthesized and tested for gene delivery carrier. Their transfection efficiency and toxicity were compared with commercially available cationic lipids, DOTMA, DOSPA, and DC-Chol. They showed efficient transfection activity and almost no toxicity on mammalian cell lines. Their ester bond degradation was monitored by $^1$H NMR.


After Felgner et al.[1] had showed that DNAs could be introduced into cells in the form of synthetic cationic lipids/DNA complexes, various cationic lipids were developed and tested for transfection reagents.[2,3] Many cationic lipids/DNA complexes are commercially available and some of them have already been used in the clinical setting. Although these cationic lipids/DNA complexes have advantages of safety and nonimmunogenesity over viral vectors, the problems of low efficiency, and cytotoxicity of cationic lipids have not been solved yet.

Since the findings that cationic cholesterol derivatives showed relatively higher efficiency,[4] much effort has been focused on the synthesis of another derivative and the understanding of the relationship between the molecular structures and the transfection efficiency. Lee et al. synthesized many cholesterol and noncholesterol derivatives, which were selected and optimized by repeated transfection experiments.[5] Cholesterol derivative having a 'T-shape' configuration of spermine was shown to be particularly efficacious. Other 'T-shape' derivatives also showed higher efficiency than those of any other configuration and commercially available lipids. They supposed that 'T-shape' polyamines resembled a cognate ligand for a cell-surface receptor and thereby facilitated attachment and entry into target cells, or the polyamines were better at promoting effective interactions with the pDNA.

There has been also much effort to overcome the other obstacle like cytotoxicity of cationic lipids. Introduction of biodegradable bonds into a cationic lipid structure could be one of many methods to do it. It is reported that cytotoxicity has been attributed to the inhibition of protein kinase C activity after incorporation of cationic amphiphiles into the plasma membrane.[4,6] Thus, if cationic and hydrophobic parts of lipids could be connected with degradable bonds, reasonable reduction of cytotoxicity would be expected. DOTAP (*N*-1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniumethyl sulfate) was developed through taking this into account, which had biodegradable ester bonds and showed lower toxicity than its homologue with ether bonds, DOTMA (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride).[7] In addition, tetraester lipids and cholesterol-based ester lipids were synthesized and characterized for transfection.[8] They also showed somewhat lower toxicity than nondegradable lipids.

By building on these concepts, we have designed novel cationic cholesterol derivatives that have a T-shape

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configuration and ester bonds. Cholesterol was conjugated with diethanolamine by carbamate bond for a T-shape configuration, and two of its hydroxyl groups were coupled with carboxyl groups of amino acids to ester bonds. The amino acids that we selected were β-alanine and lysine. Because β-alanine has one primary amine and lysine has two, we could derive the relation of toxicity and efficiency with charged groups from the comparison between two amino acids. Of course, this type of synthetic approach could be applied to other amino acids.

The synthetic strategy is illustrated in Scheme 1. Briefly, cholesterol chloroformate was added to the diethanolamine in dichloromethane/methanol. After overnight stirring, 3β-[diethanolamine-carbamoyl]-cholesterol was purified from the mixture by recrystallization. The ester bonds between 3β-[diethanolamine-carbamoyl]-cholesterol and *N*-$^t$Boc-protected amino acids were formed by well-known diisopropylcarbodiimide (DIPC) method catalyzed by DMAP (dimethylamineopyridine)/PTSA (*p*-toluenesulfonic acid).[9] The *N*-$^t$Boc groups of the conjugates, which were purified by liquid chromatography on silica gel, were deprotected by TFA (trifluoroacetic acid) to final products, $A_2$D-Chol (β-alanine$_2$-diethanolamine-cholesterol) and $K_2$D-Chol (lysine$_2$-diethanolamine-cholesterol).[10]

DNA condensing ability of $A_2$D-Chol and $K_2$D-Chol for the protection from nucleases was tested by gel retardation assay and AFM (atomic force microscopy). As shown in Figure 1A, the retardation of DNA due to lipoplex formation was observed. $A_2$D-Chol formed lipoplex with DNA at the weight ratio of around 3 (the N/P (nitrogen/phosphate) ratio of around 3) and $K_2$D-Chol did at the weight ratio of around 3 (the N/P ratio of around 5). Generally, the lipids with more charge can

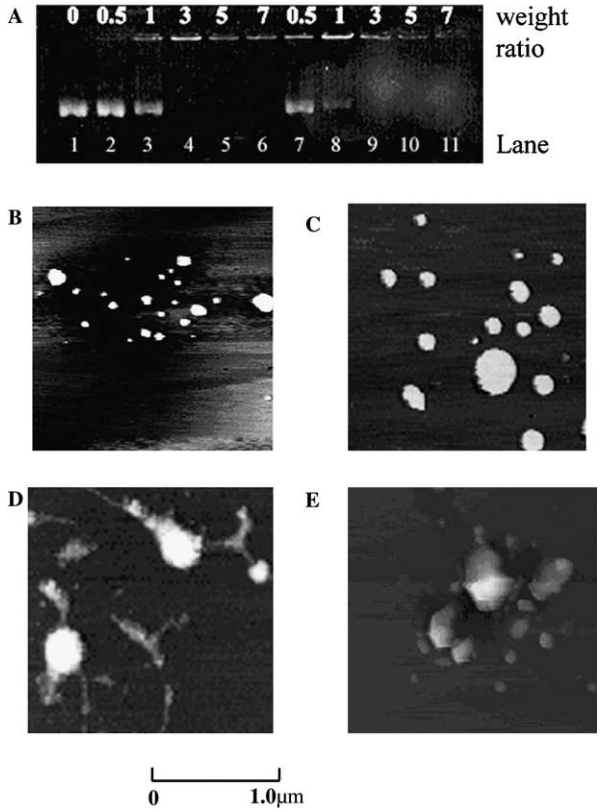
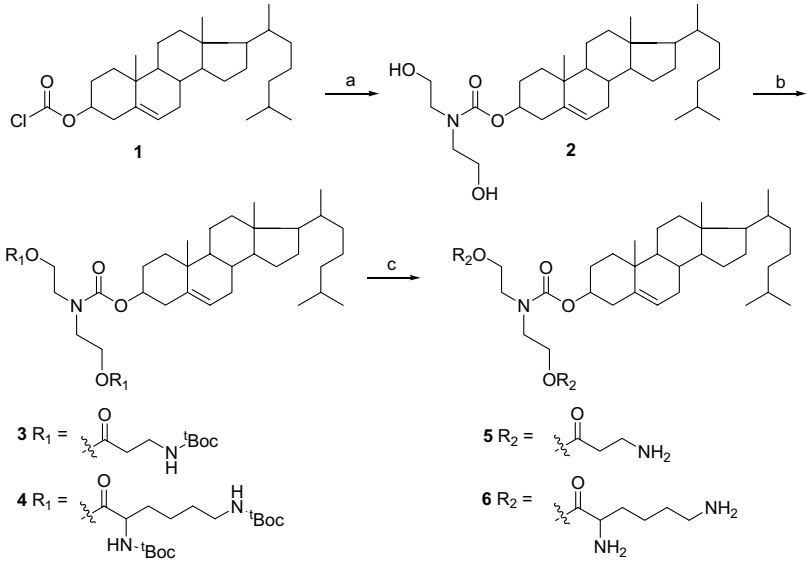



**Figure 1.** Lipoplex formation between cationic lipids and pDNAs (pSV-β-gal) after 30 min incubation in DMEM. (A) agarose gel retardation assay of $K_2$D-Chol/DNA (lane 2–6) and $A_2$D-Chol/DNA (lane 7–11). The weight ratios of lipids/DNA were shown. The N/P (nitrogen/phosphate) ratios were 0.9, 1.7, 5.1, 7.5, 11.9 for lane 2–6 and 0.5, 1, 3, 5, 7 for lane 7–11. AFM (Nanoscope IIIa system, Digital Instruments, Inc., Santa Barbara, CA) images of (B) $A_2$D-Chol/DOPE liposomes (C) $K_2$D-Chol/DOPE liposomes (D) $A_2$D-Chol liposome/DNA lipoplexes (E) $K_2$D-Chol liposome/DNA lipoplexes.




**Scheme 1.** Reagents and conditions: (a) Diethanolamine/TEA in $CH_2Cl_2$ and MeOH; (b) DMAP, PTSA, DIPC/MC, 37 °C; (c) 20% TFA in $CH_2Cl_2$ or $CHCl_3$, 0 °C.

form complexes with DNA at lower charge ratio.[11] However, from this experiment, it was observed that the binding affinity to DNA of $A_2$D-Chol per charged amine is greater than that of $K_2$D-Chol. It was suggested that all amines of $K_2$D-Chol were not effective in interaction with the phosphate groups of DNA.

AFM was also performed to confirm lipoplex formation. As shown in Figure 1B and D, the diameters of $A_2$D-Chol/DOPE (w/w = 1) liposomes ranged from ca. 30 nm to 100 nm and those of $K_2$D-Chol/DOPE (w/w = 1) liposomes were from ca. 50 nm to 300 nm. Relatively large liposomes were supposed to be multilamellar vesicles (MLV). Figure 1C and E show lipoplexes formed between $A_2$D-Chol, $K_2$D-Chol liposomes and DNA. The size of $A_2$D-Chol and $K_2$D-Chol lipoplexes ranged from 50 nm to 500 nm. And average size of $K_2$D-Chol lipoplex (300 nm) was larger than that of $A_2$D-Chol (150 nm).

Transfection efficiency of $A_2$D-Chol and $K_2$D-Chol was tested on various cell lines (Fig. 2A). DOPE (dioleoylphosphatidylethanolamine) was added to the liposome at the same weight ratio as that of cationic lipids for endosome disruption function. DC-Chol, a commercially available cholesterol-based lipid, was used as a positive control. $A_2$D-Chol and $K_2$D-Chol showed higher efficiency than DC-Chol in 293, HepG2, and NIH3T3 cell lines. Especially, they showed as much as 100–1000 fold higher efficiency than DC-Chol on NIH3T3.

It was reported that cholesterol-based cationic lipids maintained their transfection ability in the environment with serum in contrast to other types of lipids because their lipoplexes with DNA were stronger than others against the attack of serum proteins.[12] Therefore, we performed the experiment to compare the efficiency of the cationic lipids in presence of serum to that without serum on HepG2 cell lines (Fig. 2B). For the experiment, noncholesterol cationic lipids such as lipofectin(DOTMA/DOPE), and lipofectamine(DOSPA/DOPE)-PLUS, showed reduced transfection efficiencies in 10% serum condition. Especially, lipofectamine-PLUS showed 30-fold reduced transfection efficiency in presence of serum. Meanwhile, DC-Chol and $A_2$D-Chol showed similar transfection efficiency in both conditions, and $K_2$D-Chol could deliver genes into cells more efficiently in serum conditions. Our lipids may have a potential toward in vivo gene delivery applications.

The main objective of the development of $A_2$D-Chol and $K_2$D-Chol was the reduction of cellular toxicity. So, their toxicity was compared to that of DC-Chol, a nondegradable cholesterol derivative (Fig. 3). Figure 3A shows the concentration dependence of the relative cell

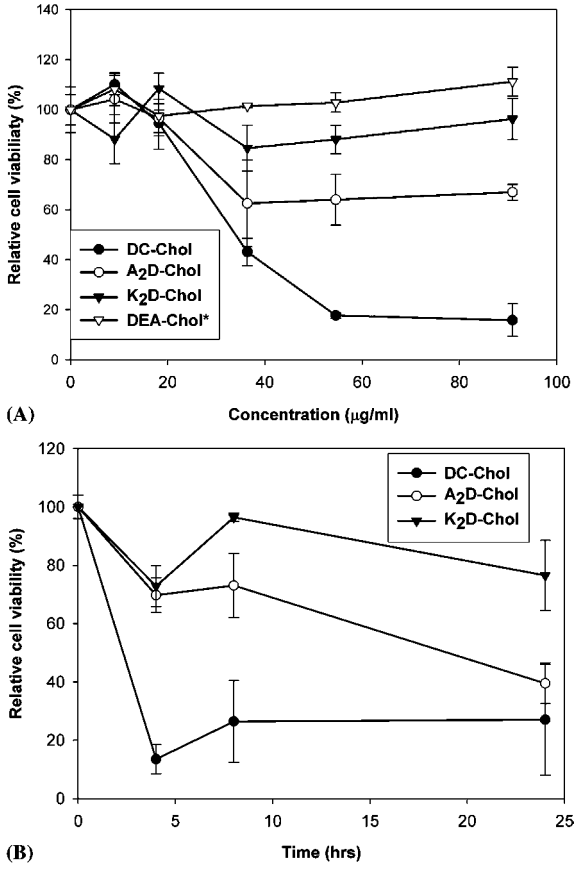



**Figure 3.** Cytotoxicity of cationic lipids on HepG2 cell lines. (A) Concentration dependence (incubation time = 4 h). (*The molarity of DEA-Chol was equal to that of $K_2$D-Chol). (B) Time dependence (concentration = 36 μg/ml). All data was measured by MTT method.

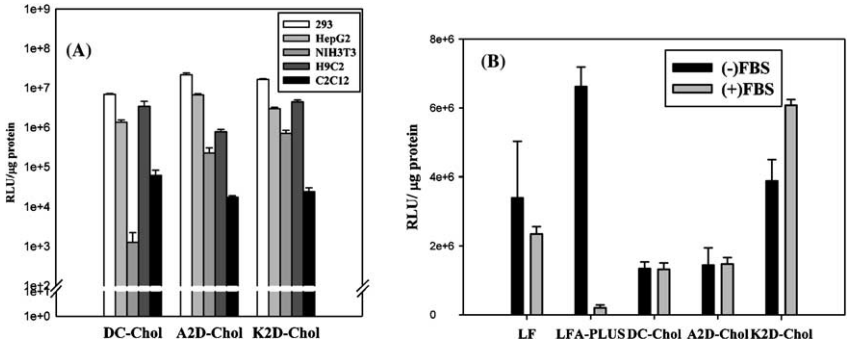



**Figure 2.** Plasmid transfection efficiency of cationic lipids to mammalian cell lines. (A) Luciferase activities of mammalian cells transfected by DC-Chol, $A_2$D-Chol, and $K_2$D-Chol. (B) Serum influence on luciferase activities of transfected HepG2. (Each transfection reagent was used at optimal lipid/pDNA ratio.)

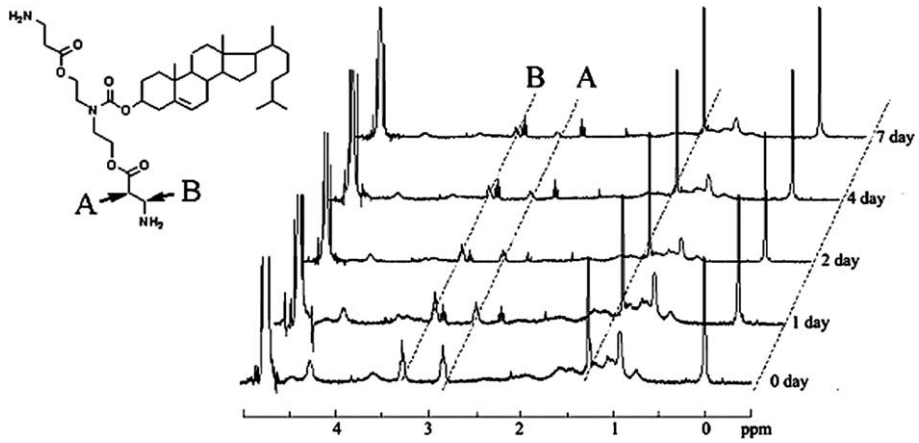



**Figure 4.** Degradation of $A_2$D-Chol ester bonds in pH 7.4 PBS. Peak at 1.24 ppm were from protons of $^t$BuOH as an internal standard. Peaks denoted as A and B were from undegraded β-alanine in $A_2$D-Chol.

viability. After 4 h incubation with cationic liposomes, the ester-linked cholesterol derivatives, $A_2$D-Chol, and $K_2$D-Chol, were much less toxic to HepG2 cells than DC-Chol. $K_2$D-Chol was almost nontoxic to cells even at 91 μg/ml and $A_2$D-Chol showed about 70% viability at the same concentration. On the contrary, HepG2 cells were severely damaged by DC-Chol and only 40% of cells could survive at the concentration of 36 μg/ml.

The incubation time dependence of cytotoxicity was also tested. At 36 μg/ml cationic lipid concentration, HepG2 cells were incubated for 4–24 h (Fig. 3B). Cells exposed to DC-Chol died as time went by. After 24 h, almost all cells were shrunken and dead (detected by microscope; data not shown) and showed no MTT signal. $A_2$D-Chol showed some toxicity to HepG2 after 24 h, and cells exposed to $K_2$D-Chol for 24 h were healthy and showed 80–100% viability. $K_2$D-Chol toxicity was examined for three days, no difference of viability between control and exposed cells was observed.

Low cytotoxicity of $A_2$D-Chol and $K_2$D-Chol shown above was supposed to be due to the degradable ester linker between charged groups and the hydrophobic cholesteryl diethanolamine. The time-dependent ester bond breakage of them was monitored by $^1$H NMR. We used a singlet proton of *tert*-butyl alcohol as an internal standard and the integration change of two triplet sets of protons in β-alanine $A_2$D-Chol was observed. At initial time, these protons showed very broad signals because they could construct a hydrophobic cluster in an aqueous environment. As incubation time increased, the hydrolyzed β-alanine units were released to water and their characteristic clear triplet peaks began to appear (Fig. 4). Peaks at 2.83 and 3.27 ppm were from α and β protons of β-alanine in $A_2$D-Chol. Peaks at 2.55 and 3.18 ppm were from those of hydrolyzed β-alanine. The integration values of the former peaks decreased, but those of the latter increased as hydrolysis proceeded. Even after seven days of incubation, the peaks of some undegraded β-alanine were detected in NMR spectrum. $K_2$D-Chol degradation was also observed by NMR (data not shown). Integrations of lysine peaks at 3.75, 3.02, 1.91, 1.71, and 1.45 ppm increased according to the degradation. After four days, no undegraded lysine peak was detected further.

The final product of degradation, 3β-[diethanolamine-carbamoyl]-cholesterol was added to HepG2 cell line at the same molar concentration as that of $K_2$D-Chol (Fig. 3A) and showed no toxicity. Therefore, it was supposed that the bond degradation between the charged and hydrophobic groups was important factor of cytotoxicity of cationic lipid and the lower toxicity of $K_2$D-Chol than that of $A_2$D-Chol was considered due to its faster degradation.

In conclusion, we synthesized new cationic ester lipids with a T-shape configuration for efficient and nontoxic gene transfer to mammalian cells. They show lower toxicity and higher efficiency than commercially available gene delivery lipids, lipofectin, and DC-Chol. Their characteristic nontoxicity was probably due to the fast degradation of ester bonds between charged groups and hydrophobic groups. Their easy synthesis could be applied to the synthesis of other amino acids derivatives of $A_2$D-Chol and $K_2$D-Chol. And their high efficiency and low toxicity would contribute to the tranfection of mamallian cells and in vivo gene delivery applications. Further research on these lipids derivatives would also enhance the understanding of the structure–function relationship in designing cationic lipids.

## Acknowledgements

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10. **3β-[Diethanolamine-carbamoyl] cholesterol (2).** 630.84 mg (6.0000 mmol) of diethanolamine, 1.40 ml (10.0 mmol) of triethylamine were diluted in the mixture of methylene-chloride and methanol ($V_{MC}/V_{methanol}$ = 1:1, 10 ml). Then cholesteryl chloroformate (898.24 mg, 2.0000 mmol) dissolved in MC (2 ml) was added dropwise into the solution at 0 °C. After 1 h stirring, the reaction was maintained overnight at room temperature. The solution was evaporated, dissolved in ethyl ether, and filtered. The filtered solution was recrystallized from ether/petroleum ether to give 0.828 g (80.0%) of **2** as a white crystalline powder. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ in ppm 0.67–2.37 (m, skeleton of cholesterol), 3.50 (br, –C$H_2$CH$_2$OH of diethanolamine), 3.83 (br, –C$H_2$OH of diethanolamine). (All NMR data were measured on a Bruker DPX-300 (300 MHz)).
    **General esterification procedure (3,4).** 0.5000 mmol (258.8 mg) of **2**, 1.500 mmol of *N*-$^t$Boc β-alanine (or *N*,*N*′-di$^t$Boc lysine), 1.500 mmol (183.3 mg) of DMAP, 0.7500 mmol (142.6 mg) of PTSA were dissolved in 8 ml MC. DIPC was added to the solution with vigorous stirring. After overnight stirring at 37 °C, the mixture was evaporated. The crude product was purified by liquid chromatography on silica gel, eluting with hexane gradually increasing to 2:1 ethyl acetate/hexane to give $^t$Boc-A$_2$D-Chol(**3**) or di$^t$Boc-K$_2$D-Chol(**4**) as colorless viscous oil. The yield of each reaction was over 90%. $^1$H NMR (300 MHz, $CDCl_3$) of $^t$Boc-A$_2$D-Chol: $\delta$ in ppm 0.67–2.37 (m, skeleton of cholesterol, $^t$Boc proton of *N*-$^t$Boc β-alanine), 2.53 (br, –OOC$H_2$CH$_2$–NH– of *N*-$^t$Boc β-alanine), 3.40 (q, –OOCH$_2$C$H_2$–NH– of *N*-$^t$Boc β-alanine), 3.54 (br, –C$H_2$CH$_2$O– of diethanolamine), 4.20 (br, –C$H_2$O– of diethanolamine). $^1$H NMR (300 MHz, $CDCl_3$) of di $^t$Boc-K$_2$D-Chol: $\delta$ in ppm 0.67–2.37 (m, skeleton of cholesterol, $^t$Boc proton of *N*,*N*′-di$^t$Boc lysine, –(C$H_2$)$_3$– of *N*,*N*′-di$^t$Boc lysine), 3.10 (br, ε-C$H_2$ of *N*,*N*′-di$^t$Boc lysine), 3.55 (br, –C$H_2$CH$_2$O– of diethanolamine), 4.30 (br, –C$H_2$O– of diethanolamine, α-C*H* of *N*,*N*′-di$^t$Boc lysine).
    **A$_2$D-Chol (5).** 0.2500 mmol (215.0 mg) of $^t$Boc-A$_2$D-Chol was dissolved in 10 ml MC. TFA (2 ml) was added to the solution at 0 °C. After 35 min stirring, TFA was evaporated under stream of $N_2$ gas, then the mixture was dried under vacuum, washed by petroleum ether to give A$_2$D-Chol as pink-colored viscous oil. Yield was over 99%. $^1$H NMR (300 MHz, $CDCl_3$) of A$_2$D-Chol: $\delta$ in ppm 0.67–2.37 (m, skeleton of cholesterol), 2.78 (br, –OOC$H_2$CH$_2$NH$_2$ of β-alanine), 3.25 (br, –OOCH$_2$C$H_2$NH$_2$ of β-alanine), 3.50 (br, –C$H_2$CH$_2$O– of diethanolamine), 4.22 (br, –C$H_2$O– of diethanolamine). MS (FAB-MASS) *m*/*z* 660.4940 ($C_{38}H_{66}O_6N_3$). (All MS data were recorded on a JEOLJMS-AX505WA spectrometer using FAB method).
    **K$_2$D-Chol (6).** 0.2500 mmol (307.4 mg) of di$^t$Boc-K$_2$D-Chol was dissolved in 10 ml chloroform. TFA (2 ml) was added to the solution at 0 °C. After 45 min stirring, TFA was evaporated under stream of $N_2$ gas, then the mixture was dried under vacuum, washed by petroleum ether to give K$_2$D-Chol as colorless viscous oil. Yield was over 99%. $^1$H NMR (300 MHz, $CD_3OD$) of K$_2$D-Chol: $\delta$ in ppm 0.69–2.35 (m, skeleton of cholesterol, –(C$H_2$)$_3$– of lysine), 2.90 (m, ε-C$H_2$ of lysine), 3.60 (br, –C$H_2$CH$_2$O– of diethanolamine), 4.35 (br, –C$H_2$O– of diethanolamine, α-C*H* of lysine). MS (FAB-MASS) *m*/*z* 774.6088 ($C_{44}H_{80}O_6N_5$).
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