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Caged compounds with a steroid skeleton: synthesis, liposome-formation and photolysis

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Abstract—The synthesis of two key precursors for caging bioactive compounds, an *o*-nitrobenzyl *p*-nitrophenyl carbonate and an *o*-nitrobenzyl diazo compound with a steroid skeleton, is reported. The former is a suitable caging group for an amino group, while the latter is appropriate for a carboxylic acid. The resulting caged compounds were irradiated at 350 nm to afford the corresponding amine and carboxylic acid. A caged compound released bioactive compounds more efficiently when embedded in liposome than when in methanol.

1. Introduction

Caged compounds[1] are biologically inert molecules that can release bioactive compounds upon photolysis. They are used to investigate and control the function of biological systems. The most popular caging groups (photolabile protecting groups) are an *o*-nitrobenzyl group and its derivatives. Although other photolabile protecting groups, such as desyl, *p*-hydroxyphenacyl[2] and coumarinylmethyl,[3] have been reported, *o*-nitrobenzyl-type caging groups have been widely used because of the ease with which derivatives can be synthesized. Some of them have electron-donating substituents on their aromatic rings to make their absorption maxima longer, and others have side chains as a linker to connect to another component.[4]

We have been studying the caged compounds of an L-leucyl-L-leucine methyl ester (LeuLeuOMe),[5] which has been reported to induce apoptosis in NK cells and macrophages.[6] Although an *o*-nitrobenzyl caged LeuLeuOMe has sufficient photochemical properties for caged compounds, some additional modifications should be made for practical biological application. For example, since bioassays are carried out in aqueous buffered solution, water-soluble compounds are desirable. For this purpose, we developed a sugar-modified caged compound.[5c] Liposome-forming caged compounds with a lipophilic substituent such as a steroid unit are also considered to be useful because macrophages are capable of endocytosis.[5d] This strategy can be applied not only to LeuLeuOMe with a free amino group but also to other bioactive compounds with other functional groups such as a carboxylic acid. In this paper, we describe detailed methods for the synthesis of steroid-substituted *o*-nitrobenzyl- and *o*-nitrophenethyl-type caged compounds and their photochemical reactivities.

2. Results and discussion

We designed two key precursors; an *o*-nitrobenzyl *p*-nitrophenyl carbonate for an amino caging group and an *o*-nitrobenzyl diazo compound for a carboxylic acid caging group. Pregnenolone acetate was used as a starting material for both types of caging groups. The ketone moiety was converted to an olefin by Wittig reaction, followed by methylation of a hydroxyl group to afford **3**. Nitrophenyl groups, a 5-hydroxy-2-nitrobenzaldehyde for an *o*-nitrobenzyl caging group and a 5-hydroxy-2-nitroacetophenone for an *o*-nitrophenethyl caging group, were introduced to the steroid skeleton after hydroboration of the olefin moiety and tosylation of an alcohol. After the reduction of a carbonyl moiety at a benzyl position to an alcohol, the mixed carbonate **8** was obtained by reacting **7** and *p*-nitrophenyl chloroformate. In this reaction, an alcohol-free chloroform should be used because ethyl *p*-nitrophenyl carbonate was obtained as a main product when we used commercially available chloroform containing ethylalcohol as a stabilizer. The mixed carbonate **8** is a useful caging group for bioactive molecules with amino groups. As a model case, a LeuLeuOMe was caged using **8**. An *o*-nitrophenethyl caged compound **9b** was obtained as a diastereomeric mixture (about 1:1). An *o*-nitrophenyl diazo compound **11** was obtained from **6a** via a tosylhydrazone **10**, the structure of which was confirmed by IR absorption at 2076 cm^{-1} due to the diazo group. The diazo compound **11** is considered to

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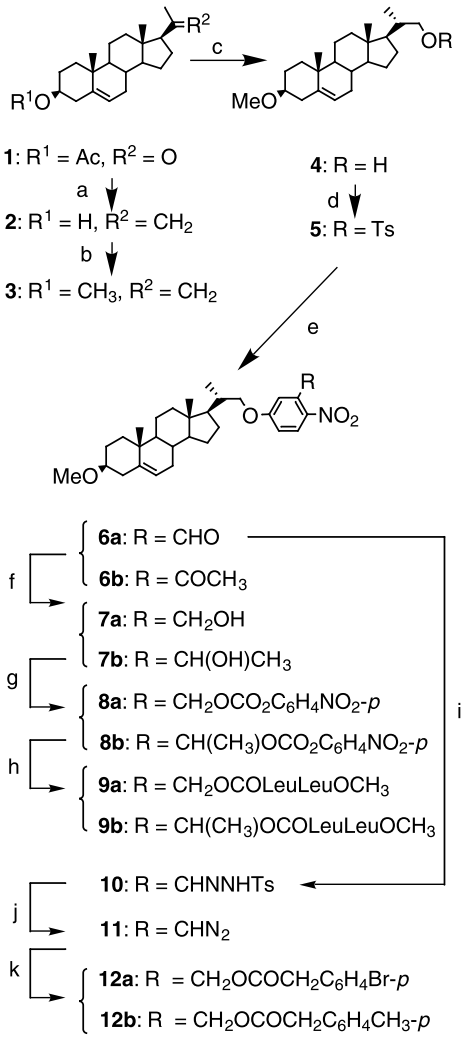

Scheme 1. Synthesis of steroid-substituted caged compounds. (a): (i) $Ph_3CH_3{}^+Br^-$, KO*t*Bu, THF, reflux 12 h, (ii) K_2CO_3, MeOH, 15 h, 95%; (b): KOH, MeI, DMSO, rt, 10 h, 88%; (c): (i) 9-BBN, THF, reflux, 13 h, (ii) 2N NaOH, 30% H_2O_2, rt, 16 h, 90%; (d): TsCl, Py, rt, 12 h, 96%; (e): 5-hydroxy-2-nitrobenzaldehyde, K_2CO_3, DMF, 110°C, 15 h, 77% for **6a**; 5-hydroxy-2-nitroacetophenone, K_2CO_3, DMF, 72% for **6b**; (f): $NaBH_4$, EtOH, rt, 20 min, 95% for **7a**, 91% for **7b**; (g): 4-nitrophenyl chloroformate, DMAP, $CHCl_3$, rt, 30 h, 61% for **8a**, 59% for **8b**; (h): LeuLeuOM·TFA, DMAP, $CHCl_3$, 4 d, 58% for **9a**, 42% for **9b**; (i): *p*-toluenesulfonyl hydrazide, EtOH, rt, overnight, 92%; (j): Et_3N, MeOH, rt, overnight, 84%; (k): *p*-bromophenylacetic acid, $CHCl_3$, reflux, overnight, 69% for **12a**; *p*-tolylacetic acid, $CHCl_3$, rt, 12% for **12b**.

be a useful caging group for carboxylic acids, such as the widely used *o*-nitrobenzyl diazomethanes.[7] Since the diazo compound **11** is a highly reactive precursor for caged compounds, we used it for the next synthetic step without extensive purification. We synthesized caged compounds of a *p*-bromophenyl and *p*-tolylacetic acid as model compounds (**12a** and **12b**).

The structures of the target molecules were confirmed by ^1H and ^{13}C NMR spectra, and solutions of **9a**, **9b**, **12a** and **12b** in methanol exhibited absorption maxima at 309, 304, 310 and 310 nm (ε 12400, 10300, 9940 and 6970) in their UV–VIS spectra, respectively.

Photolysis of the caged compounds in methanol was carried

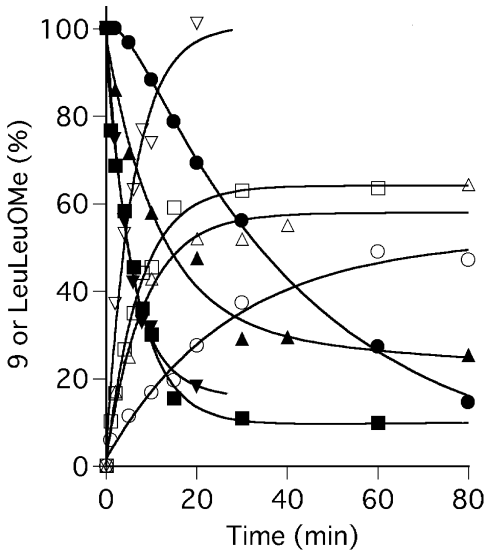

Figure 1. Time-dependent decrease in caged compounds and release of LeuLeuOMe. **9a** in MeOH: ●, **9b** in MeOH: ■, **9a** in liposome: ▲, **9b** in liposome: ▼, LeuLeuOMe from **9a** in MeOH: ○, LeuLeuOMe from **9b** in MeOH: □, LeuLeuOMe from **9a** in liposome: △, LeuLeuOMe from **9b** in liposome: ▽.

out with a Rayonet photochemical reactor (RPR 3500 Å×4). First, a photochemical reaction of **9a** was monitored by UV–VIS absorption spectra, which showed an isosbestic point for up to 40 min of irradiation.[5d] Since we intend to irradiate caged compounds for 5 min in a bioassay,[5c] the present photochemical properties are sufficient for our system.

Fig. 1 shows a time-dependent decrease in **9a** and **9b** and a concomitant increase in released LeuLeuOMe during photolysis. The amounts of caged LeuLeuOMe and released LeuLeuOMe during photolysis were estimated by HPLC analysis, and the latter was monitored by the intensity of fluorescence derived from adduct of fluorescamine[8] with released LeuLeuOMe. The *o*-nitrophenethyl caged compound **9b** showed effective photoinduced bond cleavage compared with the *o*-nitrobenzyl derivative **9a**, which is consistent with other caged compounds reported previously.[7]

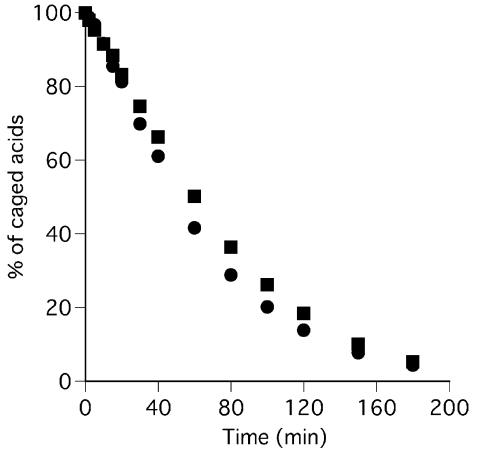

Figure 2. Time-dependent decrease in caged carboxylic acids. **12a**: ■, **12b**: ●.

Fig. 2 shows a time-dependent decrease in **12a** and **12b**. Unfortunately, the appearance of released acids could not be quantified because of overlapping of the signals due to other photoproducts in HPLC analysis. The efficiency of the photodegradation of starting materials was almost the same for **12a** and **12b** (Scheme 1).

Liposomes containing the caged LeuLeuOMe **9**, phosphatidyl choline, and dicetyl phosphate in phosphate-buffered saline (PBS) were prepared by the usual method. This combination of phospholipids was selected for liposome formation because liposomes consisting of these phospholipids have been reported to be effectively incorporated into macrophage via phagocytosis.[9] About 80% of the caged compound **9a** was considered to be incorporated in liposomes because only 20% of **9a** was detected in the supernatant after centrifugation of the liposome suspension. The caged compound **9a** embedded in liposomes is fairly stable; more than 95% of **9a** remained intact even after standing for 10 d in the dark (by HPLC analysis).

The photochemical reaction of **9a** and **9b** in liposome as well as the release of LeuLeuOMe are shown in Fig. 1 together with those in methanol. Interestingly, the rate of photocleavage for **9a** was faster in liposome than in methanol during the initial 60 min, although the intensity of UV light is considered to be reduced in the former case because of the scattering of light by phospholipids in liposome. The time-dependent release of LeuLeuOMe in the photoreaction of **9a** and **9b** shows that the reaction proceeded more efficiently in liposome than that in methanol. The present results indicates that caged compound **9a** and **9b** work well as effective precursors of the bioactive molecule even in the presence of phospholipids. The efficient photoreaction in liposome compared with that in methanol may be caused by the influence of the reaction field due to phospholipid or the desirable conformation of **9** for photoreaction in liposome.

3. Conclusion

In this paper, we reported a method for synthesizing a lipophilic caging group with a steroid skeleton that can convert a variety of bioactive compounds to their caged forms. For example, biomolecules with an amino group or a carboxyl group can be converted to caged compounds via *o*-nitrobenzyl *p*-nitrophenyl carbonate or a diazo compound, respectively. The resulting caged compound formed a liposome with phospholipids and underwent photoreaction more efficiently in liposome than in methanol.

4. Experimental

4.1. General remarks

Melting points were determined on a MEL-TEMP II (Mitamura Riken Kogyo) or a Yanaco micro melting point apparatus. All melting points were uncorrected. All solvents used in the reactions were purified by the reported methods. THF was purified by distillation from benzophenone ketyl before use. All reactions were carried out under argon atmosphere unless otherwise noted. Preparative gel permeation liquid chromatography was performed by LC-908 with JAIGEL 1H+2H columns (Japan Analytical Industry) with chloroform as solvent. Column chromatography was performed with Merck Kieselgel 60. ^{1}H NMR (270 MHz) and ^{13}C NMR (67.5 MHz) spectra were measured in $CDCl_3$ or CD_3OD with a JEOL JNM-GSX 270 spectrometer using tetramethylsilane as an external standard, or $CHCl_3$ or CH_3OH as an internal standard. EI mass spectral data were obtained on a HITACHI M-80 mass spectrometer. High resolution mass spectral data were obtained on a JEOL SX-102 mass spectrometer. Electronic spectra were recorded on a HITACHI U3210 UV/VIS spectrometer. Infrared spectra were recorded with a JASCO FT/IR 5000 spectrometer. Elemental analyses were performed by the Instrument Analysis Center of School of Pharmaceutical Sciences, Toho University. Photochemical reaction was carried out with a Rayonet Photochemial Chamber Reactor Model RPR-200 (RPR 3500 Å×4). Analytical HPLC was performed with a JASCO Crestpak C18 S or a MERCK LiChrosorb RP-18.

4.1.1. 20-Methyl-pregna-5,20-dien-3β-ol (2). A solution of methyltriphenylphosphonium bromide (16.09 g, 45.0 mmol) and potassium *t*-butoxide (5.07 g, 45.2 mmol) in THF (50 mL) was stirred for 50 min at rt under argon atmosphere. To this reaction mixture was added a solution of pregnenolone acetate (**1**; 5.18 g, 14.5 mmol) in THF (35 mL) at rt and the mixed solution was stirred for 2 h. The solution was warmed to reflux and stirred for additional 12 h. After cooled to rt, the reaction mixture was washed with water, 1N HCl, then aq. $NaHCO_3$. The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The residual solid was subsequently treated by K_2CO_3 (3.35 g, 24.2 mmol) in methanol (150 mL) for 15 h at rt. The reaction mixture was filtered through celite, and the filtrate was concentrated under reduced pressure. The residual solid was chromatographed (SiO_2/hexane–ethyl acetate=5:1) to afford **2** (4.31 g, 13.7 mmol, 94.7%). **2**: white crystals, mp 129.0–131.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.59 (s, 3H), 1.01 (s, 3H), 1.76 (s, 3H), 0.91–2.29 (m, 20H), 3.53 (m, 1H), 4.71 (br s, 1H), 4.85 (br s, 1H), 5.36 (d, J=5.4 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.8 (q), 19.5 (q), 21.2 (t), 24.3 (t), 24.7 (q), 25.5 (t), 31.6 (t), 31.9 (t), 32.2 (d), 36.6 (s), 37.3 (t), 38.7 (t), 42.2 (t), 43.1 (s), 50.3 (d), 56.5 (d), 57 (d), 71.7 (d), 110.6 (t), 121.5 (d), 140.6 (s), 145.5 (s). Anal. calcd for $C_{22}H_{34}\cdot H_2O$: C, 84.02; H, 10.90; found: C, 81.51; H, 10.66%.

4.1.2. 3β-Methoxy-20-methyl-pregna-5,20-dien (3). To a solution of **2** (335 mg, 1.13 mmol) in DMSO (30 mL) was added powdered potassium hydroxide (2.53 g, 45.0 mmol) and methyl iodide (0.70 mL, 11 mmol), and the solution was stirred for 10 h at rt. The reaction mixture was poured into ice-water and the aqueous layer was extracted with $CHCl_3$. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The crude mixture was separated by column chromatography (SiO_2/hexane–CH_2Cl_2=2:1) to give **3** (327 mg, 1.0 mmol, 88%) with recovery of **2** (17 mg, 0.054 mmol, 4.8%). **3**: white crystals, mp 96.0–97.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.59 (s,

3H), 1.01 (s, 3H), 1.76 (s, 3H), 1.04–2.38 (m, 20H), 3.07 (m, 1H), 3.35 (s, 3H), 4.71 (br s, 1H), 4.85 (br s, 1H), 5.36 (d, J=5.1 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.7 (q), 19.4 (q), 21.1 (t), 24.2 (t), 24.6 (q), 25.4 (t), 28.0 (t), 31.9 (t), 32.2 (d), 36.9 (s), 37.2 (t), 38.7 (t×2), 43.1 (s), 50.4 (d), 55.6 (q), 56.5 (d), 57.3 (d), 80.3 (d), 110.7 (t), 121.5 (d), 140.9 (s), 145.6 (s). Anal. calcd for $C_{23}H_{36}O$: C, 84.09; H, 11.04; found: C, 83.94; H, 11.19%.

4.1.3. 20α-Hydroxymethyl-3β-methoxy-5-pregnen (4). To a solution of **3** (1.40 g, 4.26 mmol) in THF (40 mL) was added a 0.5 M solution of 9-BBN in THF (30 mL, 15.0 mmol) at 0°C during 15 min under argon atmosphere. The reaction mixture was stirred at rt for 1 h, then warmed to reflux and stirred for additional 13 h. To the reaction mixture was added 2N NaOH (40 mL) and 30% H_2O_2 (40 mL) at 0°C, then the reaction mixture was stirred for 16 h at rt. After the aqueous layer was extracted with ether, the organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. Chromatographic separation (SiO_2/hexane–ethyl acetate=4:1) of the crude mixture afforded **4** (1.32 g, 3.82 mmol, 89.6%). **4**: white crystals, mp 153.0–154.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.71 (s, 3H), 1.00 (s, 3H), 1.05 (d, J=6.6 Hz, 3H), 0.89–2.42 (m, 21H), 3.06 (m, 1H), 3.35 (s, 3H), 3.35 (m, 1H), 3.64 (dd, J=10.5, 3.2 Hz, 1H), 5.36 (d, J=5.1 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.9 (q), 16.8 (q), 19.4 (q), 21.1 (t), 24.4 (t), 27.7 (t), 28.0 (t), 31.9 (d), 31.9 (t), 36.9 (s), 37.2 (t), 38.7 (t), 38.7 (d), 39.6 (t), 42.4 (s), 50.2 (d), 52.4 (d), 55.6 (q), 56.5 (d), 68.0 (t), 80.3 (d), 121.5 (d), 140.9 (s). Anal. calcd for $C_{23}H_{38}O_2$: C, 79.71; H, 11.05; found: C, 79.70; H, 11.31%.

4.1.4. 20α-(*p*-Toluenesulfonylmethyl)-3β-methoxy-5-pregnen (5). To a solution of **4** (64 mg, 0.18 mmol) in pyridine (5 mL) was added tosyl chloride (1.68 g, 8.80 mmol) at 0°C and the reaction mixture was stirred for 12 h at rt. The solution was poured into ice-cooled diluted HCl, and a white precipitate was filtered with suction. The precipitates was dissolved in CH_2Cl_2 and the solution was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The crude mixture was separated by gel permeation liquid chromatography to afford **5** (89 mg, 0.18 mmol, 96%). **5**: colorless syrup; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.64 (s, 3H), 0.99 (s, 3H), 0.99 (d, J=6.6 Hz, 3H), 0.87–1.95 (m, 19H), 2.15 (m, 1H), 2.38 (m, 1H), 2.45 (s, 3H), 3.06 (m, 1H), 3.35 (s, 3H), 3.39 (dd, J=9.3, 6.4 Hz, 1H), 3.97 (dd, J=9.3, 3.2 Hz, 1H), 5.34 (d, J=5.1 Hz, 1H), 7.34 (d, J=8.3 Hz, 2H), 7.78 (d, J=8.3 Hz, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.8 (q), 16.8 (q), 19.3 (q), 21.0 (t), 21.6 (q), 24.2 (t), 27.4 (t), 28.0 (t), 31.8 (t), 31.9 (d), 36.2 (d), 36.8 (s), 37.1 (t), 38.7 (t), 39.4 (t), 42.4 (s), 50.0 (d), 51.7 (d), 55.6 (q), 56.3 (d), 75.6 (t), 80.3 (d), 121.4 (d), 127.9 (d), 129.7 (d), 133.1 (s), 140.9 (s), 144.6 (s). Anal. calcd for $C_{30}H_{44}O_4 \cdot H_2O$: C, 69.46; H, 8.94; found: C, 69.79; H, 8.74%.

4.1.5. 20α-(3-Formyl-4-nitrophenoxymethyl)-3β-methoxy-5-pregnen (6a). A solution of **5** (746 mg, 1.49 mmol), 5-hydroxy-2-nitrobenzaldehyde (505 mg, 3.02 mmol), and K_2CO_3 (1.03 g, 7.46 mmol) in DMF (20 mL) was warmed to 110°C and stirred for 15 h under argon atmosphere. After cooled to rt, the solution was filtered through celite and the filtrate was concentrated under reduced pressure. To the residue was added CH_2Cl_2 and water, and the solution was stirred for a few hours. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The crude mixture was purified by column chromatography (SiO_2/CH_2Cl_2) to give **6a** (570 mg, 1.15 mmol, 77.2%). **6a**: pale green crystals, mp 182.0–183.0°C (decomp.); ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.75 (s, 3H), 1.01 (s, 3H), 1.15 (d, J=6.6 Hz, 3H), 0.88–2.16 (m, 20H), 2.37 (m, 1H), 3.05 (m, 1H), 3.36 (s, 3H), 3.84 (dd, J=8.9, 7.0 Hz, 1H), 4.04 (dd, J=8.9, 3.3 Hz, 1H), 5.36 (d, J=5.1 Hz, 1H), 7.13 (dd, J=9.0, 2.8 Hz, 1H), 7.30 (d, J=2.8 Hz, 1H), 8.12 (d, J=9.0 Hz, 1H), 10.49 (s, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.9 (q), 17.3 (q), 19.4 (q), 21.0 (t), 24.3 (t), 27.8 (t), 28.0 (t), 31.9 (t), 31.9 (d), 36.3 (d), 36.9 (s), 37.2 (t), 38.7 (t), 39.6 (t), 42.5 (s), 50.1 (d), 52.4 (d), 55.6 (q), 56.4 (d), 74.4 (t), 80.3 (d), 113.8 (d), 118.8 (d), 121.4 (d), 127.2 (d), 134.4 (s), 140.9 (s), 141.9 (s), 163.9 (s), 188.7 (d). Anal. calcd for $C_{30}H_{41}NO_5$: C, 72.70; H, 8.34; N, 2.83; found: C, 72.42; H, 8.53; N, 2.69%.

4.1.6. 20α-(3-Acetyl-4-nitrophenoxymethyl)-3β-methoxy-5-pregnen (6b). The procedure similar to **6a** but using a solution of **5** (473 mg, 0.95 mmol), 5-hydroxy-2-nitroacetophenone (205 mg, 1.13 mmol), and K_2CO_3 (653 mg, 4.73 mmol) in DMF (30 mL), gave **6b** (345 mg, 0.68 mmol, 71.6%). **6b**: yellow crystals mp 128.0–129.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.74 (s, 3H), 1.01 (s, 3H), 1.14 (d, J=6.9 Hz, 3H), 1.20–2.42 (m, 21H), 2.53 (s, 3H), 3.07 (m, 1H), 3.36 (s, 3H), 3.83 (m, 1H), 4.00 (m, 1H), 5.35 (d, J=4.3 Hz, 1H), 6.75 (d, J=2.6 Hz, 1H), 6.96 (dd, J=2.6, 9.2 Hz, 1H), 8.13 (d, J=9.2 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.8 (q), 17.2 (q), 19.2 (q), 20.9 (t), 24.2 (t), 27.6 (t), 27.8 (t), 30.3 (q), 31.7 (t), 31.8 (d), 36.2 (d), 36.7 (s), 37.0 (t), 38.5 (t), 39.4 (t), 42.4 (s), 45.0 (d), 52.3 (d), 55.4 (q), 56.3 (d), 74.2 (t), 80.2 (d), 112.2 (d), 115.0 (d), 121.3 (d), 126.9 (d), 137.6 (s), 140.7 (s), 141.1 (s), 164.2 (s), 200.2 (s). Anal. calcd for $C_{31}H_{43}NO_5$: C, 73.05; H, 8.50; N, 2.75; found: C, 73.07; H, 8.57; N, 2.80%.

4.1.7. 20α-(3-Hydroxymethyl-4-nitrophenoxymethyl)-3β-methoxy-5-pregnen (7a). To a solution of **6a** (39 mg, 0.079 mmol) in ethanol (5 mL) was added a solution of sodium borohydride (11 mg, 0.29 mmol) in ethanol (1 mL) at rt and the reaction mixture was stirred for 20 min. Water was added to the reaction mixture and the solution was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The residual solid was pure **7a** (37 mg, 0.074 mmol, 94.5%). **7a**: yellow crystals, mp 186.5–188.5°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.74 (s, 3H), 1.01 (s, 3H), 1.15 (d, J=6.6 Hz, 3H), 0.92–2.10 (m, 20H), 2.38 (m, 1H), 2.70 (br s, 1H), 3.07 (m, 1H), 3.36 (s, 3H), 3.82 (dd, J=9.0, 6.8 Hz, 1H), 4.02 (dd, J=9.0, 3.4 Hz, 1H), 4.99 (s, 2H), 5.36 (d, J=5.1 Hz, 1H), 6.87 (dd, J=9.2, 2.8 Hz, 1H), 7.20 (d, J=2.8 Hz, 1H), 8.17 (d, J=9.2 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.9 (q), 17.3 (q), 19.4 (q), 21.0 (t), 24.4 (t), 27.8 (t), 28.0 (t), 31.9 (t), 31.9 (d), 36.3 (d), 36.9 (s), 37.2 (t), 38.7 (t), 39.6 (t), 42.5 (s), 50.1 (d), 52. 5 (d), 55.6

(q), 56.5 (d), 63.1 (t), 73.9 (t), 80.3 (d), 113.5 (d), 114.8 (d), 121.5 (d), 128.1 (d), 140.0 (s), 140.2 (s), 140.9 (s), 164.2 (s). Anal. calcd for $C_{30}H_{43}NO_5$: C, 72.40; H, 8.71; N, 2.81; found: C, 72.12; H, 8.89; N, 2.67%.

4.1.8. 20α-[3-(1′-Hydroxyethyl)-4-nitrophenoxymethyl]-3β-methoxy-5-pregnen (7b). The procedure similar to **7a** but using a solution of **6b** (345 mg, 0.67 mmol) in ethanol (35 mL) and sodium borohydride (93 mg, 2.5 mmol) in ethanol (8 mL), gave **7b** (312 mg, 0.61 mmol, 91.0%). **7b**: yellow crystals mp 62.0–63.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.75 (s, 3H), 1.01 (s, 3H), 1.15 (d, J=6.6 Hz, 3H), 1.20–2.37 (m, 21H), 1.78 (d, J=6.3 Hz, 3H), 3.07 (m, 1H), 3.36 (s, 3H), 3.81 (m, 1H), 4.00 (m, 1H), 5.37 (d, J=3.6 Hz, 1H), 5.57 (m, 1H), 6.83 (dd, J=2.3, 8.9 Hz, 1H), 7.30 (d, J=2.3 Hz, 1H), 8.04 (d, J=8.9 Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.9 (q), 17.3 (q), 19.3 (q), 21.0 (t), 24.0 (q), {24.22, 24.27} (t), 27.6 (t), {27.7, 27.9} (t), 31.8 (t), 31.8 (d), 36.3 (d), 36.8 (s), 37.1 (t), 38.6 (t), 39.5 (t), 42.5 (s), 50.0 (d), 52.4 (d), 55.5 (q), {56.4, 56.5} (d), 65.7 (d), 73.4 (t), 80.3 (d), {112.1, 112.4} (d), {113.17, 113.24} (d), 121.38 (d), 127.44 (d), 140.0 (s), {140.7, 140.9} (s), 145.1 (s), {163.6, 163.8} (s). Anal. calcd for $C_{31}H_{45}NO_5$: C, 72.76; H, 8.86; N, 2.74; found: C, 72.82; H, 9.02; N, 2.82%.

4.1.9. 3β-Methoxy-20α-(4-nitro-3-*p*-nitrophenoxycarbonyloxymethylphenoxymethyl)-5-pregnen (8a). A solution of **7a** (98 mg, 0.20 mmol), 4-nitrophenyl chloroformate (46 mg, 0.23 mmol), and DMAP (40 mg, 0.33 mmol) in $CHCl_3$ (10 mL) was stirred at rt for 30 h under argon atmosphere. The reaction mixture was washed with 1N HCl, aq. $NaHCO_3$, then brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The residual solid was separated by gel permeation liquid chromatography to afford **8a** (80 mg, 0.12 mmol, 61.3%). **8a**: white crystals, mp 153.0–155.0°C (decomp.); ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.75 (s, 3H), 1.01 (s, 3H), 1.16 (d, J=6.6 Hz, 3H), 0.93–2.16 (m, 20H), 2.37 (m, 1H), 3.05 (m, 1H), 3.36 (s, 3H), 3.83 (dd, J=8.8, 7.1 Hz, 1H), 4.03 (dd, J=8.8, 3.2 Hz, 1H), 5.36 (d, J=4.6 Hz, 1H), 5.75 (s, 2H), 6.94 (dd, J=9.2, 2.5 Hz, 1H), 7.15 (d, J=2.5 Hz, 1H), 7.43 (d, J=9.0 Hz, 2H), 8.26 (d, J=9.2 Hz, 1H), 8.31 (d, J=9.0 Hz, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 11.9 (q), 17.3 (q), 19.4 (q), 21.0 (t), 24.3 (t), 27.8 (t), 28.0 (t), 31.9 (d), 31.9 (t), 36.3 (d), 36.9 (s), 37.2 (t), 38.6 (t), 39.6 (t), 42.5 (s), 50.1 (d), 52.4 (d), 55.6 (q), 56. 5 (d), 67.7 (t), 74.1 (t), 80.3 (d), 113.4 (d), 114.6 (d), 121.3 (d), 121.7 (d), 125.4 (d), 128.3 (d), 133.7 (s), 139.7 (s), 140.9 (s), 145.5 (s), 152.0 (s), 155.3 (s), 164.0 (s); High-resolution FAB-MS: observed m/z 663.3304. Calcd for $C_{37}H_{47}N_2O_9$ ($[M+H]^+$) 663.3282. Anal. calcd for $C_{37}H_{46}N_2O_9$: C, 67.05; H, 7.00; N, 4.23; found: C, 67.09; H, 6.99; N, 4.29%.

4.1.10. 3β-Methoxy-20α-[4-nitro-3-{1′-(*p*-nitrophenoxycarbonyloxy)ethyl}-phenoxymethyl]-5-pregnen (8b). The procedure for **8a** but using a solution of **7b** (162 mg, 0.32 mmol), 4-nitrophenyl chloroformate (75 mg, 0.37 mmol), and DMAP (65 mg, 0.53 mmol) in $CHCl_3$ (10 mL), gave **8b** (130 mg, 0.19 mmol, 59.4%). **8b**: yellow crystals, mp 76.0–77.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ {0.75, 0.77} (s, 3H), 1.02 (s, 3H), 1.16 (d, J=5.6 Hz, 3H), 1.25–2.46 (m, 21H), 1.78 (d, J=6.3 Hz, 3H), 3.07 (m, 1H), 3.36 (s, 3H), 3.81 (m, 1H), 4.00 (m, 1H), 4.36 (m, 1H), 5.37 (m, 1H), 6.55 (d, J=8.2 Hz, 1H), 6.90 (m, 1H), 7.36 (d, J=8.6 Hz, 2H), 8.12 (d, J=8.2 Hz, 1H), 8.26 (d, J=8.6 Hz, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ {11.7, 12.1} (q), 17.2 (q), {19.2, 19.4} (q), {20.8, 20.9} (t), 21.9 (q), {23.9, 24.2} (t), 26.6 (t), {27.7, 27.8} (t), 31.7 (d), 35.2 (t), 36.1 (d), {36.6, 37.0} (t), 38.4 (t), {39.3, 39.4} (t), 40.7 (s), {42.3, 42.7} (s), 45.7 (d), 49.9 (d), {55.5, 55.9} (q), 56.3 (d), 63.5 (d), 73.8 (t), 80.3 (d), {112.4, 112.5} (d), {113.3, 113.5} (d), 121.2 (d), 121.6 (d), 125.2 (d), 127.8 (d), 139.6 (s), 139.7 (s), 140.7 (s), 145.2 (s), 151.2 (s), 155.1 (s), {163.68, 163.73} (s). Anal. calcd for $C_{38}H_{48}N_2O_9$: C, 67.44; H, 7.15; N, 4.14; found: C, 67.73; H, 7.43; N, 4.01%.

4.1.11. 5-(3β-Methoxy-5-pregnen-20α-yl)methoxy-2-nitrophenylmethoxycarbonyl L-leucyl-L-leucine methyl ester (9a). A solution of **8a** (45 mg, 0.068 mmol), L-leucyl-L-leucine methyl ester trifluoroacetate (31 mg, 0.083 mmol), and DMAP (13 mg, 0.11 mmol) in $CHCl_3$ (5 mL) was stirred at rt for 4 d under argon atmosphere. The reaction mixture was washed with 1N HCl, aq. $NaHCO_3$, then brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The residual solid was separated by gel permeation liquid chromatography to afford **9a** (31 mg, 0.040 mmol, 58.4%) with recovery of **8a** (5.0 mg, 0.0075 mmol, 11%). **9a**: white crystals mp 79.0–81.0°C; ^{1}H NMR (270 MHz, CD_3OD) δ 0.78 (s, 3H), 1.02 (s, 3H), 1.16 (d, J=6.4 Hz, 3H), 0.85–2.16 (m, 38H), 2.36 (m, 1H), 3.06 (m, 1H), 3.34 (s, 3H), 3.69 (s, 3H), 3.91 (m, 1H), 4.01 (m, 1H), 4.22 (t, J=7.4 Hz, 1H), 4.46 (dd, J=5.4, 9.4 Hz, 1H), 5.36 (d, J=4.0 Hz, 1H), 5.49 (s, 2H), 6.99 (d, J=9.2 Hz, 1H), 7.14 (s, 1H), 8.18 (d, J=9.2 Hz, 1H); ^{13}C NMR (67.5 MHz, CD_3OD) δ 12.4 (q), 17.9 (q), 19.9 (q), 21.8 (q), 22.1 (q), 22.2 (t), 23.4 (q), 23.5 (q), 25.4 (t), 25.9 (d×2), 28.8 (t), 29.0 (t), 33.0 (t), 33.3 (d), 37.7 (d), 38.0 (s), 38.4 (t), 39.7 (t), 41.0 (t), 41.3 (t), 42.0 (t), 43.6 (s), 51.7 (d), 52.1 (d), 52.6 (q), 53.8 (d), 54.7 (d), 55.9 (q), 57.9 (d), 64.7 (t), 75.1 (t), 81.9 (d), 114.3 (d×2), 122.7 (d), 128.9 (d), 138.2 (s), 140.9 (s), 141.8 (s), 157.8 (s), 165.5 (s), 174.5 (s), 175.3 (s); UV λ_{max} (methanol) 309 nm (ε 12400). Anal. calcd for $C_{44}H_{67}N_3O_9$: C, 67.58; H, 8.64; N, 5.37; found: C, 67.30; H, 8.65; N, 5.34%.

4.1.12. 1-[{5-(3β-Methoxy-5-pregnen-20α-yl)methoxy-2-nitro}phenyl] ethoxy carbonyl L-leucyl-L-leucine methyl ester (9b). The procedure for **9a** but using a solution of **8b** (80 mg, 0.12 mmol), L-leucyl-L-leucine methyl ester trifluoroacetate (56 mg, 0.15 mmol), and DMAP (24 mg, 0.19 mmol) in $CHCl_3$ (10 mL), gave **9b** (40 mg, 0.05 mmol, 41.7%). **9b**: yellow crystals mp 69.0–71.0°C; ^{1}H NMR (270 MHz, CD_3OD) δ 0.77 (s, 3H), 0.94–2.19 (m, 38H), 1.02 (s, 3H), 1.14 (d, J=4.3 Hz, 3H), 1.63 (d, J=6.3 Hz, 3H), 2.37 (m, 1H), 3.08 (m, 1H), 3.39 (s, 3H), 3.74 (s, 3H), 3.94 (m, 1H), 4.12 (m, 1H), 4.35 (m, 1H), 4.69 (m, 1H), 5.20 (m, 1H), 5.35 (d, J=3.6 Hz, 1H), 6.38 (m, 1H), 6.90 (d, J=8.9 Hz, 1H), 7.02 (m, 1H), 8.26 (d, J=8.9 Hz, 1H); ^{13}C NMR (67.5 MHz, CD_3OD) δ {11.83, 11.87} (q), 17.3 (q), {19.3, 19.5} (q), 21.0 (t), {21.1, 21.4} (q), {21.7, 21.8} (q), {21.9, 22.0} (q), {22.2, 22.6} (q), {22.8, 23.1} (q), {24.0, 24.3} (t), {24.6, 24.7} (d), {24.8, 25.0} (d), 26.7 (t), {27.8, 27.9} (t), 31.8 (t), 31.8 (d), 36.3 (d), 36.8 (s), 37.1 (t), 38.5 (t), 39.5 (t), 39.6 (t), {40.9, 41.1} (t), 42.5 (s), {50.0, 50.9} (d), 51.0 (d), {52.1, 52.3} (d),

{52.4, 52.5} (q), 53.4 (d), {55.5, 56.0} (q), 56.4 (d), 63.2 (d), 73.8 (t), 80.5 (d), 113.0 (d), 115.6 (d), 121.6 (d), 126.1 (d), {127.5, 127.7} (s), 139.8 (s), 140.6 (s), 141.0 (s), 162.5 (s), 163.8 (s), 172.9 (s); UV λ_{max} (methanol) 304 nm (ε 10300). Anal. calcd for $C_{45}H_{69}N_3O_9 \cdot H_2O$: C, 66.39; H, 8.79; N, 5.16; found: C, 66.77; H, 8.72; N, 5.10%.

4.1.13. 3β-Methoxy-20α-(3-*p*-toluenesulfonylhydrazonomethyl-4-nitro-phenoxymethyl)-5-pregnen (10). A solution of 3β-methoxy-20α-(3-formyl-4-nitro-phenoxymethyl)-5-pregnen (**6a**: 391 mg, 0.789 mmol) and *p*-toluenesulfonyl hydrazide (240 mg, 1.29 mmol) in ethanol (100 mL) was stirred over night at rt. The solvent was evaporated under reduced pressure and the residual solid was purified by column chromatography to give **10** (483 mg, 0.728 mmol, 92.2%). **10**: yellow crystals mp 210.0–212.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.77 (s, 3H), 1.02 (s, 3H), 1.07–2.21 (m, 19H), 1.16 (d, 3H, *J*=6.5 Hz), 2.18 (s, 1H), 2.39 (s, 1H), 2.42 (s, 3H), 3.03–3.12 (m, 1H), 3.36 (s, 3H), 3.78–3.84 (m, 1H), 3.99–4.03 (m, 1H), 5.37 (d, 1H, *J*=4.6 Hz), 6.94 (dd, 1H, *J*=2.8, 9.1 Hz), 7.32 (d, 2H, *J*=8.1 Hz), 7.39 (d, 1H, *J*=2.8 Hz), 7.87 (d, 2H, *J*=8.1 Hz), 8.07 (d, 1H, *J*=9.1 Hz), 8.45 (s, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.0 (q), 17.4 (q), 19.4 (q), 21.1 (t), 21.7 (q), 24.4 (t), 27.9 (s), 28.0 (t), 31.90 (t), 31.94 (d), 36.4 (d), 36.9 (t), 37.2 (t), 38.7 (t), 39.6 (t), 42.6 (s), 50.1 (d), 52.5 (d), 55.6 (q), 56.5 (d), 74.1 (t), 80.3 (d), 113.2 (d), 116.4 (d), 121.5 (d), 127.6 (d), 128.0 (d), 129.8 (d), 131.0 (s), 125.3 (s), 140.5 (s), 140.9 (s), 143.2 (d), 144.5 (s), 163.4 (s). Anal. calcd for $C_{37}H_{49}N_3O_6S$: C, 66.94; H, 7.44; N, 6.33; found: C, 66.65; H, 7.46; N, 6.20%.

4.1.14. 20α-(3-Diazomethyl-4-nitro-phenoxymethyl)-3β-methoxy-5-pregnen (11). To a solution of **10** (204 mg, 0.31 mmol) in methanol (10 mL) was added a triethylamine (0.25 mL, 1.8 mmol) and stirred at rt over night. The yellow precipitates was filtered by suction to afford **11** (131 mg, 0.26 mmol, 84%). **11**: yellow crystals mp 140.0–142.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.74 (s, 3H), 0.95–2.21 (m, 20H), 1.01 (s, 3H), 1.14 (d, 3H, *J*=6.5 Hz), 2.40 (d, 1H, *J*=10.5 Hz), 3.07 (m, 1H), 3.36 (s, 3H), 3.77 (m, 1H), 3.97 (m, 1H), 5.37 (s, 1H), 6.44 (d, 1H, *J*=2.4 Hz), 6.58 (dd, 1H, *J*=2.4, 9.5 Hz), 6.69 (s, 1H), 8.22 (d, 1H, *J*=9.5 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.0 (q), 17.4 (q), 19.4 (q), 21.1 (t), 24.4 (t), 27.8 (t), 28.0 (t), 31.90 (t), 31.93 (d), 36.3 (d), 36.9 (s), 37.2 (t), 38.7 (t), 39.6 (t), 42.5 (s), 47.3 (d), 50.1 (d), 52.5 (d), 55.6 (q), 56.5 (d), 73.7 (t), 80.2 (d), 106.7 (d), 110.2 (d), 121.3 (d), 129.4 (d), 131.5 (s), 135.4 (s), 140.7 (s), 163.5 (s). IR (KBr disk) 2076 cm^{-1} ($-N_2$).

4.1.15. 20α-(3-*p*-Bromophenylmethylcarbonyloxymethyl-4-nitro-phenoxymethyl)-3β-methoxy-5-pregnen (12a). A solution of **11** (42.8 mg, 0.084 mmol) and *p*-bromophenylacetic acid (93.1 mg, 0.43 mmol) in $CHCl_3$ (2 mL) was warmed to reflux and stirred over night. The solution was washed with aq. $NaHCO_3$, dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The residue was purified by gel permeation liquid chromatography to give **12a** (40.1 mg, 0.058 mmol, 69%). **12a**: yellow crystals mp: 94.0–96.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.76 (s, 3H), 0.79–2.21 (m, 20H), 1.02 (s, 3H), 1.11 (d, 3H, *J*=7.0 Hz), 2.39 (d, 1H, *J*=12.7 Hz), 3.07 (m, 1H), 3.36 (s, 3H), 3.72 (s, 2H), 3.63–3.84 (m, 2H), 5.36 (d, 1H, *J*=3.3 Hz), 5.56 (s, 2H), 6.77 (d, 1H, *J*=2.4 Hz), 6.84 (dd, 1H, *J*=2.4, 8.9 Hz), 7.29 (d, 2H, *J*=8.4 Hz), 7.47 (d, 2H, *J*=8.4 Hz), 8.16 (d, 1H, *J*=8.9 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.0 (q), 17.4 (q), 19.4 (q), 21.1 (t), 24.4 (t), 27.8 (t), 28.0 (t), 31.8 (t), 31.9 (d), 36.3 (d), 36.9 (s), 37.2 (t), 38.7 (t), 39.6 (t), 40.8 (t), 42.6 (s), 50.1 (d), 52.4 (d), 55.6 (q), 56.5 (d), 63.8 (t), 73.8 (t), 80.3 (d), 113.3 (d), 113.7 (d), 121.1 (s), 121.4 (d), 128.0 (d), 131.1 (d), 131.8 (d), 132.6 (s), 135.2 (s), 139.7 (s), 140.9 (s), 163.8 (s), 170.2 (s). Anal. calcd for $C_{38}H_{48}BrNO_6$: C, 65.70; H, 6.96; N, 2.02; found: C, 65.97; H, 7.21; N, 1.99%. MS (70 eV): *m/z* 695 and 693 (M^+ for $C_{38}H_{48}{}^{79}BrNO_6$ and $C_{38}H_{48}{}^{81}BrNO_6$, respectively). UV/VIS (THF): λmax 310 nm (ε 9940).

4.1.16. 20α-(3-*p*-Methylphenylmethylcarbonyloxymethyl-4-nitro-phenoxymethyl)-3β-methoxy-5-pregnen (12b). The procedure for **12a** but using a solution of **11** (63.6 mg; crude products) and *p*-tolylacetic acid (95 mg, 0.63 mmol) in $CHCl_3$ (1 mL) at room temperature, gave **12b** (11.1 mg, 0.0176 mmol, 11.7% from **10**). **12b**: yellow crystals mp: 116.5–118.0°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.76 (s, 3H), 0.79–2.21 (m, 20H), 1.02 (s, 3H), 1.11 (d, 3H, *J*=6.6 Hz), 2.34 (s, 3H), 2.39 (m, 1H), 3.07 (m, 1H), 3.36 (s, 3H), 3.72 (s, 2H), 3.62–3.83 (m, 2H), 5.37 (d, 1H, *J*=3.3 Hz), 5.56 (s, 2H), 6.81 (d, 1H, *J*=2.4 Hz), 6.84 (dd, 1H, *J*=2.4, 8.9 Hz), 7.15 (d, 2H, *J*=7.9 Hz), 7.23 (d, 2H, *J*=7.9 Hz), 8.15 (d, 1H, *J*=8.9 Hz); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 12.1 (q), 17.4 (q), 19.5 (q), 21.1 (t), 21.2 (q), 24.4 (t), 27.9 (t), 28.1 (t), 31.9 (t), 32.0 (d), 36.4 (d), 36.9 (s), 37.2 (t), 38.7 (t), 39.7 (t), 41.1 (t), 42.6 (s), 50.2 (d), 52.4 (d), 55.6 (q), 56.5 (d), 63.5 (t), 73.7 (t), 80.3 (d), 113.2 (d), 113.4 (d), 121.3 (d), 127.8 (d), 129.1 (d), 129.3 (d), 130.4 (s), 135.4 (s), 136.7 (s), 139.6 (s), 140.8 (s), 163.6 (s), 170.7 (s). Anal. calcd for $C_{39}H_{51}NO_6$: C, 74.37; H, 8.16; N, 2.22; found: C, 74.07; H, 8.35; N, 2.12%. UV λ_{max} (THF) 310 nm (ε 6970).

4.2. Preparation of liposomes

Phosphatidylcholine (12.1 mg), dicetyl phosphate (4.5 mg), and the caged LeuLeuOMe **9a** (7.2 mg, 9.2 μmol) was dissolved in $CHCl_3$ (10 mL) in a round-bottomed flask. A thin film was formed on the interior of the flask by the gentle evaporation of the solvent under reduced pressure at 30°C. After the solvent was completely removed in vacuo, PBS (Phosphate Buffered Saline, 10 mL) was added to the flask. This mixture was sonicated for 5 min to afford a suspension of multilamellar vesicles.

4.3. Photolysis of the caged LeuLeuOMe in liposomes

The liposomes prepared above was divided into several portions and placed in test tube, the volume of each was 100 μL. After the sample was photolized by Rayonet Photochemimal Reactor (RPR 3500 Å×4), methanol (300 μL) was added to the solution. After the sample became clear, the amount of a remained starting material was estimated by HPLC. The amount of a released LeuLeuOMe was also estimated by HPLC after the photolized sample was treated with a solution of fluorescamine in dioxane.

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