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# Synthesis, Photoreactivity and Cytotoxic Activity of Caged Compounds of L-Leucyl-L-Leucine Methyl Ester, an Apoptosis Inducer

**Mitsuyo Odaka, Toshiaki Furuta, Yoshiro Kobayashi and Michiko Iwamura***

Department of Biomolecular Science, Faculty of Science, Toho University, Funabashi-shi, Chiba, Japan

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## ABSTRACT

**L-Leucyl-L-leucine methyl ester (Leu-Leu-OMe), an apoptosis inducer in natural killer cells and macrophages, was caged with *trans-o*-hydroxycinnamoyl (3a–d), *trans-o*-mercaptocinnamoyl (4) and *o*-nitrobenzyl derivatives (5a, b), and the photochemical reactivity of these derivatives in phosphate-buffered saline containing 1% dimethyl sulfoxide and their immunological properties were studied. All of the derivatives exhibited absorption at wavelengths longer than the UVB region. Although 3a–d and 4 were expected to isomerize to a *cis* isomer, which then cyclizes intramolecularly to give Leu-Leu-OMe and a coumarin derivative, cyclization efficiency was not satisfactory except for 3a. However, 3a itself caused necrosis (cell swelling) of U937 cells (a myeloid cell line). In contrast, 5a and b released Leu-Leu-OMe quickly and efficiently and did not affect U937 cells. Although irradiated 5b induced necrosis, irradiated 3a and 5a induced apoptosis in these cells, as evidenced by a decrease in cell size.**

## INTRODUCTION

L-leucyl-L-leucine methyl ester (Leu-Leu-OMe)† is reported to have cytotoxic activity against natural killer (NK) cells and to a lesser extent against macrophages by inducing apoptosis through the formation of a dipeptide condensation product by the action of dipeptidyl peptidase I (1). However, its application for immunological studies has been quite limited because the cytotoxic activity is suppressed in the presence of blood serum (2). If Leu-Leu-OMe is caged with a photolabile and noncytotoxic protecting group and applied to *in vivo* studies, irradiation would generate Leu-Leu-OMe, which is expected to react with neighboring NK cells and macrophages. Thus, the role of NK cells and macrophages in the immune response in the skin can be elucidated by irradiating locally administered caged Leu-Leu-OMe. We conducted this study as an attempt to develop Leu-Leu-OMe caged with the most appropriate photolabile amino protecting group and to determine the cytotoxic activity of these derivatives.

The use of photosensitive protecting groups has recently attracted great attention as a tool for the study of biological processes (3,4). Although there are numerous photosensitive protecting groups used for organic synthesis (5,6), only a limited number of them can be used as a caging group that is biologically inert and releases a biologically active molecule upon irradiation with UVA (320–400 nm) or UVB (290–320 nm) light. For the application to immunological assays, a caged group has to exhibit absorption at wavelengths longer than the UVB region, because UVB itself is known to cause immunosuppression. We chose three kinds of caged groups that satisfied the above requirement, namely (1-pyrenyl) methyloxycarbonyl (Pmoc), *trans-o*-hydroxycinnamoyl and *o*-nitrobenzyl derivatives. The peptide Leu-Leu-OMe was first caged by Pmoc, which we previously used as a photolabile and fluorescent protecting group for amino acids and peptides (7). Although the Pmoc–Leu-Leu-OMe **1** released Leu-Leu-OMe almost quantitatively upon irradiation at 340 nm in methanol, it was found to be unsuitable for immunological assays due to its extremely low solubility in phosphate-buffered saline (PBS) containing 1% dimethylsulfoxide (PBS–1% DMSO). However, because Pmoc is expected to become a unique caged group because of its high fluorescent character, an attempt to increase water solubility with a suitable substituent on the pyrene ring has been made.

*Trans-o*-hydroxycinnamoyl group is known to be effective as a photolabile protecting group for the hydroxyl group by releasing coumarin and a free OH compound (8,9). Mechanistic studies show that *trans* to *cis* isomerization takes place upon irradiation followed by the nucleophilic intramolecular cyclization in the dark. *o*-Nitrobenzyl derivatives are the most common caged groups for the phosphates such as cAMP or ATP, and their photolytic reactivity has been well established (4,10). In this report, we describe the synthesis, and the photochemical and immunological studies on Leu-Leu-OMe caged with *trans-o*-hydroxycinnamoyl, **2**, **3a–d**, a *trans-o*-mercaptocinnamoyl derivative, **4**, and *o*-nitrobenzyl-groups, **5a**, **b** (11).

*To whom correspondence should be addressed at: Department of Biomolecular Science, Faculty of Science, Toho University 2-2-1 Miyama, Funabashi-shi, Chiba 274, Japan. Fax: 81-474-75-1855; e-mail: michiko@toho-u.ac.jp.

†*Abbreviations:* DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FCS, fetal calf serum; fluorescamine, 4-phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione; GPC, gel-permeation chromatography; Leu-Leu-OMe, L-leucyl-L-leucine methyl ester; m.p., melting point; NK, natural killer; NMM, *N*-methylmorpholine; PBS, phosphate-buffered saline; PBS–1% DMSO, PBS containing 1% DMSO; Pmoc, (1-pyrenyl) methyloxycarbonyl; TCF, trichloromethyl chloroformate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UVA, 320–400 nm radiation; UVB, 290–320 nm radiation.

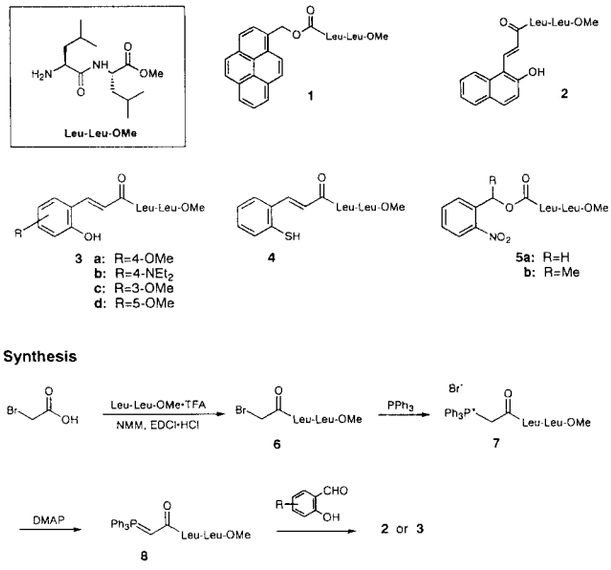


**Figure 1.**

## MATERIALS AND METHODS

*Synthesis: 2-bromoacetyl-L-leucyl-L-leucine methyl ester (6).* To a tetrahydrofuran (THF, 6 mL) solution of L-Leu-L-Leu-OMe (770 mg, 2 mmol), *N*-methylmorpholine (NMM, 440 μL, 4 mmol), bromoacetic acid (309 mg, 2.2 mmol) in THF (4 mL) and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 383 mg, 2 mmol) were added subsequently at −5°C and stirred for 2 h at the same temperature. The reaction mixture was stirred at room temperature overnight and the solvent was removed *in vacuo*. Saturated $NaHCO_3$ solution was added to the residue, which was then extracted with ethyl acetate. The extract was washed subsequently with 0.5 *M* tartaric acid, saturated $NaHCO_3$ solution, water, saturated NaCl and then dried over anhydrous $Na_2CO_3$. Removal of the solvent *in vacuo* gave light yellow oil (423 mg, 1.1 mmol) in 56% yield, which was used without further purification. $^1$H-NMR ($CDCl_3$/TMS): δ 7.03 (1H, d, J = 8 Hz, NH), 6.49 (1H, d, J = 8 Hz, NH), 4.43–4.62 (2H, m, two αCH), 4.03 (2H, s, $CH_2$), 3.75 (3H, s, $OCH_3$), 1.52–1.75 (6H, m, two $CH_2$ and two CH), 0.95–1.00 (12H, m, two $CH_2$), 0.95–1.00 (12H, m, four $CH_3$).

*2-(Triphenylphosphonio)acetyl L-leucyl-L-leucine methyl ester bromide (7).* To 2-bromoacetyl-L-leucyl-L-leucine methyl ester (**6**) (423 mg, 1.1 mmol) in benzene (5 mL), triphenylphosphine (289 mg, 1.1 mmol) was added and refluxed at 80°C for 17 h. After the removal of the solvent *in vacuo*, the residue was chromatographed on silica gel ($CH_2Cl_2$/MeOH = 20:1). Compound **7** was obtained as pale yellow oil (393 mg, 0.61 mmol, 56%). $^1$H-NMR ($CDCl_3$/TMS): δ 10.1 (1H, d, J = 7.5 Hz, NH), 7.60–7.80 (15H, m, three $C_6H_5$), 7.53 (1H, d, J = 7.5 Hz, NH), 5.11 (1H, dd, J = 13 and 6 Hz, $CH_2$), 4.87 (1H, dd, J = 13 and 6 Hz, $CH_2$), 4.44–4.54 (1H, m, αCH), 4.30 (1H, dd, J = 8 and 16 Hz, αCH), 3.65 (3H, s, $OCH_3$), 1.40–1.75 (6H, m, two $CH_2$ and two CH), 0.72–0.96 (12H, m, four $CH_3$).

*General procedure of the synthesis of* trans-*2-hydroxycinnamoyl derivatives of* L-LEUCYL-*L*-LEUCINE METHYL ESTER (**2**, **3A–D**). TO A CHLOROFORM SOLUTION (3 mL) OF 2-(TRIPHENYLPHOSPHONIO)ACETYL L-leucyl-L-leucine methyl ester bromide (**7**) (393 mg, 0.61 mmol), 4-dimethylaminopyridine (DMAP, 75 mg, 0.61 mmol) was added and stirred at room temperature for 24 h. After the removal of the solvent *in vacuo*, the crude 2-(triphenylphosphoranylidene)acetyl L-leucyl-L-leucine methyl ester (**8**) thus obtained was dissolved in 3 mL of benzene under Ar, reacted with an equimolar amount of a corresponding salicylaldehyde dissolved in benzene and stirred for 24 h in the dark at room temperature. The solvent was distilled off under the reduced pressure and the residue was chromatographed on silica gel ($CH_2Cl_2$/MeOH = 60:1) and then purified by gel-permeation chromatography (GPC) using two 20 mm × 500 mm JAIGEL-1H polystylene columns supplied by Japan Analytical Industry Co., Ltd., and $CHCl_3$ as an eluent.

Trans-*3-[1-(o-hydroxynaphthyl)acryloyl]-L-leucyl-L-leucine methyl ester (2).* Yield, 30%; melting point (m.p.) 98–101°C. $^1$H-NMR ($CDCl_3$/TMS): δ 8.28 (1H, d, J = 16 Hz, CH=), 8.03 (1H, d, J = 8 Hz, H3 of Naph), 7.74 (1H, d, J = 8 Hz, H4 of Naph), 7.70 (1H, d, J = 8 Hz, H5 of Naph), 7.45 (1H, d, J = 7 Hz, H7 of Naph), 7.33 (1H, d, J = 7 Hz, H6 of Naph), 7.19 (1H, d, J = 8 Hz, H8 of Naph), 6.79 (1H, d, J = 16 Hz, CH=), 6.65 (1H, d, J = 8 Hz, NH), 6.56 (1H, d, J = 8 Hz, NH), 4.70–4.80 (1H, m, αCH), 4.60–4.70 (1H, m, αCH), 3.75 (3H, s, $OCH_3$), 1.60–1.78 (6H, m, two $CH_2$ and two CH), 0.82–0.96 (12H, m, four $CH_3$); IR (KBr) 2958, 1657 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 354 nm (ε11 200), 319 (11 500), 234 (34 840), 202 (25 400).

Trans-*2-hydroxy-4-methoxycinnamoyl L-leucyl-L-leucine methyl ester (3a).* Yield, 28%; m.p. 81–86°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.69 (1H, d, J = 16 Hz, CH=), 7.39 (1H, d, J = 8.5 Hz, aromatic H6), 7.09 (1H, d, J = 8 Hz, NH), 6.80 (1H, d, J = 8 Hz, NH), 6.59 (1H, d, J = 16 Hz, CH=), 6.47 (1H, dd, J = 8.5 and 3 Hz, aromatic H5), 6.46 (1H, d, J = 3 Hz, aromatic H3), 4.46–4.56 (1H, m, αCH), 4.30–4.44 (1H, m, αCH), 3.75 (3H, s, $OCH_3$), 3.64 (3H, s, 4-$OCH_3$), 1.50–1.70 (6H, m, two $CH_2$ and two CH), 0.85–0.95 (12H, m, four $CH_3$); IR (KBr) 2928, 1649 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 327 nm (ε19 960), 292 (17 170), 239 (13 470); MS m/z 434 ($M^+$).

Trans-*4-diethylamino-2-hydroxycinnamoyl L-leucyl-L-leucine methyl ester (3b).* Yield, 26%; m.p. 115–116°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.95 (1H, d, J = 16 Hz, CH=), 7.27 (1H, d, J = 8 Hz, aromatic H6), 7.17 (1H, d, J = 8 Hz, aromatic H5), 6.42 (1H, d, J = 16 Hz, CH=), 6.23 (1H, s, aromatic H3), 6.21 (1H, d, J = 7 Hz, NH), 6.19 (1H, d, J = 7 Hz, NH), 4.72–4.81 (1H, m, αCH), 4.56–4.64 (1H, m, αCH), 3.70 (3H, s, $OCH_3$), 3.34 (4H, q, J = 7 Hz, two $CH_2$), 1.15–1.63 (6H, m, two $CH_2$ and two CH), 1.16 (6H, t, J = 7 Hz, two $CH_3$), 0.84–0.95 (12H, m, four $CH_3$); IR (KBr) 2924, 1657 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 366 nm (ε28 290), 261 (11 820), 207 (15 960).

Trans-*2-hydroxy-3-methoxycinnamoyl L-leucyl-L-leucine methyl ester (3c).* Yield, 51%; m.p. 78–81°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.81 (1H, d, J = 16 Hz, CH=), 7.11 (1H, d, J = 8 Hz, aromatic H5), 7.02 (1H, dd, J = 8 and 2 Hz, aromatic H6), 6.82 (1H, br, NH), 6.79 (1H, dd, J = 8 and 2 Hz, aromatic H4), 6.76 (1H, d, J = 16 Hz, CH=), 6.58 (1H, br, NH), 4.72–4.84 (1H, m, αCH), 4.54–4.66 (1H, m, αCH), 3.90 (3H, s, 3-$OCH_3$), 3.70 (3H, s, $OCH_3$), 1.51–1.80 (6H, m, two $CH_2$ and two CH), 0.81–0.99 (12H, m, four $CH_3$); IR (KBr) 3282, 2962, 1655 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 284 nm (ε21 770), 226 (23 050).

Trans-*2-hydroxy-5-methoxycinnamoyl L-leucyl-L-leucine methyl ester (3d).* Yield, 52%; m.p. 73–75°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.92 (1H, d, J = 16 Hz, CH=), 7.28 (1H, d, J = 8 Hz, NH), 6.89 (1H, d, J = 2.5 Hz, aromatic H6), 6.87 (1H, d, J = 9 Hz, aromatic H3), 6.81 (1H, d, J = 8 Hz, NH), 6.76 (1H, dd, J = 9 and 2.5 Hz, aromatic H4), 6.74 (1H, d, J = 16 Hz, CH=), 4.77–4.88 (1H, m, αCH), 4.56–4.66 (1H, m, αCH), 3.74 (3H, s, 5-$OCH_3$), 3.72 (3H, s, $OCH_3$), 1.50–1.76 (6H, m, two $CH_2$ and two CH), 0.82–0.96 (12H, m, four $CH_3$); IR (KBr) 3274, 2962, 1655 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 351 nm (ε8060), 277 (18 190), 246 (12 190).

Trans-*2-mercaptocinnamoyl L-leucyl-L-leucine methyl ester (4).* 4-Dimethylaminopyridine (100 mg, 0.81 mmol) was added to a chloroform solution (3 mL) of 2-(triphenylphosphoranylidene) acetyl L-leucyl-L-leucine methyl ester (**8**) (300 mg, 0.47 mmol) and stirred overnight at room temperature. After removal of the solvent *in vacuo*, the reaction mixture, which was dissoved in 3 mL of benzene, was reacted with *o*-mercaptobenzaldehyde (60 mg, 0.42 mmol) in benzene (2 mL) under Ar and stirred over night in the dark at room temperature. After removal of the solvent *in vacuo*, the reaction mixture was chromatographed on silica gel ($CH_2Cl_2$/MeOH = 40:1) and purified by GPC (JAIGEL-1H, $CHCl_3$). Light yellow solid (70 mg, 0.17 mmol) was obtained in 39% yield. M.p. 182–187°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.87 (1H, d, J = 16 Hz, CH=), 7.47–7.52 (1H, m, aromatic H), 7.33–7.38 (1H, m, aromatic H), 7.13–7.23 (3H, m, aromatic H and NH), 7.05 (1H, d, J = 7 Hz, NH), 6.37 (1H, d, J = 16 Hz, CH=), 4.52–4.74 (2H, m, two αCH), 3.73 (3H, s, $OCH_3$), 1.53–1.80 (6H, m, two $CH_2$ and two CH), 0.83–1.05 (12H, m, four $CH_3$); IR (KBr) 3272, 2962, 1655 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ 276 nm (ε16 340), 250 (15 200); MS m/z 421 ($M^+$).

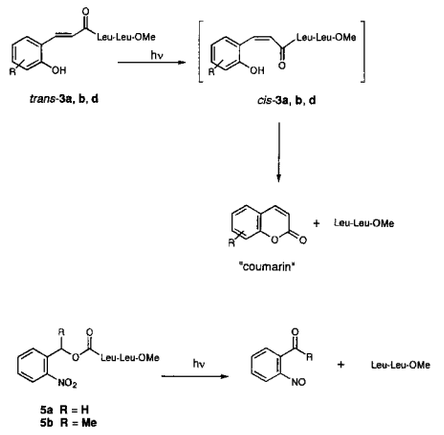


**Figure 2.** Photolysis of caged compounds **3** and **5**.

*2-Nitrobenzyloxycarbonyl L-leucyl-L-leucine methyl ester (**5a**).* To 2-nitrobenzyl alcohol (306 mg, 2 mmol) in THF (2 mL), trichloromethyl chloroformate (TCF, 480 mL, 4 mmol) in THF (10 mL) was added dropwise under Ar and refluxed for 4 h. Removal of the solvent *in vacuo* gave crude 2-nitrobenzylchloroformate as viscous oil, which was used without further purification. To 2-nitrobenzylchloroformate (203 mg, 1 mmol) in $CHCl_3$ (2 mL), Leu-Leu-OMe trifluoroacetic acid (TFA, 387 mg, 1 mmol) and triethylamine (560 mL, 4 mmol) were added and stirred in the dark at room temperature for 30 h. After the removal of the solvent *in vacuo,* the reaction mixture was chromatographed on silica gel ($CH_2Cl_2$/MeOH = 150:1) and then purified by GPC. White solid was obtained in 30% yield. M.p. 76–82°C. $^1$H-NMR ($CDCl_3$/TMS): δ8.10 (1H, broad d, J = 8 Hz, aromatic H3), 7.65 (1H, dt, J = 1.5 and 8 Hz, aromatic H6), 7.59 (1H, broad d, J = 8 Hz, aromatic H5), 7.47 (1H, dt, J = 1.5 and 8 Hz, aromatic H4), 6.35 (1H, d, J = 8 Hz, NH), 5.53 (1H, s, $CH_2$), 5.54 (1H, s, $CH_2$), 5.42 (1H, d, J = 8 Hz, NH), 4.56–4.65 (1H, m, αCH), 4.17–4.27 (1H, m, αCH), 3.72 (3H, s, $OCH_3$), 1.48–1.75 (6H, m, two $CH_2$ and two CH), 0.82–1.02 (12H, m, four $CH_3$); IR (KBr) 3298, 1657, 1528 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ 259 nm (ε3910).

*1-(2′-Nitrophenyl)ethoxylcarbonyl L-leucyl-L-leucine methyl ester (**5b**).* 1-(2′-Nitrophenyl) ethylchloroformate (990 mg, 4.5 mmol), which was made from 1-(2′-nitrophenyl)ethanol and TCF by the same procedure as that of 2-nitrobenzylchloroformate was dissolved in 4 mL of $CHCl_3$ and reacted with Leu-Leu-OMe TFA (1.73 g, 4.5 mmol) and triethylamine (2 mL, 14 mmol) for 43 h in the dark at room temperature. After the removal of the solvent *in vacuo,* the reaction mixture was chromatographed on silica gel ($CH_2Cl_2$/MeOH = 200:1 and then 10:1) and then purified by GPC. White solid was obtained in 12% yield. M.p. 100–103°C. $^1$H-NMR ($CDCl_3$/TMS): δ7.93 (1H, ddd, J = 8, 8 and 2 Hz, aromatic H3), 7.60–7.65 (2H, m, aromatic H5 and H6), 7.43 (1H, m, J = 4 Hz, aromatic H4), 6.18–6.34 (2H, m), 5.20–5.35 (1H, m), 4.45–4.63 (1H, m), 4.05–4.20 (1H, m), 3.70, 3.73 (3H, s), 1.45–1.69 (6H, m), 1.63, 1.61 (1H, d, J = 6 Hz), 0.76–0.97 (12H, m); IR (KBr) 3300, 1657, 1528 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ 257 nm (ε4280).

*Photolytic reactions.* A 5 mL PBS–1% DMSO solution of a caged Leu-Leu-OMe at 0.1–0.5 m*M* was in a Pyrex test tube (φ 10 mm) capped with a rubber septa was irradiated with a 100 W medium pressure mercury lamp for 1 h at room temperature. The photolyzed solution was kept in the dark at room temperature and subjected to analysis by HPLC on a C-18 column using 80% methanol. For the quantitative analysis of the released Leu-Leu-OMe, the following procedure was employed (12). To 10 mL of the photolyzed solution, which was diluted with 140 mL of PBS, 50 mL of 4-phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione (fluorescamine) solution (30 mg of fluorescamine in 100 mL of dioxane) was added with vigorous stirring. The solution was then subjected to analysis by HPLC using a mixture of methanol and sodium acetate buffer (50 m*M*, pH 4.4) (7:3, vol/vol) and detected with emission at 475 nm and excitation at 390 nm.

*Immunological assay.* The U937 cells (a human myeloid tumor cell line), Daudi cells (a B cell line) and YTN cells (an NK cell line) were, respectively, maintained with RPMI-1640 medium containing 10% fetal calf serum (FCS). Before assays, cells were washed with 20 m*M* PBS (pH 7.0) three times, and suspended in PBS containing 1% glucose at $1.5 \times 10^6$ cells/mL. The peptide Leu-Leu-OMe, the caged derivatives, and those irradiated were added to the suspension in which the final concentration of DMSO was adjusted to 1%, followed by incubation at room temperature for 20 min. After the addition of a four-fold volume of RPMI-1640 medium containing 10% FCS, cells were further incubated for 4 h. Changes in cell size were assessed with flow cytometry (FACScan, Becton Dickinson) using the paramaters of forward scatter and side scatter. Viability was determined by staining with trypan blue dye.

**Table 1.** Ultraviolet absorption of caged compounds in methanol

| Caged compound | $\epsilon_{340}$* | $\lambda_{max}(\epsilon)$† |
|---|---|---|
| **2** | 7900 | 354 (11 200) |
| **3a** | 13 600 | 327 (19 960) |
| **3b** | 17 100 | 366 (28 290) |
| **3c** | 5400 | 284 (21 770) |
| **3d** | 7200 | 351 (8060) |
| **5a** | 260 | 259 (3910) |
| **5b** | 310 | 257 (4280) |

*Molar absorptivity (dm$^3$ mol$^{-1}$ cm$^{-1}$) at 340 nm.
†Absorption maxima at longer wavelength with molar absorptivity in parentheses.

## RESULTS

### Synthesis and absorption spectra of caged-Leu-Leu-OMe

All of the *trans-o*-hydroxycinnamoyl caged Leu-Leu-OMe were obtained by the Wittig reaction of the corresponding arylaldehyde with 2-(triphenylphosphoranylidene)acetyl-Leu-Leu-OMe (Fig. 2). The large molar absorptivity at 340 nm (ε > 5000) of all the cinnamoyl derivatives, **2** and **3**, as shown in Table 1 satisfied the requirement for use in immunological assays. *Trans*-3-[1-(*o*-hydroxynaphthyl)acryloyl] caged Leu-Leu-OMe **2** was synthesized in an attempt to shift its absorption to the longer wavelength region than those of the cinnamoyl derivatives **3**, however, it turned out to have almost no effect. For instance, a cinnamoyl derivative, **3b**, showed an absorption maximum (366 nm) longer than that of **2** (354 nm) as shown in Table 1. *o*-Nitrobenzyl caged Leu-Leu-OMe, **5a**, **b**, were obtained by the reaction of *o*-nitrobenzylalcohol with TCF followed by the reaction with Leu-Leu-OMe TFA. Compounds **5a** and **b** showed broad absorption with maxima at 257 nm (ε 4280), which had a small absorptivity (ε 310) at 340 nm.

### Irradiation in PBS–1% DMSO

For immunological assays, the substrate has to be dissolved at 0.5 m*M* in PBS or PBS–1% DMSO: no more than that amount of DMSO can be used because of cytotoxcity. Although all the caged compounds, **2** and **3**, were soluble in acetonitrile up to *ca* $10^{-4}$ *M*, **2** and **3b** were almost insoluble in PBS–1%DMSO. Compounds **3a** and **d** gave milk-white solutions that became transparent upon irradiation; **3c** showed the solubility in between of the above two groups, namely **2**, **3b** and **3a**, **3b**. Irradiation was carried out on $10^{-4}$ *M* PBS–1% DMSO solutions of **2** and **3a–d** regardless of

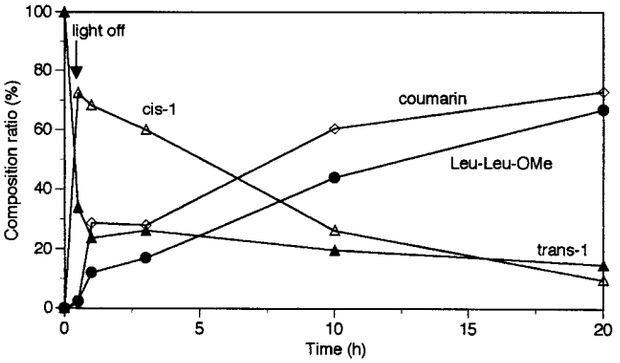


**Figure 3.** Formation of Leu-Leu-OMe and 7-methoxycoumarin from *cis*-**3a** upon irradiation of *trans*-**3a** in PBS–1% DMSO. The *trans*-isomer was irradiated for 1 min and the photolysate was kept in the dark.

their solubility in order to examine the photoreactivity under the conditions to be used in the immunological assay. A *cis*-isomer, Leu-Leu-OMe and a coumarin derivative are obtained as the major reaction products as detected either by HPLC or TLC (Fig. 3). In some cases, small HPLC peaks of minor by-products are detected, but structural determinations were not attempted. Table 2 shows the results on irradiation with a 100 W medium pressure mercury lamp through a Pyrex filter for 1 h. The yields of a *cis*-isomer and a coumarin derivative were calculated based on a *trans*-isomer consumed. We assume that the yield of Leu-Leu-OMe is similar to that of the coumarin derivative, according to the reaction scheme shown in Fig. 2. Compound **3a** gave the highest yield for the coumarin formation, and the consumed **3a** was converted to the *cis*-isomer and the coumarin derivative quantitatively. On the other hand, although **2** showed the highest conversion ratio of 84%, the coumarin derivative and the remaining *cis*-isomer amounted only to 41% of the substrate consumed. Compound **3c** was almost inert toward irradiation under these conditions. Similar but somewhat better results are obtained from the irradiation of these caged compounds in acetonitrile perhaps due to the better solubility. From the point of view of both solubility and photoreactivity, **3a** is most promising among **2** and **3a–d**. A more detailed study on **3a** reveals that the yields of both Leu-Leu-OMe and the coumarin derivative increased after irradiation at the expense of *cis*-**3a** as shown in Fig. 3. Although the amount of Leu-Leu-OMe in Fig. 3 is less than that of the coumarin probably because of the difference in the analytical methods employed, we assume they are essentially equal. The release of Leu-Leu-OMe from the *cis*-isomer is concluded to be rather slow, because the amount of Leu-Leu-OMe reached a plateau nearly after 20 h.

An attempt to improve the yield and rate of coumarin formation by replacing *o*-hydroxy by more nucleophilic mercapto group as in **4** was not successful: the irradiation of **4** in PBS–DMSO (3:1) or in acetonitrile gave more than 10 HPLC peaks of similar height whose structures were not determined.

*o*-Nitrobenzyl-caged Leu-Leu-OMe, **5a** and **b**, were more soluble than **3** in PBS–1% DMSO as well as in methanol or in acetonitrile, **5a** being somewhat more soluble in PBS–1% DMSO than **5b**. Upon irradiation of a 0.5 m*M* PBS–1% DMSO solution of **5a** with a 100 W medium pressure mercury lamp through a Pyrex filter, nearly 80% of **5a** was consumed within 40 min and the release of Leu-Leu-OMe was at most 40% based on the HPLC fluorometric analysis using fluorescamine. There is a possibility that the low yield of Leu-Leu-OMe might be due to the reaction with 2-nitrosobenzaldehyde formed by photolysis to give an imine that we have not identified (13). Irradiation of a 0.5 m*M* PBS–1% DMSO solution of **5b** for 20 min under the same conditions resulted in the consumption of *ca* 80% of **5b** along with the formation of Leu-Leu-OMe in 60% yield. Because any other products from **5b** were detected by HPLC and TLC, the yields of Leu-Leu-OMe may be essentially the same as that of the substrate consumed. The results are illustrated in Fig. 4. Comparison of Figs. 3 and 4 clearly shows that *o*-nitrobenzyl derivatives, **5a** and **b**, release Leu-Leu-OMe more quickly and efficiently than *o*-hydroxycinnamoyl derivative, **3a**.

**Table 2.** Photolysis of **2** and **3a–d** to *cis*-isomers and coumarin derivatives*

| Substrate | Conversion/% | *Cis*/%† | Coumarin/%† |
|---|---|---|---|
| **2** | 84 | 33 | 8 |
| **3a** | 76 | 64 | 36 |
| **3b** | 51 | ND | 15 |
| **3c** | 0 | — | — |
| **3d** | 53 | 5 | 10 |

*Compounds **2** and **3a–d** ($10^{-4}$ *M*) in PBS–1% DMSO were irradiated with a 100 W medium pressure Hg lamp through Pyrex filter for 1 h, followed by HPLC analysis.
†Based on the substrates consumed.

## Immunological assay

Although apoptosis is associated with chromosome condensation and DNA fragmentation, it is also characterized with a decrease in cell size (14). We, therefore, used a flow cytometer (FACScan) to assess apoptosis in U937 cells (a myeloid cell line), Daudi cells (a B cell line) and YTN cells (an NK cell line) induced by Leu-Leu-OMe. The dose-dependent induction of apoptosis by Leu-Leu-OMe was prominent for U937 cells, and almost absent for Daudi cells

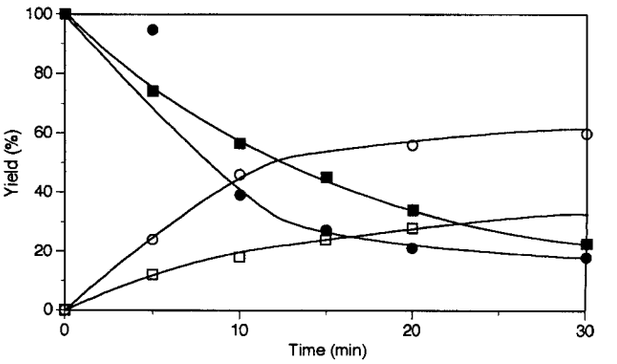


**Figure 4.** Formation of Leu-Leu-OMe from **5a** and **5b** in PBS–1% DMSO upon irradiation. (——■——) decrease of **5a**; (——□——) formation of Leu-Leu-OMe from **5a**; (——●——) decrease of **5b**; (——○——) formation of Leu-Leu-OMe from **5b**.

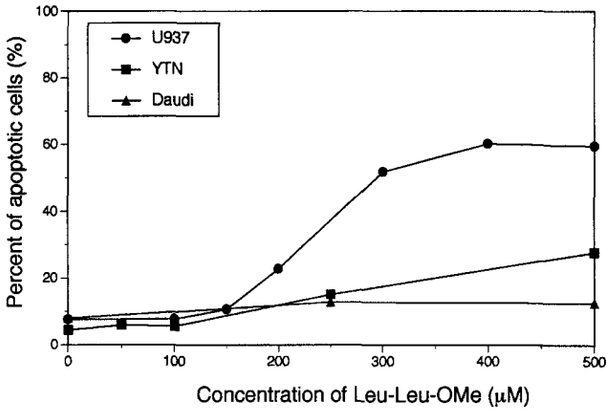


**Figure 5.** Induction of apoptosis by Leu-Leu-OMe. The U937, YTN and Daudi cells were treated with various concentrations of Leu-Leu-OMe according to the method described in the Materials and Methods. The results were expressed as % of cells with a decreased size (apoptotic cells).

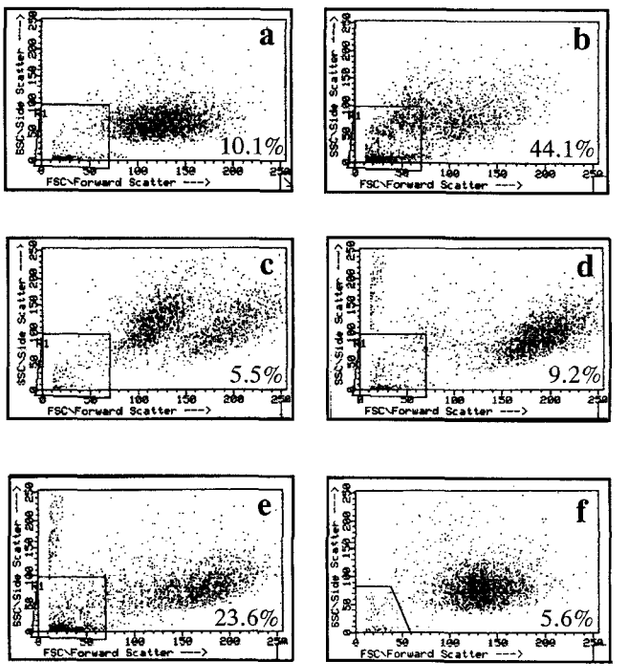


**Figure 6.** Induction of apoptosis of U937 cells by **3a**. The U937 cells were treated with Leu-Leu-OMe, **3a** and 7-methoxycoumarin (a product of photolysis of **3a**). (a) Control (1% DMSO), (b) Leu-Leu-OMe at 0.5 m*M*, (c) **3a** at 0.5 m*M*, (d) **3a** at 0.5 m*M* added to the cells immediately after irradiation for 0.5 h, (e) **3a** at 0.5 m*M* photolyzed for 0.5 h and set aside in the dark for 24 h before addition, (f) 7-methoxycoumarin at 0.5 m*M*. The results are expressed as % of cells with a reduced size, which are gated with lines in each panel.

(Fig. 5). Chromosome condensation was also observed in U937 cells treated with Leu-Leu-OMe under fluorescence microscopy using propidium iodide (data not shown).

Even though the release of Leu-Leu-OMe from **3a** is slow and not quantitative, we assumed that it could be used for immunological study if the photolyzed **3a**, but not **3a** and the coumarin derivative, has cytotoxic activity. When U937 cells were incubated with 0.5 m*M* of **3a**, which was photolyzed beforehand for 30 min and kept in the dark for 24 h (75% of **3a** decomposed and at most 0.375 m*M* of Leu-Leu-OMe released), FACScan showed the cell shrinkage (apoptosis) of as much as 50% of that caused by the addition of 0.5 m*M* of Leu-Leu-OMe (Fig. 6b and e). Although 7-methoxycoumarin, a by-product of photolysis, did not cause the change in the size of U937 cells (Fig. 6f), the unphotolyzed **3a** itself was found to induce cell swelling (necrosis) as shown in Fig. 6c, which was confirmed to be associated with the cell death by means of the trypan blue dye exclusion test. A similar phenomena was observed when **3a** was photolyzed for 30 min (at most 0.06 m*M* of Leu-Leu-OMe released) just before the addition to U937 cells (Fig. 6d). Thus, it was concluded that **3a** is not appropriate to our purpose.

In contrast, the addition of **5a** or **b** to U937 cells did not cause any effect; cytotoxic activity of Leu-Leu-OMe was completely deleted by the *o*-nitrobenzyl-caged groups (Fig. 7c and f). When the irradiated **5a**, 90% of which was photolyzed, was added to U937 cells, apoptosis was very efficient as detected by decrease in size of the cells (Fig. 7b and d; 39.8% *vs* 37.2%). Apoptosis was also observed to a lesser extent when **5a** was irradiated in the presence of U937 cells (Fig. 7e and d; 14.8% *vs* 39.8%). On the other hand, although addition of the irradiated **5b**, 70% of which was photolyzed, caused cell swelling rather than cell shrinkage (Fig. 7g and b; 11.7% *vs* 37.2%), irradiation of **5b** in the presence of U937 cells caused nearly no change in size of the cells (Fig. 7h).

## DISCUSSION

In this paper, we described the synthesis of new caged compounds of Leu-Leu-OMe, **2**, **3a–d** and **5a**, **b**, and examined their photochemical and immunological properties. Although all the *trans-o*-hydroxycinnamoyl derivatives, **2**, **3a–d**, exhibited absorption at wavelengths longer than 320 nm, their solubility in PBS–1% DMSO was not sufficient for immunological studies except for **3a** and **d**. They all gave a *cis*-isomer, Leu-Leu-OMe and a coumarin derivative upon irradiation either in an organic solvent such as acetonitrile or in PBS–1% DMSO; however, cyclization of the *cis*-isomer to release Leu-Leu-OMe was not so efficient. Although **3a** gave the best result for the release of Leu-Leu-OMe, **3a** itself induced necrosis (cell swelling) of these cells. In contrast, the *o*-nitrobenzyl derivatives, **5a** and **b**, satisfied the requirements for both absorption and solubility, released Leu-Leu-OMe faster and more efficiently than **2** and **3** did, and did not cause almost any change in the sizes of U937 cells.

To our knowledge, this is the first report of synthesis of caged compounds potentially applicable to immunological study. Caged compounds have been synthesized in order to analyze the very early phase of biochemical reaction. Therefore, such compounds are expected to release uncaged substance spontaneously on irradiation. In this sense, **2** and **3a–d** belong to a different category from such caged compounds: they can release an uncaged substance for a long span of time after irradiation. Although caged compounds with such properties might be used for immunological study, **3a** was proved not to be applicable to this study by inducing necrosis of the cells.

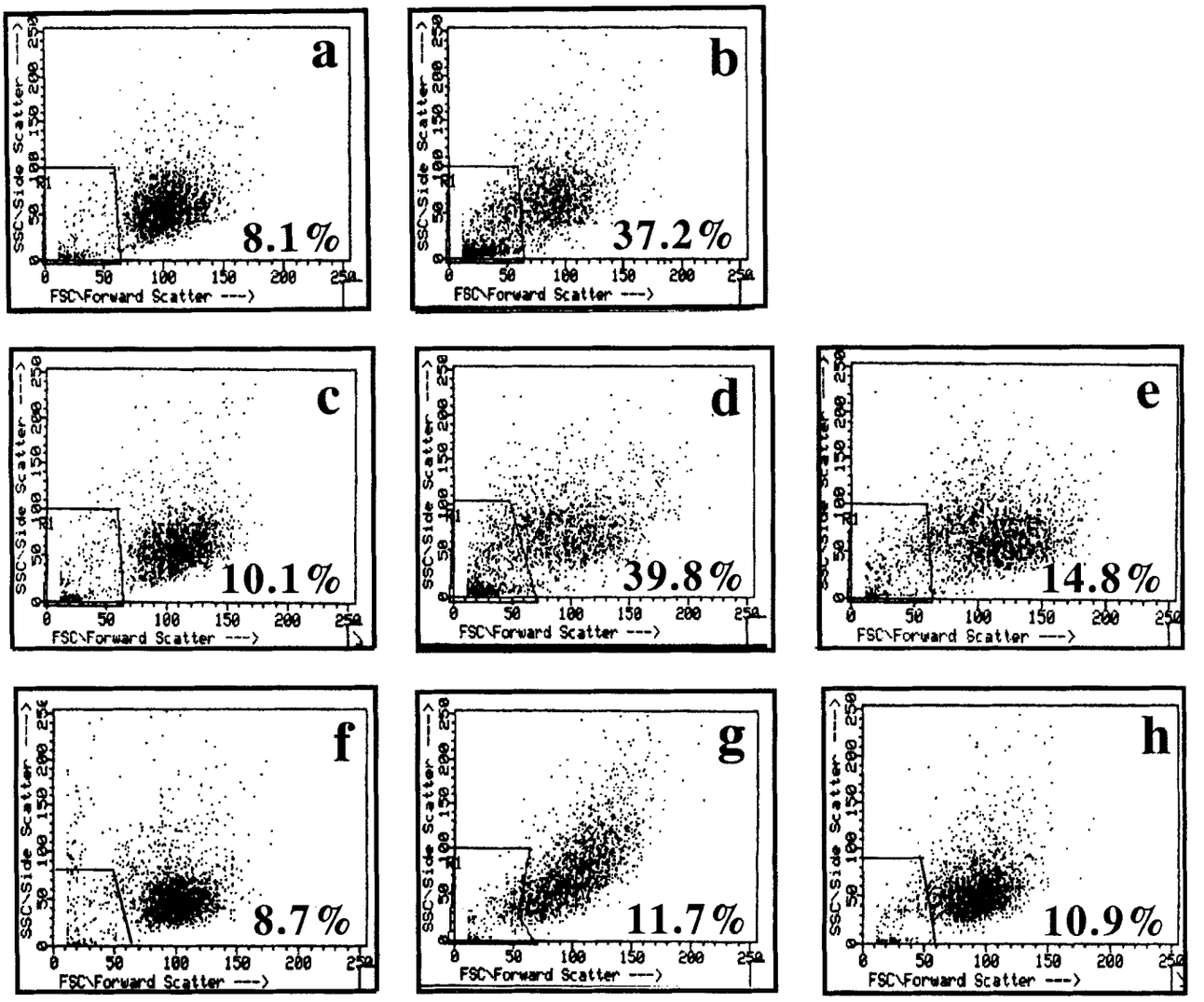


**Figure 7.** Induction of apoptosis of U937 cells by **5a** and **b**. The U937 cells were treated with Leu-Leu-OMe, **5a**, **b**. (a) Control (1% DMSO), (b) Leu-Leu-OMe at 0.5 m*M*, (c) **5a** at 0.5 m*M*, (d) **5a** at 0.5 m*M* photolyzed for 30 min, (e) **5a** at 0.5 m*M* photolyzed for 15 min in the presence of U937 cells, (f) **5b** at 0.5 m*M*, (g) **5b** at 0.5 m*M* photolyzed for 30 min, (h) **5b** at 0.5 m*M* photolyzed for 15 min in the presence of U937 cells. The results are expressed as % of cells with a reduced size, which are gated with lines in each panel.

The peptide Leu-Leu-OMe has been shown to be selectively toxic to NK cells, killer T cells and macrophages (3). We confirmed this by using a myeloid cell line (U937 cells), an NK cell line (YTN cells) and a B cell line (Daudi cells). The peptide Leu-Leu-OMe induced apoptosis in U937 cells and YTN cells, but not Daudi cells, as evidenced by a decrease in cell size (data not shown), being compatible with the report (2). Although photolyzed **3a** and **5a** caused apoptosis in U937 cells as expected, it is unexpected that, when **5a** was irradiated in the presence of cells, the efficacy for induction of apoptosis was reduced. It may be due to uptake of **5a** into the cells, although we do not know the reason. It is also noted that the cytotoxic activities of **5b** are quite different from those of **5a**: photolyzed **5b** induced necrosis and irradiation of **5b** in the presence of cells did not cause any change in the size of the cells.

Taken together, **5a** is a good candidate for a future study on the immunological role of macrophages and NK cells in the skin. Whether or not **5a** deteriorates the function of macrophages and NK cells more efficiently than cell survival awaits further experiments.

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