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Synthesis and Cytotoxic Studies of 5-O-(β–Glucopyranosyl)-2-nitrobenzyl Caged L-Leucyl-L-Leucine Methyl Ester with Increased Solubility in PBS Containing 1% DMSO

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We report the synthesis of a new type of a caged L-leucyl-L-leucine methyl ester, a sugar derivative 2, and study its photochemical and immunological properties. Compared with those of the previously reported *o*-nitrobenzyl caged compound, 1, and another new 4, 5-dimethoxy-2-nitorobenzyl caged compound 3, 2 was found to be almost 30 times more soluble in PBS containing 1% DMSO, and released leucyl-leucine methyl ester upon irradiation more efficiently than 1. Efficiency of induction of apoptosis of HL60 cells by irradiation of a solution containing 2 was only slightly lower than that by leucyl-leucine methyl ester itself.

L-Leucyl-L-leucine methyl ester (Leu-Leu-OMe) is known to cause the killing of cytotoxic lymphocytes and myeloid cells by inducing apoptosis through the formation of dipeptide condensation products, $(Leu\text{-}Leu)_n$-OMe, by action of dipeptidyl peptidase I (1). Although it gives a promising method to remove these cells from human peripheral blood mononuclear cells, its application for immunological studies has been quite limited because of the suppression of the cytotoxic activity in the presence of blood serum (2). We have been interested to synthesize caged compounds of Leu-Leu-OMe to elucidate the mechanism of the cytotoxic activity and to explore its application to the immunological study. Several photolabile derivatives of Leu-Leu-OMe have been tested to find that only *o*-nitrobenzyl derivative **1** was promising for further immunological study, the rests being either hardly soluble in PBS containing 1%DMSO or inducing necrosis of the cells (3,4). However, a clear-cut result on the immunological assay was not obtained as to the effect of the released Leu-Leu-OMe from the irradiated **1,** because the solubility in PBS containing 1% DMSO was not enough. While carboxyl or phosphoric groups are often used to modify the caged groups to increase the solubility in physiological salt solution (5,6), a saccharide is another candidate because it may be benign to biological systems. However, such an attempt has not been appeared. In this report, we describe the synthesis of 5-O-(β-glucopyranosyl)-2-nitrobenzyl caged Leu-Leu-OMe **2,** its increased solubility and superb characteristics as a caged Leu-Leu-OMe.

MATERIALS AND METHODS

Synthesis. 5-O-(β-Glucopyranosyl)-2-nitrobenzyl caged Leu-Leu-OMe **2** was synthesized from β-D-glucose following the scheme shown in Fig. 1. Detailed procedures of the synthesis will be published elsewhere. **2** was obtained from the reaction of leucyl-leucine methyl ester with the carbonic ester derivative **6,** followed by purification by HPLC using Merck Hibar RT 250-25 column with 60% methanol as an eluent. 4,5-Dimethoxy-2-nitrobenzyl caged Leu-Leu-OMe **3** was synthesized from 4,5-dimethoxy-2-nitorobenzaldehyde in a similar sequence through 4-nitrophenyl-4,5-dimethoxy-2-nitrobenzyl-carbonate.

Determination of solubility. Absorption spectra were taken on PBS solutions containing 1%DMSO saturated with the caged compounds, **1, 2** and **3.** From the absorptivity at several wavelengths, the averaged concentration (solubility) was determined.

Photochemical reaction. A 0.1 mM PBS/1%DMSO solution (5 mL) of **1, 2** or **3** in a Pyrex test tube (o.d.10 mm) capped with a rubber

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Abbreviations: L-Leucyl-L-Leucine methyl ester, Leu-Leu-OMe; dimethylsulfoxide, DMSO; fetal calf serum, FCS; phosphate-buffered saline, PBS.

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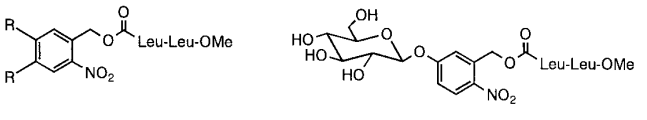


1: R = H
3: R = OMe

2

FIG. 1. Structures of caged derivatives of Leu-Leu-OMe.

septa was irradiated either with 100W medium pressure mercury lamp or using a Rayonet photochemical reactor with four RPR 3500Å lamps. The progress of the reaction was monitored by HPLC with a C-18 column using 60% MeOH at 254 nm. For the quantitative analysis of the released Leu-Leu-OMe, the following procedure was used.(7) To a 10 mL of the photolysed solution which was diluted with 140 mL of PBS, a 50 mL of fluorescamine (4-Phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione) solution (3.0 mg of fluorescamine in 10 mL of dioxane), was added with vigorous stirring. The solution was analyzed by a fluorometric HPLC using MeOH/50 mM sodium acetate buffer (pH4.1) = 7:3 (v/v) and detected by emission at 475 nm with excitation at 390 nm.

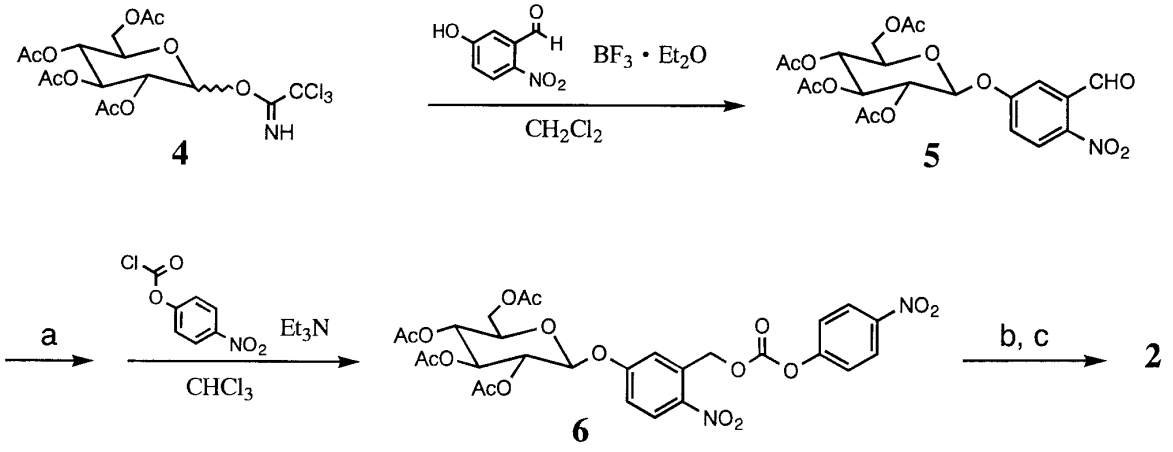
Apoptosis assessed by flow cytometry. HL60, a human myeloid tumor cell line, was maintained with RPMI 1640 medium containing 10% fetal calf serum (FCS). Before assay, cells were washed with 20 mM phosphate-buffered saline (PBS, pH 7.0) three times, and suspended in PBS containing 1% glucose at 1.5×10^6 cells/mL. Leu-Leu-OMe, the caged derivatives, and those irradiated were added to the suspension, followed by incubation at room temperature for 20 min. After the addition of a 4-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 parameters of forward scatter and side scatter.

RESULTS

For the synthesis of 5-O-(β-Glucopyranosyl)-2-nitrobenzyl caged Leu-Leu-OMe **2,** the methodology reported by R. R. Schmidt *et al.*, (8) to prepare the conjugate glycoside *via* C-1 imidate was used with β-D-glucose as a starting material (Figure 2). One of the key intermediates was the glycoside **5** which was obtained in 57% yield from the reaction of trichloroacetimidoyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside **4** with 5-hydroxy-2-nitrobenzaldehyde in the presence of boron trifluoride-diethyl ether in dichloromethane. The formyl group of **5** was reduced by sodium borohydride to afford the corresponding alcohol, which was then reacted with 4-nitrophenylchloroformate in the presence of triethylamine to give another key intermediate, the carbonic acid derivative **6** in 70% yield. **6** was reacted with Leu-Leu-OMe in the presence of triethylamine in dioxane to give the protected caged Leu-Leu-OMe in 51% yield, and then reacted with sodium methoxide in methanol to afford the deprotected caged Leu-Leu-OMe **2** in 70% yield.

Solubility of **2** in PBS containing 1%DMSO is about 240 μM, which is more than 30 times higher than those of **1** and **3** (Fig. 3). Although this solubility of **2** was not sufficient for the immunological assay, **2** gave a clear solution of over 500 μM when its DMSO stock solution was added to PBS to make up the 1% DMSO solution.

Upon irradiation of a solution of **2** at 100 μM in PBS containing 1%DMSO with a medium pressure mercury lamp through a Pyrex filter, nearly 80% of **2** was consumed within 40 min, a result similar to the irradiation of **1** as shown in Figure 4. The quantum yield for the decrease of **2** at 350 nm was determined to be 0.044 using potassium ferrioxalate as a chemical actinometer. The release of Leu-Leu-OMe from the irradiated **2** was at most 75% and more efficient than that from **1.** Thus, by introduction of a sugar group to 2-nitrobenzyl



Reagents: a) $NaBH_4$, EtOH, THF b) Leu-Leu-OMe TFA, Et_3N, dioxane c) NaOMe, MeOH

FIG. 2. Synthesis of 5-O-(β-glucopyranosyl)-2-nitrobenzyl caged Leu-Leu-OMe 2.

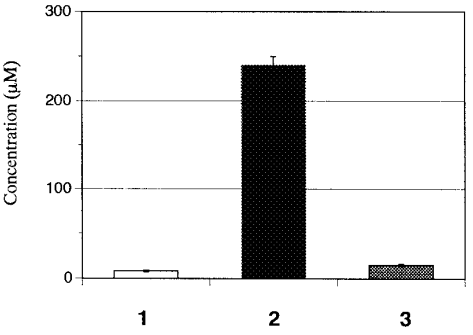


FIG. 3. Solubility of caged derivatives of Leu-Leu-OMe in PBS containing 1%DMSO. The solubilities were determined from the UV absorptivity of the saturated solution according to the method described under Materials and Methods.

group, **2** was formed to increase solubility in PBS, efficient photolytic reactivity as well as efficient release of Leu-Leu-OMe.

Previously, we showed that apoptosis was induced with Leu-Leu-OMe most prominently in U937 cells (a human myeloid cell line) among the three cell lines examined, others being Daudi cells (a B cell line) and YTN cells (an NK cell line) (4). HL60 cells (a human myeloid cell line) were used in this study because they were found to be more sensitive to Leu-Leu-OMe than U937 cells: the apoptosis of HL60 cells were induced at 100 μM of Leu-Leu-OMe up to the same extent as that at 500 μM (4,9). The addition of **2** at 500 μM to HL60 cells did not cause any effect, while the addition of photolysed **2** induced efficient apoptosis, which was reflected by a decrease in size of the cells observed by flow cytometry. Although data are not shown, Leu-Leu-OMe induced chromatin condensation, another marker of apoptosis. Either **1** or **2** at 500 μM in the presence of HL60 cells was irradiated in a Rayonet photochemical reactor with four RPR 3500 Å lamps for 5 min, based on the observation that irradiation of HL60 cells for less than 10 min did not cause UV-induced apoptosis. Figure 5 shows the ratios of cells with a reduced size before and after irradiation along with the result of the addition of Leu-Leu-OMe to HL60 cells. Irradiation of **2** in the presence of HL60 cells (photolytic consumption of **2:** less than 15%) induced apoptosis nearly similarly well as that induced by Leu-Leu-OMe itself (57.7% *vs* 67.6%). On the other hand, apoptosis induced by **1** (26.3% and 28.7%, before and after irradiation, respectively) was as little as those induced by the control, *i.e.* without caged Leu-Leu-OMe, (17.8% and 21.3%).

DISCUSSION

In this report, we described the synthesis of 5-O-(β-glucopyranosyl)-2-nitrobenzyl caged Leu-Leu-OMe **2,** its increased solubility and superb characteristics as a caged Leu-Leu-OMe. Of note was that irradiation of **2** in the presence of HL60 cells induced apoptosis nearly similarly well as that induced by Leu-Leu-OMe itself at 500 μM, although the amount of the released Leu-Leu0OMe was estimated to be less than 75 μM. Additionally, **2** has fairly large absorptivity at UVA region (ϵ_{350} = 1275 L mol^{-1}cm^{-1}) where the light can be penetrated into the skin without immunosupression. From these results we plan to conduct *in vivo* studies on the roles of macrophages and NK cells in the immune response in the skin by irradiating locally administered **2.**

2 improved solubility in PBS containing 1% DMSO more than 30 times better than other 2-nitrobenzyl caged Leu-Leu-OMe, **1** and **3.** Especially important to mention is that **2** gives a clear 500 μM solution when its DMSO stock solution is added to PBS to make up 1%DMSO solution, which is requisite for our immunological study. On the other hand, **3,** supposed to be more polar than **1** due to two methoxy groups, did not improve the solubility as had been expected.

2 was synthesized via 9 steps with moderate to good yields from β-D-glucose. The carbonic ester derivative **5** was the key intermediate to introduce the carbamate bond between the caged group and Leu-Leu-OMe: any other methods such as the reaction of the 2-nitrobenzyl alcohol derivative, Leu-Leu-OMe and triphosgene (bis-(trichloromethyl)-carbonate) was not successful. This synthetic procedure will be applicable to synthesis of 2-nitrobenzyl caged peptides modified with various kinds of sugar groups. We believe that some of them may be taken up by phagocytic cells via lectin-like molecules. Mature macrophages express a 162-kDa mannose receptor which binds mannose- and fucose-BSA with high affinity (10) and plays an important role in phagocytosis of bacteria (11). On the other hand, neutrophils, which lack the mannose receptor, are capable of ingesting bacteria perhaps via β-glucan receptor (12). Therefore Leu-Leu-OMe caged with glucose or mannose derivatives may be taken up by macrophages

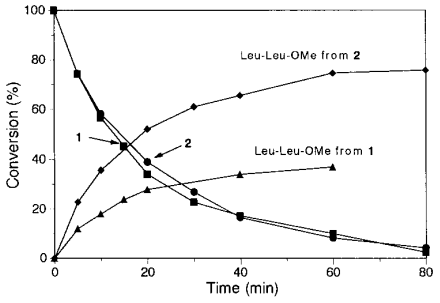


FIG. 4. Formation of Leu-Leu-OMe upon irradiation of 1 and 2 in PBS containing 1% DMSO. The solution was irradiated and the percentage of conversion was determined according to the method described under Materials and Methods.

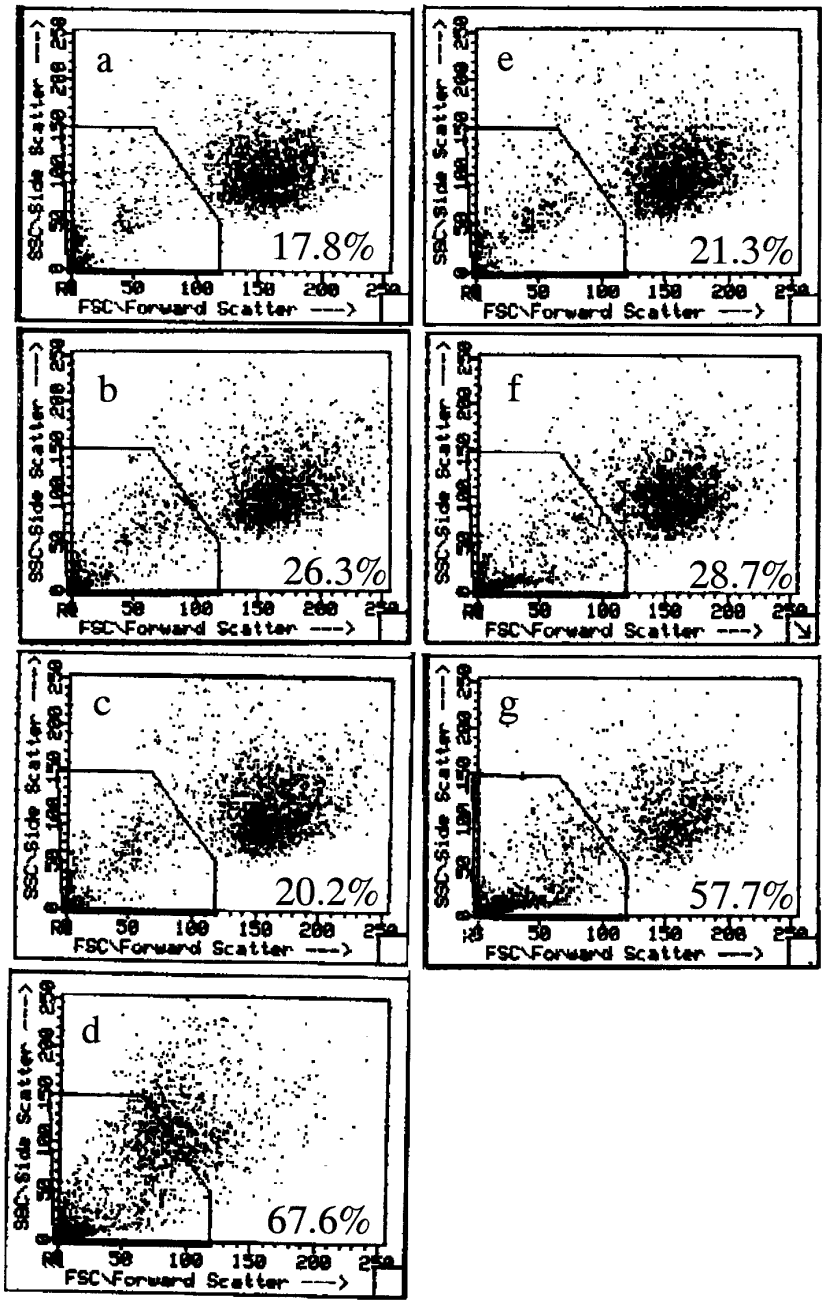


FIG. 5. Induction of apoptosis in HL60 cells. HL60 cells were treated with Leu-Leu-OMe and its derivative (**1** and **2**) according to the method described under Materials and Methods. Before irradiation; (a) control (PBS-1%DMSO), (b) **1** at 500 μM, (c) **2** at 500 μM, (d) Leu-Leu-OMe at 500 μM. After irradiation for 5 min; (e) control, (f) **1** 500 μM, (g) **2** at 500 μM. The results were shown as the percentage of cells with a reduced size, which were squarely delineated with lines in each panel.

or neutrophils through these receptors, synthesis of which are now in progress in our laboratory.

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