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Design, Synthesis, Photochemical Properties and Cytotoxic Activities of Water-Soluble Caged L-Leucyl-L-leucine Methyl Esters that Control Apoptosis of Immune Cells

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Abstract—L-Leucyl-L-leucine methyl esters (LeuLeuOMe) is a lysosomotropic agent that induces apoptosis of certain immune cells. Glucose-carrying 2-nitrobenzyl (2-NB) and 2-nitrophenethyl (2-NPE) caged LeuLeuOMe, **1a** and **b**, were synthesized and their photochemical and immunological properties were studied. Caged glycine methyl esters (GlyOMe), **2a**,**b**, were also prepared to examine the cytotoxic activity of the photolytic byproducts from **1a**,**b**. All the caged compounds were soluble in PBS containing 1% DMSO more than 400 μM, and efficiently released the substrates upon irradiation at 350 nm. While both **1a** and **1b** were not toxic to HL60 cells, **1b** released LeuLeuOMe more quickly and induced apoptosis of HL60 cells far more efficiently than **1a**. Although GlyOMe was not cytotoxic, **2a** and **b** were slightly toxic before and after irradiation almost to the similar extent. Therefore, the photolytic products from the caging groups appear to be not toxic to the cells, and the apoptosis inducing activity of **1a** and **b** may be for the most part due to LeuLeuOMe.

Introduction

Caged compounds are biologically inert substrates due to modification with caging (photoremovable protecting) groups that release their substrates in high concentration upon irradiation at longer than 350 nm. Since they can release the substrates at any particular time and place one desires, they are being increasingly used for neurobiological studies.[1] However, there have been few attempts to use these characteristics in immunological or medicinal chemistry studies.[2] We have been interested in the design and synthesis of new caging groups, and in applying them as tools for studying the two areas mentioned above.

L-Leucyl-L-leucine methyl ester (LeuLeuOMe) is known to induce apoptosis of NK cells and macrophages through the formation of dipeptide condensation products, $(LeuLeu)_n$–OMe, by the action of dipeptidyl peptidase I.[3] Recently, it was demonstrated that LeuLeuOMe induces apoptosis in HL60 cells through the activation of a caspase 3-like protease.[4] LeuLeuOMe has attracted considerable attention due to its selective cytotoxic activity, and has been expected to be an alternative immunosuppressive reagent to cyclosporin A, which has serious side effects, such as renal toxicity.[5] We designed and synthesized caged compounds of LeuLeuOMe with the intent to elucidate the mechanism of its cytotoxic activity and to develop it as a new type of prodrug to be used as an immunosuppressive reagent. It would also be applicable to chronic inflammation, such as rheumatoid arthritis, which often becomes serious due to the overexpression of macrophages. To use caged LeuLeuOMe in an immunological study, the following requirements must be satisfied: (1) sufficient solubility (~500 μM) and stability in phosphate-buffered saline (PBS) containing 1% DMSO, (2) efficient release of LeuLeuOMe upon irradiation at a wavelength longer than 350 nm, and (3) absence of cytotoxic activity.

We synthesized LeuLeuOMe caged with newly developed 1-pyrenylmethyl,[6] and 2-hydroxycinnamoyl and conventional 2-nitrobenzyls to examine their solubility, stability, photochemical reactivity and cytotoxic activity.[7] Among them, only 2-nitrobenzyl (NB)- and

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2-nitrophenethyl (NPE)-caged LeuLeuOMes were not cytotoxic to U937 cells. However, their ability to induce apoptosis was unclear due to their very low solubility in PBS containing 1% DMSO. Therefore, to increase their solubility in water, we designed and synthesized Leu-LeuOMe caged with 5-*O*-(β-D-glucopyranosyl)-2-NB (**1a**). It showed adequate solubility and efficiently induced the apoptosis of HL60 cells upon irradiation at 350 nm.[8] Since 2-nitrosobenzaldehyde, a known by-product of an irradiated NB-caged compound, has been reported to be toxic to cells,[9] the cytotoxicity of the by-products from **1a** should be examined. To examine the cytotoxic activities of the photolytic byproducts of our system, it would be useful to have a biologically inactive substrate caged with the same glucose-carrying 2-NB. For that purpose, we chose glycine methyl ester (GlyOMe), which by itself has been shown to be not cytotoxic to HL60 cells.

We report here the synthesis, photochemistry and cytotoxic activity of LeuLeuOMe and GlyOMe caged with glucose-carrying 2-NB and 2-NPE groups (**1** and **2**; Fig. 1). We also report the synthesis of glucose-carrying 5-methoxy-2-nitorophenethyl-caged LeuLeuOMe (**3**) which may undergo photolysis at a longer wavelength.

Results and Discussion

Chemistry

Synthesis. 5-*O*-(β-D-Glucopyranosyl)-2-NB- and-2-NPE-caged compounds **1a**, **b** and **2a**, **b** were synthesized via five steps from 5-*O*-(β-D-glucopyranosyl) trichloroacetimidate using 5-hydroxy-2-nitrobenzaldehyde **4a**, or 5-hydroxy-2-nitroacetophenone **4b** as a glycosyl acceptor (Scheme 1). 4-*O*-(β-D-Glucopyranosyl)-5-methoxy-2-nitrophenethyl-caged LeuLeuOMe **3** was similarly synthesized from 4-hydroxy-5-methoxy-2-nitroacetophenone **5** which was obtained from acetovanillone via three steps. Glycosylation gave the β-anomer stereoselectively. Total yields after five steps were between 10 and 15%. Compound **4b** was obtained by nitration of 3-hydroxyacetophenone with cuprous nitrate in a mixture of acetic acid and acetic anhydride.[10] In spite of the low yield, usually less than 20%, and the formation of 4- and 6-nitro derivatives, we used this procedure because no other nitration procedures worked well. The stereochemistry at the glucose anomeric position was β throughout the reactions, as evidenced by ^{1}H NMR coupling constants. Reduction of the benzoyl of **6b** gave a roughly 1:1 mixture of diastereomers, which were used for further study without separation.

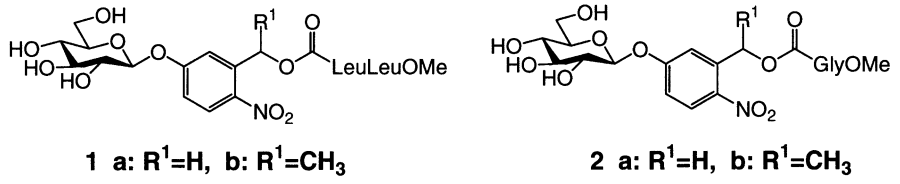
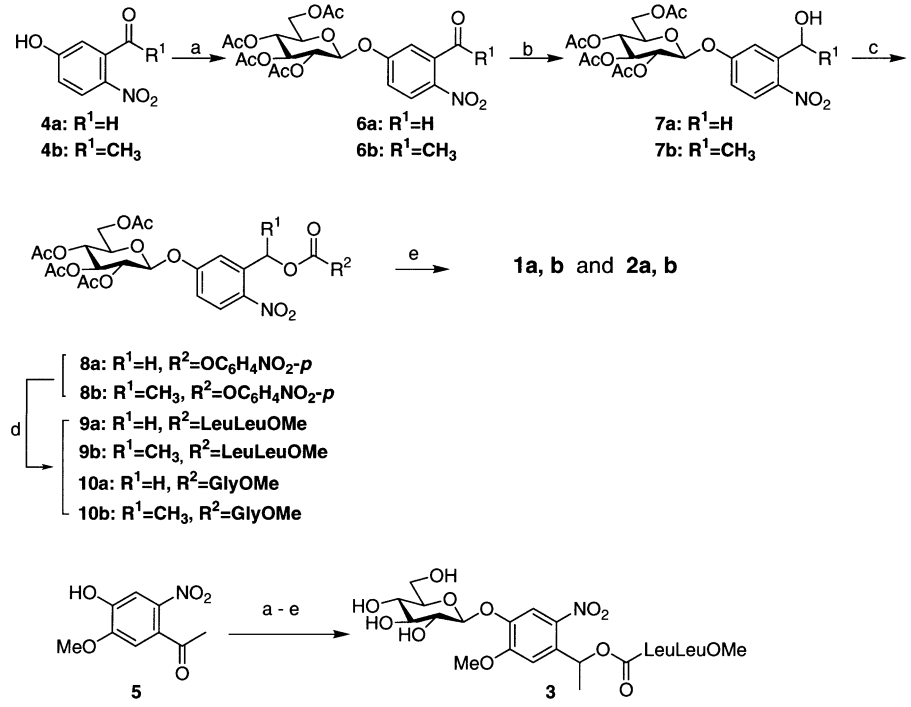


Figure 1. Structures of caged compounds.



Scheme 1. Synthesis: (a) tetra-*O*-acetylglucopyranosyl trichloroacetimidate, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (b) $NaBH_4$, EtOH; (c) 4-nitrophenyl chloroformate, DMAP, $CHCl_3$; (d) LeuLeuOMe·TFA, or GlyOMe·HCl, DMAP, $CHCl_3$; (e) NaOMe, MeOH.

Solubility and stability. Solubilities of **1a**,**b** and **3** in PBS containing 1% DMSO were about 490, 400 and 390 μM, respectively, which were more than 30 times higher than that of the simple 2-NB-caged LeuLeuOMe (Table 1). More hydrophilic caged glycine methyl esters, **2a** and **b**, were more soluble. **1**, **2** and **3** were stable in PBS containing 1% DMSO for over a week at 0 °C in the dark.

Photochemistry. Table 1 shows the absorption spectral properties of the caged compounds **1–3** in PBS containing 1% DMSO. They are almost the same as those of the conventional 2-NB derivatives, and show adequate absorptivities at 350 nm for photolysis. Compared with **1** and **2**, **3** has an additional absorption maxima at longer wavelength with greater absorptivity, resulting in greater absorptivity at 350 nm. However, no great difference was observed between the absorptivities of **3** and **1b** at 400 nm.

A 100 μM solution of the caged compound in PBS containing 1% DMSO was irradiated at 350 nm using a Rayonet photochemical reactor with four 3500 Å lamps, and the progress of the photolysis was monitored by HPLC, as shown in Figure 2. Although both 2-NPE and 2-NB caged compounds are photolysed via aci-nitroso derivatives to give either 2-nitrosoacetophenone or 2-nitrosobenzaldehyde along with the substrates, 2-NPE derivatives are known to be photolysed more quickly and release the substrates more efficiently than 2-NB derivatives.[11] The same trend was observed in our caged compounds. For example, after 5 min of irradiation, 17 and 64% of the caged compounds were consumed, and 7 and 64% of LeuLeuOMe was released for **1a** and **1b**, respectively. The quantum yields for the decrease in **1a** and **1b** at 350 nm were determined to be 0.0025 and 0.033, respectively, using potassium ferrioxalate as a chemical actinometer. Although the profiles of time-dependent decrease in **2a** and **b** almost overlapped those of **1a** and **b**, the release of GlyOMe was less efficient than that of LeuLeuOMe, possibly due to the analytical method we applied. Due to its greater absorptivity, the photolysis of **3** at 350 nm had been expected to proceed more quickly and efficiently than that of **1b**. Contrary to this expectation, the profile of the decrease in **3** upon irradiation at 350 nm was almost the same as that of **1b** (Fig. 2a). We also found that both **1b** and **3** were photolysed at 400 nm, albeit less efficiently than at 350 nm.

HPLC analysis at 254 nm of a photolysed solution of **1a** and **b** showed almost the same results as those of photolysed **2a** and **b**, respectively, which showed a large peak accompanied by several very small peaks. Therefore, our strategy for evaluating the cytotoxic activity of the photolytic by-products of **1a** and **b** by using caged GlyOMe was shown to be valid. ^{1}H NMR spectra of photolysed **1a** and **1b** in methanol-d_4, which showed HPLC profile similar to those photolysed in PBS

Table 1. Properties of the caged compounds in PBS containing 1% DMSO

			Absorption		
	Solubility (μM)	λ_{max} (nm)	ε_{max} (cm^{-1} M^{-1})	ε_{350} (cm^{-1} M^{-1})	ε_{400} (cm^{-1} M^{-1})
1a	490	300	8430	2200	250
1b	400	295	7010	2700	300
2a	990	300	8100	2070	240
2b	890	295	6900	2500	290
3	390	310, 328	4650, 4670	4000	480

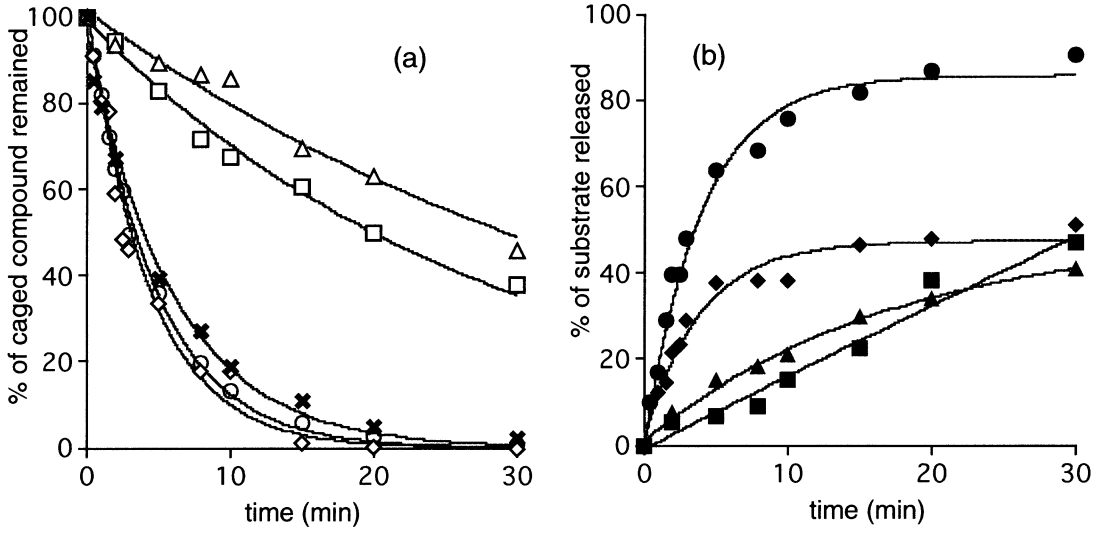


Figure 2. Photolysis of caged compounds in PBS containing 1% DMSO at 350 nm: (a) decrease of caged compounds; (b) release of LeuLeuOMe or GlyOMe (□; **1a**, ○; **1b**, △; **2a**, ◇; **2b**, ×; **3**, ■; release of LeuLeuOMe from **1a**, ●; release of GlyOMe from **1b**, ▲; release of GlyOMe from **2a**, ◆ release of GlyOMe from **2b**.

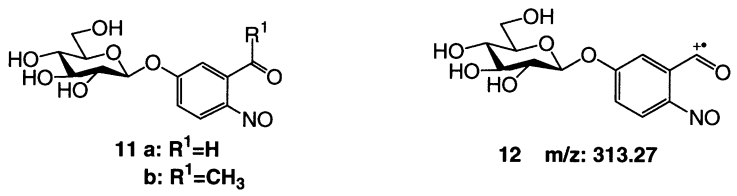


Figure 3. Structures of photolytic byproducts and the MS fragmentation product.

containing 1% DMSO, exhibited signals assigned to 5-glucose-carrying 2-nitrosobenzaldehyde **11a** and 2-nitrosoacetophenone **11b**, respectively (Fig. 3, CHO at 10.03 ppm and $COCH_3$ at 2.58 ppm). After 70% of **1b** was photolysed, the yield of **11b** was calculated to be 45% of the theoretical amount assuming the complete conversion of **1b** to **11b** and LeuLeuOMe. The collected fractions of the larger HPLC peak of the photolysed **1b** showed FABMS fragmentation of *m/z*: 313.27. This was assigned to $(M+H)^+$ of the glucose-carrying 2-nitrosobenzoyl cation **12**, which might be derived from **11b**. The same fragmentation was observed in the FABMS of the collected fractions of photolysed **1a**. These results suggested that the photolytic by-products were mainly the nitroso compounds **11a** and **b**.

Biological assay

Previously, we examined the dose-dependency of the induction of apoptosis with LeuLeuOMe in U937 cells (a human myeloid cell line), Daudi cells (a B cell line), YTN cells (an NK cell line) and HL60 cells (a human myeloid cell line), and found that HL60 cells were the most sensitive.[3,8] Although LeuLeuOMe-induced apoptosis of HL60 cells was detected by both DNA ladder formation and flow cytometry, the latter gave more quantitative results.[4] Therefore, the in vitro cytotoxic activities of **1a**,**b** and **2a**,**b** were examined on HL60 cells by flow cytometry. Based on the observation that irradiation of HL60 cells in a Rayonet photochemical reactor with four RPR 3500 Å lamps for less than 10 min did not cause UV-induced apoptosis, the caged compounds at 500 μM in the presence of HL60 cells were irradiated for 5 min. Swelling of the cells, which is characteristic of cell necrosis, was not observed throughout the study. Therefore, we evaluated the efficiency in inducing apoptosis (Fig. 4) from eq (1), where % of 'viable cells' was determined with a flow cytometer by setting a gate for the cells which had the same sizes as 'cells in control (viable cells)'. Efficiency of photochemical release of the substrates under the same irradiation condition was also shown in Figure 4.

$$\%\ \text{of apoptoic cells} = [1 - (\%\ \text{of 'viable cells'})/(\%\ \text{of 'cells in control'})] \times 100 \quad (1)$$

While the addition of LeuLeuOMe at 500 μM induced apoptosis effectively, GlyOMe caused almost no change in the size of the cells. Only slight change was observed in HL60 cells upon addition of **1a** and **b**, therefore, we confirmed that the cytotoxic activity of LeuLeuOMe was suppressed by caging with sugar-carrying 2-NB and -NPE. In other words, **1a** and **b** are not toxic to the cells. Compound **1b** induced apoptosis upon irradiation almost to the same extent as LeuLeuOMe itself (79%), while 36% induction was observed on **1a** under the same conditions. The results indicate that the efficiency of the induction of apoptosis is roughly parallel the amount of released LeuLeuOMe (35 μM from **1a** vs 320 μM from **1b**). On the other hand, while GlyOMe is not toxic to the cells, Figure 4 suggests that both **2a** and **b** (9 and 18%, respectively) are slightly toxic. However, the reason for this is not clear at present. Moreover, the apoptosis-inducing activity of the photolysed **2a** and **b** (13 and 23%, respectively) is not significantly different from those without irradiation. Therefore, we presume that **2a** and **b** have the weak apoptosis-inducing ability, but the photolytic products from the glucose-carrying 2-NB and 2-NPE caging groups may be not toxic to the

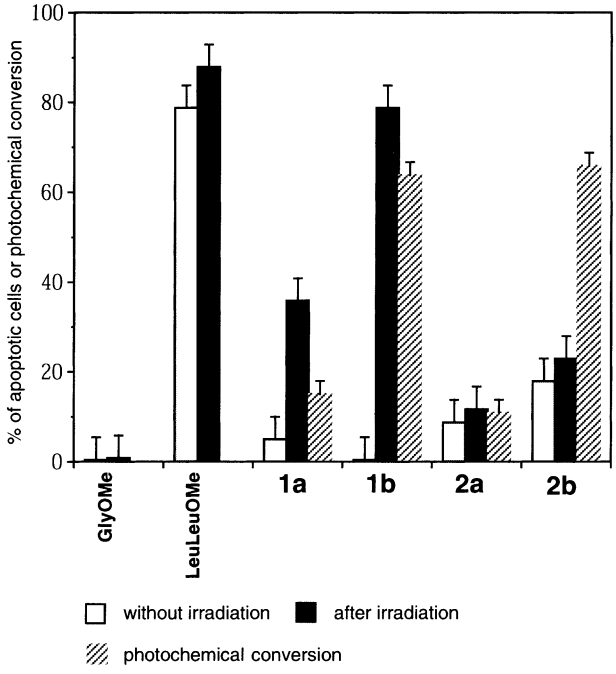


Figure 4. Induction of apoptosis (%) in HL60 cells and photolytic conversion (%) of the caged compounds. HL60 cells were treated with caged compounds according to the method described under Experimental. Without irradiation; the cells were added to GlyOMe or LeuLeuOMe or the caged compounds at 500 μM, and incubated without irradiation. After irradiation: the cells were added to GlyOMe or LeuLeuOMe or the caged compounds, and irradiated for 5 min before incubation. Photochemical conversion; the caged compounds without cells were irradiated for 5 min under the same conditions. Experiments were carried out in triplicates, and the results contained less than ±5% of standard error. The results of bioassay were shown as% of the apoptotic cells calculated from [1−(% of 'viable cells')/(% of 'cells in control')]. Photochemical conversion was expressed by the % of the caged compounds decreased.

cells. In this sense, the apoptosis inducing activity of photolysed **1a** and **b** appears to be solely due to Leu-LeuOMe.

Conclusion

We have developed glucose-carrying 2-NB, 2-NPE and 3,4-dimethoxy-2-nitrophenethyl groups as water-soluble photoremovable protecting groups (caging groups). LeuLeuOMe caged with these caging groups was synthesized via five steps in moderate to good yields from 5-*O*-(β-D-glucopyranosyl)trichloroacetimidate. The carbonic ester derivative **8** was the key intermediate to introduce the carbamate bond between the caging group and LeuLeuOMe or any other bioactive substrates containing either amino or hydroxyl groups. Using other sugars such as mannose instead of glucose, this synthetic route can be applied to the synthesis of a caged compound with cell-recognition ability.

All of the caged compounds are fairly stable in PBS containing 1% DMSO, and are more than 30 times more soluble than unmodified 2-NB caged Leu-LeuOMe. They have fairly large absorptivity in the UVA region where light can penetrate the skin without immunosuppression. The 2-NPE derivative **1b** has much higher photolytic efficiency than the 2-NB derivative **1a**. Although **3**, which has an additional elelctron-donating methoxy group at the aromatic ring, shows the greatest absorptivity at 350 nm and a slightly greater absorptivity at 400 nm, it was photolysed at almost the same rate as **1b**. All of the caged compounds release the substrates effectively. As for the main photolytic products other than the substrates, 5-glucopyranosyl-2-nitroso derivatives of benzaldehyde **11a** from **1a** and acetophenone **11b** from **1b** were identified. In addition to these, several minor photoproducts were found by HPLC analysis.

While the caged compounds, **1a** and **1b**, are not toxic to HL60 cells, they induce apoptosis upon irradiation at 350 nm. Since **1b** is photolysed more quickly and releases LeuLeuOMe more efficiently than **1a**, it induces apoptosis of HL60 cells very efficiently. The cytotoxic activity in the photolytic products other than Leu-LeuOMe from **1a** and **b** was examined by using caged compounds of GlyOMe, **2a** and **b**. While GlyOMe is not toxic to HL60 cells, the effects of **2a** and **b** were slightly significant both before and after irradiation. However, these effects appear mainly due to the caged compounds, **2a** and **b**, themselves. Therefore, the apoptosis-inducing activity of **1a** and **b** may be for the most part due to LeuLeuOMe.

It is known that mature macrophages express a 162-kDa mannose receptor which binds mannose– and fucose–BSA with high affinity[12] and plays an important role in phagocytosis of bacteria.[13] On the other hand, neutrophils, which lack the mannose receptor, are capable of ingesting bacteria, perhaps via β-glucan receptor.[14] Therefore, we believe that LeuLeuOMe caged with glucose or mannose derivatives may be taken up by macrophages or neutrophils through these receptors. The design and synthesis of caged compounds suitable for this application, that is sugar-carrying caged dendrimers, are now in progress in our laboratory.[15]

Experimental

General chemistry methods

NMR spectra were mostly recorded in $CDCl_3$ and were referenced to Me_4Si (δ 0.00, ^1H NMR), $CDCl_3$ (δ 77.0, ^{13}C NMR), except for those of the compounds **1**–**3**, which were recorded in CD_3OD (δ 49.00, ^{13}C NMR). Preparative HPLC was performed on a LC-908 recycling preparative HPLC using JAIGEL GS310 or (1H + 2H) column (supplied by Japan Analytical Industry Co. Ltd). To determine the solubility, absorption spectra were taken on PBS solutions containing 1% DMSO saturated with the caged compounds and the averaged concentration (solubility) was determined from the absorptivity at several wavelengths.

4-Hydroxy-5-methoxy-2-nitroacetophenone (5). Benzyl bromide (4.0 mL, 34 mmol) was added to a solution of acetovanillone (4.70 g, 28.3 mmol), potassium carbonate (8.05 g, 58.3 mmol) and potassium iodide (0.20 g, 1.2 mmol) in acetonitrile (60 mL) at room temperature under Ar. The reaction mixture was warmed to reflux and stirred for 1 day. After the reaction mixture was cooled to room temperature, the inorganic salt was filtered off through Celite. The solvent was removed under reduced pressure and the residue was separated by column chromatography (SiO_2/hexane: CH_2Cl_2 = 1:2) to afford 7.07 g (27.6 mmol, 98%) of 4-benzyloxy-3-methoxyacetophenone: white crystals (from methanol), mp 83.0–84.0 °C ^1H NMR (270 MHz, $CDCl_3$) δ 2.54 (s, 3H), 3.94 (s, 3H), 5.23 (s, 2H), 6.89 (d, J = 8.2 Hz, 1H), 7.31–7.54 (m, 7H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 26.1 (q), 55.9 (q), 70.6 (t), 110.3 (d), 111. 9 (d), 122.8 (d), 127.0 (d), 127.9 (d), 128.4 (d), 130.5 (s), 136.0 (s), 149.2 (s), 152.1 (s), 196.4 (s).

Fuming nitric acid (1.5 mL, 36 mmol) was added to a solution of 4-benzyloxy-3-methoxyacetophenone (1.24 g, 4.83 mmol) in acetic acid (15 mL); during the addition, the reaction mixture was cooled by ice-water bath. The reaction mixture was stirred at room temperature for 1 day and then poured into ice-water. The yellow precipitate was filtered and was purified by column chromatography (SiO_2/hexane–CH_2Cl_2 = 1:2) to afford 979 mg (3.25 mmol, 67%) of 4-benzyloxy-5-methoxy-2-nitroacetophenone: pale yellow crystals (from methanol–CH_2Cl_2), mp 142.0–143.0 °C ^1H NMR (270 MHz, $CDCl_3$) δ 2.49 (s, 3H), 3.98 (s, 3H), 5.22 (s, 2H), 6.76 (s, 1H), 7.35–7.47 (m, 5H), 7.67 (s, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 30.4 (q), 56.6 (q), 71.3 (t), 108.7 (d), 108.7 (d), 127.4 (d), 128.4 (d), 128.7 (d), 132. 9 (s), 135.1 (s), 138.0 (s), 148.3 (s), 154.3 (s), 199. 8 (s).

A solution of 4-benzyloxy-5-methoxy-2-nitroacetophenone (5.19 g, 17.2 mmol) in trifluoroacetic acid (50 mL) was stirred at room temperature for 14 h. After the solvent was removed under reduced pressure, 1 N NaOH

and ethyl acetate were added to the residue. The aqueous layer was acidified by addition of 6 N HCl and was extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure. The residual solid was recrystallized from $CHCl_3$–hexane to give **5** (3.24 g, 15.3 mmol, 89%). **5**: yellow crystals (from hexane–$CHCl_3$), mp 151.5–152.5 °C, ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.49 (s, 3H), 4.02 (s, 3H), 5.96 (s, 1H), 6.79 (s, 1H), 7.67 (s, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 30.4 (q), 56.8 (q), 108.5 (d), 110.6 (d), 131.8 (s), 139.4 (s), 146.5 (s), 150.9 (s), 199.6 (s). Anal. calcd for $C_9H_9NO_5$: C 51.19, H 4.30, N 6.63%; found C 51.05, H 4.33, N 6.61%.

General procedure for the synthesis of aroyl derivatives. The synthesis of 5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzaldehyde **6a** is described as an example. To 2,3,4,6-tetra-*O*-acetylglucopyranosyl trichloroacetimidate[16] (2.90 g, 5.89 mmol) and 5-hydroxy-2-nitrobenzaldehyde (1.15 g, 6.33 mmol), and 5 g of molecular sieves (3 A) in dichloromethane (80 mL), trifluoroboron etherate (1.27 mL, 10.1 mmol) was added with stirring under Ar. The resulting mixture was stirred for 13 h at room temperature and then filtered. The filtrate was washed with 1 N HCl, brine and water, and dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (10:1 dichloromethane/AcOEt) to give pure **6a** as a white solid (2.70 g, 5.44 mmol).

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzaldehyde (6a). Yield 32%; mp 55–56 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 2.05 (s, 3H), 2.08 (s, 3H×2), 2.11 (s, 3H), 4.01 (m, 1H), 4.22 (m, 2H), 5.16 (m, 1H), 5.33 (m, 3H), 7.28 (dd, 1H, J=8.9 and 2.0 Hz), 7.46 (d, 1H, J=2.0 Hz), 8.16 (d, 1H, J=8.9 Hz), 10.45 (s, 1H); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.4 (q×4), 61.8 (t), 67.9 (d), 70.7 (d), 72.3 (d), 72.5 (d), 97.8 (d), 115.5 (d), 121.2 (d), 127.0 (d), 133.8 (s), 144.1 (s), 160.4 (s), 169.1 (s), 169.3 (s), 170.0 (s), 170.6 (s), 187.7 (d); IR (KBr) 1750 (C=O), 1698 (–CHO), 1524 (–NO_2) cm^{-1}. Anal. calcd for $C_{21}H_{23}NO_{13}$: C 50.71, H 4.66, N 2.82%; found C 50.61, H 4.66, N 2.93%.

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitroacetophenone (6b). Yield 92%; mp 126–127 °C (from diethyl ether); ^{1}H NMR (270 MHz $CDCl_3$) δ 2.05 (s, 3H), 2.07 (s, 3H×2), 2.09 (s, 3H), 2.53 (s, 3H), 3.93 (m, 1H), 4.21 (m, 2H), 5.16 (m, 1H), 5.25 (m, 1H), 5.33 (m, 2H), 6.92 (d, 1H, J=1.2 Hz), 7.11 (dd, 1H, J=9.4 and 1.2 Hz), 8.14 (d, 1H, J=9.4 Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.53 (q, 2C), 20.57 (q), 20.59 (q), 30.3 (q), 61.8 (t), 67.9 (d), 70.8 (d), 72.3 (d), 72.5 (d), 97.9 (d), 114.6 (d), 117.4 (d), 126.9 (d), 140.1 (s), 140.9 (s), 160.7 (s), 169.2 (s), 169.4 (s), 170.1 (s), 170.5 (s), 199.4 (s). IR (KBr) 1758 (C=O), 1524 (–NO_2) cm^{-1}. Anal. calcd for $C_{22}H_{25}NO_{13}$: C 51.67, H 4.93, N 2.74%; found C 51.38, H 5.02, N 2.81%.

General procedure for the synthesis of alcohol derivatives

1-[5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl]ethanol (**7b**) is described as an example. Compound **6b** (3.75 g, 7.57 mmol) in THF (100 mL) was treated with sodium borohydride (0.947 g, 3.98 mmol) in ethanol (70 mL) under Ar at rt. The reaction mixture was stirred for 30 min at rt, quenched with iced water and then extracted with dichloromethane. The combined organic layer was washed with 1 N HCl, brine and water, and dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (6:1 dichloromethane/AcOEt) to give **7b** as a mixture of diastereomers (1.93 g, 3.87 mmol).

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzyl alcohol (7a). Yield 98%; mp 65–66 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 2.05 (s, 3H), 2.07 (s, 3H×2), 2.08 (s, 3H), 3.96 (m, 1H), 4.23 (m, 2H), 5.02 (s, 2H), 5.18 (m, 1H), 5.30 (m, 3H), 6.99 (dd, 1H, J=9.0 and 2.5 Hz), 7.38 (d, 1H, J=2.5 Hz), 8.16 (d, 1H, J=9.0 Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.50 (q, 2C), 20.53 (q), 20.58 (q), 61.8 (t), 62.2 (t), 68.0 (d), 70.9 (d), 72.3 (d), 72.4 (d), 97.8 (d), 115.4 (d), 116.1 (d), 127.5 (d), 140.7 (s), 141.8 (s), 160.6 (s), 169.3 (s), 169.4 (s), 170.2 (s), 170.7 (s); IR (KBr) 3496 (–OH), 1756 (C=O), 1520 (–NO_2) cm^{-1}. Anal. calcd for $C_{21}H_{25}NO_{13}$: C 50.50, H 5.05, N 2.80%; found C 50.21, H 5.14, N 2.92%.

1-[5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl]ethanol (7b). Yield 51% (obtained as a mixture of diastereomers); mp 64–68 °C (from diethyl ether); ^{1}H NMR (270 MHz $CDCl_3$) δ 1.53 (d, 3H, J=6.2 Hz), 2.05 (s, 3H), 2.07 (s, 3H), 2.07 (s, 3H), 2.08 (s, 3H), 3.97 (d, 1H, J=8.9 Hz), 4.22 (s, 2H), 5.16 (t, 1H, J=8.9 Hz), 5.29 (s, 3H), 5.51 (q, 1H, J=6.2 Hz), 6.95 (dd, 1H, J=8.2 and 2.3 Hz), 7.46 (d, 1H, J=2.3 Hz), 7.99 (d, 1H, J=8.2 Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.48 (q), 20.51 (q), 20.57 (q), 20.61 (q), [24.3 (q), 24.4 (q)], [61.7 (t), 61.9 (t)], [65.4 (d), 65.5 (d)], [67.95 (d), 67.99 (d)], 70.9 (d), [72.27 (d), 72.34 (d)], [72.38 (d), 72.41 (d)], [97.7 (d), 97.9 (d)], [114.6 (d), 114.7 (d)], [115.3 (d), 115.5 (d)], 127.0 (d), 142.1 (s), 145.0 (s), 160.2 (s), 169.2 (s), 169.4 (s), 170.1 (s), 170.6 (s); IR (KBr) 3388 (–OH), 1754 (C=O), 1522 (–NO_2) cm^{-1}.

General procedure for the synthesis of carbonates

The synthesis of 1-[5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl]ethyl 4-nitrophenyl carbonate (**8b**) is shown as an example. Compound **7b** (1.93 g, 3.87 mmol) and 4-nitrophenyl chloroformate (2.08 g, 3.46 mmol) were dissolved under Ar at rt into dichloromethane (150 mL) which was pretreated with alumina, molecular sieves 3 A, and then molecular sieves 4 A. Triethylamine (2.3 mL, 31 mmol) was added and the reaction mixture was stirred for 17 h at rt. The mixture was washed with 1 N HCl, brine and water, and dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (9:1 dichloromethane/AcOEt) to give a mixture of diastereomers of **8b** as a light yellow solid (1.53 g, 2.25 mmol).

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzyl 4-nitrophenyl carbonate (8a). Yield 53%; mp 61–62 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 2.05 (s, 3H), 2.06

(s, 3H×2), 2.07 (s, 3H), 3.96 (m, 1H), 4.18 (dd, 1H, J=11.9 and 2.1 Hz), 4.31 (dd, 1H, J=11.9 and 4.0 Hz), 5.20 (m, 1H), 5.3 (m, 3H), 5.74 (s, 2H), 7.07 (dd, 1H, J=8.9 and 2.7 Hz), 7.30 (d, 1H, J=2.7 Hz), 7.44 (d, 2H, J=8.9 Hz), 8.25 (d, 1H, J=8.9 Hz), 8.31 (d, 2H, J=8.9 Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.55 (q, 2C), 20.60 (q, 2C), 61.7 (t), 67.2 (t), 67.9 (d), 70.9 (d), 72.4 (d), 72.5 (d), 97.9 (d), 115.6 (d), 116.7 (d), 121.7 (d), 125.4 (d), 128.0 (d), 133.9 (s), 141.8 (s), 145.6 (s), 152.0 (s), 155.2 (s), 160.5 (s), 169.2 (s), 169.3 (s), 170.1 (s), 170.4 (s); IR (KBr) 1758, 1528 cm^{-1}. Anal. calcd for $C_{28}H_{28}N_2O_{17}$: C 50.61, H 4.25, N 4.22%; found C 50.31, H 4.43, N 4.03%.

1-[5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl]ethyl 4-nitrophenyl carbonate (8b). Yield 58% (obtained as a mixture of diastereomers); mp 54–55 °C (from ethanol); ^{1}H NMR (270 MHz $CDCl_3$) δ 1.78 (d, 3H, J=6.2 Hz), 2.06 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H×2), 2.09 (s, 3H), 4.00 (m, 1H), 4.26 (m, 2H), 5.21 (q, 1H, J=8.9 Hz), 5.33 (s, 3H), 6.49 (q, 1H, J=6.2 Hz), 7.06 (d, 1H, J=8.9 Hz), 7.36 (d, 2H, J=8.1 Hz), 7.41 (s, 1H), 8.09 (d, 1H, J=8.9 Hz), [8.25 (d, 2H, J=8.1 Hz), 8.26 (d, 2H, J=8.1 Hz)]; ^{13}C NMR (67.5 MHz CDCl3) δ 20.41 (q), 20.46 (q, 2C), 20.51 (q), [21.7 (q), 21.9 (q)], [61.5 (t), 61.7 (t)], 67.7 (d), 67.8 (d), [70.79 (d), 70.83 (d)], [72.2 (d), 72.4 (d)], 73.2 (d), [97.7 (d), 97.9 (d)], 114.9 (d), [115.3 (d), 115.7 (d)], 121.5 (d), 125.2 (d), 127.4 (d), [139.6 (s), 139.7 (s)], [141.9 (s), 142.0 (s)], 145.3 (s), 151.3 (s), 155.1 (s), 160.4 (s), 169.1 (s), 169.2 (s), 167.0 (s), 170.3 (s); IR[?0x81?]iKBr) 1760, 1528 cm^{-1}. Anal. calcd for $C_{29}H_{30}N_2O_{17}\cdot 0.5H_2O$: C 50.66, H 4.54, N 4.07%; found C 50.59, H 4.60, N 4.01%.

General procedure for the synthesis of amino acid derivatives

The synthesis of 1-[5-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl] ethoxy carbonyl L-leucyl-L-leucine methyl ester (**9b**) is shown as an example. Compound **8b** (1.42 g, 2.09 mmol) and trifluoroacetic acid salt of LeuLeuOMe (4.60g, 12.4 mmol) in 1,4-dioxane (20 mL) were treated with triethylamine (6.0 mL, 43 mmol) under Ar at 0 °C. The reaction mixture was stirred for 3 days at rt in the dark, and then diluted with dichloromethane. The mixture was washed with 1 N HCl, brine, dried over $MgSO_4$, filtered and concentrated. The residue was chromatographed on silica gel (4:1 dichloromethane/AcOEt) to afford **9b** as a diastereomeric mixture.

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzyloxycarbonyl L-leucyl-L-leucine methyl ester (9a). Yield 51%; mp 60.0–62.5 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 0.92–0.99 (m, 12H), 1.55–1.73 (m, 6H), 2.05 (s, 3H×2), 2.07 (s, 3H×2), 3.74 (s, 3H), 4.01 (br s, 1H), 4.23 (m, 3H), 4.61 (br s, 1H), 5.14–5.31 (m, 4H), 5.42–5.47 (m, 1H), 5.61–5.73 (m, 2H), 6.32 (d, 1H, J=7.6 Hz), 6.99 (d, 1H, J=8.6 Hz), 7.20 (s, 1H), 8.17 (d, 1H, J=8.6 Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.5 (q, 2C), 20.6 (q, 2C), 21.8 (q), 21.9 (q), 22.6 (q), 22.8 (q), 24.6 (d), 24.7 (d), 41.2 (t), 41.3 (t), 50.7 (d), 52.2 (q), 53.5 (d), 62.2 (t), 63.4 (t), 68.3 (d), 70.8 (d), 71.9 (d), 72.3 (d), 97.7 (d), 115.3 (d), 115.4 (d), 127.5 (d), 136.2 (s), 141.5 (s), 155.3 (s), 160.3 (s), 169.2 (s), 169.3 (s), 167.0 (s), 170.7 (s), 171.8 (s), 173.0 (s); IR(KBr) 1754, 1584 cm^{-1}. Anal. calcd for $C_{35}H_{49}N_3O_{17}\cdot H_2O$: C 52.43, H 6.41, N 5.24%; found C 52.36, H 6.12, N 4.82%.

1-[5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl] ethoxycarbonyl L-leucyl-L-leucine methyl ester (9b). Yield 80%; mp 74.0–76.0 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 0.80–0.94 (m, 12H), 1.51–1.64 (m, 6H), 1.56 (d, 3H, J=6.2Hz), 2.04 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H×2), [3.70 (s, 3H), 3.73 (s, 3H)], 4.0–4.3 (m, 4H), [4.48 (br s, 1H), 4.59 (br s, 1H)], 5.10–5.50 (m, 5H), 6.1–6.4 (m, 2H), 6.94 (d, 1H, J=9.2Hz), 7.18 (s, 1H), [8.00 (d, 1H, J=9.2Hz), 8.04 (d, 1H, J=9.2Hz)]; ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.6 (q, 2C), 20.68 (q), 20.76 (q), [21.71 (q), 21.87 (q)], [22.0 (q), 22.2 (q)], [22.4 (q), 22.7 (q)], 22.8 (q), 24.6 (d), 24.7 (d), 24.8 (q), 41.2 (t), [41.1 (t), 41.5 (t)], [50.7 (d), 50.8 (d)], [52.28 (q), 52.34 (q)], 53.5 (d), [62.1 (t), 62.3 (t)], 68.4 (d), [68.8 (d), 69.2 (d)], [70.9 (d), 71.1 (d)], 72.2 (d), 72.5 (d), 98.1 (d), [114.2 (d), 115.1 (d)], [115.3 (d), 115.5 (d)], [127.0 (d), 127.4 (d)], 142.1 (s), 142.2 (s), 155.1 (s), [160.2 (s), 160.4 (s)], 169.2 (s), 169.4 (s), 170.1 (s), 170.7 (s), 171.8 (s), [173.0 (s), 173.1 (s)]; IR (KBr) 1752, 1522 cm^{-1} . Anal. calcd for $C_{36}H_{51}N_3O_{17}$: C 54.20, H 6.44, N 5.27%; found C 53.91, H 6.43, N 5.25%.

5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrobenzyloxy carbonyl glycine methyl ester (10a). Yield 51%; mp 59.0–61.0 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 2.03 (s, 3H), 2.05 (s, 3H), 2.06 (s, 3H), 2.11 (s, 3H), 3.84 (br s, 1H), 3.84 (s, 3H), 4.21 (s, 3H), 4.47 (br s, 1H), 5.08 (t, 1H, J=3.2Hz), 5.32 (d, 2H, J=8.4Hz), 5.55 (t, 1H, J=3.2Hz), 5.67 (d, 1H, J=7.8Hz), 5.88 (d, 1H, J=16.7Hz), 6.05 (br s, 1H), 6.97 (d, 1H, J=8.6Hz), 7.12 (s, 1H), 8.18 (d, 1H, J=8.6Hz); ^{13}C NMR (67.5 MHz $CDCl_3$) δ 20.5 (q), 20.6 (q, 2C), 20.8 (q), 42.5 (t), 52.7 (q), 63.5 (t), 63.6 (t), 69.5 (d), 70.6 (d), 70.7 (d), 72.2 (d), 96.8 (d), 111.6 (d), 116.6 (d), 127.7 (d), 137.4 (s), 140.9 (s), 156.1 (s), 160.9 (s), 169.3 (s), 169.5 (s), 170.1 (s), 171.1 (s), 171.7 (s); IR (KBr) 1752, 1522 cm^{-1}. Anal. calcd for $C_{25}H_{30}N_2O_{16}\cdot 0.5H_2O$: C 48.16, H 5.01, N 4.49%; found C 48.20, H 4.98, N 4.29%.

1-[5-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-nitrophenyl] ethoxycarbonyl glycine methyl ester (10b). Yield 80% (obtained as a diastereomeric mixture); mp 68.0–70.0 °C; ^{1}H NMR (270 MHz $CDCl_3$) δ 1.60 (d, 3H, J=6.5 Hz), 2.03 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 2.06 (s, 3H), [3.73 (s, 3H), 3.81 (s, 3H)], 3.92 (d, 1H, J=5.1 Hz), 4.1–4.3 (m, 3H), 4.47 (br s, 1H), 5.09 (t, 1H, J=9.5 Hz), 5.33 (m, 2H), 5.56 (t, 1H, J=7.0 Hz), 5.8 (m, 1H), [6.36 (q, 1H, J=6.5 Hz), 6.40 (q, 1H, J=6.5 Hz)], [6.93 (d, 1H, J=9.2 Hz), 6.96 (d, 1H, J=9.2Hz)], 7.24 (s, 1H), [8.03 (d, 1H, J=9.2 Hz), 8.08 (d, 1H, J=9.2Hz)]; ^{13}C NMR (67.5 MHz $CDCl_3$)δ 20.51 (q), 20.54 (q), 20.6 (q), 21.0 (q), [21.8 (q), 22.2 (q)], [42.3 (t), 42.4 (t)], [52.3 (q), 52.7 (q)], [61.8 (t), 63.6 (t)], [69.3 (d), 67.0 (d)], 70.8 (d), [70.9 (d), 71.0 (d)], [72.1 (d), 72.4 (d)], [98.1 (d), 98.3 (d)], [111.5 (d), 114.7 (d)], [115.6 (d), 116.8 (d)], [127.2 (d), 127.7 (d)], [141.3 (s), 142.0 (s)], [142.1 (s), 142.8 (s)], [155.2 (s), 155.6 (s)], [160.4 (s), 161.5 (s)], [169.25 (s),

169.30 (s)], [169.33 (s), 169.5 (s)], [170.0 (s), 170.1 (s)], [170.3 (s), 170.6 (d)], [171.2 (s), 171.5 (s)]; IR (KBr) 1754, 1522 cm^{-1}. Anal. calcd for $C_{26}H_{32}N_2O_{16}$: C 49.68, H 5.13, N 4.46%; found C 49.56, H 5.01, N 4.36%.

General procedure for the synthesis of deprotected caged amino acid methyl esters

The synthesis of 1-[5-*O*-(β-D-glucopyranosyl)-2-nitrophenyl]ethoxycarbonyl L-leucyl-L-leucine methyl ester (**1b**) is shown as an example. To **9b** (1.32 g, 1.67 mmol) in methanol (60 mL), sodium methoxide (542 mg, 10.0 mmol) in methanol (50 mL) was slowly added and stirred for 10 min under Ar at rt. The reaction mixture was treated with cation exchange resins (Dowex 50×8, H^+-form, 24 g) to make pH of the solution at 6–7, and then filtered them off. After concentration, an almost pure diastereomeric mixture was obtained.

5-*O*-(β-D-Glucopyranosyl)-2-nitrobenzyloxycarbonyl L-leucyl-L-leucine methyl ester (1a). Yield 83%; mp 98.5–100.5 °C; ^{1}H NMR (600 MHz CD_3OD) δ 0.88 (d, 3H, J = 6.5 Hz), 0.93 (d, 3H, J = 6.5 Hz), 0.95 (d, 3H, J = 6.5 Hz), 0.99 (d, 3H, J = 6.5 Hz), 1.56–1.81 (m, 6H), 3.35 (m, 2H), 3.52 (t, 2H, J = 4.3 Hz), 3.68 (s, 3H), 3.69 (m, 1H), 3.93 (dd, 1H, J = 12 and 6.2 Hz), 4.20 (dd, 1H, J = 12 and 6.2 Hz), 4.45 (dd, 1H, J = 13 and 4.3 Hz), 5.02 (d, 1H, J = 6.2 Hz), 5.44 (d, 1H, J = 7.5 Hz), 5.49 (d, 1H, J = 7.5 Hz), 7.16 (dd, 1H, J = 9.1 and 2.3 Hz), 7.40 (d, 1H, J = 2.3 Hz), 8.16 (d, 1H, J = 9.1 Hz); ^{13}C NMR (67.5 MHz CD_3OD) δ 21.8 (q), 22.0 (q), 23.3 (q), 23.5 (q), 25.8 (d, 2C), 41.3 (t), 42.2 (t), 52.1 (d), 52.6 (q), 54.7 (d), 62.6 (t), 64.4 (t), 71.4 (d), 74.7 (d), 77.9 (d), 78.6 (d), 101.9 (d), 116.56 (d), 116.62 (d), 128.5 (d), 137.6 (s), 142.4 (s), 157.7 (s), 163.2 (s), 174.5 (s), 175.4 (s); IR (KBr) 3330, 1719, 1520 cm^{-1}. Anal. calcd for $C_{27}H_{41}N_3O_{13}\cdot H_2O$: C 51.18, H 6.84, N 6.63%; found C 51.27, H 6.59, N 6.59%.

1-[5-*O*-(β-D-Glucopyranosyl)-2-nitrophenyl]ethoxycarbonyl L-leucyl-L-leucine methyl ester (1b). Yield 97%; mp 108.0–109.5 °C; ^{1}H NMR (600 MHz CD_3OD) δ [0.84 (d, 3H, J = 6.6 Hz), 0.85 (d, 3H, J = 6.6 Hz)], 0.93 (d, 3H, J = 6.6 Hz), [0.93 (d, 3H, J = 6.6 Hz), 0.94 (d, 3H, J = 6.6 Hz)], [0.95 (d, 3H, J = 6.6 Hz), 0.96 (d, 3H, J = 6.6 Hz)], 1.48–1.72 (m, 6H), 1.58 (d, 3H, J = 6.5 Hz), 3.33–3.38 (m, 2H), 3.45–3.55 (m, 3H), [3.62 (s, 3H), 3.69 (s, 3H)], [3.93 (dd, 1H, J = 12 and 1.9 Hz), 3.95 (dd, 1H, J = 12 and 1.4 Hz)], 4.06–4.09 (m, 1H), [4.35 (dd, 1H, J = 9.6 and 4.8 Hz), 4.45 (dd, 1H, J = 9.6 and 5.3 Hz)], [4.97 (d, 1H, J = 7.3 Hz), 5.05 (d, 1H, J = 6.9 Hz)], [6.23 (q, 1H, J = 6.5 Hz), 6.25 (q, 1H, J = 6.5 Hz)], [7.12 (dd, 1H, J = 9.1 and 2.5 Hz), 7.13 (dd, 1H, J = 9.1 and 2.5 Hz)], [7.36 (d, 1H, J = 2.5 Hz), 7.43 (d, 1H, J = 2.5 Hz)], [8.01 (d, 1H, J = 9.1 Hz), 8.04 (d, 1H, J = 9.1 Hz)]; ^{13}C NMR (67.5 MHz CD_3OD) δ 21.9 (q), 22.1 (q), 23.3 (q), 23.5 (q), 25.7 (d), 25.8 (d), 25.9 (q), [41.3 (t), 41.4 (t)], [41.9 (t), 42.0 (t)], 52.1 (d), 54.5 (q), [54.5 (d), 54.6 (d)], [62.6 (t), 62.7 (t)], [69.9 (d), 70.1 (d)], [71.4 (d), 71.5 (d)], [74.7 (d), 74.8 (d)], [77.87 (d), 78.0 (d)], [78.6 (d), 78.8 (d)], [101.8 (d), 102.1 (d)], [114.9 (d), 115.5 (d)], [116.9 (d), 117.1 (d)], [128.0 (d), 128.4 (d)], [142.8 (s), 143.0 (s)], 143.3 (s), 157.3 (s), [163.0 (s), 163.2 (s)], 174.4 (s), 175.4 (s); IR (KBr) 3350, 1709, 1657, 1522 cm^{-1}. Anal. calcd for $C_{28}H_{43}N_3O_{13}\cdot 0.5H_2O$: C 52.66, H 6.94, N 6.58%; found C 52.90, H 7.00, N 6.59%.

5-*O*-(β-D-Glucopyranosyl)-2-nitrobenzyloxycarbonyl glycine methyl ester (2a). Yield 99%; mp 93.0–95.0 °C; ^{1}H NMR (270 MHz CD_3OD) δ 3.34 (s, 2H), 3.49–3.69 (m, 4H), 3.74 (s, 3H), 3.89 (m, 2H), 5.16 (d, 1H, J = 7.3 Hz), 5.46 (d, 1H, J = 16 Hz), 5.59 (d, 1H, J = 16 Hz), 7.15 (dd, 1H, J = 2.4 and 8.9 Hz), 7.39 (d, 1H, J = 2.4 Hz), 8.18 (d, 1H, J = 8.9 Hz); ^{13}C NMR (67.5 MHz CD_3OD) δ 43.2 (t), 52.8 (q), 62.6 (t), 64.5 (t), 71.4 (d), 74.7 (d), 77.8 (d), 78.3 (d), 101.6 (d), 115.7 (d), 116.9 (d), 128.5 (d), 138.0 (s), 142.2 (s), 158.6 (s), 163.3 (s), 172.4 (s); IR (KBr) 3380, 1719, 1518 cm^{-1}; Anal calcd for $C_{17}H_{22}N_2O_{12}\cdot H_2O$: C 43.97, H 5.21, N 6.03%; found C 44.11, H 5.13, N 5.53%.

1-[5-*O*-(β-D-Glucopyranosyl)-2-nitrophenyl]ethoxycarbonyl glycine methyl ester (2b). Yield 82% (obtained as a diastereomeric mixture); mp 88.0–90.0 °C; ^{1}H NMR (270 MHz CD_3OD) δ 1.59 (d, 3H, J = 6.5 Hz), 3.36 (s, 2H), 3.51 (m, 3H), 3.79 (s, 3H), 3.80–4.00 (m, 3H), [5.08 (d, 1H, J = 7.3 Hz), 5.13 (d, 1H, J = 7.3 Hz)], [6.25 (q, 1H, J = 6.5 Hz), 6.27 (q, 1H, J = 6.5 Hz)], [7.12 (dd, 1H, J = 2.2 and 4.3 Hz), 7.14 (dd, 1H, J = 2.2 and 4.3 Hz)], [7.38 (d, 1H, J = 2.2 Hz), 7.44 (d, 1H, J = 2.2 Hz)], [8.04 (d, 1H, J = 4.3 Hz), 8.10 (d, 1H, J = 4.3 Hz)]; ^{13}C NMR (67.5 MHz CD_3OD) δ [22.2 (q), 22.5 (q)], [43.0 (t), 43.1 (t)], 52.7 (q), [62.5 (t), 62.7 (t)], 70.1 (d), 70.4 (d), 74.8 (d), 77.9 (d), 78.3 (d), [101.7 (d), 101.9 (d)], [114.8 (d), 115.3 (d)], [116.9 (d), 117.2 (d)], 128.2 (d), 142.6 (s), 143.4 (s), 158.1 (s), 163.4 (s), 172.3 (s); IR (KBr) 3378, 1713, 1518 cm^{-1}; Anal calcd for $C_{18}H_{24}N_2O_{12}\cdot 0.5H_2O$: C 46.06, H 5.37, N 5.97%; found C 46.20, H 5.35, N 5.75%.

1-[4-*O*-(β-D-Glucopyranosyl)-5-methoxy-2-nitrophenyl]ethoxycarbonyl L-leucyl-L-leucine methyl ester 3. Yield 97% (obtained as a diastereomeric mixture); mp 148.0–150.0 °C; ^{1}H NMR (270 MHz, CD_3OD) : δ 0.52–0.98 (m, 12H), 1.45–1.61 (m, 9H), 3.44–3.47 (m, 4H), 3.65–3.69 (m, 4H), 3.87 (m, 1H), [3.96 (s, 3H), 3.99 (s, 3H)], 4.10 (m, 1H), 4.38 (m, 1H), 4.98 (m, 1H), 6.25 (m, 1H), 7.21 (s, 1H), [7.86 (s, 1H), 7.87 (s, 1H)]. ^{13}C NMR (67.8 MHz, CD_3OD): δ 21.71 (q), [21.85 (q), 21.92 (q)], 22.15 (q), [22.38 (q), 22.53 (q)], [23.31 (q), 23.36 (q)], 25.72 (d), [25.80 (d), 25.87 (d)], 41.32 (t), [41.97 (t), 42.02 (t)], [51.90 (d), 52.04 (d)], 52.61 (q), [54.37 (d), 54.45 (d)], [56.93 (q), 57.09 (q)], [62.22 (t), 62.30 (t)], 70.01 (d), [71.03 (d), 71.11 (d)], [74.64 (d), 74.70 (d)], [77.71 (d), 77.75 (d)], [78.16 (d), 78.30 (d)], [102.39 (d), 102.44 (d)], 109.76 (d), [114.08 (d), 114.36 (d)], [136.62 (s), 136.69 (s)], [140.44 (s), 140.84 (s)], [146.22 (s), 146.28 (s)], [155.52 (s), 155.57 (s)], [157.01 (s), 155.57 (s)], 174.22 (s), [174.95 (s), 175.02 (s)]; Anal calcd for $C_{29}H_{45}N_3O_{14}\cdot H_2O$: C 51.40, H 6.99, N 6.20%; found C 51.15, H 6.88, N 6.11%.

Photochemical reaction

A 0.1 mM PBS/1% DMSO solution (5 mL) of the caged LeuLeuOMe or GlyOMe in a Pyrex tube (10×160 mm)

capped with a rubber septa was irradiated using a Rayonet photochemical reactor with four RPR 3500 Å 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-LeuOMe and GlyOMe, the following procedure was used.[17] To a 10 μL of the photolysed solution which was diluted with 140 μL of PBS, a 50 μL 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 (pH 4.1) = 7:3 (v/v) and detected by emission at 475 nm with excitation at 390 nm. For determination of the quantum yields for the decrease of the caged compounds, potassium ferroxalate was used as a chemical actinometer according to the procedure described in the literature.

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. The suspended solution was taken into Eppendorf tubes at 200 μL each, to which Leu-LeuOMe, or the caged derivatives were added to make the concentration at 500 μM, followed by incubation at room temperature for 20 min. Two samples for each caged compound were prepared to compare the results of those with and without irradiation. One of the each two samples was irradiated for 5 min in a Rayonet photochemical reactor using four RPR 3500 Å lamps. Then a 4-fold volume of RPMI 1640 medium containing 10% FCS was added to the all the samples, and 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. Experiments were carried out in triplicate.

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