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SYNTHESIS OF CAGED PEPTIDES USING CAGED LYSINE: APPLICATION TO THE SYNTHESIS OF CAGED AIP, A HIGHLY SPECIFIC INHIBITOR OF CALMODULIN-DEPENDENT PROTEIN KINASE II

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Abstract: N_α-Fmoc-N_ε-(2-nitrobenzyloxycarbonyl)-lysine has been prepared and used in the solid-phase synthesis of caged peptides. The synthesized caged AIP (cagedKcagedKALRRQEAVDAL) showed characteristics required for caged peptides including a significantly reduced inhibitory activity to calmodulin-dependent protein kinase II and instantaneous recovery of the activity with photo-irradiation.

Keywords: Enzyme inhibitors; Peptides and polypeptides

Caged peptides whose activities are masked by the introduction of photocleavable groups have been recently recognized as a useful tool to elucidate various biological phenomena even in living cells with highly spatial and temporal resolution,[1-5] because active peptides can be generated at the desired time and position in a sample specimen by making a photochemical reaction using pulsed and focused UV-light irradiation, respectively. Various amino acid residues are involved in the activities of peptides, but the building block for the solid phase peptide synthesis (SPPS) of caged peptides has been reported only for tyrosine.[1,2] Although photocleavable groups have been introduced into other amino acid residues by chemical modification (Arg^3 and Cys^4) or by the cell-free protein synthesis system using the chemically aminoacylated suppressor tRNA (Ser^5), the advantage of SPPS is simplicity, rapidity, and versatility in the synthesis of various peptide sequences. We now report the synthesis of a novel building block for a caged lysine residue and the application to the SPPS of AIP, the autocamtide-2 related inhibitory peptide.[6] Synthesized caged AIPs showed characteristics required for caged peptides that included a significantly reduced inhibitory activity and instantaneous recovery of the activity with photo-irradiation as illustrated in scheme 1.

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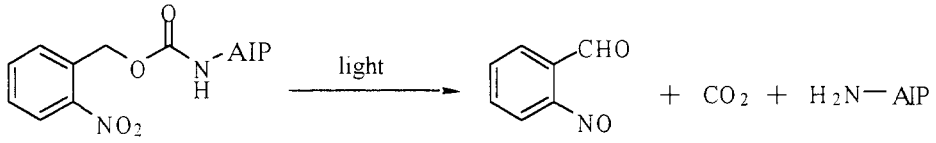
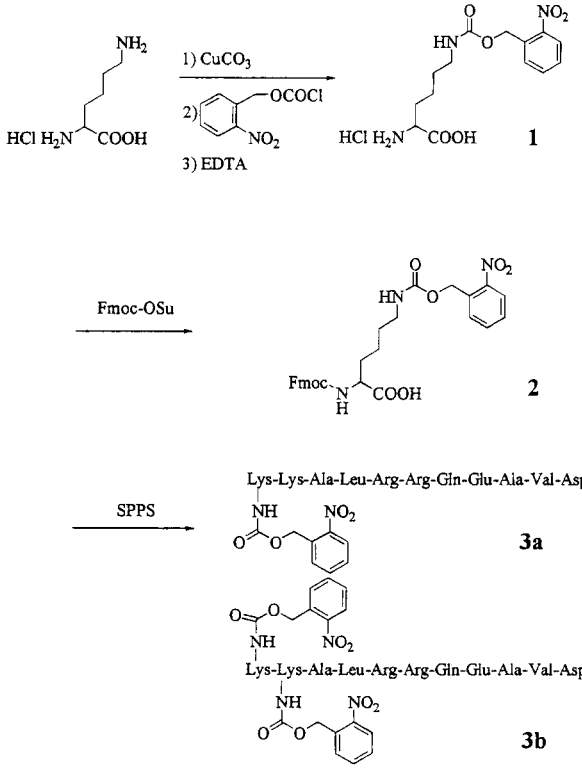
The caged-lysine building block was synthesized in two steps (Scheme 2).[7-9] The incorporation of the caged lysine into the peptide sequence was carried out using the standard Fmoc chemistry with an automated peptide synthesizer.[10] The photocleavable linkage was stable against 30% piperidine in DMF during deprotection of the Fmoc group and trifluoroacetic acid during C-terminal cleavage from the resin. The desired caged AIPs, **3a** and **3b**, were obtained as a single major product by RP-HPLC. In all the sequences we synthesized, the elution time for the caged peptide was significantly longer than that for the intact peptide, presumably due to the hydrophobicity of the photocleavable group. Therefore, any trace of the intact peptide, which might be produced during the synthesis, could be easily removed by RP-HPLC. To avoid the photolysis, the purification of the caged peptides was carried out by preparative RP-HPLC without use of a UV-detector.



Scheme 1. Photolysis of caged lysine residue



Scheme 2. Synthesis of caged-lysine building block and caged AIP

Upon irradiation of the caged AIPs with UV light, the generation of the intact AIP was observed by RP-HPLC and mass spectrometry. The kinetics of the photocleavage were studied by measurements of the transient absorption in the same manner as that of the caged peptides containing caged tyrosine.[1] When the caged AIP in 5 mM TES buffer (pH7.5) at 23°C was irradiated with the third-harmonic of a Nd:YAG laser, a rapid increase and a slow decay occurred in the absorption at 420 nm due to the formation of a *aci*-nitro compound as an intermediate. The half life of the intermediate during photolysis was calculated to be ca. 2.0 μs.

The absorption maximum of the nitrobenzyloxycarbonyl group was at 265 nm (ε = 5580 cm^{-1} M^{-1}). The irradiation with near-visible 405-nm light did not cause the photocleavage of **3a**, but that with 350 nm caused photocleavage to some extent. Therefore, **3a** and **3b** can be safely used under ordinary room illumination.

Next, we examined the masking effect of the photocleavable group on the biological activity of the peptides. AIP was found to inhibit calmodulin-dependent protein kinase II (CaMKII) in a highly specific manner.[6]

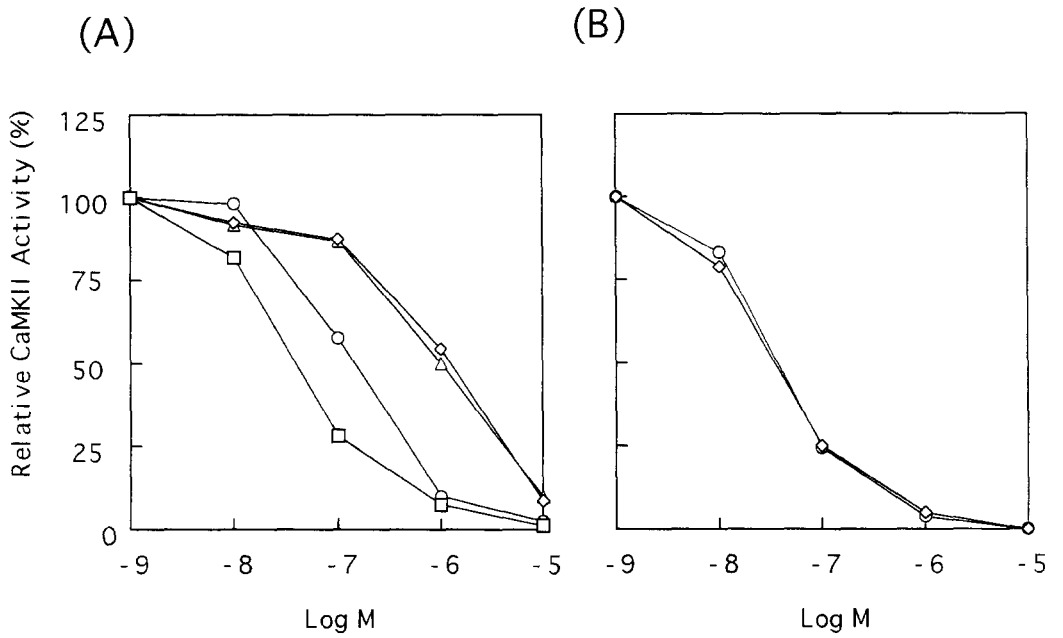


Figure 1. Inhibitory activity of caged AIPs before (A) and after (B) UV-irradiation. The activity of CaMKII was measured in the presence of AIP (□), des[$Lys^{1,2}$]AIP (△), **3a** (○), **3b** (◇).

Our previous study indicated the importance of the first two lysine residues in the inhibitory activity of AIP on CaMKII,[11] and this was supported by the result that the deletion of these residues resulted in the decrease in the activity by ca. 2 digits; des[Lys^{1,2}]AIP (IC_{50} = 1.0 x 10^{-6} M) and AIP (IC_{50} = 3.2 x 10^{-8} M) (Fig. 1a).[12] The introduction of nitrobenzyloxycarbonyl group to the lysine residues resulted in the decrease of the inhibitory activity as expected, and the IC_{50} value for **3b** (1.2 x 10^{-6} M) was almost same as that for des[Lys^{1,2}]AIP. By irradiation of the aqueous solution of the caged peptides with UV light, the inhibitory activity was restored to the same level as that of the intact AIP as shown in Fig. 1b (IC_{50} of the photolysate of **3a** = 3.6 x 10^{-8} M, IC_{50} of the photolysate of **3b** = 3.4 x 10^{-8} M), showing that nitrosobenzaldehyde, the by-product of the photolysis of caged AIP, does not interfere with the activity.

Protein kinases are key enzymes in the signal transduction pathways of many important cellular functions, and therefore, their caged inhibitors are expected to be useful probes to explore the spatial and temporal cross-talk between different transduction pathways in intact living cells which remains unclear using conventional methods. CaMKII is abundant in brain and known to be a multifunctional protein kinase, which plays important roles in controlling a variety of cellular functions including memory in response to an increase in intracellular Ca^{2+} [13,14] Therefore, caged AIPs would provide a useful method for the elucidation of the temporal and spatial behaviors of CaMKII in memory, etc.

As a concluding remark, it should be noted that any caged derivative of peptides whose lysine residues are crucial in their activities can be obtained by a facile SPPS using the building block for the caged-lysine residue **2**.

References and notes

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7. 2-Nitrobenzylalcohol was refluxed with 2 equivalents of phosgene-dimer in THF for 2 h and the solvent and excess reagent were evaporated to give the chloroformate. It was used without purification.
8. **N_ε-2-Nitrobenzyloxycarbonyl-lysine (1)**: mp 176-180°C, $[\alpha]_D^{29.0}$ 15.9° (*c* 3.2, 1N HCl), ^{1}H NMR (500 MHz, D_2O) δ =8.11(1H, d, *J* = 8Hz), 7.75(1H, t, *J* = 8Hz), 7.61 (1H, d, *J* = 8Hz), 7.57 (1H, t, *J* = 8Hz), 5.42 (2H, s), 4.09 (1H, t, *J* = 6Hz), 3.15 (2H, t, *J* = 6Hz), 2.00 (1H, m), 1.94 (1H, m), 1.56 (2H, m), 1.46 (2H, m).
9. **N_α-(9-Fluorenylmethoxycarbonyl)-N_ε-(2-nitrobenzyloxycarbonyl)-lysine (2)**: mp 99-104°C, $[\alpha]_D^{28.5}$ 11.3° (*c* 4, $CHCl_3$), ^{1}H NMR (500 MHz, DMSO-d_6) δ =8.10 (1H, d, *J* = 7 Hz), 7.88 (2H, d, *J* = 7 Hz), 7.78 (1H, t, *J* = 7 Hz), 7.72 (2H, d, *J* = 7 Hz), 7.64 (2H, m), 7.41 (2H, t, *J* = 7Hz), 7.33 (2H, t, *J* = 7 Hz), 5.35 (2H, s), 4.28 (2H, d, *J* = 7 Hz), 4.22 (1H, t, *J* = 7 Hz), 3.91 (1H, br), 3.00 (2H, m), 1.69 (1H, m), 1.60 (1H, m), 1.41 (2H, m), 1.34 (2H, m), FABMAS calcd [M] for $C_{29}H_{29}N_3O_8$: 547.1955; found [M + H$^+$] *m / z* 548.1997.
10. Ten equivalents each of the Fmoc-protected amino acids, benzotriazolyloxy-tris-pyrrolidino-phosphonium-hexafluorophosphate, 1-hydroxybenzotriazole, and *N*-methylmorpholine were used.
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12. The IC_{50} was defined as the concentration of the peptide which gave 50% inhibition and measured by the method described in ref. 6.
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