A Photolabile Carboxyl Protecting Group for Solid Phase Peptide Synthesis

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A new kind of photolabile protecting group (PLPG) for carboxyl moieties was designed and synthesized as the linker between resin and peptide. This group can be used for the protection of amino acid carboxyl groups. The peptide was synthesized on Nph (2-hydroxy-3-(2-nitrophenyl)-heptanoic acid)-derivatized resins and could be cleaved under UV exposure, thus avoiding the necessity for harsh acid-mediated resin cleavage. The PLPG has been successfully used for solid-phase synthesis of peptides.

1. Introduction

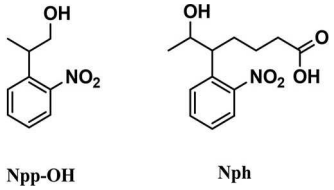
Protecting groups are indispensable tools in organic chemistry.[1] Photochemical reactions are very appealing from both an economical and environmental perspective. Indeed, light is a very cheap reagent, and it does not generate chemical waste.[2] Photochemical methods are valuable alternatives to conventional approaches that often employ acids, bases, or reducing/oxidizing reagents.[3]

Photolabile protecting groups (PLPGs) are protecting groups that can produce free radicals or isomerize molecules under light of a certain wavelength. They can concurrently release target molecules.[4] Compared to traditional protecting groups, PLPGs avoid the use of strong acid[5] and strong bases condition. They can release the protecting substrate only under irradiation at a specific wavelength.

The past two decades have witnessed the continuous emergence of new PLPGs and their creative applications, such as o-nitrobenzyl groups, coumarin groups, arylcarbonylmethyl groups, and arylmethyl groups.[6] The 2-nitrobenzyl derivatives are the most commonly used PLPG.[7] The photochemical cleavage mechanism of the (o-nitrobenzyl)oxy function is triggered by the abstraction of a benzylic H-atom by the excited nitro group.[8] In our early work,[9] 2-(2-nitrophenyl)propan1-ol (Npp–OH) (Figure 1) was successfully synthesized as a PLPG. It can protect the carboxylic acid group of amino acids and be quickly removed[10] after UV irradiation for 30 minutes. However, there are still some disadvantages to this protecting group. Its primary alcohol is unstable after being treated with strong nucleophilic reagents, and it can have easy concurrent side reactions with diketopiperazine. Thus, for the first time, we designed and synthesized a novel carboxyl PLPG (**Nph**) (Figure 1) that can be applied in peptide solid-phase synthesis as a linker between resin and amino acid. It was used as the linker between the resin and the amino acid by protecting the carboxyl group of the amino acid versus Npp–OH. The linker is stable in alkaline conditions and can also avoid the occurrence of diketopiperazine reaction and be quickly removed with light.[6,7,8,9,10]

2. Results and Discussion

Here, 2-fluoronitrobenzene was the starting material, and ethyl acetoacetate was the substitute in DMSO (dimethyl sulfoxide); **2** was obtained after heating in 80 °C. The initial strategy for synthesis of **4** was to get **3** by inserting the side chain ethyl bromobutyrate directly to **2**, and **3** was then decarboxylated to obtain **4** to avoid the double substituted side chain products. However, the subsequent decarboxylation of **3** did not occur. Therefore, in the alternative strategy, **2** was decarboxylated to **3'**, and then the side chain was inserted to get **4**.[11] Intermediate **5** was obtained via reduction of **4** with sodium borohydride in an ice bath in methanol. Finally, target product



Npp-OH Nph

Figure 1. Structure of Npp–OH and Nph.

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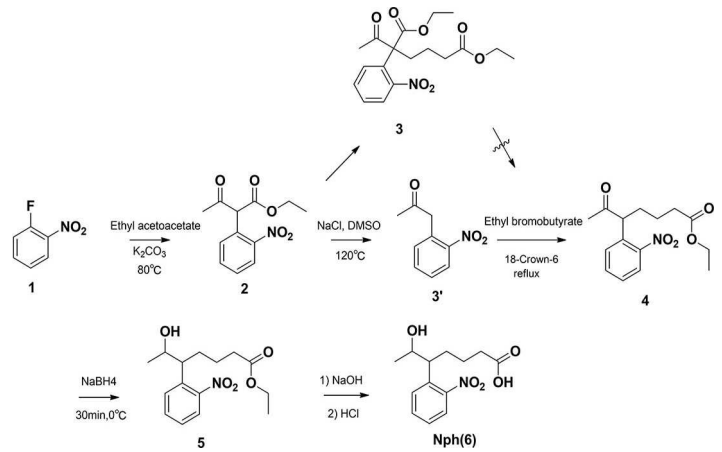
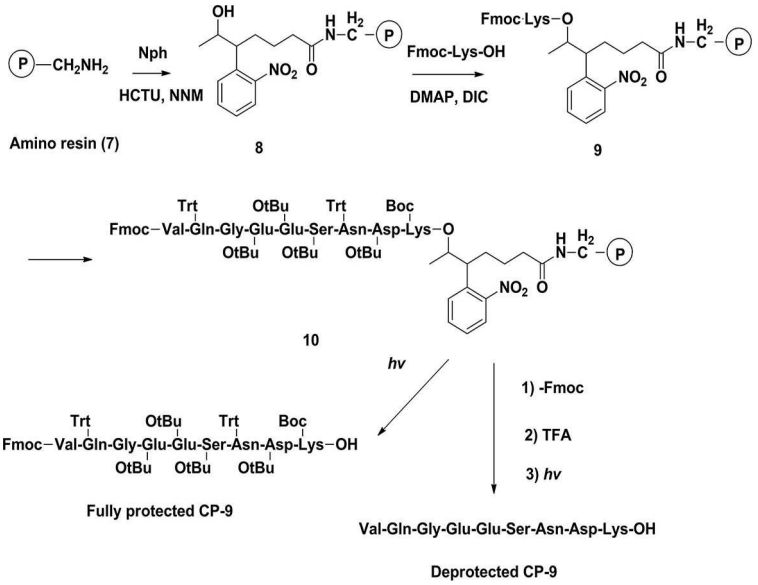


Scheme 1. Synthesis of **Nph**.



Scheme 2. Synthesis of fully protected **CP-9** and deprotected **CP-9** with **Nph**.

Nph (**6**) was obtained from **5** after hydrolysis and acidification. Their structures were verified by NMR/MS (Scheme 1).[11]

A series of tests involving amino acid loading rate, photolysis time, photolysis efficiency, acid, and alkali resistance on Nph were performed to prove that it could protect the carboxyl group for peptide chain assembly as the linker of amino acid and aminomethyl resin. This step involved the anti-radiation peptide CBLB612 (**CP-9**) (Figure 2) with Nph.

CBLB612 is an anti-radiation peptide with the peptide chain Val-Gln-Gly-Glu-Glu-Ser– Asn-Asp-Lys-OH (**CP-9**).[12] The synthesis of **CP-9** is very important, and we have studied its synthesis in which the amino acids of **CP-9** were loaded with **Nph** as the PLPG.[12]

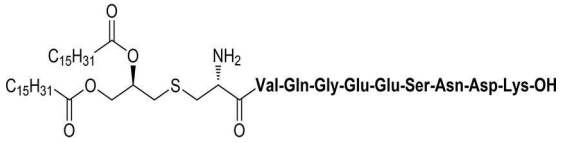


Figure 2. Structure of CBLB612.

Nph was connected to amino resin (**7**) with HCTU (N,N,N′,N′-tetramethyl-O- (6-chloro-1H-benzotriazol-1-yl) uraniumhexafluorophosphate) and NMM (4-methylmorpholine) in DMF. When connected completely, the amino acid lysine was loaded in DCM-DMAP (4-(N,N-dimethylamino) pyridine) condition to obtain **9**. The loading of other amino acids followed the method of solid-phase synthesis to get **10**, which was photolyzed to obtain fully protected **CP-9**. This was then deprotected and then photolyzed to obtain deprotected **CP-9** (Scheme 2). In the synthesis of **CP-9**, the loading ratios of amino acids were tested. The general procedure was that the resin with one amino acid was washed, dried, and weighed. The loading ratio was calculated as follows: loading ratio (%) = (M2-m)/M1*100% (M2: total weight of resin and amino acid, m: weight of resin, M1: theoretical weight gain of amino acids). The loading ratios of

Table 1. Loading ratio of Nph to amino acid.

Entry	Amino acid	Loading ratio (%)
1	Lys	96.5
2	Asp	95.2
3	Asn	97.2
4	Ser	95.3
5	Glu	91.7
6	Gly	94.4
7	Gln	93.2
8	Val	95.7

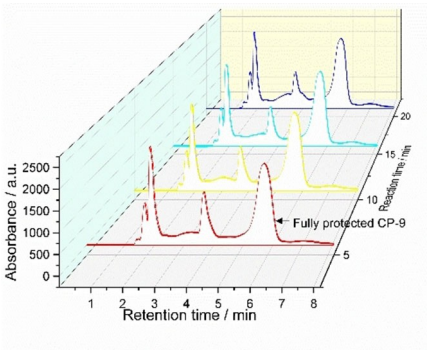


Figure 3. HPLC of fully protected **CP-9** under different photolysis times.

lysine (Lys), aspartic acid (Asp), asparagine (Asn), serine (Ser), glutamic acid (Glu), glycine (Gly), glutamine (Gln), and valine (Val) were calculated by the same method (Table 1).

The photolysis efficiency was evaluated by **10**. Here, 0.1 g **10** in 5 mL THF was exposed to a 365 nm ultraviolet lamp for 5, 10, 15, or 20 min. A 400 μL of the irradiation solution was aliquoted and analyzed by HPLC. The eluent was 90% aqueous acetonitrile. The HPLC results showed that the peak strength of peptide did not increase any more at 15 min (Figure 3), indicating that the peptide was almost completely photolyzed at 15 min.[13] After purification, the purified fully protected **CP-9** was obtained.[13]

Next, 0.2 g of **10** was put into a 10 mL peptide synthetic tube, and 20% piperidine-DMF was used to remove the Fmoc protective group and cleavage solution (1.8 mL trifluoroacetic acid, 0.1 mL water, and 0.1 mL triisopropylsilane) to remove other protective groups. After 5 mL THF was added, the samples (400 μL) were collected after light exposure for 5, 10, 15, 20, 25, or 30 min. The mobile phase was 3% acetonitrile-water solution. The spectra showed that the peptide content does not increase further after 25 minutes of light exposure (Figure 4). Thus, we concluded that the peptide release was complete after 20 minutes of illumination. The pure product of deprotected **CP-9** was obtained after purification.

Additional experiments verified the stability of the protecting group under acidic and alkaline conditions. Fmoc-Gly-OH was selected because it had no other protective groups, which minimized adverse influence on the results. Here, 0.1 g of the peptide resin was taken into an EP tube with 1 ml TFA. After standing, 100 μL samples were collected at 2, 4, 6, and 12 h and then dried with argon and dissolved with 0.5 mL 50% acetonitrile water solution for liquid phase analysis. The liquid phase was 50% acetonitrile-water solution. The data of the peak area showed that the protective group could tolerate 6 h under acidic conditions (Figure 5).

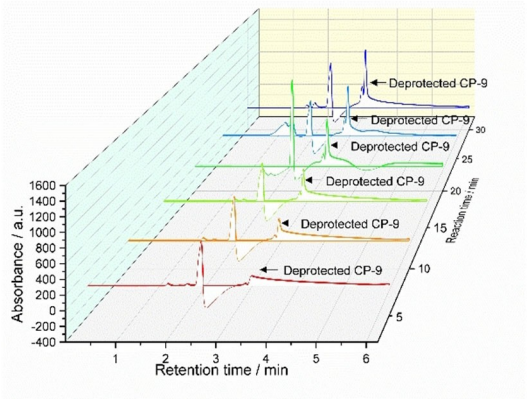


Figure 4. HPLC of deprotected **CP-9** under different photolysis times.

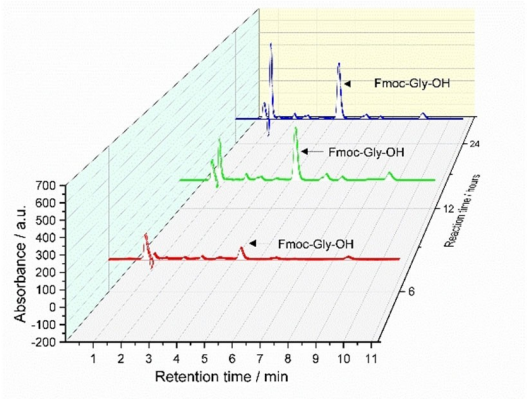
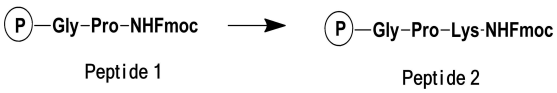


Figure 5. HPLC of Fmoc-Gly-OH in trifluoroacetic acid for different times.



Scheme 3. Synthesis of Peptide 2 from Peptide 1.

The side effects of diketopiperazine occur under alkaline conditions when the first amino acid or the second amino acid is Gly or Pro. Therefore, the two peptides (Peptide 1 (Gly-Pro-NHFmoc) and Peptide 2 (Gly-Pro-Lys-NHFmoc) (Scheme 3)) that meet these conditions were selected as the research objects, and solid-phase synthesis was performed with Nph as the linker to investigate the tolerance in alkaline conditions.

Peptide 1 and 2 were successively synthesized, and the absorbance of Fmoc from their deprotection solution was measured under UV. These consistent absorbance values indicated that no diketopiperazine side reaction occurred. Variable absorbance values indicated that the diketopiperazine side reaction occurred.

After Peptide 1 was synthesized, 5 mL of 20% piperidine DMF solution was added into the peptide synthesis vessel for

deprotection. This was then shaken for 1 h at room temperature and allowed to stand for for 5 min. Next, 100 μL of deprotection solution was aspirated to 10 mL, and the absorbance was measured by UV.

After extruding the deprotection solution, the resin was cleaned with DMF and loaded with amino acid Lys. After detection with ninhydrin, the 5 mL of 20% piperidine DMF solution was added into the peptide synthesis vessel for deprotection. The resin was shaken at room temperature for 1 h and then allowed to stand for 5 min. Next, 100 μL of deprotection solution was absorbed and diluted to 10 mL and then measured for UV absorbance.

The UV spectra of the two deprotection solutions showed that the Fmoc absorption values of the two deprotection solutions were almost the same (Figure 6 Figure 7). This indicates that the concentrations of Fmoc in the two samples are the same. This result indicated that there was no side reaction of diketopiperazine, which proved that **Nph** had good tolerance to alkaline environments.

3. Conclusion

The use of 2-fluoronitrobenzene as a starting material to design and synthesize PLPG **Nph** as a linker between the resin and peptide has been evaluated. It can protect the carboxyl group of amino acids. The linker was synthesized from commercially available inexpensive materials with high yield.[14] A loading ratio experiment used eight amino acids and photolysis. The results suggested that the linker has high protection/deprotection efficiencies and remarkable stability. Both the protection and deprotection reactions of **Nph** can be carried out in a neutral environment. Furthermore, **Nph** is tolerant of both acid and alkali conditions; it can be applied to the solid-phase synthesis of peptides without other chemical reagents. The deprotected peptide and whole protected peptide were connected to the resin by **Nph** and could be removed rapidly at 365 nm, thus avoiding cutting the peptide and removing other protective groups under acid conditions. Thus, **Nph** has broad application prospects for carboxyl protection in solid-phase peptide synthesis.[14]

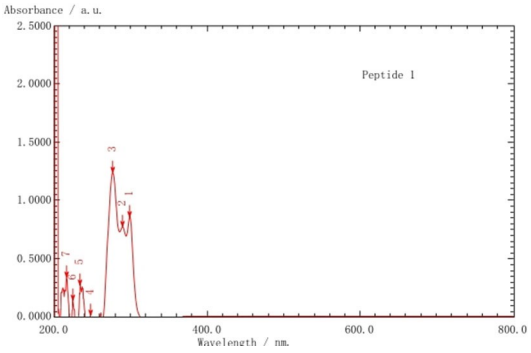


Figure 6. UV absorbance at different Wavelengths of Peptide 1.

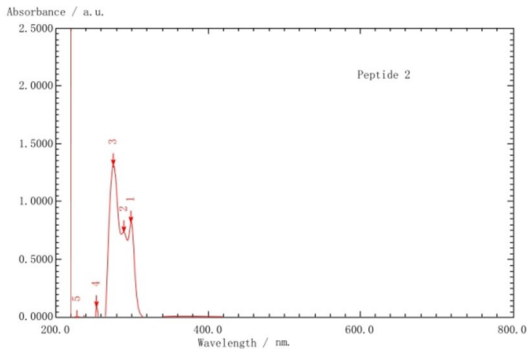


Figure 7. UV absorbance at different Wavelengths of Peptide 2.

Experimental Section

General

All chemicals and solvents were of analytical grade and used without further purifications. All organic solutions were concentrated by rotary evaporation under reduced pressure. Flash column chromatography was performed with 230–400 mesh silica gel. The reaction progress was monitored by silica gel thin layer chromatography (TLC) plates. UV-visible spectra were obtained using a Shimadzu UV-2501PC UV-visible Recording spectrophotometer with a sample concentration of $8.56*10^{-5}$ M. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 600 spectrometer and Bruker 400 NMR spectrometer; chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to the residual protium in the NMR solvent (DMSO-d_6: 2.50). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances), and integration of coupling constant in hertz (Hz). Photolysis used a 250 W UV lamp (365 nm).

1-(2-Nitrophenyl) Propan-2-one (3′)

Ethyl acetoacetate 7.05 g (50.0 mmol) was dissolved in 60 mL DMSO. Anhydrous potassium carbonate 20.7 g (150.0 mmol) and 2-fluoronitrobenzene 113.0 g (100.0 mmol) were added and heated at 80 °C. The reaction was monitored completely by TLC. After the reaction, the reaction solution was cooled to room temperature and poured into ice water. The reaction was neutralized to pH 5–6 with 1 M hydrochloric acid and then extracted with ethyl acetate (50 mL × 3). The organic phase combined and dried with anhydrous sodium sulfate. It was filtered and concentrated to get crude product **2**, which was directly placed in the next reaction without purification.

Next, **2** was dissolved in 40 mL DMSO with 10 mL saturated sodium chloride solution. The solution was heated at 120 °C; 50 mL water was added to dilute the reaction solution when complete. The solution was extracted with DCM (30 mL × 3) and washed with saturated brine twice. Drying of the collected organic layer over Na_2SO_4 was followed by concentration of the product. Product **3′** was purified by flash chromatography (petroleum ether: ethyl acetate = 10/1). 7.75 g (86.6%); yellow oil Rf = 0.2 (petroleum ether/ethyl acetate = 6/1); ^{1}H NMR (400 MHz, DMSO) δ 8.07 (dd, J = 8.4, 1.4 Hz, 1H), 7.71 (td, J = 7.56, 1.4 Hz, 1 H), 7.55 (td, J = 8.4, 1.68 Hz, 1 H), 7.45 (dd, J = 7.56, 1.12 Hz, 1 H), 4.24 (s, 2 H), 2.22 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 149.12, 134.26, 134.13, 130.94, 128.92, 125.12, 48.02, 40.59, 40.39, 40.18, 39.97, 39.76, 39.55, 39.34. HRMS (ESI): *m/z* calcd for $C_9H_9NO_3 + H$: 180.06, found 180.0656.

2-Carbonyl-3-(2-Nitrophenyl)-Ethyl Heptanoate (4)

1.8 g (10.0 mmol) **3'** was dissolved in 50 mL of anhydrous acetonitrile; 11.6 g (85.0 mmol) of anhydrous potassium carbonate and 15 mg of 18-crown-6 were added to the solution. Ethyl bromobutyrate in 5.0 mL of acetonitrile was dropped into the solution slowly and then refluxed. The reaction was monitored completely by TLC (for about 16 h). When the reaction was complete, the solvent was evaporated to dryness, and citric acid water was added and extracted with ethyl acetate (50 mL×3). The combined organic phase was washed with saturated sodium chloride solution and dried with the anhydrous sodium sulfate. Product **4** was purified by flash chromatography (petroleum ether: ethyl acetate = 10/1) 2.45 g (83.6%) yellow oil Rf = 0.3 (petroleum ether/ethyl acetate = 10/1) ^{1}H NMR (400 MHz, DMSO) δ 7.97 (dd, J = 8.16, 1.12 Hz, 1 H), 7.73 (td, J = 7.56, 1.4 Hz, 1 H), 7.56 (td, J = 8.12, 1.4 Hz, 1 H), 7.47 (dd, J = 7.6, 1.12 Hz, 1 H), 4.13 (dd, J = 9.0, 5.6 Hz, 1 H),4.01 (dd, J = 14.32, 7.0 Hz, 2 H), 2.27 (td, J = 7.56, 2.52 Hz, 2 H), 2.09 (s, 3H), 2.07–1.99 (m, 1 H), 1.73–1.64 (m, 1 H), 1.46–1.39 (m, 1 H), 1.31–1.28 (m, 1 H), 1.15 (t, J = 18.76 Hz, 3 H). ^{13}C NMR (100 MHz, DMSO) δ 206.35, 172.98, 150.18, 133.86, 132.94, 130.81, 129.01, 124.94, 60.19, 53.52, 40.61, 40.40, 40.19, 39.98, 39.77, 39.56, 39.36, 33.60, 30.26, 29.28, 22.92, 14.54. HRMS (ESI): *m/z* calcd. for $C_{15}H_{19}NO_5 + Na^+$: 316.13 $[M + Na]^+$, found 316.1153.

2-Hydroxy-3-(2-Nitrophenyl)-Heptanoic acid (**Nph**): Here, **4** was dissolved in 50 mL of methanol, and 0.38 g of sodium borohydride were added slowly in an ice bath and stirred. The reaction was monitored and completed after 30 min, as shown by TLC. The reaction solution was evaporated to dryness to obtain the crude product of **5** (2.53 g).

5 was dissolved in the mixed solvent (5:1) of EtOH and water with 0.4 g NaOH. It was stirred at room temperature for 12 h. The reaction was monitored completely by TLC. The solvent was then evaporated when the reaction was complete. The crude product was washed with hydrochloric acid solution to pH 1 and extracted with DCM. Drying collected the organic layer over Na_2SO_4, followed by concentration. The product **Nph** was purified by flash chromatography (dichloromethane/ methanol = 25/1) as light yellow solid; 1.91 g (85.6%) light yellow solid Rf = 0.6 (dichloromethane/ methanol = 20/1) ^{1}H NMR (400 MHz, DMSO) δ 11.95 (s,1H), 7.68 (dd, J = 7.84, 0.84 Hz, 1H), 7.63-7.55 (m, 2 H), 7.38 (td, J = 7.0, 1.68 Hz, 1 H), 4.64 (d, J = 3.08 Hz, 1 H), 3.73 (d, J = 1.68 Hz, 1 H), 2.87 (dd, J = 13.72, 7.0 Hz, 1 H), 2.13 (dd, J = 13.16, 6.72 Hz, 2 H), 1.69 (dd, J = 15.68, 7.84 Hz, 2H), 1.39-1.28 (m, 1 H), 1.25-1.16 (m, 1 H), 0.89 (d, J = 6.44 Hz, 3 H). ^{13}C NMR (100 MHz, DMSO) δ 174.15, 152.53, 136.21, 132.29, 130.02, 127.47, 123.33, 69.21, 46.09, 34.07, 31.22, 23.01, 22.05. HRMS (ESI): *m/z* calcd. for $C_{13}H_{17}NO_5$-H, 266.11$[M–H]^-$, found 266.1034.

The resin loading of fully protected CP-9 (**10**): The linker (**Nph**) was placed on the amino resin: 0.1 mmol (0.217 g) of amino resin in DCM for swelling for 20 min with **Nph** (0.5 mmol), HCTU (0.5 mmol), and NMM (0.5 mmol). These were dissolved in 6 ml DMF and shaken at room temperature for 2 h. The reaction was monitored by ninhydrin at 100 °C for 5 min. Loading the first amino acid: Fmoc-Lys-OH (0.5 mmol) and DMAP (0.0122 g, 0.10 mmol) were dissolved in 5 mL DCM (0.5 mL DMF for solubilization). Diisopropylcarbodiimide (0.06 g, 0.5 mmol) was slowly dropped into the reaction solution. The solution was shaken at room temperature for 6 h and then fed again.

Sealing: The resin was shaken in 5 mL (1 mL acetic anhydride, 1 mL pyridine, 3 mL DMF) and mixed for 1 h after washing with DMF three times at room temperature.

Deprotection: The resin was shaken in 5 mL of 20% piperidine-DMF solution for 15 min at room temperature and then washed with DMF three times.

Loading with other amino acids: The resin was shaken in the DMF solution containing Fmoc-AA-OH (0.5 mmol), HCTU (0.5 mmol) and NMM (0.5 mmol) for 2 h at room temperature. The reaction was monitored by ninhydrin at 100 °C for 5 min.

Fully Protected CP-9 and Deprotected CP-9 from 10

10 (0.1 g) was dissolved into THF (5 mL) and exposed to a 365 nm ultraviolet lamp for 5, 10, 15, or 20 min; 400 μL of the irradiated solution was removed and detected by HPLC. The liquid phase was 90% acetonitrile-water solution. After purification, the fully protected **CP-9** was obtained (photolysis rate was 74.6%) and identified by mass spectrometry. TOF-MS: *m/z* calculated for 2058.0311$[M + Na]^+$ found 2058.8354.

10 (0.2 g) was added to 20% piperidine-DMF (6 mL) to remove Fmoc protection and allow cleavage (1.8 mL trifluoroacetic acid, 0.1 mL water and 0.1 mL triisopropylsilane) to remove other protective groups. The resin after treatment was put into 5 mL THF and exposed to a 365 nm ultraviolet lamp for 5, 10, 15, 20, or 25 min; 400 μL of the irradiated solution was removed and studied by HPLC at each time point. The liquid phase was 3% acetonitrile-water solution. After purification, the deprotected **CP-9** was obtained (photolysis rate was 67.0%) and identified by mass spectrometry. TOF-MS: *m/z* calculated for 1005.4411$[M + H]^+$, found 1005.4479.

Supporting Information: Further detailed experimental procedures and copies of ^{1}H and ^{13}C NMR spectra as well as other chromatograms disseminated are available as supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: linker molecules · peptides · protecting groups · photolabile compounds · solid-phase synthesis

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