Synthesis of Pentafluorophenyl Esters of Nitroveratryloxycarbonyl-Protected Amino Acids

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Received 16 September 2009

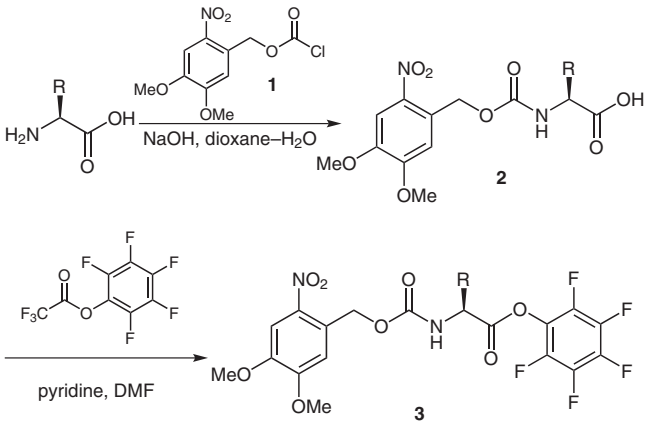
Abstract: For efficient peptide synthesis on a glass chip, 20 kinds of pentafluorophenyl (Pfp) esters of nitroveratryloxycarbonyl (NVOC)-protected amino acids were synthesized by using Pfp trifluoroacetate. Simple purification step gave moderate to high yield. The first loading time of each amino acid on glass surface was 30–60 min. The UV cleavage of the NVOC group was completed within 10 minutes.

Key words: NVOC amino acid, Pfp ester, in situ synthesis, peptide microarray

With the increasing demand for high throughput screening for drug discovery, the diagnosis of disease and immunological assays, the fabrication of biomolecule microarrays has become a crucial technology.[1] Among the techniques used for the fabrication of biomolecule microarrays, photolithographic in situ synthesis on a surface is a powerful method in the field of combinatorial chemistry because a number of molecules can be addressed densely on surface.[2] For the in situ synthesis of peptide microarrays, activated amino acid ester monomers protected by photolabile protecting groups are required.[3] However, when an automated system of solid-phase peptide synthesis is used, the mixtures of coupling agents and protected amino acids are liable to lead to undesired side reactions when they are stored for a long time. Therefore, we designed a more stable active ester of the amino acid monomer for peptide synthesis on a chip in an automated system (Scheme 1).

Several research groups have reported methods of synthesizing pentafluorophenyl (Pfp) esters because they are known to be somewhat stable in aqueous solution and highly active with amino groups.[4] Moreover, Pfp esters can be easily adapted to automated peptide synthesis systems. For example, Pfp esters were used for solid-phase peptide nucleic acid synthesis.[5] Kisfaludy and Schon first reported on the Pfp ester synthesis of 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids. The Fmoc amino acid was coupled with pentafluorophenol by using dicyclohexylcarbodiimide (DCC) as a coupling agent.[6] However, this method required further purification steps, the removal of the insoluble dicyclohexyl urea and the recrystallization of the product. After several years, a new, efficient synthetic method was introduced using Pfp trifluoroacetate.[7] According to the proposed mechanism, no byproducts were formed by virtue of the absence of any coupling reagent. Therefore, the product could be separated with high purity by simple extraction.

In our previous work, we synthesized nitroveratryloxycarbonyl (NVOC) 6-aminocaproic acid and used it as a spacer for protein patterning by photolithography.[8] In this report, 20 kinds of amino acids protected by NVOC **2** were synthesized by the conventional Schotten–Baumann reaction.[9] The amino acids containing a hydrophobic side chain gave no problem in the workup step using aqueous HCl solution. Some of the products having side-chain protecting groups, for example the trityl- or *tert*-butyl-containing amino acids, were difficult to extract with EtOAc. In this case, the insoluble solids were suspended in solution after being treated with aqueous HCl solution, because the side-chain protecting groups were acid labile. However, the products were extracted more easily when they were acidified with acetic acid than when using hydrochloric acid. Their yields were all above 90%.



Scheme 1

The NVOC amino acid Pfp esters were synthesized using Pfp trifluoroacetate. In the case of the Fmoc amino acids, the unreacted Pfp trifluoroacetate and pentafluorophenol were washed out using 5% aqueous $NaHCO_3$.[7] However, we observed that the NVOC amino acid Pfp esters were partially hydrolyzed even in 1% aqueous $NaHCO_3$ solution. Therefore, we tried mild conditions for the workup procedures; 0.1% aqueous $NaHCO_3$ solution was used for the extraction of the byproducts. Alternatively, if the byproducts could not be removed thoroughly using the

SYNLETT 2009, No. 20, pp 3307–3310
Advanced online publication: 18.11.2009
DOI: 10.1055/s-0029-1218369; Art ID: U09309ST

above method, the NVOC amino acid Pfp esters were precipitated with ice water because the products were stable and insoluble in cold water.

The synthetic yields and characterization results of the NVOC amino acid Pfp esters are summarized in Table 1. Glu(*t*-Bu) and Val gave especially low yields because the precipitated products were difficult to separate.

The products were easily characterized by ^{1}H NMR spectroscopy after the reaction. All of the peaks in the NMR spectra of the products **3** showed almost the same proton chemical shifts as the starting materials **2**. However, the chemical shift of the α-carbon proton of the amino acid was obviously changed. The average difference in its chemical shift was ca. 0.3.

To measure the first loading time of each amino acid on a glass chip, NVOC-AA-Pfp ester was spotted on an aminated glass substrate, whose background of which was blocked by a hydrophobic perfluoroalkyl silane.[10] After the reaction, the remaining amino groups were stained with RBITC (rhodamine B isothiocyanate; Figure 1). Based on the fluorescence image, the optimum coupling time of each amino acid was calculated as shown in Table 2. Val, Pro, and Trp showed longer coupling times than the other hydrophobic chain containing amino acids. The coupling times of Asn(Trt) and Asp(*t*-Bu) were longer than those of Glu(Trt) and Gln(*t*-Bu). The amino acids containing bulky side chains such as Arg(Pbf), Lys(Boc), and Tyr(*t*Bu) showed longer coupling times than the other amino acids (over 45 min).

To check the efficiency of the photocleavage of the NVOC group on the surface, NVOC-Ala-Pfp was coupled on the same patterned amino-group-containing surface. After irradiating the NVOC-protected surface with UV using fiber optics, RBITC was attached to the free amino groups and the fluorescence image was obtained from a

Table 1 Characterization of Pfp Esters of NVOC-Protected Amino Acids

Amino acid	Calculated [M + Na$^+$]	Found[a] [M + Na$^+$]	R_f[b]	^{1}H NMR chemical shift of α-carbon proton (ppm)[c]		Yield (%)
				2	**3**	
Gly	503.05	503.12	0.66	4.07	4.40	73
Ala	517.06	517.10	0.71	4.46	4.77	73
Val	545.10	545.15	0.80	4.35	4.70	39
Leu	559.11	559.08	0.86	4.42	4.75	93
Ile	559.11	559.10	0.86	4.42	4.73	74
Pro	543.08	543.13	0.60	4.46	4.75	73
Phe	593.10	593.08	0.77	4.69	5.02	56
Trp	632.11	632.04	0.57	4.24[d]	4.46[d]	41
Met	577.07	577.04	0.74	4.56	4.95	66
Cys(Trt)	791.15	791.04	0.89	4.24	4.40	87
Tyr(*t*-Bu)	665.15	665.03	0.80	4.68	5.00	78
Ser(*t*-Bu)	589.12	589.15	0.77	4.46	4.88	37
Thr(*t*-Bu)	603.14	603.09	0.57	4.36	4.47	99
Lys(Boc)	674.17	674.05	0.60	4.38	4.65	82
Arg(Pbf)	854.21	854.12	0.20	4.41	4.56	90
His(Trt)[e]	825.20	–	–	–	–	–
Asp(O*t*-Bu)	617.12	617.04	0.77	4.65	4.99	25
Glu(O*t*-Bu)	631.13	631.07	0.70	4.40	4.76	89
Asn(Trt)	802.18	802.06	0.71	4.01[d]	4.29[d]	80
Gln(Trt)	816.20	816.11	0.60	4.20	4.65	68

[a] Molecular weight was measured by MALDI-TOF MS.
[b] TLC was performed using hexane–EtOAc (1:1).
[c] NMR solvent was $CDCl_3$, unless otherwise stated.
[d] NMR solvent was DMSO-d_6.
[e] His(Trt) Pfp ester was hydrolyzed under ambient conditions.

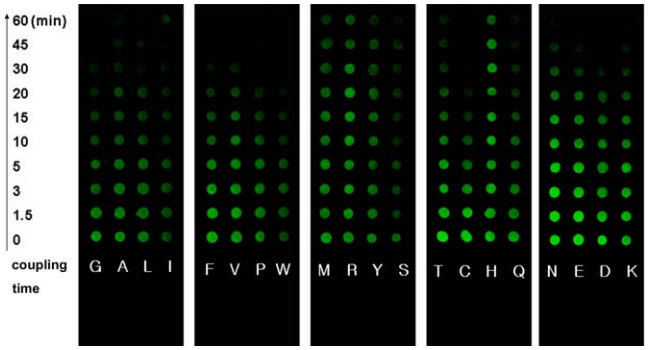


Figure 1 Measurement of the first amino acid coupling time on a patterned glass surface obtained from microarray scanner

Table 2 First Amino Acid Coupling of NVOC-AA-Pfp Esters on the Aminated Surface

Amino acid	Time (min)	Amino acid	Time (min)
Gly	30	Tyr(*t*-Bu)	45
Ala	30	Ser(*t*-Bu)	30
Val	45	Thr(*t*-Bu)	30
Leu	30	Lys(Boc)	45
Ile	30	Arg(Pbf)	>60
Pro	40	His(Trt)	decomp.
Phe	30	Asp(*t*-Bu)	45
Trp	45	Glu(*t*-Bu)	30
Met	30	Asn(Trt)	45
Cys(Trt)	30	Gln(Trt)	30

microarray scanner (Figure 2, a). It was found that the NVOC cleavage reaction was completed within 10 minutes by analyzing the fluorescence intensity (Figure 2, b).

In conclusion, we have presented the simple preparation of NVOC amino acid Pfp esters. The workup procedure provided in this paper gives high product yields with a simple purification step. The first amino acid coupling of each NVOC-AA-Pfp ester on the aminated surface was checked, and the photocleavage of the NVOC group on the surface was completed within 10 minutes. From the result, it was confirmed that NVOC amino acid Pfp esters can be used for automated peptide synthesis systems.

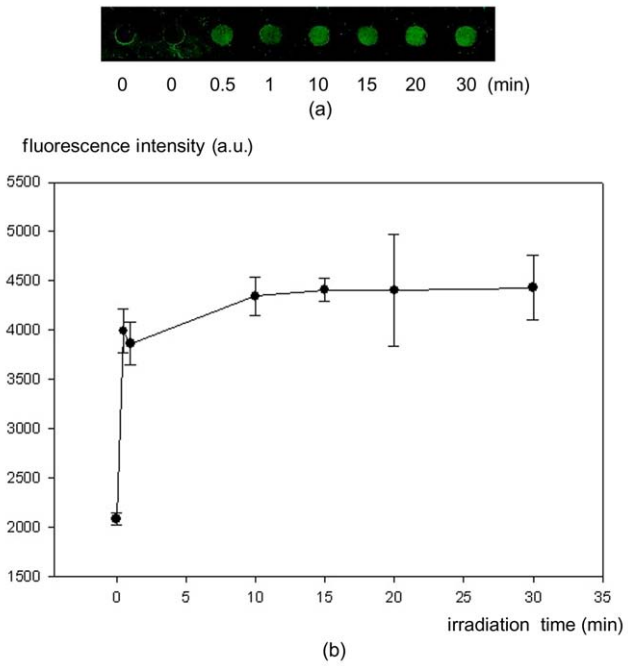


Figure 2 Measurement of photocleavage efficiency on a patterned glass chip: (a) fluorescence image; (b) profile of fluorescence intensity

General Experimental Procedure

Synthesis of NVOC Chloride 1

4,5-Dimethoxy-2-nitrobenzyl alcohol (10.3 g, 48.3 mmol) was suspended/mixed with THF in an ice bath. Phosgene in toluene (20% w/v, 58 mL, 112.6 mmol) was poured into the solution and magnetically stirred. After 1 h, the ice bath was removed, and the reaction solution/mixture was stirred at 25 °C for 12 h. Nitrogen gas was then bubbled for 2 h, and the solvent was evaporated. (13.1 g, yield 97%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.77 (s, 1 H), 7.01 (s, 1 H), 5.74 (s, 2 H), 4.06 (s, 3 H), 3.98 (s, 3 H).

Synthesis of NVOC Amino Acids 2

The amino acids (2 mmol) were dissolved in 2 N aq NaOH solution (1 mL) in an ice bath. NVOC chloride (662 mg, 2.4 mmol) in 1,4-dioxane (1 mL) and CH_2Cl_2 (4 mL) and 2 N aq NaOH solution (1.1 mL) were inserted in 10 times by turns. After stirring for 1 h, the ice bath was removed, the solution continuously stirred for a further 4–12 h and monitored by TLC. The organic layer was removed, and the aqueous layer was acidified to pH 3–4 using 5 N aq HCl solution. The side-chain-protected amino acids were acidified by AcOH. The product was extracted with EtOAc, dried with $MgSO_4$, and the solvent was evaporated. In the case of the products acidified by AcOH, the small amount of AcOH residue was evaporated azeotropically three times with hexane.

Synthesis of NVOC Amino Acid Pfp Esters 3

The NVOC amino acid (1 mmol) was dissolved in DMF (2 mL). Pyridine (95 mg, 1.2 mmol) and Pfp trifluoroacetate (336 mg, 1.2 mmol) were added to the solution, which was then stirred magnetically for 1 h at r.t. After the reaction, EtOAc (100 mL) was added to the solution. The organic layer was washed with 0.1 N aq HCl and 0.1% aq $NaHCO_3$, dried with $MgSO_4$, and evaporated. Another approach to the workup method was also attempted, in which the solution was poured into ice water and removed by filtration. The insoluble residue was collected, washed with water, and dried in vacuo.

Glass-Slide Patterning

Glass slides (7.5 cm × 2.5 cm) were treated with H_2SO_4–H_2O_2 (4:1) for 10 min and cleaned with H_2O and EtOH. The slides were patterned with photoresist (PR, AZ 4330) by selective photolithography using a photomask and then treated with 0.1% 1*H*,1*H*,2*H*,2*H*-perfluorodecylmethyldichlorosilane in isooctane at 25 °C for 20 min.[11] The slides were washed with hexane, and the PR was finally removed using acetone. Epoxidation with 3-glycidoxypropyltrimethoxysilane (GPTS) was carried out at 45 °C in 5% $CHCl_3$ solution for 12 h. To remove the noncovalently adsorbed silane molecules, sonication in $CHCl_3$ was performed for 10 min. The GPTS-introduced glass slides were treated with 1% (w/v/v) chitosan and

AcOH in H_2O to generate amino groups on the spots. The slides were rinsed with 1% (v/v) AcOH in H_2O and MeOH and dried under nitrogen gas.

First Amino Acid Loading on a Patterned Glass Slide

NVOC-AA-Pfp (5 mM)/DIEA in NMP solution was spotted on the aminated surface with gradually increasing reaction times. The slide was rinsed with NMP and MeOH and dried under nitrogen gas. Then, droplets of DIEA (10 mM) and RBITC/NMP were added to the reaction spots to label free amino groups. The slide was rinsed with NMP and MeOH and dried under nitrogen gas. The fluorescence image was obtained using a microarray scanner (Axon Instrument, GenePix 4000B).

Photocleavage Reaction on a Patterned Glass Slide

The amino-modified region of the glass substrate was treated with NVOC-Ala-Pfp (5 mM)/DIEA in NMP for 30 min. The slide was rinsed with NMP and MeOH and dried under nitrogen gas. The unreacted amino groups were capped with 10% Ac_2O/10% pyridine in NMP for 10 min. The specific site in the solution of H_2SO_4 (5 mM)/1,4-dioxane was irradiated with UV in the proper direction by using an optical fiber (Hamamatsu Photonics, Mercury-Xenon lamp # L2570; 360 nm, 30 mW/cm^2). The slide was rinsed with 0.1% DIPEA/NMP and MeOH and dried under nitrogen gas. After the RBITC reaction, the fluorescence image was obtained by the same method.

Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2007-357-D00057), the WCU (World Class University) program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology (grant number R32-2008-000-10213-0), Seoul R & BD program (10538), the Nano-Systems Institute-National Core Research Center (NSI-NCRC) program and Korea Biotech R&D Group of next-generation growth engine project of the Ministry of Education, Science and Technology.

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