Facile Synthesis of *N*-(9-Fluorenylmethyloxycarbonyl)-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic Acid as a Photocleavable Linker for Solid-Phase Peptide Synthesis

Jaehi Kim,* San Kyeong, Dong-Sik Shin, Sewon Yeo, Joonhyuk Yim, Yoon-Sik Lee

School of Chemical and Biological Engineering, Seoul National University, Gwanak-Gu, Seoul 151-744, Korea
Fax +82(2)8769625; E-mail: yslee@snu.ac.kr

Received: 26.12.2012; Accepted after revision: 05.02.2013

Abstract: A photocleavable linker, *N*-(9-fluorenylmethyloxycarbonyl)-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic acid was synthesized from veratraldehyde, with simple reaction and separation steps. This linker was stable under the normal solid-phase peptide synthesis conditions and rearranged to a labile form when irradiated with UV light (λ = 365 nm). A model peptide sequence (YGGFL) was synthesized and released from solid supports with high efficiency by UV irradiation.

Key words: photochemistry, photocleavable linker, solid-phase synthesis, amino acid derivative, peptides

Since its invention by R. B. Merrifield in 1963,[1] the solid-phase peptide synthesis (SPPS) method has been widely used. By virtue of the SPPS method, the construction of one-bead one-compound (OBOC) combinatorial peptide libraries, for screening of various ligands or enzymatic substrates, has been possible.[2] For successful SPPS and bioassay, a linker that connects peptides to polymer matrices should be orthogonally stable to the reaction conditions, for building up the peptide sequences on the polymer supports. Various linkers that are labile to acid, base, or chemicals have been developed to release peptides from the polymer supports.[3] However, a bioassay system by the released peptides could be damaged under these cleavage conditions. Since photolabile linkers are stable with chemicals, and produce a minimum amount of byproducts, they are more attractive than other linkers in the SPPS and bioassay.

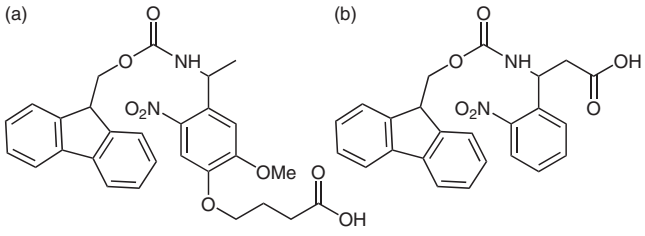
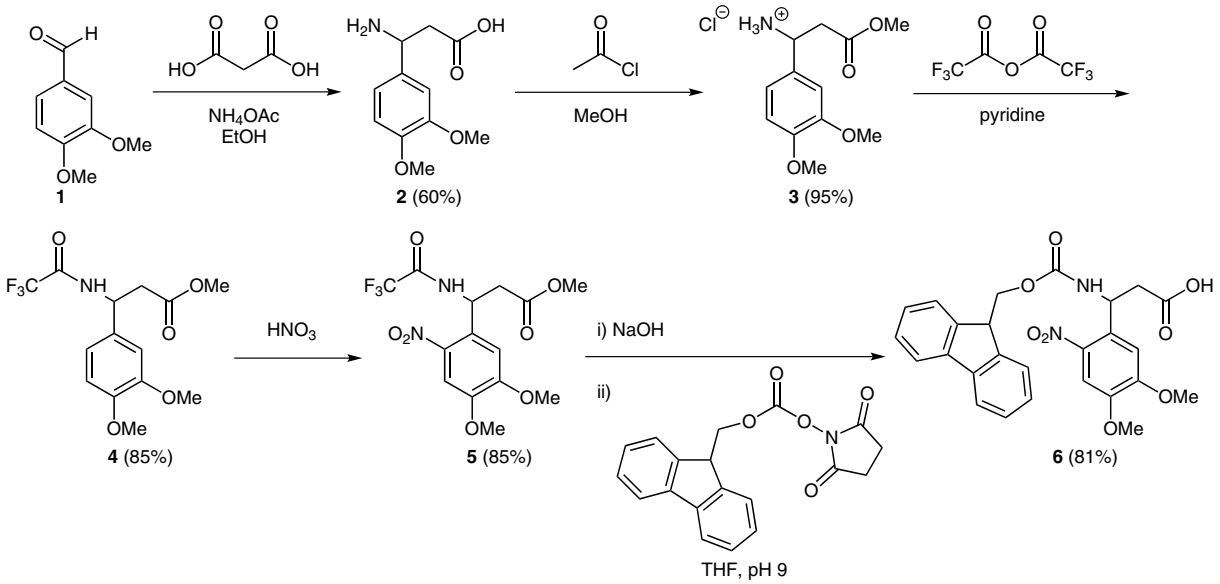


Figure 1 Photoreactive linkers

Holmes et al. and Geysen et al. reported the linkers that can be rearranged to the labile form by UV irradiation.[4] 4-{4-[1-(9-Fluorenylmethyloxycarbonylamino)ethyl]-2-methoxy-5-nitrophenoxy}butanoic acid (Fmoc-photolabile linker) was synthesized from acetovanillone via condensation reaction with hydroxylamine (Figure 1, a),[4a] and *N*-(9-fluorenylmethyloxycarbonyl)-3-amino-3-(2-nitrophenyl)propionic acid (Fmoc-ANP linker) was synthesized from 2-nitrobenzaldehyde via Perkin-type condensation reaction (Figure 1, b).[4b] These linkers can release synthesized peptides from solid phase, by radical rearrangement of the *o*-nitrobenzyl group, when they are exposed to UV light (λ = 365 nm). Since the peptides that are released from the polymer supports are of stable amide form, the linkers are quite suitable for OBOC peptide library synthesis. However, these linkers have not been widely used, because their synthetic routes are troublesome. In the synthesis of the Fmoc-photolabile linker, the hydrogenation of ketoxime to amine was tedious and time-consuming.[4a] The synthetic route of the Fmoc-ANP linker was quite simple, compared to the Fmoc-photolabile linker. However, the Perkin-type condensation reaction of 2-nitrobenzaldehyde with malonic acid gave low yield (27%), because of the electron-withdrawing effect of the *o*-nitro group.[4b] Therefore, in this paper, we report on the novel synthesis method of a photocleavable linker that has high photocleavable efficiency and its application in SPPS.

Starting from veratraldehyde, our synthetic pathway for a photocleavable linker consists of serial six-step reactions (Scheme 1). Compared to 2-nitrobenzaldehyde, the aldehyde group of veratraldehyde is less hindered, and two methoxy groups are expected to boost the Perkin-type condensation reaction by their electron-donation effect.[5] Taking these into consideration, 3-amino-3-(3,4-dimethoxyphenyl)propionic acid was synthesized under mild conditions, in higher yield than from 3-amino-3-(2-nitrophenyl)propionic acid. Prior to performing nitration, the carboxylic acid and the amino groups were protected with conventional methods (95% and 85%, respectively). After nitration, we found that the nitro group was introduced only on the 2-position, in the same way as veratraldehyde was nitrated. The nitration was selectively performed because of the electron-donating effect of *m*-methoxy groups and steric hindrance. After deprotection of the trifluoroacetyl group and hydrolysis of methyl ester by saponification, 9-fluorenylmethyloxycarbonyl (Fmoc) group was introduced to the free amino group under the Schotten–Baumann conditions. After following these steps, *N*-(9-fluorenylmethyloxycarbonyl)-3-amino-3-

SYNLETT 2013, 24, 0733–0736
Advanced online publication: 04.03.2013
DOI: 10.1055/s-0032-1318314; Art ID: ST-2012-U1101-L



Scheme 1 Synthetic pathway of the Fmoc-PCA linker

(4,5-dimethoxy-2-nitrophenyl) propionic acid (Fmoc-PCA linker, named after photocleavable amino acid) was synthesized in overall 33% yield. It is notable that the overall yield through our synthetic strategy is higher than that from the literature,[4] and no chromatographic separation is required during all the reaction steps.

As a preliminary test, the photocleavage yield of the PCA linker was estimated by measuring the amount of released product, Fmoc-Phe-NH_2. For this, PCA linker and Fmoc-Phe-OH were serially introduced to the core-shell-type HiCore resin, which was proved to be ideal for solid-phase photochemical reaction.[6] Then, Fmoc-Phe-NH_2 was released by UV irradiation ($\lambda = 365$ nm) on the resin. The photocleavage yield of Fmoc-Phe-NH_2 increased over time, and reached ca. 50% in 20 minutes. This result is comparable to those with a commercially available Fmoc-photolabile linker.[6,7] Similar or better efficiency of photocleavage reaction confirms that the PCA linker is applicable to on-bead assays.[2b,c]

As a performance test of the PCA linker in the solid-phase peptide synthesis, Leu-enkephaline (YGGFL) was synthesized on the HiCore resin with Fmoc/*t*-Bu chemistry (see experimental procedures for details). After building up the peptide on the resin, Leu-enkephaline was released as an amide form from the resin by UV irradiation. The purity of the released peptide was confirmed by HPLC (Figure 2) and MALDI-TOF MS analyses {$[M + H]^+$: 555.62, $[M + Na]^+$: 577.56, Figure 3}. Overall, this result proves that the PCA linker is stable under acidic and basic conditions in Fmoc/*t*-Bu chemistry, including piperidine or trifluoroacetic acid treatment.

In conclusion, we successfully synthesized the Fmoc-PCA linker from veratraldehyde in six steps, without any column chromatography. The Fmoc-PCA linker was stable under typical solid-phase peptide synthesis conditions, and ca. 50% of the synthesized peptide was released by 20 minutes of UV irradiation. Based on these results, we envision that the Fmoc-PCA linker can be used in solid-phase peptide synthesis for on-bead assay. Moreover, our

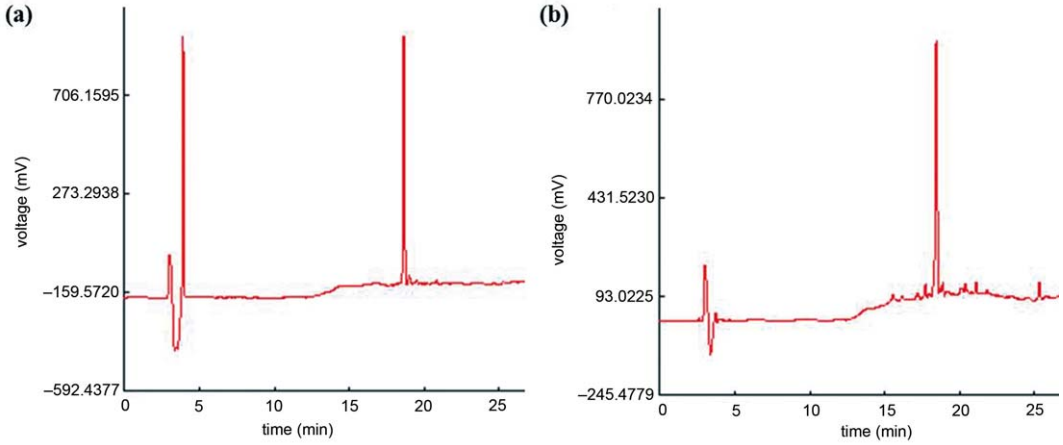


Figure 2 (a) HPLC analysis data of purified H-YGGFL-NH_2 and (b) photocleaved H-YGGFL-NH_2 by using the Fmoc-PCA linker

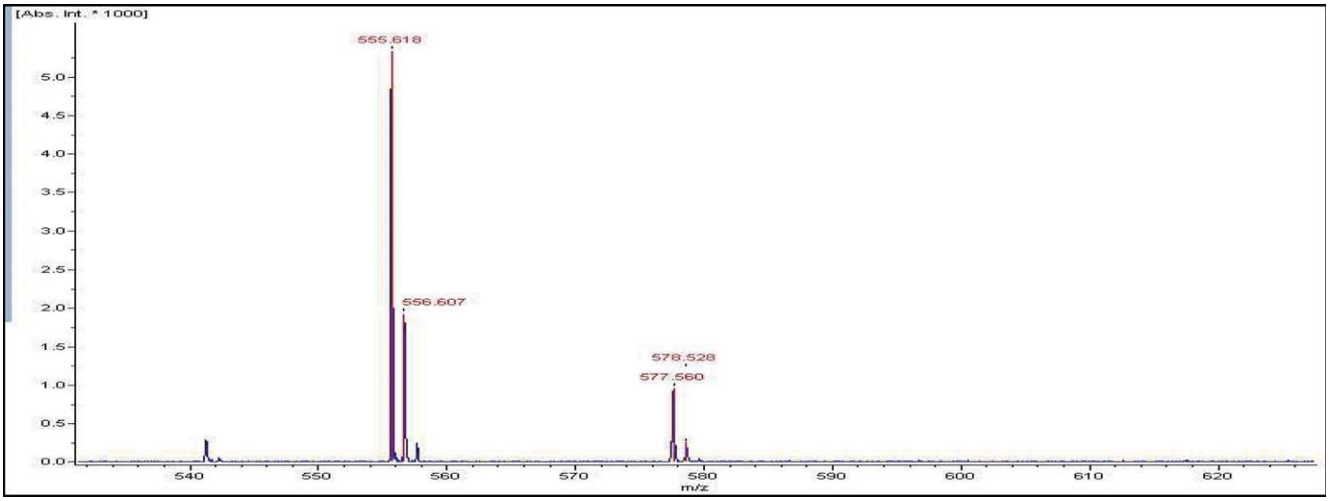


Figure 3 MALDI-TOF MS analysis of H-YGGFL-NH_2 synthesized from HiCore resin by using Fmoc-PCA linker

synthetic strategy of the PCA linker is more convenient and efficient than the previously reported Fmoc-photolabile linkers. Therefore, we expect that the PCA linker can be widely used in SPPS for on-bead assay.

3-Amino-3-(3,4-dimethoxyphenyl)propionic Acid (2)

Well-crushed veratraldehyde (2.5 g, 15.05 mmol), NH_4OAc (4.64 g, 60.18 mmol), and malonic acid (6.26 g, 60.18 mmol) were dissolved in EtOH (150 mL). The reaction solution was refluxed during 18 h. After reaction, the white solids could be separated by filtration. This white compound was gently washed with cold EtOH about three or more times till the filtrate become colorless, and dried in vacuo. 3-Amino-3-(3,4-dimethoxyphenyl)propionic acid (225.24 g/mol) was obtained as a white powder, after drying; yield: 2.046 g (60%); mp 213–216 °C. ^{1}H NMR (400 MHz, D_2O/K_2CO_3): δ = 7.04–6.99 (m, 3 H), 4.55 (t, *J* = 7.1 Hz, 1 H), 3.83 (s, 3 H), 3.81 (s, 3 H), 2.86 (dd, *J* = 7.9, 9.1 Hz, 1 H), 2.75 (dd, *J* = 6.8, 9.9 Hz, 1 H). ^{13}C NMR (100 MHz, D_2O/NaOH): δ = 183.00, 150.78, 149.84, 140.76, 121.70, 114.64, 112.88, 58.58, 58.52, 55.47, 49.80 ppm. ESI-HRMS (–): *m/z* calcd: 225.24; found: 224.0928.

1-(3,4-Dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium Chloride (3)

3-Amino-3-(3,4-dimethoxyphenyl)propionic acid (1.6 g, 7.10 mmol) was suspended in anhyd MeOH (150 mL), and acetyl chloride (1.5 mL, 21.37 mmol) was added to the suspension. The reaction mixture was stirred and refluxed for 3 h. 1-(3,4-Dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium chloride (275.73 g/mol) was obtained as white salt form, after evaporation of MeOH and drying in vacuo; yield: 1.860 g (95%); mp 183–185 °C. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.52–6.86 (m, 3 H), 4.66 (t, *J* = 7.1 Hz, 1 H), 3.84 (s, 3 H), 3.80 (s, 3 H), 3.65 (s, 3 H), 3.17 (dd, *J* = 7.9, 9.1 Hz, 1 H), 3.01 (dd, *J* = 6.8, 9.9 Hz, 1 H). ^{13}C NMR (100 MHz, CD_3OD): δ = 171.86, 151.38, 150.97, 129.88, 121.37, 113.17, 112.22, 56.87, 56.64, 53.12, 52.88, 39.42 ppm. ESI-HRMS (+): *m/z* calcd: 275.73; found: 240.1229.

Synthesis of Methyl 3-(3,4-Dimethoxyphenyl)-3-(2,2,2-trifluoroacetamido)propanoate (4)

Methyl 1-(3,4-dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium chloride (1.860 g, 6.75 mmol) was dispersed into pyridine (80 mL). The reaction solution was cooled to 0 °C with an ice bath, and TFAA (1.2 mL, 8.50 mmol) was added to the vigorously stirred solution. After 1 h, the reaction solution was poured into a separation funnel with EtOAc. This solution was acidified with 6 M HCl until the pH value of the solution reached about 2–3, and the resulting two layers were partitioned. The aqueous layer was thrown, and the remaining organic layer was washed with aq 0.5 N $NaHCO_3$ solution, collected, and dried by using $MgSO_4$. The dried organic layer was filtered and evaporated under reduced pressure. As a result, methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoroacetamido)propanoate (335.28 g/mol) was obtained as yellow crystals; yield: 1.921 g (85%); mp 85–86 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.01–6.86 (m, 3 H), 5.24 (qr, *J* = 7.7 Hz, 1 H), 3.76 (s, 3 H), 3.74 (s, 3 H), 3.60 (s, 3 H), 2.99 (dd, *J* = 9.3, 9.6 Hz, 1 H), 2.87 (dd, *J* = 6.1, 5.4 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 170.45, 148.84, 148.41, 132.91, 118.61, 111.73, 110.50, 55.51, 51.50, 50.06 ppm. ESI-HRMS (+): *m/z* calcd: 335.28; found: 336.1055.

Synthesis Methyl 3-(4,5-Dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoroacetamido)propanoate (5)

Nitration of methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoroacetamido)propanoate was performed as follows. About 20 mL of 70% HNO_3 was slowly added to methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoroacetamido)propanoate (1.921 g, 5.73 mmol) in an ice bath. The solution was stirred in a dark place for 2 h, and the resulting orange-colored reaction solution was quenched by pouring cold H_2O (ca. 500 mL, about ten or more times the HNO_3 volume) into the reaction solution. A light yellow solid was obtained from the solution by chilling at 4 °C overnight and filtration. The collected solid was washed with H_2O three times. Methyl 3-(4,5-dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoroacetamido)propanoate (380.27 g/mol) was obtained as ivory powder after drying in vacuo; yield: 1.844 g (85%); mp 167–169 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.57 (s, 1 H), 7.28 (s, 1 H), 5.24 (qr, *J* = 7.7 Hz, 1 H), 3.76 (s, 3 H), 3.74 (s, 3 H), 3.60 (s, 3 H), 2.99 (dd, *J* = 9.3, 9.6 Hz, 1 H), 2.87 (dd, *J* = 6.1, 5.4 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 169.99, 153.28, 147.89, 140.15, 129.90, 109.98, 107.63, 56.27, 56.09, 39.93, 39.51 ppm. ESI-HRMS (+): *m/z* calcd: 380.27; found: 381.0859.

Synthesis of Fmoc-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic Acid (6)

Methyl 3-(4,5-dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoroacetamido)propanoate (1.844 g, 4.85 mmol) was dissolved into 0.1 M NaOH solution (150 mL). The solution was stirred and refluxed for 5 h and cooled to r.t. After that, the pH of the solution was adjusted to about 7 by adding 6 M HCl, until the color of solution changed to yellow. The resulting solution was stirred in an ice, followed by the addition of DIPEA (1.2 mL). After that, a solution of 1.083 g of Fmoc-OSu in THF (50 mL) was added slowly to the reaction mixture and stirred for 1 h in an ice bath, and overnight at r.t. After THF was evaporated from the reaction mixture by reduced pressure, the remaining aqueous solution was slowly acidified to pH 2–3 with 6

M HCl, and the precipitated product was separated by centrifugation. Fmoc-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic acid (Fmoc-PCA linker) was obtained as a brown powder after freeze drying; yield: 1.942 g (81%); mp 210–215 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.38 (s, 1 H), 8.12 (d, *J* = 8.0 Hz, 1 H), 7.88–7.24 (m, 10 H), 5.57 (qr, *J* = 7.4 Hz, 1 H), 4.27 (d, *J* = 5.8 Hz, 2 H), 4.18 (t, *J* = 6.7 Hz, 1 H), 3.87 (s, 3 H), 3.85 (s, 3 H), 2.70 (d, *J* = 6.6 Hz, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.37, 155.28, 153.17, 147.38, 143.93, 143.54, 140.73, 140.69, 139.91, 133.08, 127.61, 126.98, 126.90, 125.04, 120.14, 120.10, 109.90, 107.31, 65.41, 56.20, 56.04, 47.56, 46.62 ppm. ESI-HRMS (+): *m/z* calcd: 492.48; found: 493.1613.

Preparation of Fmoc-Phe-NH_2 for the Calculation of the Photocleavage Yield

HiCore resin (0.3 mmol/g, 100 mg) was swollen in DMF. Fmoc-PCA linker was introduced on the resin with the BOP coupling method for 2 h, and the Fmoc group was removed by using 20% (v/v) piperidine in DMF for 1 h. Fmoc-Phe-OH was coupled onto the resin with BOP coupling in the same manner. After perfect drying, about 10 mg of resin was transferred into 2 mL microtubes, and DMF (1 mL) was added. The resin was irradiated by UV light (λ = 365 nm, 50 mW) for a certain period of time (1–20 min). Samples were analyzed by HPLC, and the amount of Fmoc-Phe-NH_2 was calculated with standard curve.

Preparation and Analysis of H-YGGFL-NH_2

HiCore resin (0.3 mmol/g, 100 mg) was swollen in DMF. Fmoc-PCA linker was introduced on the resin with the BOP coupling method for 2 h, and the Fmoc group was removed by using 20% (v/v) piperidine in DMF for 1 h. The reaction mixture of the resulting resin and 4 equiv of Fmoc-amino acids, BOP, HOBt, and 8 equiv of DIPEA in DMF was shaken for 2 h. When each coupling reaction was ended, the resins were washed with DMF, CH_2Cl_2, and MeOH about three times. The completion of coupling was confirmed by Kasier's ninhydrin test. After whole peptide sequences were coupled on the resin, the side-chain protecting groups were removed by using the cleavage cocktail (TFA–3,6-dioxa-1,8-octanedithiol–H_2O–triisopropylsilane = 94:2.5:2.5:1, volume ratio) for 1 h. The resins were washed with TFA, CH_2Cl_2, and MeOH, about three times. About 10 mg of the resin was sampled in 2 mL microtube, and MeOH (1 mL) was added. UV light (λ = 365 nm, 50 mW) was irradiated to the microtube for 20 min. The peptide MeOH solution was sampled and analyzed by HPLC and MALDI-TOF.

Acknowledgment

This research was supported by the Pioneer Research Center Program, through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (Grant Number 2012-0000460).

References

(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.
(2) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature (London)* **1991**, *354*, 82. (b) Kim, Y. G.; Shin, D. S.; Kim, E. M.; Park, H. Y.; Lee, C. S.; Kim, J. H.; Lee, B. S.; Lee, Y. S.; Kim, B. G. *Angew. Chem. Int. Ed.* **2007**, *119*, 5408. (c) Shin, D. S.; Kim, Y. G.; Kim, E. M.; Kim, M.; Park, H. Y.; Kim, J. H.; Lee, B. S.; Kim, B. G.; Lee, Y. S. *J. Comb. Chem.* **2008**, *10*, 20.
(3) (a) Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787. (b) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 538. (c) Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1971**, 636.
(4) (a) Holmes, C. P.; Jones, D. G. *J. Org. Chem.* **1995**, *60*, 2318. (b) Brown, B. B.; Wagner, D. S.; Geysen, H. M. *Mol. Diversity* **1995**, *1*, 4.
(5) Tan, C.; Weaver, D. *Tetrahedron* **2002**, *58*, 7449.
(6) Kim, H.; Cho, J. K.; Chung, W. J.; Lee, Y. S. *Org. Lett.* **2004**, *6*, 3273.
(7) Cho, J. K.; Park, B. D.; Lee, Y. S. *Tetrahedron Lett.* **2000**, *41*, 7481.

