Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 2507–2510

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Tag Introducible (3-Trifluoromethyl)phenyldiazirine Based Photoreactive Phenylalanine

Makoto Hashimoto,[a,*] Yasumaru Hatanaka,[b] Yutaka Sadakane[b] and Kensuke Nabeta[a]

[a]*Department of Bioresource Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080-8555, Hokkaido, Japan*

[b]*Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2360 Sugitani, Toyama, 930-0194 Japan*

Received 23 May 2002; accepted 3 July 2002

Abstract—An efficient synthesis of tag introducible (3-trifluoromethyl)phenyldiazirine based phenylalanine derivatives is described. Alkylation of a chiral glycine equivalent with a spacer containing (3-trifluoromethyl)phenyldiazirinyl bromides enables us to create photoreactive L-phenylalanine derivatives. After introduction of biotin at the spacer, the biotinylated and photoreactive amino acid was applied for L-amino acid oxidase and incorporated into a substrate binding site. These compounds will be powerful tools not only for photoaffinity labeling to elucidate properties of bioactive peptides but also as trifunctional photophors to introduce a ligand skeleton.

Photoaffinity labeling is a powerful method in the study of biological structure and function.[1–6] It will be suitable for analysis of biological interactions in vivo because it is based on the affinity of the ligand moiety. However, because of the low photolabeling yield, purification and isolation of labeled components are hampered.[6–8] We have been encouraged to try to resolve these difficulties with a combination of avidin-biotin systems (photoaffinity biotinylation)[9–15] or immune-interaction of photolabeling reagents.[16] The combinations enable us to detect and isolate photolabeled proteins and peptides without use of radioisotopes. It is well known that many peptides are potentially bioactive to receptors and other biomolecules. Several papers have reported that photoaffinity labeling could be utilized to investigate the functions of these bioactive peptides.[17,18] Two classes of photoreactive peptides have been used, synthetic peptides containing photoreactive amino acids[17] and photoactivatable derivatives of peptides made by chemical modification of natural products.[18] In both cases tag moieties (biotin or fluorescent group etc.) were individually introduced at the N- or C- terminal of the photoreactive peptides by chemical methods.

Various photophors such as phenyldiazirine, arylazide and benzophenone, have been used. Comparative irradiation studies of these three photophors for living cells suggested that a carbene precursor (3-trifluoromethyl)phenyldiazirine is the most promising.[8] Recently, photoreactive amino acid derivatives containing benzophenone units have been utilized since the compounds are commercially available. However, comparative incorporation studies of photoreactive plasminogen activator peptide with benzophenone or (3-trifluoromethyl)phenyldiazirine at the same position revealed that yield of the latter peptide was higher than the former.[19] Furthermore, it has been reported that the higher specific labeling by (3-trifluoromethyl) phenyldiazirine results from its closer proximity to the target biomolecule than that by benzophenone that limits the reaction radius.[19] There are only few reports on the synthesis of (3-trifluoromethyl)phenyldiazirine based amino acids, because of the complicated synthesis of the diazirinyl three membered ring. One method was the synthesis of racemic *N*-acetyl amino acid followed by selective deacetylation of the L-isomer by acylase.[20,21] Another involved the diastereoselective alkylation of the glycine moiety of the complex **4** with diazirinylx benzyl halide. This afforded the optically pure photoreactive L-phenylalanine.[22] Both methods were applied to the unsubstituted (3-trifluoromethyl) phenyldiazirinyl compound (Fig. 1).

*Corresponding author. Tel.: +81-155-49-5542; fax: +81-155-49-5577; e-mail: hasimoto@obihiro.ac.jp

0960-894X/02/$ - see front matter
PII: S0960-894X(02)00522-X

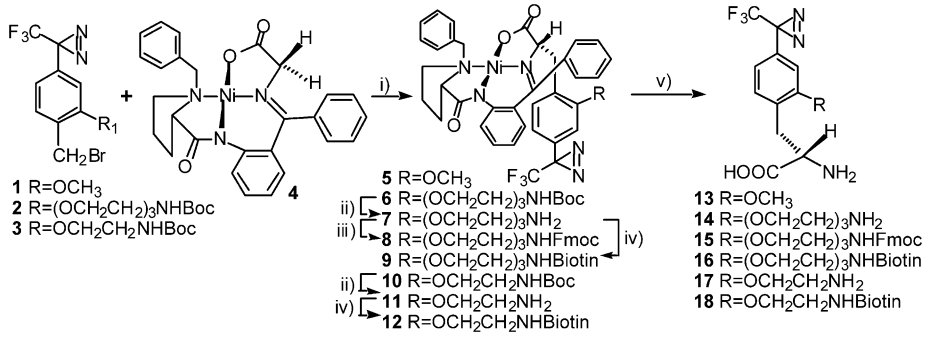

Figure 1. Synthesis of tag introducible photoreactive amino acid derivatives. (i) NaOH, CH_3CN, room temperature, 75, 70 and 68% for **5**, **6** and **10**, respectively, (ii) 50% trifluoroacetic acid–CH_2Cl_2, room temperature, 85 and 92% for **7** and **11**, (iii) Fmoc–OSu, CH_2Cl_2, triethylamine, room temperature, 85%, (iv) Biotin-OSu, DMF, triethylamine, room temperature, 87 and 75% for **9** and **12**, (v) 1 N HCl, methanol, reflux, 65–72%.

In this report, we describe the enantioselective synthesis of tag introducible (3-trifluoromethyl)phenyldiazirinyl based phenylalanine derivatives to achieve one step introduction of a tag group into an amino acid. Furthermore, we tested the properties of biotinylated phenylalanine derivatives.

We synthesized amino acid derivatives of the 3-methoxy substituted compound, the parent skeleton in our diazirine synthesis. The condensation of diazirinyl bromide **1**[23] and the Ni complex **4**[24] proceeded in the presence of powdered sodium hydroxide in acetonitrile at room temperature to afford the alkylated compound **5** without diastereomer. The Ni complex **5** was heated with 1 N HCl in methanol to yield the photoreactive amino acid derivative **13**.[25]

We have already reported that introduction of a poly (ethyleneglycol)-type spacer between the photophor and the tag moiety is useful to increase the solubility in aqueous solution,[9] although it cannot be applied for the azide photophor.[26] Hence we tried to establish useful synthetic routes in which the introduction of tag components is carried out at a later stage.

The Boc protected diazirinyl bromides **2** and **3**[27] were treated with **4** as described above to produce compounds **6** and **10**. These compounds contained two acid labile parts (the Ni complex and the Boc group). The conditions for releasing amino acid (1 N HCl–methanol, reflux) deprotected both the Ni complex and Boc groups to afford the diamino compounds **14** and **17**. It was difficult to perform mono amino acylation for these compounds. Selective deprotection of the Boc group in the Ni complex was achieved with 50% TFA–CH_2Cl_2 at room temperature to afford the amino containing Ni complexes **7** and **11**. FmocOSu was easily reacted with in the usual manner. Subsequent acid hydrolysis decomposed the Ni complex affording Fmoc protected the amino acid derivative **15**.

The amino group at the spacer moiety in the Ni complex enables us to introduce any tags via the amide bond.

For example, the amino Ni compounds **7** and **11** were reacted with (+)-biotin *N*-hydroxysuccinimide in the presence of triethylamine at room temperature. HCl treatment of the biotinylated Ni complexes **9** and **12** afforded the biotinylated phenyldiazirinyl amino acids **16** and **18**, respectively.[25] Both compounds have typical diazirine UV spectra, with broad maxima near 350 nm. This adsorption peak diminished smoothly on irradiation with black light. (Fig. 2 for **18**, $t_{1/2}$ 2.3 and 2.6 min for **16** and **18**, respectively.)

Determination of the optical purity of these derivatives was attempted by chiral HPLC before and after Boc protection at the α-amino group. However, optimization is difficult due to the ethylene glycol spacers. To assign the stereochemistry correctly, these compounds were subjected to L- and D-amino acid oxidase reactions. The

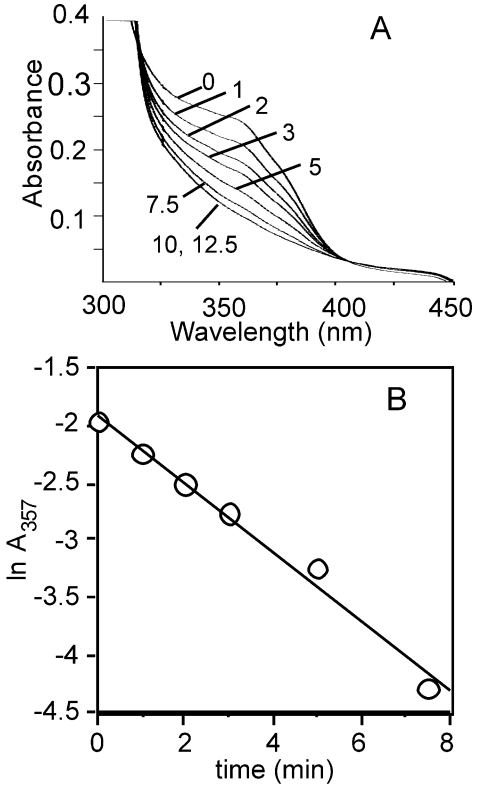

Figure 2. Photolysis of amino acid analogue **18** in methanol. (A) UV spectra of the photolysis reaction mixture at times (in min) indicated with numbers. (B) The decay of the absorbance at 357 nm as a function of time of photolysis in a semi-log representation.

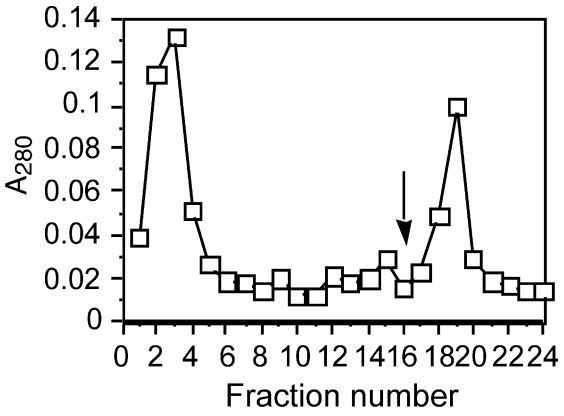

Figure 3. Purification of biotinylated photoreactive amino acid **18** on an immobilized monomeric avidin column (2 mL gel, 50 nmol equivalent). Compound **18** (1.6 μmol) in methanol–water (1:4, 0.25 mL) was loaded onto the gel at room temperature, then washed with 0.1 M sodium phosphate (pH 7.0). The arrow indicates treatment of the column with 2 mM d-biotin in 0.1 M sodium phosphate (pH 7.0). One mL fractions were collected.

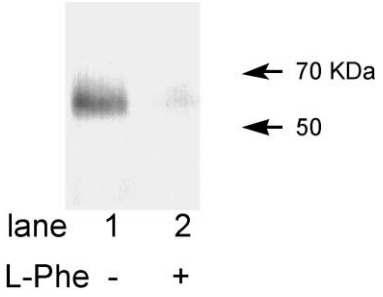

Figure 4. Photoaffinity labeling of L-amino acid oxidase with compound **18**. The compound **18** (15 μM) was incubated with the enzymes (0.2 μM) at 37 °C for 5 min, then irradiated at 0 °C for 20 min. The irradiated samples were subjected to SDS-PAGE (10% gel), blotting to PVDF membrane. The transferred membrane was treated with chemiluminescence detection as previously reported.[10] Lane 1 irradiated with compound **18** without L-phenylalanine, lane 2 with 0.1 M L-phenylalanine.

amino acid oxidases catalyze the oxidative deamination of the optically pure amino acids to yield their corresponding α-keto acids.[28] Those with shorter spacers, **13** and **18** were easily converted to α-keto acid with L-amino acid oxidase, whereas that with the longer spacer **16** was not. On the other hand, these compounds were not substrates for D-amino acid oxidase and the optical rotations did not change after enzymatic reactions. The results show that compounds **13** and **18** are optically pure (calculated as over 90%ee from optical rotations before and after enzymatic reactions), but the compound with long spacer caused loss of affinity for L-amino acid oxidase.

To ensure the biotin properties of the synthetic amino acids, the compound **18** was applied to an immobilized monomeric avidin column and treated as described in our previous work.[10] The UV adsorption profile indicated that the compound was absorbed on the column and easily eluted with excess biotin (Fig. 3)

L-Amino acid oxidase is a dimeric glycoprotein containing two noncovalently bound flavin adenine dinucleotides (FAD) per protein molecule.[29] Some affinity probes of flavin analogues were used to elucidate the flavin binding sites for typical flavoproteins.[30–33] The results provide very important information in determining the role of flavin compounds in enzymatic reactions. Less information, however, is available on the binding sites of enzyme substrates. The enzymatic resolution studies indicated that biotinylated compound 18 was suitable for photoaffinity labeling of L-amino acid oxidase. The diazirinyl biotinylated amino acid 18 was incubated with L-amino acid oxidase derived from *Crotalus adamanteus venom* at 37 °C for 5 min, then irradiated at 0 °C for 20 min. The irradiated sample was subjected to SDS-PAGE, blotting to PVDF membrane and chemilminescence detection of biotinylated components as previously reported.[15] The biotinylation of the enzyme was detected at near 68 kDa as a single band[34] (Fig. 4, lane 1). The signal was completely inhibited in the presence of large amounts of L-phenylalanine (Fig. 4 lane 2). The biotinylated amino acid analogue 18 was recognized as substrate for L-amino acid oxidase.

We conclude that the biotinylated photoreactive phenylalanine will be useful for photoaffinity labeling of bioactive peptides in binding site studies. The shorter spacer one was recognized as substrate for L-amino acid oxidase. The compound should be useful in elucidating functions of enzymes that recognize single amino acids. Furthermore, these photoreactive reagents have trifunctional groups, two amines and a carboxylic acid, in their molecules. They should therefore be useful in elucidating mult-ligand recognition in complex systems with photoaffinity labeling.

Acknowledgements

This research was partly supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Encouragement of Young Scientists, 12780433 (M.H.) and 13771410 (Y.S.), for Scientific Research 12470504 (Y.H.) and for Scientific Research on Priority Areas (C) 'Genome Science', No. 13202023 (Y.S.). M. H. thanks the Akiyama Foundation and Uehara Memorial Foundation for a partial financial support of this work.

References and Notes

1. Bayley, H. In *Laboratory Techniques in Biochemistry and Molecular Biology: Photogenerated Reagents in Biochemistry and Molecular Biology*; Work, T. S., Burdon, R. H., Eds.; Elsevier: Amsterdam, 1983; pp 25–44.
2. Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483.
3. Kotzyba-Hilbert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296.
4. Dorm, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64.
5. Hatanaka, Y.; Sadakane, Y. *Curr. Top. Med. Chem* **2002**, 271.
6. Hatanaka, Y.; Nakayama, H.; Kanaoka, Y. *Rev. Heteroatom Chem.* **1996**, *14*, 213.
7. Hatanaka, Y.; Kanaoka, Y. *Heterocycles* **1998**, *47*, 625.

8. Gillingham, A. K.; Koumanov, F.; Hashimoto, M.; Holman, G. D. In *Detection and Analysis of Glucose Transporters Using Photolabelling Techniques*; Baldwin, S. A., Ed.; Membrane Transport: A Practical Approach; Oxford University Press: Oxford, 2000; p 193.
9. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *Bioorg. Med. Chem.* **1994**, *2*, 1367.
10. Hatanaka, Y.; Hashimoto, M.; Nishihara, S.; Narimatsu, H.; Kanaoka, Y. *Carbohydr. Res.* **1996**, *294*, 95.
11. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *J. Am. Chem. Soc.* **1998**, *120*, 453.
12. Hashimoto, M.; Hatanaka, Y. *Chem. Pharm. Bull.* **1999**, *47*, 667.
13. Hashimoto, M.; Hatanaka, Y.; Nabeta, K. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2481.
14. Hashimoto, M.; Yang, J.; Holman, G. D. *Chem. Biochem.* **2001**, *2*, 52.
15. Hashimoto, M.; Hatanaka, Y.; Yang, J.; Dhesi, J.; Holman, G. D. *Carbohydr. Res.* **2001**, *331*, 119.
16. Hashimoto, M.; Hatanaka, Y.; Nabeta, K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 89.
17. Marie, J.; Seyer, R.; Rombard, C.; Desarnaud, F.; Aumelas, A.; Jard, S.; Bonnafous, J.-C. *Biochemistry* **1990**, *26*, 8943.
18. Farby, M.; Schaefer, E.; Ellis, L.; Kojro, E.; Fahrenholz, F.; Brandenburg, D. *J. Biol. Chem.* **1992**, *267*, 8950.
19. Ploug, M.; Ostergaard, S.; Hansen, L. B. L.; Holm, A.; Dano, K. *Biochemistry* **1998**, *37*, 3612.
20. Nassal, M. *J. Am. Chem. Soc.* **1984**, *106*, 7540.
21. Beewen, L.; Bayley, H. *Anal. Biochem.* **1985**, *144*, 132.
22. Fishwick, C. W. G.; Sanderson, J. M.; Findlay, J. B. C. *Tetrahedron Lett.* **1994**, *35*, 4611.
23. Hashimoto, M.; Kanaoka, Y.; Hatanaka, Y. *Heterocycles* **1997**, *46*, 119.
24. Belokon', Y. N.; Bulychev, A. G.; Vitt, S. V.; Struchkov, Y. T.; Batsanov, A. S.; Timofeeva, T. V.; Tsyryapkin, V. A.; Ryzhov, M. G.; Lysova, L. A.; Bakhmutov, V. I.; Belikov, V. M. *J. Am. Chem. Soc.* **1985**, *107*, 4252.
25. Compound **13** $[\alpha]_D = -39.4$ ($c = 1.8$, methanol), FAB-MS m/z 304 ($[M+H]^+$), ^{1}H NMR (CD_3OD) δ 7.37 (1H, d, $J = 7.9$ Hz), 6.88 (1H, d, $J = 7.9$ Hz), 6.78 (1H, s), 3.92 (3H, s), 3.85 (1H, dd, $J = 8.9$, 4.6 Hz), 3.41 (1H, dd, $J = 14.2$, 4.6 Hz), 3.02 (1H, dd, $J = 14.2$, 8.9 Hz). Compound **16** $[\alpha]_D = +13.8$ ($c = 2.4$, methanol), FAB-MS m/z 647 ($[M+H]^+$), ^{1}H NMR (CD_3OD) δ 7.27 (1H, d, $J = 7.9$ Hz), 6.78 (1H, d, $J = 7.9$ Hz), 6.64 (1H, s), 4.38 (1H, dd, $J = 7.9$, 5.0 Hz), 4.19 (1H, dd, $J = 7.9$, 5.0 Hz), 4.12 (1H, m), 3.77 (3H, m), 3.61 (2H, m), 3.55 (2H, m), 3.45 (2H, m), 3.26 (5H, m), 3.08 (1H, m), 3.00 (1H, dd, $J = 14.5$, 8.6 Hz), 2.81 (1H, dd, $J = 12.5$, 5.0 Hz), 2.59 (1H, d, $J = 12.5$ Hz), 2.11 (2H, t, $J = 7.3$ Hz), 1.53 (4H, m), 1.33 (2H, m). Compound **18** $[\alpha]_D = +23.2$ ($c = 0.7$, methanol), FAB-MS m/z 559 ($[M+H]^+$), ^{1}H NMR (CD_3OD) δ 7.40 (1H, d, $J = 7.9$ Hz), 6.80 (1H, d, $J = 7.9$ Hz), 6.63 (1H, s), 4.40 (1H, dd, $J = 7.9$, 5.0 Hz), 4.20 (1H, dd, $J = 7.9$, 5.0 Hz), 4.00 (1H, m), 3.77 (1H, m), 3.61 (2H, m), 3.45 (3H, m), 3.08 (3H, m), 3.00 (1H, dd, $J = 14.5$, 8.6 Hz), 2.81 (1H, dd, $J = 12.5$, 5.0 Hz), 2.59 (1H, d, $J = 12.5$ Hz), 2.20 (2H, t, $J = 7.3$ Hz), 1.53 (4H, m), 1.33 (2H, m).
26. Witkowski, A.; Witokowska, H. E.; Knudsen, J.; Smith, S. *Anal. Biochem.* **1999**, *267*, 412.
27. Hatanaka, Y.; Kempin, U.; Park, J.-J. *J. Org. Chem.* **2000**, *65*, 5639.
28. Meister, A.; Wellner, D. Flavoprotein Amino Acid Oxidase. In *The Enzymes*, 2nd ed.; Boyer, P. D, Ed.; 1963; p 609.
29. Curti, B.; Ronchi, S.; Simonetta, M. P. In *Chemistry and Biochemistry of Flavoenzymes*; Muller, F, Ed.; 1992; Vol. 3, p 69.
30. Ghisla, S.; Massey, V. *Biochem. J.* **1986**, *236*, 1.
31. Raibekas, A. A.; Jorns, M. S. *Biochemistry* **1994**, *33*, 12649.
32. Raibekas, A. A.; Jorns, M. S. *Biochemistry* **1994**, *33*, 12656.
33. Murthy, Y. V. S. N.; Massey, V. *J. Biol. Chem.* **1995**, *270*, 28586.
34. Raibekas, A. A.; Massey, V. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7546.