Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 881–884

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

POLYMER-BOUND *N*-HYDROXYSUCCINIMIDE ESTERS: A COLUMN-FREE FLUORESCENT-LABELING METHOD

Miho Katoh and Mikiko Sodeoka*

Sagami Chemical Research Center, Nishi-Ohnuma, Sagamihara, Kanagawa 229-0012, Japan

Received 12 February 1999; accepted 15 February 1999

Abstract: Polymer-bound *N*-hydroxysuccinimide esters of 1-pyrenebutyric acid, 6-carboxyfluorescein diacetate, and biotin were efficiently prepared. Column-free fluorescent- and biotin-labeling reactions of various amines using these resins were successfully demonstrated.

Fluorescent-labeling is becoming one of the most important techniques in biological studies.[1] Fluorescent-labeled compounds can be applied to fluorescence microscopy experiments, confocal laser scanning microscopy studies, collisional fluorescence quenching experiments, fluorescence-monitored equilibrium binding studies, flow cytometric studies, or enzyme assays. The reaction of *N*-hydroxysuccinimide (NHS) ester of fluorescent compounds is known to selectively react with amines, and has been widely used for the fluorescent-labeling of low molecular weight compounds, peptides, and proteins.[2] The reaction itself is, in many cases, very clean and quantitative, but purification of the product, such as column chromatography, HPLC, or dialysis, is necessary to remove the excess reagent and by-produced *N*-hydroxysuccinimide. To avoid this time-consuming purification step, we planned to develop novel polymer-bound NHS esters of fluorescent compounds. Some polymer-bound NHS esters had been developed for peptide synthesis,[3] but most of them have used copolymer of maleic anhydride or *N*-hydroxymaleimide derivatives. Since the polymerization step may affect the chemical and physical properties of the polymer, we believed that the use of a commercially available and well-characterized resin would be more efficient, and that the key NHS ester should be connected to the polymer with an appropriate spacer to achieve high reactivity. Based on these considerations, we selected the PS-Thiophenol resin (ARGONAUT TECHNOLOGIES) and planned to connect the NHS ester group by the Michael-type addition reaction of the thiol to maleimide esters.

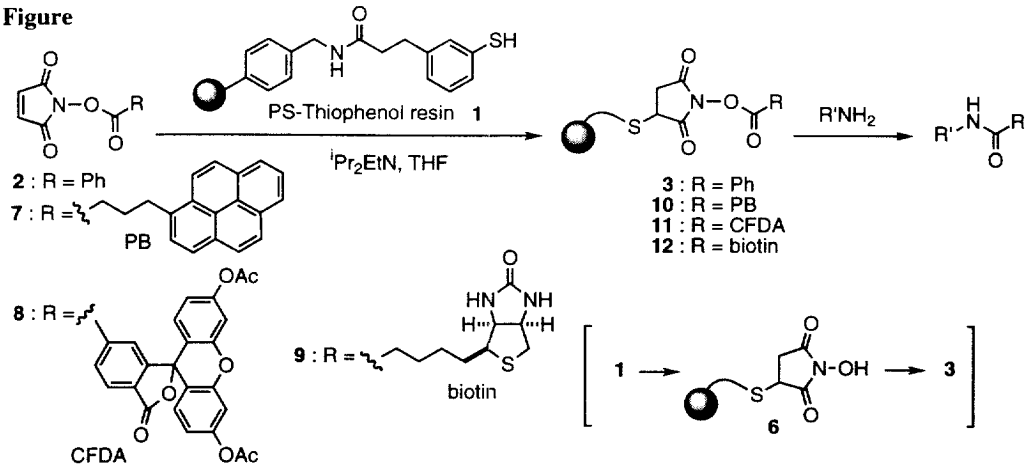
After examining various reaction conditions using *N*-benzoyloxymaleimide (prepared from benzoyl chloride and *N*-hydroxymaleimide) and thiophenol as a soluble model, we found that the desired active ester resin was efficiently prepared according to the following procedure. Namely, PS-Thiophenol resin **1** (1.26 mmol/g) was treated with *N*-benzoyloxymaleimide (**2**) (2 equiv) and *N,N*-diisopropylethylamine (DIPEA) (0.4 equiv) in THF at 23 °C for 2.5 h. After washing the resin with THF, 0.98 equiv of **2** was recovered indicating the almost complete conversion of **1** to the *N*-benzoyloxysuccinimide resin **3**.[4] The active ester resin **3** was next treated with tetradecylamine (**4**) (0.5 equiv) in THF at 23 °C for 40 min, and washed with THF. Removal of THF from the combined filtrates afforded *N*-tetradecylbenzamide (**5**) in a good chemical yield (89% based on the amine). It is noteworthy that treatment of this "used" resin with another **4** (0.3 equiv), again, gave **5** in an 80% yield. ^{1}H-NMR analysis revealed the purity of the product at >95%, and no

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00091-8

starting amine or hydrolyzed product, such as benzoic acid, was detected in the product. These facts indicate the high loading and high reactivity of the active ester resin.

We also tested a second possible method for making the active ester resin. Namely, PS-Thiophenol resin was first treated with *N*-hydroxymaleimide (2.0 equiv) and DIPEA (0.4 equiv) in THF to give the *N*-hydroxysuccinimide resin **6** in quantitative yield. This NHS resin was then treated with DCC (1 equiv) and benzoic acid (1 equiv) in THF at 0 °C. In this case, however, undesired *N*-benzoyl-*N,N'*-dicyclohexylurea was formed in a 51% yield, and the treatment of the resin with tetradecylamine (1 equiv) afforded only a 42% yield of the amide, indicating that the yield of the esterification was less than 50%. Even though 3 equiv of benzoic acid and DCC were used for the esterification, the amide yield did not increase. Thus the loading level of the resin from the second method was not satisfactory, and we decided to choose the first method.

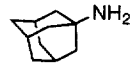
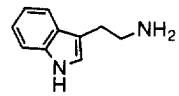
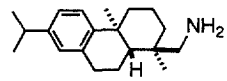
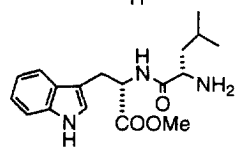
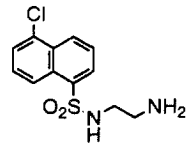
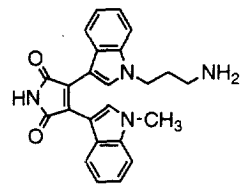
Figure



With these basic results in hand, we finally applied this methodology to the fluorescent compounds. Fluorescent-labeling resins **10** and **11** were similarly prepared from PS-Thiophenol resin, and the *N*-hydroxymaleimide esters **7** and **8** prepared from corresponding carboxylic acids.[5] As shown in Table 1, the reaction of active ester resin of 1-pyrenebutyric acid (PB) **10** (~1.5 equiv) with various amines proceeded smoothly to give a variety of pyrene-labeled compounds **19–24** in good chemical yields without any column purification.[6] Furthermore, the active ester resin of 6-carboxyfluorescein diacetate (CFDA) **11** also gave satisfactory results. Fluorescein diacetate-labeled compounds are known to be useful cell membrane-permeable probes which are hydrolyzed by cell esterase after entering the cell and become highly fluorescent. Bisindolylmaleimide VIII (**18**) is known as a powerful protein kinase C (PKC) inhibitor, and the fluorescent-labeled compound **26** has been used for studies on PKC.[7] Biotin-labeling resin **12** was also prepared from **1** and **9**, and efficiently used for the biotinylation of **14**.[8]

In conclusion, high-loading polymer-supported NHS esters of fluorescent compounds were efficiently prepared, and "column-free" fluorescent-labeling of various compounds using these novel resin reagents was achieved affording highly pure products. This novel methodology may not be limited to fluorescent-labeling and biotinylation, but could also be applied to various other modifications such as the introduction of photoreactive groups.[9] This polymer-supported NHS ester method would be applicable to peptide or protein labeling by using more hydrophilic resin. It is also noteworthy that this methodology may be of use

Table 1. Acylation using the active ester resins [a]

entry	resin	amine		amide	yield (%)
1	**10**	tetradecylamine (**4**)	$CH_3(CH_2)_{13}NH_2$	**19**	85
2	**10**	1-adamantanamine (**13**)		**20**	92
3	**10**	tryptamine (**14**)		**21**	91
4	**10**	dehydroabietylamine (**15**)		**22**	93
5	**10**	H-L-Leu-L-Trp-OMe (**16**)		**23**	95
6	**10**	A3 (**17**)		**24**	92
7	**11**	A3 (**17**)		**25**	89
8	**11**	bisindolylmaleimide VIII (**18**)		**26**	83
9	**12**	tryptamine (**14**)		**27**	81

[a] The active ester resin (1.5 equiv) was treated with a THF solution of the amine at 23 °C for 1 h.

in the creation of a biased library of small molecules. Combinatorial chemistry is now becoming an important technology for developing novel biologically active compounds, and recently the importance of a target-oriented library instead of random library has been recognized as well.[10] In the process of lead optimization, a library whose compounds share a common key structure essential for the interaction with an active site, and that possess structural variation for achieving selectivity and higher affinity to the specific target is required. In such case, a library of compounds easily preparable by the reaction of the key structure's NHS ester with various amines would be a good "biased" library for the specific target.

After completion of this work, reports describing the preparation of NHS resin from *N*-hydroxymaleimide and SH-derivatized Merrifield resin and ArgoPore® resin and their use for active ester formation and coupling with amines appeared.[11] The approach described in these reports is quite similar to our second method in that they used ethyl (3-dimethylaminopropyl) carbodiimide in DMF as a coupling reagent. Although these reports did not mention the yield of the coupling reaction, a large excess (6 equiv) of the resin reagent and long reaction period (20 h) was employed for the key amide formation reaction.

Acknowledgment: We thank ARGONAUT TECHNOLOGIES for generous gift of the PS-Thiophenol resin. This work was partially supported by Kato Memorial Bioscience Foundation.

References and Notes

1. Mayer, A.; Neuenhofer, S. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1044, and references cited therein.
2. Brinkley, M. *Bioconjugate Chem.* **1992**, *3*, 2, and references cited therein.
3. (a) Fridkin, M.; Patchornik, A.; Katchalski, E. *Biochemistry* **1972**, *11*, 466. (b) Akiyama, M.; Shimizu, K.; Narita, M. *Tetrahedron Lett.* **1976**, 1015. (c) Andreev, S. M.; Tsiryapkin, V. A.; Samoilova, N. A.; Mironova, N. V.; Davidovich, Y. A.; Rogozhin, S. V. *SYNTHESIS* **1977**, 303, and references cited therein.
4. Completion of the reaction was also confirmed by the disappearance of the S-H absorption band at 2570 cm^{-1} in the infrared spectrum of **3**. IR (KBr) **1**: 2570, 1660 cm^{-1}; **2**: 1770, 1740 cm^{-1}; **3**: 1780, 1745, 1670 cm^{-1}. Normally, use of 1.1 equiv of the *N*-hydroxymaleimide ester was sufficient for the reaction as shown below.
5. **A representative procedure for preparation of the active ester resins.** To a suspension of **1** (0.29 g, 0.365 mmol) in THF (2 mL) and water (0.1 mL) was added tri-n-butylphosphine (0.4 mL). The mixture was stirred at 23 °C for 1 h under an argon atmosphere. The solution was removed, and the resin was washed with degassed THF. To a suspension of this freshly reduced resin in THF (2 mL) was added DIPEA (20 μL, 0.146 mmol) and a solution of **7** (147 mg, 0.383 mmol, 1.1 equiv to **1**) in THF (2.2 mL). After stirring at 23 °C for 30 min, the solution was removed, and the resin was washed with THF and dried under vacuum to give **10** (0.47 g, *ca.* 0.78 mmol/g).
6. **A representative procedure for the labeling reactions.** To a suspension of **10** (0.1 g, 0.078 mmol) in THF (2 mL) was added a solution of **4** (11.2 mg, 0.052 mmol) in THF (1.4 mL), and the mixture was stirred at 23 °C for 1 h. After filtration, the resin was further washed with THF. The filtrates were combined and concentrated to give **19** (21.7 mg, 85%).

   Satisfactory IR, ^{1}H-NMR, and mass spectral data were obtained for all products.
7. Chen, C.-S.; Poenie, M. *J. Biol. Chem.* **1993**, *268*, 15812.
8. For review on the biotin technology, see: Wilchek, M.; Bayer, E. A. *Methods in Enzymol.* **1990**, *184*, 123.
9. Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483, and references cited therein.
10. For examples, see (a) Gordon, E. M.; Gallop, M. A.; Patel, D. V. *Acc. Chem. Res.* **1996**, *29*, 144. (b) Combs, A. P.; Kapoor, T. M.; Feng, S.; Chen, J. K.; Daudé-Snow, L. F.; Schreiber, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 287, and references cited therein.
11. (a) Adamczyk, M.; Fishpaugh, J. R.; Mattingly, P. G. *Tetrahedron Lett.* **1999**, *40*, 463. (b) *idem Bioorg. Med. Chem. Lett.* **1999**, *9*, 217.