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Synthesis of N-Hydroxysuccinimide Esters Using Polymer Bound HOBT.

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Abstract: *The preparation of the N-hydroxysuccinimide esters via reactions mediated by polymer bound 1-hydroxybenzotriazole (HOBT) is reported.*

The use of active esters of carboxylic acids, including N-hydroxysuccinimide esters (NHS esters) and pentafluorophenyl esters (PFP esters), for solid-phase peptide synthesis[1] and the modification of proteins and peptides has become a routine procedure.[2] We have utilized bifunctional crosslinking agents containing an NHS moiety for the efficient synthesis of peptide bundles.[3] In subsequent studies, we have found it necessary to prepare the NHS esters from the corresponding carboxylic acids directly. NHS esters of carboxylic acids are typically prepared by reaction of the carboxylate moiety with NHS using dicyclohexylcarbodiimide (DCC), or by reaction of the acid chloride with NHS.[4] The resulting NHS esters, as well as PFP esters, can sometimes be purified by recrystallization. Unfortunately, the use of silica gel chromatography, often results in low isolated yields or decomposition of product.[5]

To avoid difficulties in the purification of NHS esters prepared by solution phase methods, we sought an alternate method for their preparation. A recent report describes the use of polystyrene-supported EDAC (ethyl dimethylaminopropylcarbodiimide) for the efficient synthesis of NHS and pentafluorophenyl esters from carboxylic acids.[6] Although the reaction is efficient, the polymer-bound EDAC reagent must be kept under anhydrous conditions, and can only be regenerated by dehydration.[7]

A review of the literature revealed that polystyrene-supported 1-hydroxybenzotriazole (**P**-HOBT) has been used as a highly reactive N-acylating agent for the formation of peptide bonds[8] and simple amides.[9] We have reported the use of **P**-HOBT for the synthesis of medium-ring lactams from linear ω-amino acid precursors.[10] As a result, we felt **P**-HOBT was reactive enough for the convenient preparation of activated esters of carboxylic acids using N-hydroxysuccinimide or pentafluorophenol as the nucleophile. We prepared the **P**-HOBT using Bio-Rad SM-2 dried macroporous beads (polystyrene-divinylbenzene copolymer resin, 100-200 mesh, MW cutoff 2000) according to the method of Fridkin and Patchornik.[8] The number of active sites on the resin was determined by first reacting **P**-HOBT with acetic anhydride in the presence of pyridine to form the polymer-bound acetate ester. Subsequent treatment with isopropylamine (large excess) yielded N-isopropylacetamide and recovered polymer. Based on the yield of recovered N-isopropylacetamide, two batches of **P**-HOBT were prepared with

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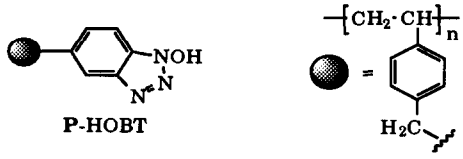
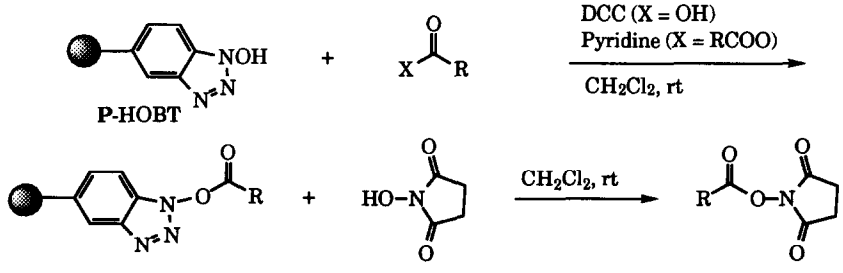
activities of 0.27 mmol/g and 0.25 mmol/g, respectively. The first batch (0.27 mmol/g) was prepared and stored undessicated at room temperature for two years. At this time, the activity of the polymer was re-evaluated and determined to be 0.24 mmol/g. The polymer retained 89% of its original activity over two years, an indication of its stability as compared to polymer-bound EDAC which must be stored under nitrogen at 0°C and has a shelf-life of only several months.[7]



With the polymer available, the synthesis of a variety of NHS esters, including commonly used protein/peptide modification reagents (NHS esters of 3-maleimido propionic acid, pyrene carboxylic acid and 3-(4-hydroxyphenyl)propionic acid), was carried out as shown in **Scheme 1**. For ease of synthesis and potential automation, the reactions were carried out in 8 mL Extract-Clean™ solid phase extraction tubes (from Alltech Associates, Inc.), equipped with a disposable inlet cap and a one-way stopcock on the outlet. Reactions were mixed by gentle rocking and filtration was carried out using a 12-port solid phase extraction manifold connected to a water aspirator. In general, the polymer-bound ester was formed by the addition of the acid anhydride and pyridine to the immobilized HOBT group, or by coupling of the free carboxylic acid to the polymer using DCC (or diisopropylcarbodiimide, DIC) as catalyst. The solvent in both cases was CH_2Cl_2. After washing the activated resin thoroughly, it was suspended in a solution of CH_2Cl_2 containing 0.8 equivalents of NHS based on polymer activity. The reaction mixture was rocked for 4 - 7 hours at room temperature, at which time filtration of the resin, and subsequent concentration of the filtrate yielded the N-hydroxysuccinimide ester as the only observable product.[11,12] Interestingly, the attempted use of pentafluorophenol (PFP-OH) as the nucleophile did not afford any activated ester product. This is in contrast to the report utilizing polymer-bound EDAC as catalyst.[6] A summary of these results is given in **Table 1**.



Scheme 1. General method for NHS ester synthesis

Table 1. Formation of NHS Esters

Entry	Carboxylic acid (derivative)	Reaction Time (h)	Yield (%)
1	acetic anhydride	4	88
2	benzoic acid	4	88
3	*trans*-cinnamic acid	7	86
4	3-maleimido propionic acid	4	100
5	3-maleimido propionic acid[a]	4	99
6	3-(4-hydroxyphenyl)propionic acid	5	68
7	pyrenecarboxylic acid[b]	4	74
8	N_{α}-Boc proline[b]	4	71
9	dehydrocholic acid[b]	20	56

[a] used reactivated polymer
[b] used recycled polymer

In contrast to the use of polymer-bound EDAC, the polymer is completely recyclable. We have developed two protocols for re-use of the polymer bound HOBT reagent. The first involves use of the spent **P**-HOBT to synthesize the same NHS ester again. We have utilized this protocol for the repetitive synthesis of 3-maleimidopropionic acid NHS ester. After the 3-maleimidopropionic acid NHS ester product from entry **4** (**Table 1**) was collected, the spent resin was washed with 3x5mL portions each of CH_2Cl_2, MeOH, DMF, MeOH, CH_2Cl_2 and diethyl ether to remove any remaining unbound reagents. The washed **P**-HOBT was reactivated using 3-maleimidopropionic acid and DIC, followed by subsequent treatment with NHS to yield the same product in excellent yield (entry **5**, **Table 1**).

Alternately, the used **P**-HOBT can be used to synthesize a different NHS ester. A convenient two step procedure which involves washing the used polymer with a simple amine to remove any unreacted active ester, followed by rigorous washing of the polymer to remove unwanted reagents is sufficient to yield active, clean polymer. To demonstrate the recyclability of **P**-HOBT, the used polymer from the synthesis of the NHS ester of 3-(4-hydroxyphenyl)propionic acid (entry **6**, **Table 1**) was reacted with a large excess of isopropylamine (based on polymer activity) in CH_2Cl_2 for two hours. The polymer was subsequently filtered and washed with 3x5mL portions each of CH_2Cl_2, MeOH, DMF, MeOH, NMP, MeOH, CH_2Cl_2 and diethyl ether to ensure removal of all amide and unreacted amine products. The polymer was then activated with pyrenecarboxylic acid and subsequently reacted with NHS to provide the desired product in good yield with no contamination by other products (entry **7**, **Table 1**).

In addition, the **P**-HOBT reaction is carried out in two steps, formation of the acylated intermediate, followed by reaction with NHS to form the product. As a result, the parallel preparation of several different acylated polymers is easily accomplished, followed by the

parallel or one pot formation of NHS esters if desired. The process should be easily amenable to automation.

In summary, a convenient method has been developed for the synthesis of N-hydroxysuccinimide esters from carboxylic acid precursors using polymer-supported HOBT as a catalyst. The utility of this method has been exploited in the preparation of several useful protein/peptide modifying agents, including the NHS esters of 3-maleimidopropionic acid, pyrenecarboxylic acid and 3-(4-hydroxyphenyl)propionic acid. The polymer-bound HOBT reagent, which is highly reactive, stable for extended periods (≥ 2 years), completely recyclable, and amenable to automation represents a significant improvement over the use of polymer-bound EDAC, as well as solution phase methods for the preparation of NHS esters of interest.

References and Notes

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11. Experimental procedure for the preparation of 3-maleimidopropionic acid NHS ester (entry **4**, **Table 1**): A solution of 3-maleimidopropionic acid (0.0608 g, 0.359 mmol, 2.85 equiv.) in CH_2Cl_2 (5 mL) was rocked with polymer-bound HOBT (0.506 g, 0.25 mmol/g, 0.126 mmol) and DIC (0.050g, 0.062mL, 0.40mmol, 3.2 equiv.) for 1 h at 25°C. The polymer was filtered, washed with CH_2Cl_2 (3x5 mL), DMF (3x5 mL), CH_2Cl_2 (3x5 mL), and dry Et_2O (3x5 mL). The dried polymer was suspended in CH_2Cl_2 (5 mL) followed by the addition of NHS (0.0111g, 0.0964 mmol, 0.76 equiv. based on activated polymer). The suspension was rocked at 25°C for 4 h. The polymer was then filtered and washed with CH_2Cl_2 (4x5 mL). The filtrate and washings were combined and concentrated to yield 0.027 g (99%) of 3-maleimidopropionic acid NHS ester. [^{1}H NMR ($CDCl_3$): δ 2.82 (s, 4H, C(O)C$\underline{H}_2$C$\underline{H}_2$C(O)), 3.02 (t, 2H, J = 7.5 Hz, C$\underline{H}_2$COO), 3.93 (t, 2H, J = 7.5 Hz, NC$\underline{H}_2$), 6.74 (s, 2H, C$\underline{H}$=C$\underline{H}$)]. The ^{1}H NMR data are consistent with reported spectral data.[13]
12. ^{1}H NMR data are as follows: acetic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H, C$\underline{H}_3$), 2.85 (s, 4H, C$\underline{H}_2$C$\underline{H}_2$)]; benzoic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 2.92 (s, 4H, C$\underline{H}_2$C$\underline{H}_3$), 7.52 (m, 2H, Ar$\underline{H}$), 7.69 (m, 1H, Ar$\underline{H}$), 8.16 (m, 2H, Ar$\underline{H}$)]; *trans*-cinnamic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 2.89 (s, 4H, C$\underline{H}_2$C$\underline{H}_3$), 6.60 (d, 1H, J = 16Hz, C$\underline{H}$COOR), 7.43 (m, 3H, Ar$\underline{H}$), 7.59 (m, 2H, Ar$\underline{H}$), 7.93 (d, 2H, J = 16Hz, ArC$\underline{H}$)]; 3-(4-hydroxyphenyl)propionic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 2.82 (s, 4H, NC(O)C$\underline{H}_2$C$\underline{H}_3$C(O)), 2.85 (m, 2H, Ar $CH_2C\underline{H}_3$), 2.96 (m, 2H, Ar C$\underline{H}_2$CH$_3$), 6.75 (m, 2H, Ar$\underline{H}$), 7.05 (m, 2H, Ar$\underline{H}$)]; pyrenecarboxylic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 3.00 (s, 4H, C$\underline{H}_2$C$\underline{H}_3$), 8.00-8.40 (m, 8H, Ar$\underline{H}$), 8.79 (d, 1H, J = 8.1Hz, Ar$\underline{H}$), 9.08 (d, 2H, J = 9.3Hz, Ar$\underline{H}$)]; N_α-Boc-proline NHS ester [^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), 2.00 (m, 2H), 2.35 (m, 2H), 2.85 (s, 4H), 3.50 (m, 1H), 3.60 (m, 1H), 4.55 (m, 1H)]; dehydrocholic acid NHS ester [^{1}H NMR ($CDCl_3$) δ 0.85 (d, 3H, J = 6.8 Hz), 1.10 (s, 3H), 1.15 - 1.70 (m, 6H), 1.40 (s, 3H), 1.80 - 2.45 (2 m, 14H), 2.50 - 2.75 (m, 2H), 2.85 (s, 4H), 2.90 - 3.00 (m, 2H); FAB LRMS calcd for $C_{28}H_{38}O_7N$ 500 [M + 1]$^+$, found 500 [M + 1]$^+$].
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