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A NEW CLASS OF REAGENTS FOR THE CHEMICAL MODIFICATION OF PROTEINS‡

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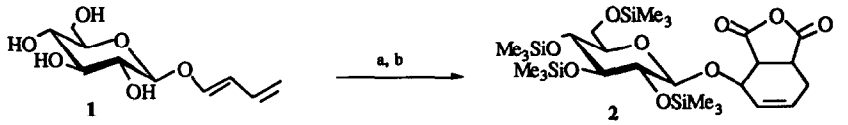
Abstract. Highly reactive substituted anhydrides were synthesized by Diels-Alder cycloadditions of maleic anhydride with various substituted 1,3-dienes. To emphasize the scope of the method, two reagents were prepared : one, very hydrophilic derived from D-glucose, and another one, very hydrophobic, bearing two fatty-acid chains. Bovine serum albumin and two enzymes were extensively modified by these reagents.

There is a constant need for new efficient reagents for the chemical modification of proteins by coupling small molecules. Enhancement of antigenicity of haptens, stabilisation of enzymes against denaturation and modification of some of their physicochemical properties are among the aims of such operations. We assumed that an ideal reagent must both be very reactive and allow a great versatility regarding the nature of the groups that will be grafted onto the protein.

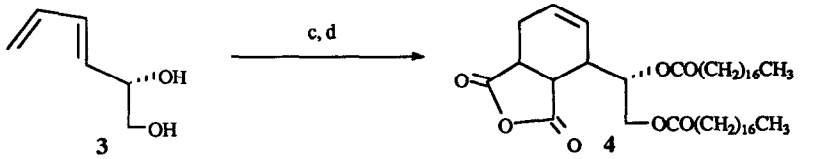
We report in this letter the first applications of highly reactive anhydrides obtained by cycloaddition reactions of maleic anhydride with substituted 1,3-dienes. This methodology can be used with a large variety of molecules, as long as stable butadienyl derivatives can be synthesized. The general strategy for chemical derivatization of proteins is summarized in the following scheme:

We chose at first to attach functional groups of very opposite nature, such as glucosyl residues (very hydrophilic) and di-fatty acyl chains (very hydrophobic). Two compounds were synthesized from dienes recently prepared in our laboratory by original procedures[1]. For the fixation of glucose residues, we used compound **2** (as a mixture of 3 diastereoisomers, as shown by ^1H NMR) prepared quantitatively from the known diene **1.**



a: HMDS, pyridine, TFA, room temperature, then distillation ($Eb_{0.1\ mm}$: 130-140°, 93%) b: maleic anhydride (1 eq.), THF, 40°C, 12h.

Fatty acid derived reagent **4** (as a mixture of diastereoisomers identified through ^1H, ^{13}C and 2D NMR) was prepared from diene **3.**



c: stearoyle chloride,pyridine, room temperature (78 %) d: maleic anhydride (2eq), THF, 80°C, 24h, then sublimation of the excess of anhydride

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A solution of each reagent in dry THF was shown to be stable for weeks at 4° C and was used directly for the modification of proteins.

Since it is commonly used as acceptor of small molecules in immunological methods (immunisation of animals, ELISA tests ...), bovine serum albumin (BSA) was first chosen to react with **2** and **4** (native BSA has 60 free amino groups [2])

a) A large excess of **2** was gradually added to a cold (7°C) stirred solution of the BSA in a phosphate buffer (pH 8, 20 % acetone). After one night (the silyl groups being spontaneously hydrolyzed), the protein was purified by chromatography on Sephadex G50 followed by dialysis against water.The glucose content of the modified protein was determined enzymatically after acid hydrolysis[3]. Up to 70 molecules of glucose per molecule of protein were fixed.

b) Treatment of a buffered solution (pH 6, 20% THF) of the BSA with a large excess (20 fold/NH_2 groups) of **4** resulted in the precipitation of the modified protein. The precipitate was thoroughly washed with THF to remove the excess of **4** and the corresponding hydrolyzed product. The amount of stearic acid liberated after saponification of the modified protein was estimated by HPLC, using myristic acid as an internal standard. Up to 100-110 groups per molecule of protein were fixed, which correspond to 200-220 chains of fatty-acid. A lower excess of reagent **4** (5/NH_2 group) permitted the recovery of a soluble modified protein bearing 5-8 groups. In order to demonstrate that the reagent had been covalently linked to the protein, the dicarboxylic acid derived from **4** by hydrolysis of the anhydride function was added to a buffered solution of the BSA. After 48 h., no protein had precipitated. The reaction medium was evaporated and the residue was washed with THF. No stearic acid was liberated after hydrolysis of this sample of BSA.

Two enzymes of industrial and medical importance were then chosen as acceptors :

-Horse radish peroxydase (HRP, mixture of isoenzymes) was treated with **2** in a phosphate buffer (pH 8, 20% acetone). This resulted in the binding of 3-4 molecules of glucose per molecule of enzyme (the native enzyme has 5.5 free amino groups[4]) with no loss of enzymic activity. The HRP could also be modified with **2** in non aqueous conditions (THF : Pyridine 80:20). In this way, up to 20 molecules of glucose were fixed, but only 20% of enzymic activity remained. HRP was also treated with reagent **4** in a phosphate buffer (pH8, 20% THF) ; 50% of the protein remained soluble and was found to bear 0.7 group per molecule of enzyme. The precipitated protein contained 1.7 group per molecule.

-Bovine superoxide dismutase (SOD, 20 free amino groups[5]) was treated with **4.** Surprisingly, this protein was found to be much less reactive. No modification was observed in buffers up to pH 9. However, treatment of SOD with **4** in a water : pyridine mixture (1:1, pH 10.6) resulted in the precipitation of 45% of the enzyme, this fraction bearing 14 groups per molecule. The soluble protein was recovered unmodified and 100% active.

As illustrated by the modification of the BSA, this new class of reagents can be quite reactive. Grafting one hundred or so residues per molecule of BSA in one single operation is exceptional. Other established methods[6] (water soluble carbodiimides, acylazides, NHS-esters, reductive amination, isothiocyanates...) moreoften allow the coupling of only 20-50 (or less) small molecules on the BSA. Very different functionnalities can be introduced, as illustrated by the use of **2** or **4.** Moreover, it should be possible to extend to other 1,3-dienes and to any compound bearing a free hydroxyl group the method used for the synthesis of **2**[1].
Physicochemical and catalytic properties of the modified enzymes are being investigated. First results indicate that a modified HRP bearing 3-4 molecules of glucose is indeed stabilized against thermal denaturation, since its half-life at 60° C is five fold that of the native enzyme.

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