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DIRECT ESTERIFICATION OF NEUTRAL AMINO ACIDS TO POLYMERIC ALCOHOLS

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A number of methods have been devised for formation of ester linkages between amino acid derivatives and supporting media. In the original Merrifield scheme of solid-phase peptide synthesis, chloromethylated styrene-divinylbenzene copolymers were refluxed with salts of N-protected amino acid derivatives in a suitable solvent[1,2]. In other procedures carboxyl-activated, N-protected amino acid derivatives have been coupled to supports containing hydroxyl groups[3-6].

We wish to report the direct acid-catalyzed esterification of neutral amino acids to soluble and insoluble polymeric alcohols in an adaptation of methods used for the synthesis of benzyl esters and nitrobenzyl esters of amino acids[7-9]. The introduction and removal of amine protecting groups is bypassed and the activation of the carboxyl function by diimides or other coupling agents is avoided. Direct attachment of amino acids to the polymer obviates the microchemistry required for dealing with isotopically labelled amino acids or other expensive amino acids prior to attachment to the supporting medium.

Polyethyleneglycol Esters of Amino Acids: A) An amino acid (100 mg) was suspended in a solution of toluenesulfonic acid hydrate (200 mg) and polyethyleneglycol (1g, mw 6,000) in benzene (50 ml). Arginine and histidine were introduced either as hydrochloride salts or as free bases along with an equivalent additional amount of toluenesulfonic acid. The reaction mixture was held at reflux for 2-3 days with condensate returning to the pot through a bed of Molecular Sieves 4A. The reaction mixture was filtered to remove any residual amino acid salts and the filtered solution was evaporated to dryness. The residue was dissolved in 10 ml water and dialyzed against four changes of a liter dialysis bath (dialysis tubing had a molecular weight cutoff of 3500). The dialyzed solutions were evaporated to dryness and the products were recrystallized from CH_2Cl_2/ether or from acetone. Polymer derivatives were recovered in essentially quantitative yield as white, ninhydrin reactive[10] powders. Samples hydrolyzed in 6 M HCl at 110° for 22 hours were analyzed quantitatively for amino acid content. The substitutions of amino acids on the various polymer derivatives are recorded in Table 1. No alloisoleucine was observed in hydrolysates of the isoleucine-polymer derivative.

B) Polyethyleneglycol (1.0g) was esterfied with phenylalanine using the procedure described above except that the amount of amino acid in the reaction mixture was decreased to 0.1 mmole and the amount of toluenesulfonic acid hydrate was decreased to 100 mg. After the reaction the mixture was clear and free of suspended material. A sample of the reaction mixture was analyzed and found to contain no free phenylalanine. Phenylalanine esterified to the isolated polymer ester derivative accounted for 85% of the amino acid put into the esterification reaction.

Hydroxymethylated Styrene-divinylbenzene Copolymer Esters of Amino Acids: Hydroxymethyl substituted copolystyrene-2%-divinylbenzene (100 mg) and an amino acid (10 mg) were suspended in

a solution of 20 mg toluenesulfonic acid hydrate in 50 ml CCl_4. The reaction mixture was held at reflux for 3 days with continual trapping of water from the reflux condensate. The resin samples were collected by filtration and washed thoroughly with dimethylformamide and hot water. Dried samples of the ninhydrin reactive polymer beads were subjected to acid hydrolysis in 12 M HCl:dioxane (1:1 by volume) mixtures. Quantitative analysis for amino acids in the hydrolysates provided the data for calculation of the substitution levels presented in Table 1. No alloisoleucine was detected in hydrolysates of the isoleucine polymer ester. Since little glycine was esterified to the resin in the foregoing procedure, the reaction was repeated using benzene as refluxing solvent. There was no significant improvement in substitution.

Table 1. Millimoles of Esterified Amino Acid per Gram of Polymer Ester

	Polyethyleneglycol Ester	Merrifield Resin Ester
Arginine	< 0.01	< 0.01
Glycine	0.11	0.01
Histidine	< 0.01	< 0.01
Isoleucine	0.16	0.33
Methionine	0.06	--[a]
Leucine	0.08	0.19
Norleucine	0.14	0.14
Phenylalanine	0.14, 0.085[b]	0.14
Proline	0.08	0.18

[a]Hydrolysate contained a complex mixture of ninhydrin reactive products.
[b]Esterification carried out using phenylalanine as limiting reactant.

t-Butyloxycarbonylglycylphenylalanylpolyethyleneglycol: The tosyl salt of phenylalanylpolyethylene glycol (300 mg) was converted to the free base with Amberlyst A-21 resin, dissolved in CH_2Cl_2, and treated with t-butyloxycarbonylglycine N-hydroxysuccinimide ester (150 mg)[11] for 20 hr. The product was precipitated by addition of ether, dialyzed exhaustively against water, and crystallized from CH_2Cl_2/ether. An acid hydrolyzed sample was analyzed for amino acids and was found to contain equimolar amounts of phenylalanine and glycine.

Benzyloxycarbonylleucylphenylalanylpolyethyleneglycol: The tosyl salt of phenylalanylpolyethyleneglycol (10 g) was converted to the free base with Amberlyst A-21 and dissolved in a mixture of 20 ml CH_2Cl_2 and 20 ml DMF at 0°. Benzyloxycarbonylleucine nitrophenyl ester[12] (4 mmol) was added. After 4 hr the mixture was still ninhydrin positive, so 200 mg of 1-hydroxybenzotriazole[13] was added. Within a short time the mixture was ninhydrin negative. Solvents were removed by rotary evaporation. The residue was dissolved in water, centrifuged to remove a small amount of suspended solids and dialyzed against water. The solution was

evaporated to dryness and crystallized from CH_2Cl_2/ether. Analysis of an acid hydroysate yielded phenylalanine and leucine in the molar ratio of 1 to 1.1.

Chymotryptic Hydrolysis of t-Butyloxycarbonylglycylphenylalanylpolyethyleneglycol: The title compound (170 mg) was dissolved in 2 ml 0.1 M tris/HCl buffer, pH 7 containing 0.05 M $CaCl_2$. Chymotrypsin (5 mg) was added and the mixture was stirred at room temperature for 17 hr. The solution was dialyzed against water, filtered through Celite, and evaporated to a film on the rotary evaporator. The dried residue was taken up in toluene, filtered, and reevaporated. After recrystallization from CH_2Cl_2/ether a sample of the product was acid-hydrolyzed and analyzed for amino acids. No amino acids were found in the acid hydrolysate.

Benzyloxycarbonylleucylphenylalanine. Benzyloxycarbonylleucylphenylalanylpolyethyleneglcyol (10 g) was saponified by the method of Tilak and Hollinden[14]. Benzyloxycarbonylleucylphenylalanine recovered from this cleavage (500 mg) was crystallized from ethyl acetate-petroleum ether. M.p.108-10°, $(\alpha)^{23}_{546}$-17.2 (c 1, in methanol). Acid hydrolysate contained leucine and phenylalanine in equimolar ratio. Weygand[15] has reported m.p.102-4°, $(\alpha)^{20}_{546}$-12.7° (c 1, in methanol). Other melting points reported in the literature[16-18] range from 116° to 123°.

Discussion

The treatment of polymeric alcohols with neutral amino acids under conditions commonly used for formation of amino acid esters gives polymer derivatives which are ninhydrin positive and from which free amino acids are recovered by acid hydrolysis. Treatment of one of these polymeric materials, the phenylalanine-polyethyleneglycol derivative, with benzyloxycarbonylleucine nitrophenyl ester yielded a product in which leucine and phenylalanine were recovered in equimolar ratio after acid hydrolysis. Saponification of the polymer derivative under conditions routinely applied to cleavage of peptidepolyethyleneglycol esters yielded benzyloxycarbonylleucylphenylalanine whose physical constants correspond to those reported in the literature. From these considerations we conclude that the amino acid attached directly to the polymer is linked through an ester bond.

The complete cleavage of t-butyloxycarbonylglycylphenylalanine from the polyethyleneglycol ester by chymotrypsin provides further evidence for the ester linkage and suggests that the stereochemical integrity of the amino acid residue is preserved during the esterification. This experiment also reconfirms our earlier observation that amino acid and peptide derivatives bound to polyethyleneglycol are freely accessible to enzymes in aqueous solution[19]. This is of considerable importance where polyethyleneglycol may be used as a supporting medium during synthesis or semi-synthesis of polypeptides using enzyme-labile blocking groups[20-22].

The esterification of phenylalanine to polyethyleneglycol under conditions where the amount of amino acid in the reaction is the limiting factor points up the advantages of this method when the amino acid must be conserved. Approximately 85% of the phenylalanine used in the reaction was recovered as polymer ester without prior micromanipulations to block the amino group.

The acid catalyzed esterification of amino acids by polymeric alcohols requires no covalent protection of the amino function, no subsequent removal of the protecting group, and no activation of the carboxyl function. The procedure described here is limited by practical con-

siderations to the esterification of neutral amino acids. It should be especially useful for handling isotopically labelled amino acids. Both the expense and the multiple reaction steps associated with reversible blocking of the amino group are avoided as is the increased danger of racemization which may accompany activation of the carboxyl function.

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