PEPTIDE SYNTHESIS IN PARTIALLY AQUEOUS SOLUTION

Preparation of a Leu–Ala–Gly–Val using symmetrical anhydrides of *N*-alkoxycarbonylamino acids in aqueous *N,N*-dimethylformamide

N. Leo BENOITON and Francis M. F. CHEN
Department of Biochemistry, University of Ottawa, Ottawa, K1N 9A9, Canada

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1. Introduction

Peptide synthesis by incremental addition is normally carried out in organic solvents in which the activated residue is soluble and coupling reactions proceed smoothly. Addition of a single residue to amino groups in proteins, however, is less straightforward because the acylating agents are not soluble in, and the usual coupling procedures are not compatible with, the aqueous solvents required to dissolve proteins. The practise has been to use mixed solvents, the most popular activated form of the protected amino acid being an activated ester (review [1]). Large excesses of reagent have to be used; water-soluble activated esters have been introduced to try to overcome their inefficiency in coupling in aqueous solvents [2]. Mixed anhydrides will acylate amino groups in partially aqueous solution, however secondary acylation products are formed [3,4]; *N*-carboxyanhydrides can lead to the addition of two residues [5]. A coupling procedure which is efficient in partially aqueous solution and which leads to only one acylation product would be valuable since it would allow the synthesis of products requiring a minimum of purification. We describe here a method of acylating amino groups in partially aqueous solution which satisfies these criteria. It involves the use of symmetrical anhydrides of *N*-alkoxycarbonylamino acids in 80% aqueous *N,N*-dimethylformamide.

Abbreviations: Boc, *t*-butoxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl, DMF, *N,N*-dimethylformamide; EDC, *N*-ethyl-*N'*-(γ-dimethylaminopropyl)-carbodiimide hydrochloride; TosOH, toluene-*p*-sulfonic acid; $(Boc\text{-}Ala)_2O$, symmetrical anhydride of Boc-alanine. Amino acid symbols represent the L-isomer, except for glycine

2. Materials and methods

Chemically pure crystalline (except $[Z\text{-}Leu]_2O$ which is an oil) symmetrical anhydrides were prepared by reaction of the *N*-alkoxycarbonyl-amino acid with the soluble carbodiimide EDC followed by washing the dichloromethane solution with aqueous acid and $NaHCO_3$ [6]. The Boc group was removed using hydrogen chloride in ether at 23°C for 1 h. Benzyl and Z groups were removed by catalytic hydrogenation over 5% palladium-on-charcoal for 18 h. Thin-layer chromatography (TLC) was carried out on precoated silica gel G-60 plates using ethyl acetate/hexane (4:1) as solvent, except where indicated.

Symmetrical anhydride was added to a stirred solution of the amino acid or peptide ester salt (1 mmol) and *N*-methylmorpholine (1 mmol) in DMF/H_2O (4:1) (5 ml/mmol) and the mixture was stirred at 23°C overnight, or for 3 h for the analytical experiments. When excess symmetrical anhydride had been used, 1-amino-3-dimethylaminopropane (1 mmol) [7] was then added to convert the excess into water-soluble products and stirring was continued for 1 h. Dichloromethane (100 ml) was added, and the solution was washed successively with 50 ml each of H_2O (×2), 10% aqueous citric acid, H_2O, saturated aqueous $NaHCO_3$, and H_2O and then dried ($MgSO_4$). The solvent was removed by evaporation under reduced pressure leaving the protected peptide which did not require further purification. Yields are based on the component used in lesser amount.

3. Results

Acylation of H–Leu–OBzl · TosOH (1 mmol)

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with 1 equiv. $(Boc\text{-}Phe)_2O$ in various solvent mixtures gave the following yields of Boc-Phe–Leu–OBzl, m.p. 75–77°C, TLC one spot R_F 0.88 (chloroform/methanol, 9:1), R_F 0.96 (ethyl acetate/pyridine/acetic acid/H_2O, 60:30:6:11): DMF/H_2O, 4:1, 91%; DMF/H_2O, 3:2. 91%; DMF/H_2O, 1:1, 60% dimethylsulfoxide/H_2O, 4:1, 43%; tetrahydrofuran/H_2O, 4:1, 45%. Acylation was therefore efficient and clean in 80% or 60% aqueous DMF and clean but less efficient in the other solvents. That no racemization occurred during the couplings was established by reaction of $(Boc\text{-}Val)_2O$ with H–Lys(Z)–OBzl · HCl and subsequent two-step deprotection and analysis for the diastereomeric Val–Lys dipeptides with an amino acid analyzer [8]. Not more than 0.2% of D–L isomer could be detected. The valyl (or isoleucyl) residue is the most susceptible to racemization when couplings are carried out in polar solvents [9].

The model peptide L-leu–L-alanylglycyl–L-valine [10] was obtained as described, no attempt having been made to optimise the yields. Acylation of H–Val–OBzl · TosOH (4 mmol) with $(Boc\text{-}Gly)_2O$ (3.9 mmol) gave Boc-Gly–Val–OBzl, 93% yield, oil, TLC one spot R_F 0.75.

Cleavage of the Boc-group gave H–Gly–Val–OBzl · HCl, 92% yield after washing with dichloromethane, m.p. 180–181°C, which was coupled (2 mmol) with $(Boc\text{-}Ala)_2O$ (2.2 mmol) to give Boc-Ala–Gly–Val–OBzl, 94.4% yield, m.p. 123–124°C, $[\alpha]_D^{23}$ −20° (*c* 2, chloroform), TLC one spot R_F 0.50.
Anal. calc. for $C_{22}H_{33}N_3O_6$: C, 60.67; H, 7.64; N, 9.65.
Found: C, 60.83; H, 7.59; N, 9.56.

Cleavage of the urethane followed by evaporation after the addition of hexane gave H–Ala–Gly–Val–OBzl · HCl, 90% yield, as a hygroscopic powder, which was reacted (0.9 mmol) with $(Z\text{–}Leu)_2O$ (1.5 mmol). Crystallisation of the product from dichloromethane/light petroleum gave Z–Leu–Ala–Gly–Val–OBzl, 90% yield, m.p. 125–126°C, $[\alpha]_D^{23}$ −21.3° (*c* 2, chloroform), TLC one spot R_F 0.31.
Anal. calc. for $C_{31}H_{42}N_4O_7$: C, 63.90; H, 7.27; N, 9.62.
Found: C, 63.92; H, 7.14; N, 9.64.

Catalytic hydrogenation of the protected tetrapeptide (0.4 mmol) gave after filtration and evaporation of the solvent H–Leu–Ala–Gly–Val–OH · H_2O as a homogeneous (amino acid analyzer) powder, 97% yield, m.p. 155–160°C, $[\alpha]_D^{23}$ +22.7° (*c* 2, ethanol), TLC one spot R_F 0.54 (*n*-butanol/acetic acid/water, 4:1:1), lit. $[\alpha]_D$ +23.7° (*c* 0.85, ethanol) [11].
Amino acid analysis (12 N HCl, 110°C, 18 h): Gly 1.00, Ala 1.01, Val 1.00, Leu 1.00.
Anal. after drying at 56°C for 8 h.
Calc. for $C_{16}H_{30}N_4O_5$: C, 53.61; H, 8.44; N, 15.63.
Found: 53.41; H, 8.51; N, 15.32.

Lit. m.p. 140–145°C, $[\alpha]_D^{25}$ +23.0° (*c* 1, ethanol), R_F 0.45 (*n*-butanol/acetic acid/water, 4:1:1) for the anhydrous peptide [12]. The product and protected intermediates showed appropriate ^1H NMR spectra (deuterochloroform).

4. Discussion

Modified versions of the Merrifield solid-phase method of peptide synthesis involve the use of the 'preformed' symmetrial anhydrides as the activated form of the residue to be coupled [12,13], see [6]. The protected amino acid and *N,N'*-dicyclohexylcarbodiimide are mixed to form the anhydride, the urea is filtered off, and the solution is added to the amino-containing component on the support. We have shown that symmetrical anhydrides are surprisingly insensitive to water and consequently can be obtained in a pure state if the soluble carbodiimide EDC is used [6]. All the by-products of the reaction become soluble in water. This work is a logical extension of the recognition of the properties of symmetrical anhydrides and was prompted by the report that mixed anhydrides are not satisfactory for the acylation of amino groups in proteins in partially aqueous solution because they also give rise to a second acylation product, the *N*-alkoxycarbonyl derivative [4].

We have shown by the synthesis of the tetrapeptide Leu–Ala–Gly–Val, which is a sequence frequently used to assess modified methods of synthesis [11,12], that symmetrical anhydrides of *N*-alkoxycarbonyl-amino acids couple efficiently in 80% aqueous DMF. Standard work-up of reaction mixtures, consisting of washing them with aqueous solutions followed by removal of the solvent, gave products requiring no additional purification. Evidently only one acylation product is formed. Using little or no excess of acylating agent, the yield of peptide after 6 steps corresponded to 63.5%. This compares favourably with the yields of final products obtained both by solution and solid phase methods, in the latter case especially

if one takes into account the large excess of reagents which are employed for solid phase synthesis. The method is somewhat time-consuming, partly because the symmetrical anhydrides have to be prepared; however this drawback is amply compensated for by the fact that the products obtained require a minimum of purification.

The synthesis of peptides in aqueous solution has long been a principle aim of peptide chemistry. However, despite the numerous reagents and methods at their disposal, researchers have yet to describe a method which is generally applicable even in partially aqueous solution. We suggest that the use of symmetrical anhydrides in aqueous DMF holds promise as a general method for the efficient and unambiguous synthesis of peptides in partially aqueous solution. We also suggest that the method might prove suitable for the addition of single residues to amino groups in proteins.

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