Study of Hydrogenolytic Cleavage of Peptide-Resin Benzyl Ester Bonds for Synthesis of Protected Fragments of the Human Leukocyte Interferon

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Three Boc-protected fragments of the human leukocyte interferon, LeIFA, have been synthesized: I, BOC LeIFA (99–105) (BOC-Val-Ile-Gln-Gly-Val-Gly-Val); II, BOC LeIFA (116–119) (BOC-Ile-Leu-Ala-Val); III, BOC LeIFA (123–126) (BOC-Phe-Gln-Arg(Tos)-Ile). The presence of the Boc-group was desired so that these peptides could serve as intermediates in fragment condensation toward larger segments of interferon. To achieve these intermediates, a study was desirable on three methods of cleavage of the benzyl ester-polymer bonds. It was found that hydrogenolysis with hydrogen and palladium generated *in situ* was distinctly superior to transfer hydrogenation with ammonium formate or cyclohexene. An effective swelling of the peptide-resin to allow penetration of palladium acetate into the matrix and mobility of the peptide chains on the resin to expose the benzyl ester bond to hydrogenolysis appear to be essential conditions for the best cleavage.

Introduction

Recently, accounts have been made concerning the hydrogenolytic cleavage of peptides connected to the polymeric support by benzyl ester bonds. Different sources of hydrogen have been used; hydrogen gas [1, 2], ammonium formate [3], cyclohexene [4] and cyclohexadiene [5]. The catalyst has in all cases been palladium black generated *in situ* from palladium(II) acetate. Using transfer hydrogenation with cyclohexadiene and a nitrated benzhydrylamine resin, the peptide amide thymosin α_1 has been synthesized in good yield [6].

If only groups susceptible to hydrogenolysis like benzyl, benzyloxycarbonyl or nitro are present as side-chain protection, the free peptides are obtained under very mild conditions. The method has also been considered for the preparation of N_zFmoc/side chain *t*-butyl protected peptides for fragment condensation [7] but since it was shown that the Fmoc group is not completely stable towards hydrogenolysis, this approach was replaced by a new protecting group combination [8].

Catalytic hydrogenation is however, a most suitable method for the preparation of BOC-protected peptides containing amino acids which have groups which are stable to acid and hydrogenolysis, *e.g.* Arg(Tos).

Experimental

General. The BOC-amino acids were purchased from Peninsula Laboratories, San Carlos, CA. For Arg, the tosyl derivative was used. The chloromethyl resin was from Sigma Chemical Company, St. Louis, MO. The palladium(II) acetate and cyclohexene were from Aldrich Chemical Company, Milwaukee, WI. The ammonium formate was purchased from MCB Manufacturing Chemists Inc., Cincinnati, OH. The amino acid analyses were carried out on a 118 CL amino acid analyser from Beckman Instruments Inc., Palo Alto, CA, after hydrolysis in 6 N HCl/110 °C for 24 h. The HPLC was performed on an instrument from Waters Associates, Milford, MA, which was equipped with a Model 660 solvent programmer and a Partisil PXS 10/25 ODS-2 column from Whatman Inc., Clifton, NJ. The flow rate was 1.5 ml/min and a linear gradient from 10% to 50% acetonitrile in 0.02 M NH₄HCO₃ in 30 min was used. Absorption was recorded at 210 nm. The TLC was performed on EM 0.25 mm silica gel plates 60 F254 in the following solvents: A, 70% aqueous ethanol; B, 1-butanol-acetic acid-water 4:1:5 upper phase; C, 1-butanol-pyridine-acetic acid-water 30:10:3:12. The UV measurement was done on a Beckman Acta III Spectrophotometer.

Synthesis. The first amino acid was attached to the resin according to the method of Gisin [9]. The load of Val was 0.69 mmol/g and that of Ile 0.76 mmol/g. The peptides were synthesized on a Beckman 990 peptide synthesizer using the DCC method and a standard protocol [10] except that reversed addition of DCC and amino acid was employed [11]. The glutamine residue was coupled as its *p*-nitrophenyl ester with one equivalent of HOBT in 50% DMF in CH₂Cl₂ [15]. The completeness of the couplings was checked by the Kaiser

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ninhydrin test [12] and, if necessary, a double coupling was performed. From 10 g of Val-resin, there was obtained 13.83 g of peptide I-resin and from 8 g of I-resin there was obtained 10.12 g of peptide II-resin. Similarly, from 10 g of Ile-resin, there was obtained 14.40 g of peptide III-resin. These yields indicate that essentially quantitative couplings had taken place.

Hydrogenations. The transfer hydrogenations with ammonium formate [3] and cyclohexene [4] were carried out as described. The hydrogenations with H were slight modifications of the method of Schlatter *et al.* [1] and Jones [2]. Palladium acetate (0.4 g) was dissolved in 4 ml of DMF with heating to about 60 °C. After cooling, 1 g of peptide-resin was allowed to swell in this solution for 2.5 h with occasional swirling. Then the mixture was hydrogenated for about 10 min at room temperature in a Parr apparatus at 60 psi in order to reduce the Pd(II). Then another 8–10 ml of DMF was added in order to facilitate agitation, and hydrogenation was continued for 40 h under the same conditions. Then, the resin was filtered and washed with 4 × 10 ml of DMF and for peptide I, with 3 × 40 ml 0.02 M NH₄HCO₃. Lyophilization or evaporation gave the crude peptides. The gum obtained after evaporation of the DMF solution was dissolved in 0.02 M aqueous NH₄HCO₃ and the solution was lyophilized. For peptides I and II, this process was repeated with 0.2 g fresh palladium acetate. The crude yields are in Table I.

Table I. Yields of Peptides.

Peptide	Method A	Yields % ^a	
		Method B	Method C
I	3	13	50
II	5	n.d. ^b	65
III	12	52	~ 100

^a The crude yields are based on the hydrogenation of 1 g samples of the resin-peptides. In method C, peptide-resins I and II were hydrogenated twice and peptide-resin III once (see Experimental section). The crude I was 85–90% pure and II and III were ~95% pure;

^b not determined.

Purification and characterization of the peptides

BOC-Val-Ile-Gln-Gly-Val-Gly-Val (I). The crude peptide was purified by partition chromatography on Sephadex G-25 with the solvent system 1-butanol-pyridine-0.1% acetic acid, 5:3:11. After equilibration with the lower and upper phases, 170 mg of peptide was dissolved in 8 ml of the upper phase and the solution was applied on a 2.5 × 100 cm column and chromatographed with the upper phase; flow rate, 0.5 ml/min; yield, 124 mg; *R_f*A = 0.90; *R_f*B = 0.67; *R_f*C = 0.66; >97% pure by HPLC with a retention time of 14.4 min. Amino acid analysis: Glx 1.02(1); Gly 2.07(2); Val 2.76(3);

Ile 0.75(1). The low Ile value is due to the Val-Ile linkage which is known to be a resistant to hydrolysis [13].

BOC-Ile-Leu-Ala-Val (II). Purification was achieved by chromatography on Sephadex LH-20 with 0.02 M NH₄HCO₃ as the eluant; flow rate, 0.5 ml/min; 192 mg of crude peptide gave 180 mg of pure material; *R_f*A = 0.93; *R_f*B = 0.85; *R_f*C = 0.69; >98% pure on HPLC with a retention time of 20.0 min. Amino acid analysis: Ala 1.10(1); Val 1.06(1); Ile 0.91(1); Leu 0.93(1).

BOC-Phe-Gln-Arg(Tos)-Ile (III). Purification was performed as for peptide II; 189 mg of crude material gave 158 mg of pure peptide; *R_f*A = 0.92; *R_f*B = 0.77; *R_f*C = 0.67; >99% pure on HPLC with a retention time of 18.7 min. Amino acid analysis: Glx 1.01(1); Ile 1.0(1); Phe 1.05(1); Arg 0.94(1). Measurement of the UV absorption at 263 nm gave $\epsilon = 709.8$, $c = 0.785$ g/l in EtOH, $d = 1$ cm; calculated (1 Phe, 1 Tos) $\epsilon = 673$ [14]. This result shows that the tosyl group is completely stable under the hydrogenation conditions.

Results and Discussion

We have synthesized three BOC-protected fragments of the human leukocyte interferon, LeIFA [15] which are:

- BOC LeIFA (99–105)
- BOC-Val-Ile-Gln-Gly-Val-Gly-Val (I)
- BOC LeIFA (116–119)
- BOC-Ile-Leu-Ala-Val (II)
- BOC LeIFA (123–126)
- BOC-Phe-Gln-Arg(Tos)-Ile (III)

To achieve these peptides, we investigated three of the published methods for cleavage of benzyl ester-polymer bonds, namely, the transfer hydrogenations with ammonium formate [3, 4] (A) and cyclohexene (B) and a modification of the hydrogenation with hydrogen gas [1, 2] (C). The yields varied considerably both with the structure of the peptide and the method (Table I). For the three methods, the yields were I < II < III, and for the three peptides, the yields were A < B < C (for peptide II, method B was not tried). Peptide III was the most easily liberated, and method C was the best method. The transfer hydrogenation by ammonium formate gave low yields in contrast to the 95% yield reported for the synthesis of Leu-enkephalin [3].

Two criteria may have to be fulfilled in order for the hydrogenation to proceed at a reasonable rate: (a) an effective swelling property of the resin-peptide in order to allow the palladium acetate to penetrate the matrix; (b) good mobility of the peptide chains

in order to expose the benzyl ester bond to the catalyst [16]. These factors are interrelated since it has been shown [17] that the swelling property of a peptide-resin is dependent on the peptide, especially at high peptide loadings. The better a peptide interacts with the solvent, the more it contributes to the swelling property of the peptide-resin, and the more exposed the benzyl ester bonds are toward hydrogenolysis. Consequently, the influence of the structure of the peptide on the hydrogenation rate is two-fold.

The low yields obtained in the ammonium formate reductions may be due to the fact that the ammonium formate is added as a concentrated aqueous solution. This procedure could promptly shrink the resin-matrix, especially considering the lipophilic nature of these peptides. In method B, the addition of cyclohexane, which is a very good swelling solvent for the polystyrene resin evidently causes somewhat less shrinking. A higher temperature, *i.e.* 70 °C *vs.* room temperature, should also increase the reaction rate. The best solvent seemed to be pure DMF [17]. In the reactions with hydrogen, the palladium was precipitated inside and on the resin. No palladium mirror was formed which is always the case with the transfer hydrogenations, and may be a consequence of the solvents.

Since the yields for peptide III were invariably much higher than those for the other peptides it

seems reasonable that this peptide may have the best mobility within the matrix. Perhaps, peptide III has less tendency to adopt a rigid, tight conformation than I and II. The very lipophilic peptide chains might coil up together, almost α -helix-like [18]. This property could diminish swelling and, indeed, it was observed that the peptide I-resin swelled considerably less than the peptide III-resin. This behavior could be even more pronounced in the aqueous environment of method A. Peptides I and II did not give a positive chlorine test on TLC in the solvent systems butanol-acetic acid-water 4:1:5 upper phase and 70% aqueous ethanol. Evidently, in these solvents, the peptides may exist as neutral molecules, and adopt a conformation that does not allow amide hydrogen-chlorine replacement with hypochlorite. Consequently, they appear as white spots on spraying with *o*-tolidine/potassium iodide.

It thus seems reasonable to believe that the yields of cleavage of peptide-resin benzyl ester bonds by hydrogenolysis vary in such a way that the more compact conformation expected of the peptide, the lower the yield. Moreover, the hydrogenation with hydrogen gas in DMF seems superior to transfer hydrogenation.

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