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Fragment Couplings with Stepwise Couplings in Solid-Phase Synthesis. Synthesis of a Fragment of Human Leukocyte Interferon

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A fragment of the human leukocyte interferon, AcLeIFA(116–137)–NH_2, Ac–Ile–Leu–Ala–Val–Arg–Lys–Tyr–Phe–Gln–Arg–Ile–Thr–Leu–Tyr–Leu–Lys–Glu–Lys–Lys–Tyr–Ser–Pro–NH_2, has been synthesized on solid-phase. In addition to stepwise couplings, two BOC-protected tetrapeptides were added to the growing chain. These peptides, BOC–Phe–Gln–Arg(Tos)–Ile and BOC–Ile–Leu–Ala–Val were prepared on the chloromethyl resin and released by hydrogenolysis. The crude peptide, obtained after HF-cleavage, was purified by gel filtration, partition chromatography, and chromatography on LH-20. The ease of purification was no doubt the result of the reduced number of chemical transformations achieved by the use of the fragments. The use of simple peptides, prepared by hydrogenolysis, can be of significant value to increase the quality and yield by peptides synthesized mainly by stepwise methods.

Stepwise solid-phase peptide synthesis [1] is a very fast and convenient method for small peptides up to about ten amino acid residues. For larger peptides, however, the formation of numerous side products can occur. The number of both failure and truncated sequences [2] increase rapidly with increasing chain length. Side products also form in the repetitive chemical transformations along the synthesis, *e.g.* deprotection and neutralization.[3]

Fragment coupling, *i.e.* coupling of preformed, purified protected peptides would ameliorate the situation in two ways. The number of chemical transformations and the possibilities for side reactions would decrease, and the difference in chain length between the target peptide and failure and truncated sequences would increase, and would contribute to the formation of a purer peptide.

RESULTS AND DISCUSSION

Earlier fragment couplings have largely used fragments synthesized in solution [4–6] because the solid-phase method had not been developed for the synthesis of protected peptides. However, there are examples of protected fragments synthesized on solid support.[7–9] The cleavage from the resin was achieved by hydrazinolysis,[7,8] and 2-dimethylaminoethanol,[9] but such cleavage restricts side-chain functions.

Recent achievements include the work by Birr and Voss [10] and Sheppard *et al.*[11,12] The former used the very acid labile α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz) protection for the α-amino protection, *t*-butyl side-chain protection, and basic cleavage of the protected peptide from a phenacyl resin. Sheppard *et al.* used the base-labile fluorenylmethoxycarbonyl-(Fmoc) group for α-amino protection, *t*-butyl side-chain protection, and a very acidlabile dialkoxybenzylalcohol polyamide resin. The multidetachable resins developed by Merrifield *et al.*[13] should also facilitate the synthesis of protected peptides.[13]

These methods are at the forefront of this research, and use sophisticated procedures and materials and are not likely to be routinely

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Ac–Ile[116]–Leu–Ala–Val–Arg[120]–Lys–Tyr–Phe–Gln–Arg[125]–Ile–Thr–Leu–Tyr–Leu[130]–Lys–Glu–Lys–Lys–Tyr[135]–Ser–Pro[137]–NH_2

Fig. 1. AcLeIF(116–137)–NH_2.

adapted for some time in standard peptide laboratories. There is, however, a simple way to use fragment couplings to improve the quality of larger peptides synthesized mainly by stepwise methodology, *i.e.* BOC protection of the α-amino group and benzyl type side-chain protection. It is well-known that BOC-protected peptides can be liberated from the Merrifield resin by hydrogenolysis.[14,15] Small peptides, prepared in this very mild way, are normally very easily purified[14–16] and are thus suitable for fragment coupling. Hydrogenolysis excludes the use of hydrogenolyzable side-chain protection and, since the fragment coupling normally is to be followed by stepwise couplings, *t*-butyl side-chain protection is not acceptable. Consequently, the peptides to be synthesized in this way can only include amino acids with non-functional side-chains, and Gln, Asn and Arg(Tos). (Whether His(Tos) would be acceptable is unknown to us, but it is likely that it would. In most peptides, however, there are limited sequences that fulfill these requirements.

We have used this approach for the synthesis of an acetylated fragment of human leukocyte interferon A,[17] AcLeIFA(116–137)-NH_2, Fig. 1. The synthesis is outlined in Scheme 1. First, Pro[137] was attached to the benzhydrylamine resin at a substitution of 0.237 mmol/g. Then, stepwise couplings were performed to Thr[127], followed by coupling of the fragment BOC–LeIFA(123–126), stepwise couplings of the residues 122–120 and fragment coupling with BOC–LeIFA (116–119). Deprotection, acetylation and HF cleavage gave the crude peptide. Originally it was planned to synthesize the fragment BOC–Ile–Leu–Ala–Val–Arg(Tos), [BOC–LeIFA(116–120)], but this approach was abandoned since the reaction of the cesium salt of BOC–Arg(Tos) with the chloromethyl resin[18] proceeded in very low yield, ~0.09 mmol/g.

Amino acid analyses during the synthesis indicated that the fragment couplings had proceeded smoothly (Table 1). The ninhydrin reaction[19] was negative after both fragment couplings.

Dicyclohexylcarbodiimide/2 eq. 1-hydroxybenzotriazole[20] was a satisfactory coupling reagent. The combination carbonyldiimide/2 eq. 1-hydroxybenzotriazole, advocated by Birr[10] gave

BOC–Pro[137]–BHA resin

↓ Stepwise couplings

BOC–(127–137)–BHA resin

↓ (1) Deprotection
↓ (2) 5 eq BOC–Phe[123]–Gln–Arg(Tos)–Ile[126]/DCC/2HOBt

BOC–(123–137)–BHA resin

↓ Stepwise couplings

BOC–(120–137)–BHA resin

↓ (1) Deprotection
↓ (2) 5 eq BOC–Ile[116]–Leu–Ala–Val[119]/DCC/2HOBt

BOC–(116–137)–BHA resin

↓ (1) Deprotection
↓ (2) Acetylation
↓ (3) HF-cleavage

AcLeIF(116–137)–NH_2 (I)

Scheme 1. Synthesis of AcLeIF(116–137)–NH_2.

Table 1. Amino acid analyses of the peptide-resin at the different stages of the synthesis.

Residues No.	127–137	123–137	120–137	116–137
Thr	1.10(1)	0.95(1)	0.94(1)	0.86(1)
Ser	0.57(1)	0.58(1)	0.59(1)	0.72(1)
Glx	1.12(1)	2.16(2)	2.07(2)	2.12(2)
Pro	1.15(1)	1.24(1)	1.25(1)	1.33(1)
Ala				0.94(1)
Val				0.93(1)
Ile		0.70(1)	0.65(1)	1.61(2)
Leu	2.01(2)	2.11(2)	2.21(2)	3.21(3)
Tyr	2.09(2)	2.18(2)	3.13(3)	3.19(3)
Phe		0.90(1)	0.91(1)	0.95(1)
Lys	2.98(3)	3.31(3)	4.51(4)	4.37(4)
Arg		0.90(1)	1.74(2)	1.81(2)

negligible yields, according to amino acid analysis of the peptide-resin. It might have been that the activation of the carboxyl group was slower than expected and that unreacted CDI blocked the free amino group. This is supported by the fact that the ninhydrin reaction was negative.

The coupling of the fragment BOC–Phe–Gln–Arg(Tos)–Ile to the amino group of Thr(O–Bzl) proceeded smoothly considering the steric bulk of both Ile and Thr(O–Bzl).

Purification included gel filtration, partition chromatography and chromatography on LH-20. The gel filtration showed a product with very small amounts of shorter peptides, which indicated that all couplings had proceeded in high yields. After partition chromatography the purity was >90 % (HPLC). This material could be conveniently purified by chromatography on LH-20 with 0.1 % acetic acid as the eluant. The success of the latter chromatography is no doubt due to the adsorptive properties of the gel towards the four aromatic residues of the peptide.

The yield of purified material was 45 mg with a purity of >97 %, corresponding to a yield of 22.3 % based on crude material corrected for the fact that only half of the gel filtrated material was further purified.

These results show that preparation of simple, BOC-protected peptides by hydrogenolysis from the Merrifield resin and coupling them on solid-phase can be of value to enhance the quality of peptides synthesized mainly by standard solid-phase methods.

EXPERIMENTAL

General. The BOC-amino acids were purchased from Peninsula Laboratories, San Carlos, CA. Side-chain protection was provided by benzyl groups for Glu, Ser and Thr. The guanidino group of Arg was protected as the tosyl derivative, and the phenolic hydroxyl of Tyr and the ε-amino group of Lys were protected as the Br–Z and Cl–Z derivatives, respectively. The benzhydrylamine hydrochloride resin was obtained from Beckman Instruments, Inc., Palo Alto, CA. The amino acid analyses were carried out on a 118 CL amino acid analyzer from Beckman after hydrolysis for 24h/110 C in 1:1 HCl-propionic acid for the resinbound peptides, and for 48h in 6N HCl for the free peptides. The TLC was performed on EM 0.25 mm silica gel plates, 60F 254, in the following solvents: 1-butanol–pyridine–acetic acid–water 30:10:3:12 (A) and 3:5:1:4 (B); 1-butanol–acetic acid–water 4:1:2 (C). All chromatography was monitored by TLC in solvent system A. The spots were visualized with the chlorine–*o*-tolidine reagent. The HPLC was performed on an instrument from Waters Associates, Milford, MA, equipped with a 660 solvent programmer and a μ-Bondapak C_{18} column 5×250 mm. The flow rate was 1.5 ml/min and the absorption was measured at 230 nm. Solvent A was 0.1 % trifluoroacetic acid and solvent B was 80 % acetonitrile and 20 % A. A gradient of 30–80 % B in 30 min was used.

Synthesis. The stepwise couplings were carried out on a Beckman Model 990 peptide synthesizer. First, BOC–Pro was attached to the BHA resin as the symmetrical anhydride[21] to give a substitution of 0.237 mmol/g. The excess amino groups were acetylated, and 2.7 g of this resin, containing 0.64 mmol Pro was then subjected to stepwise couplings until Thr[127] had been incorporated. A standard protocol was used[22] except that the amino acids were added as their corresponding symmetrical anhydrides. Also, diisopropylethyl amine was used in the neutralization step instead of triethylamine.

At this point, the resin-peptide weighed 4.43 g. 1.89 g of this material, theoretically 0.27 mmol peptide, was used for further couplings. Next, the fragment BOC–Phe–Gln–Arg(Tos)–Ile, prepared as described,[16] was coupled: 1.1 g (1.35 mmoles) of the peptide and 0.365 g (2.7 mmol) of HOBt were dissolved in 15 ml DMF. Then, at 0 °C, 278 mg (1.35 mmol) of DCC was added and the flask swirled to dissolve the DCC. Then this mixture was allowed to stand for 15 min at room temperature. Small amounts of dicyclohexylurea precipitated. This solution was, without filtra-

tion, added to the deprotected and neutralized resin-peptide, preswollen in 15 ml of DMF. The resulting mixture was then left for 72 h at room temperature with gentle swirling in a Gyrotory water bath shaker, model G76, from New Brunswick Scientific, Edison, NJ. After this period of time, the resin was filtered, washed with 3×30 ml DMF, 2×30 ml CH_2Cl_2, 3×30 ml EtOH, 3×30 ml DMF and 3×30 ml CH_2Cl_2. The ninhydrin reaction[19] was negative. The resinpeptide was subsequently returned to the synthesizer for stepwise couplings of residues 122–120. After this, 1.35 mmoles of the fragment BOC–Ile–Leu–Ala–Val[16] was coupled using the same procedure as above yielding 2.4 g peptide-resin. 1.4 g of this materal was deprotected and acetylated.

Purification and Characterization of the Peptide. 1.4 g acetylated peptide-resin was cleaved and side-chain deprotected by treatment with doubly distilled HF.[23] HF(g) from the cylinder was condensed by cooling with acetone/dry ice into a vessel containing CoF_3 and a magnetic stirring bar. It was then distilled from this vessel with stirring and gentle warming with warm water into another vessel cooled with acetone/dry ice. The latter vessel contained the peptide-resin and *ca.* 2.5 g anisole. About 20 ml HF(1) was distilled. The temperature was then raised to 0 °C and the peptide-resin stirred at this temperature for 1 h. Still at 0 °C, the HF was evaporated with a water aspirator. The last traces of HF were removed in a vacuum desiccator over KOH at 1 mmHg overnight. The crude material was then stirred with 4×30 ml ether with subsequent filtrations in order to remove the non-peptidic cleavage products. The resulting material was then stirred with 5×30 ml 5 % acetic acid with filtration in order to dissolve the peptide. This solution was stirred during 20 min with 30 g anion exchanger, AG1–X8, Bio–Rad, acetate form, filtered and lyophilized to give 390 mg crude peptide. Gelfiltration of this material, in two runs, on a G50 column, 2.5×110 cm, eluted with 5 % acetic acid, 0.5 ml/min, gave 320 mg peptide in fractions 58–86 (6 ml each). Then, 165 mg of this material was chromatographed by partition on G50 with the solvent system 1-butanol–pyridine–0.1 % acetic acid 5:3:11. The column size, flow rate and fraction size were as above. The desired peptide appeared in fractions 115–152 and was ca 90 % pure by HPLC. An earlier fraction, 102–114, 17 mg, was 70 % pure. The main fraction, 80 mg, was finally purified by chromatography on LH 20 with 0.1 % acetic acid as the eluant under the same conditions as above. The purified peptide appeared in fractions 59–64; yield 45 mg; R_fA=0.19; R_fB=0.73; R_fC=0.26.

Amino Acid Analysis. Thr 0.97(1); Ser 0.97(1); Glu 2.07(2); Pro 1.01(1); Ala 0.97(1); Val 0.92(1); Ile 1.72(2); Leu 3.11(3); Tyr 3.13(3); Phe 1.00(1); Lys 4.16(4); NH_3 1.99(2); Arg 1.99(2).

Purity by HPLC. >97 %. Retention time, 17.1 min.

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