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NEW NONSOLIDPHASE METHOD FOR QUICK, QUANTITATIVE SYNTHESIS OF ANALYTICALLY PURE PEPTIDES WITHOUT INTERMEDIATE OR FINAL PURIFICATIONS: I. SYNTHESIS OF A NONAPEPTIDE

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1.0 Recent improvements in peptide synthesis methodology have been successfully employed in the synthesis of materials with full or partial hormonic and enzymatic activities. However, the procedures are either extremely laborious or the products are complex mixtures of difficultly separable components.

1.1 We have developed a procedure for rapid and quantitative synthesis of oligopeptides which are analytically and chromatographically pure without isolation or purification. As a demonstration of the simplicity of our method we are reporting the synthesis of a nonapeptide used by Wünsch(1) in the synthesis of glucagon.

1.2 In peptide coupling reactions an excess of one reactant usually must be used to drive the reaction to completion. The resulting contamination of the desired product with starting material necessitates purification with attendant loss of yield. Activation procedures often produce side products which are difficultly removable from the desired product. No known method of peptide synthesis(2,3,4) combines all the advantages of quantitative yields, ease of handling, purity of the final product, and use of stable, readily available derivatives which permit mild deblocking. Thus, what has been needed for simplification of peptide synthesis is a process allowing easy removal of excess reactants and side products.

1.3 To achieve this goal we investigated approaches which would (a) produce complete coupling through the use of excess of suitable activated amino acid derivative, (b) enable quantitative removal of the excess of reactants at completion of coupling by simple extraction procedures, (c) not produce difficultly separable side-products such as dicyclohexylurea or nitrophenol and (d) utilize readily available, simply prepared amino acid derivatives.

2.0 Mixed (5) and symmetrical anhydrides (6) seemed promising. F. Weygand

et al (6) have obtained high yields of peptides using an excess of the symmetrical anhydrides of Boc amino acids. After coupling, excess anhydride was reacted with an excess of N-(2-aminoethyl)piperazine, and the products were removed from the peptide with citric acid/H_2O and $NaHCO_3/H_2O$ extractions. But because symmetrical anhydrides are difficult to obtain and use twice as much of the expensive amino acid derivatives, we chose mixed anhydrides. Under the conditions we use, mixed anhydride couplings are known to be racemization-free (7). We have confirmed this by enzymic digestion of a tetra- (reported here) and a hexapeptide (to be reported) made by our method.

2.1 Efforts to hydrolyze the excess mixed anhydride with a warm (40-50°) DMF*/ deionized H_2O (containing CO_2) mixture were not successful as determined by IR. We found that quantitative hydrolysis of excess mixed anhydride was possible with $KHCO_3/H_2O$ at 0°C in 1/2 hr. The resulting Z-amino acid(8) K salt can be removed with H_2O wash of the product and reclaimed from H_2O by acidification.

2.2 Experiments using several ratios of reactants showed that a 0.4 molar excess of Z-Phe isobutylcarbonic mixed anhydride(5) gave complete reaction with Phe-OMe detected by TLC. Concentration, temperature, steric hindrances, and solvents affect the excess needed for complete coupling.

2.3 Procedure: We react the amino component (cooled to -15°C or lower in DMF) with a 0.5 molar excess of Z-amino acid isobutylcarbonic mixed anhydride for 2-4 hrs. The mixed anhydride is formed in DMF at -15° or lower in 10-15 minutes using ~6% excess Z-amino acid derivative and N-methylmorpholine over isobutyl chloroformate to avoid side reactions due to unreacted chloroformate. (With DCHA salts, the Z-amino acid is obtained as the free acid by extraction into EtOAc from 10% citric acid/H_2O. The EtOAc phase is carefully washed free of citric acid with H_2O and is evaporated to obtain the Z-amino acid derivative.) The excess mixed anhydride is then hydrolyzed at 0° by raising and maintaining the pH at ~8 with a slight excess of sat. $KHCO_3/H_2O$ and stirring for 1/2 hour. The peptide is precipitated with cold 90% sat. NaCl/H_2O; the Z-amino acid K salt is removed by filtering the peptide and washing it with NaCl/H_2O and H_2O. Non-filterable peptides are extracted into EtOAc, and the extracts are washed with NaCl/H_2O, dried, and

*All abbreviations at end of references.

evaporated. The crude peptide thus obtained is used directly in further syntheses.

2.4 The Z group is hydrogenolyzed in MeOH, DMF, HOAc, or mixtures using 100-500 mg Pd/C catalyst per mmole of peptide. CO_2 evolution is tested with $Ba(OH)_2$ solution. With dipeptides and N-terminal glutamine peptides, one equivalent of aq. HCl is added to prevent side reactions. After filtering (Whatman paper #5 or #50) and washing the catalyst, the filtrate is evaporated exhaustively in vacuo (10^{-4}cm Hg; Büchi, Model VE50) at <30° adding benzene to accomplish the azeotropic removal of MeOH. The deblocked peptide is then ready for the next coupling after neutralizing HCl, if present.

2.5 Advantages and flexibility: Using this procedure with Z-amino acids, we have synthesized several different sequences at an average rate of one residue per day. For example, the synthesis of the hexapeptide(V) reported in the experimental section was completed in 5 1/2 days. One coupling cycle can be conducted in 6-8 hrs. We have consistently obtained high yields of pure peptides without intermediate or final purification. All the usual criteria of purity: total elemental analysis (CHNO), thin layer chromotography in 4 systems, and amino acid analysis were used for each nonpurified intermediate. (Although elemental analysis of consecutive protected peptides are similar, the difference between the deblocked and the subsequent protected peptide is significant.) In combination, these criteria offer strong evidence of purity. Since the peptides are not attached to a solid support, our procedure allows recrystallization and purification of intermediates, if needed, to prevent accumulation of impurities. Our method requires smaller excesses (1:1.6) for quantitative coupling than solid phase (1:4), and the required reagents are comparatively less expensive and more readily obtained. Since yields have been in excess of 96% on each step in a nonapeptide reported here, stepwise synthesis of long chain peptides appears feasible. We have successfully used Nα-Boc amino acids(9) and expect this method to be applicable to substituted benzyl-[benzhydryl(10)], -oxy-carbonyl, phthaloyl, and other amino protections and combinations compatible with mixed anhydride activation. Use of compatible side chain protection is essential for purity; we have used O-t-butyl, O-acetyl, O-benzyl, N-ε-BOC, nitro, Z_2-guanido, and amido xanthyl. We expect that substituted

benzyl, benzhydryl(10), trityl, and O-acyl (for -OH, -SH, -COOH) could be used. Our procedure is also adaptable to pivalic and other mixed anhydrides. No other method of peptide synthesis has shown the results that are being reported here. For protective groups and references to the preparations of the various amino acid derivatives used in the following, see Shröder and Lübke, The Peptides, Vol. I pp. 246-270(1966).

3.0 Synthesis of a Nonapeptide; Z-Thr(Bu^t)-Ser(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)OEt: To assure purity, reactant quantities and experimental conditions(temp., activation time, pH during washings, exhaustive hydrogenolysis, complete solution of reactant) must be observed exactly. Couplings were conducted overnight for convenience. However, 2-4 hrs is more than sufficient.

3.1 Z-Leu-Asp(OBu^t)-OEt(I): 10 mmoles (3.15g) Z-Asp(OBu^t)-OEt in 200 ml MeOH, 9.9 ml 1N aq HCl, and 1 ml HOAc was hydrogenolyzed at 0° and worked up (Sec. 2.4). Residue (in 25 ml DMF) was neutralized with 9.9 mmole (1.10 ml) N-methylmorpholine and reacted at -15° with 15 mmole Z-Leu mixed anhydride [obtained from 16 mmole (4.24 g oil) Z-Leu]. Work up [Sec. 2.3. (EtOAC extraction)]gave dipeptide(I).

3.2 Z-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (II): Crude (I) in 50 ml MeOH, 9.9 ml 1N aq HCl and 1 ml HOAc was hydrogenolyzed at 0° and worked up (Sec. 2.4). Residue (in 25 ml DMF) was neutralized with 9.9 mmole N-methylmorpholine and reacted at -15° with 15 mmole Z-Tyr(Bu^t) mixed anhydride [obtained from 16 mmole (8.84 g) Z-Tyr(Bu^t), DCHA see (Sec. 2.3)]. Work up [Sec. 2.3 (EtOAC extract)]gave tripeptide(II).

3.3 Z-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (III): Crude (II) in 50 ml MeOH was hydrogenolyzed and worked up (Sec. 2.4) Residue in 25 ml DMF was reacted with 15 mmole Z-Lys(Boc)-mixed anhydride [obtained from 16 mmole (9.0 g) Z-Lys(Boc), DCHA see Sec. 2.3]. Work up (Sec. 2.3) gave filterable ppt. of (III). 1 spot in 4 TLC systems. Crude elemental analysis: Theory C 63.20, H 8.06, N 7.67, O 21.05; Found C 63.21, H 8.19, N 7.64, O 20.97. Amino acid analysis. Lys 1.06, Tyr 1.00, Leu 1.10, Asp .95 $[\alpha]_D^{23}$= -25.42, C=3 in DMF; amino acid analysis of amino peptidase digest (fully deblocked peptide) Tyr 1.0, Leu 1.13, Asp 1.08, Lys .91 (on diff. column).

3.4 Z-Ser(Bu^t)-Lys(Boc)Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (IV): Crude (III) in MeOH hydrogenolyzed and worked up (Sec. 2.4). Residue (in 25 ml DMF) was reacted at

-15° with 15 mmole Z-Ser(Bu^t)-mixed anhydride [obtained from 16 mmole (7.64 g) Z-Ser(Bu^t) DCHA see Sec. 2.3]. Work up (Sec. 2.4) gave filterable ppt.(IV). 1 spot in 4 TLC systsms. m.p. 170-173°. Crude elemental analysis. Theory C 62.59, H 8.21, N 7.96, O 21.22; Found C 62.29, H 8.39, N 7.78, O 21.40. Amino acid analysis Lys .86, Tyr 1.0, Leu 1.0, Asp .84, Ser .98; $[\alpha]_D^{23}$= -16.94, C=1.1 MeOH.

3.5 Z-Tyr(Bu^t)-Ser(Bu^t)-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt: Crude(IV) in 100 ml DMF/250 ml MeOH was hydrogenolyzed and worked up(Sec. 2.4). Residue(in 25 ml DMF) reacted at -15° with 15 mmole Z-Tyr(Bu^t)-mixed anhydride [obtained from 16 mmole (8.84 g) Z-Tyr(Bu^t), DCHA (see Sec. 2.3)]. Worked up (Sec. 2.3 - filterable ppt) to give (V). 1 large and 1 minor TLC spot. 94% yield (based on C-terminal amino acid, $\tilde{=}$98.7% on each step), m.p. 184 sint., 194-197° d. Crude elemental analysis Theory C 64.07, H 8.14, N 7.69, O 20.08; Found C 64.16, H 7.90, N 7.64, O 20.38. Amino acid analysis Lys 1.00, Asp .85, Ser .82, Leu 1.05, Tyr 1.90.

3.6 Z-Asp(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (VI): One-half of crude (V) in DMF/MeOH hydrogenolyzed and worked up (Sec. 2.4). Residue (in DMF) was reacted at ~15°C with 7.5 mmoles Z-Asp(OBu^t)-mixed anhydride. [Z-Asp(OBu^t) was obtained by saponifying Z-Asp(OBu^t)-OEt]. Worked up (Sec. 2.3-filterable ppt.) to give (VI). Yield 95% based on (V). 1 spot in 4 TLC systems. m.p. 209-211d. Crude elemental analysis. Theory C 63.13, H 8.09, N 7.75, O 21.03; Found C 63.16, H 8.29, N 8.01, O 21.27.

3.7 Z-Ser(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (VII): Crude (VI) in ~150 ml DMF/MeOH was hydrogenolyzed and worked up (Sec. 2.4). Residue (in 25 ml DMF) was reacted at -15°C with 7.5 mmole Z-Ser(Bu^t)-mixed anhydride [obtained from 8.0 mmole (3.8 g) Z-Ser(Bu^t), DCHA]. Worked up (Sec. 2.3-filterable ppt) to give (VII). 1 spot in 4 TLC systems; m.p. 215-216°d. Crude elemental analysis. Theory C 62.74, H 8.18, N 7.93, O 21.15; Found C 62.70, H 8.16, N 7.93, O 21.16. Amino acid analysis Lys 1.06, Asp 1.90, Ser 1.66, Leu 1.13, Tyr 2.0.

3.8 Z-Thr(Bu^t)-Ser(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(Boc)-Tyr(Bu^t)-Leu-Asp(OBu^t)-OEt (VIII): Crude (VII) in 50 ml DMF, 100 ml MeOH hydrogenolyzed and worked up (Sec. 2.4). Residue (in 25 ml DMF) reacted at -15° with 7.5 mmole Z-Thr(Bu^t)-mixed anhydride [obtained from 8.0 mmole (3.9 g) Z-Thr(Bu^t), DCHA].

Worked up (Sec. 2.3-filterable ppt.) to give VIII. Yield ~79% based on C-terminal amino acid (i.e. >96% on each step) 1 spot in 4 TLC systems. m.p. 205-210°. Crude elemental analysis. Theory C 62.59, H 8.31, N 8.02, O 21.07; Found C 62.34, H 8.49, N 8.21, O 21.30. Amino acid analysis Lys 1.03, Asp 1.96, Thr 1.06, Ser 1.58, Leu 1.10, Tyr 2.00.

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Abbreviations used are as follows: BOC=tertiary butyl-oxycarbonyl, DCHA=dicyclohexylamine, DMF=dimethyl formamide, EtOAC=ethyl acetate, HOAC=acetic acid, MeOH=methanol, Bu^t=tertiary butyl, Z=N-α-carbobenzoxy, TLC=thin layer chromatogram.