quantitative). Recrystallization from pentane gave a yellow crystalline solid, which was identified to be **11**: mp 119–119.5 °C; IR (CCl_4) ν (CN) 2242 cm^{-1}; ^1H NMR δ ($CDCl_3$) 7.20–7.62 (m, 5 H, aromatic), 4.45 (d, 1 H, J_{HH} = 8 Hz, benzylic), 2.70–3.55 (m, 3 H, remaining azetidine ring protons), 0.89 (s, 9 H, *tert*-butyl).

The compound was analyzed as its picrate derivative, mp 181–181.5 °C.

Anal. Calcd for $C_{20}H_{21}N_5O_7$: C, 54.18; H, 4.74; N, 15.80. Found: C, 53.98; H, 4.70; N, 15.57.

***trans*-1-*tert*-Butyl-2-phenyl-3-hydroxymethylazetidine (14).** A 0.6-g (0.0025 mol) sample of **8** was refluxed in a suspension of 1 g of lithium aluminum hydride in a mixture of 15 mL of dioxane and 60 mL of ether for 57 h. The $LiAlH_4$ in excess was destroyed by adding water to it. The organic layer was separated, and the aqueous layer was extracted with diethyl ether. The combined organic extract was dried over anhydrous magnesium sulfate. Evaporation of the solvent in vacuo yielded 490 mg (89%) of a slightly yellow oil, which was identified to be **14**: IR ν (OH) 3400 cm^{-1}; ^1H NMR δ ($CDCl_3$) 7.10–7.58 (m, 5 H, aromatic), 4.13 (d, 1 H, J_{HH} = 7 Hz, benzylic), 3.66 (d, 2 H, J_{HH} = 5.5 Hz, $-CH_2OH$), 2.20–3.50 (m, 3 H, remaining azetidine ring protons), 0.89 (s, 9 H, *tert*-butyl); high-resolution MS M^+ 219.1620; molecular weight, calcd for $C_{14}H_{21}NO$ = 219.1623.

Azetidine 14 from 7a. A 500-mg (0.0021 mol) sample of **7a** was stirred in a suspension of 350 mg of lithium aluminum hydride in 75 mL of anhydrous ether for 46.5 h at room temperature. The $LiAlH_4$ in excess was destroyed by adding water to the mixture. The organic layer was separated and the aqueous layer was extracted several times with ether. The combined ethereal extract was dried over anhydrous magnesium sulfate. Evaporation of the solvent yielded 300 mg (65.2%) of a light yellow oil, which was spectrally equivalent to **14** prepared by a different method as described in the previous section.

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Registry No.—**1a**, 53059-43-1; **2b** HBr, 62029-87-2; **4**, 59728-94-8; **7a**, 62029-88-3; **7a** picrate, 62029-89-4; **7b**, 62029-90-7; **7b** picrate, 62029-91-8; **8**, 62029-92-9; **9**, 62029-93-0; **10**, 62029-94-1; **11**, 62029-95-2; **11** picrate, 62029-96-3; **11a**, 62059-32-9; **12**, 62029-97-4; **13**, 10235-75-3; **13-*d***, 13943-11-8; **14**, 62029-98-5; *tert*-butylamine, 75-64-9.

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(5) Under basic condition *cis*-1-*tert*-butyl-2-phenyl-3-benzoylazetidine epimerizes to its trans isomer.[2b]
(6) Compared to sodium methoxide and potassium *tert*-butoxide in the appropriate alcohol which fail to catalyze epimerization or deuterium exchange of azetidinyl ester **7**, lithium aluminum hydride and the bases produced during hydrolysis are much weaker. Therefore, under the condition described, base-catalyzed epimerization of azetidinyl ester **7a**, acid **8**, and alcohol **14** is unlikely.
(7) A report on the x-ray crystallographic studies of the picrates of the series of 1-*tert*-butyl-2-phenyl 3-substituted azetidines is under preparation and will be published elsewhere. A preliminary report was presented at the 12th Midwest Regional Meeting (Organic) of the American Chemical Society, University of Missouri, Kansas City, Mo., October 1976.
(8) The ring closure of a γ-haloamine in the presence of base is one of the most commonly used methods for the synthesis of azetidines. This reaction involves an internal nucleophilic displacement by an amino group of the halogen atom at the γ position of a three-carbon chain. For a review, see J. A. Moore in "Heterocyclic Compounds with Three and Four-Membered Rings", Part II, A. Weissberger, Ed., Interscience, New York, N.Y., 1964, p 885.
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Excess Azide Method of Peptide Synthesis

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A new procedure of peptide synthesis using a large excess of protected amino acid azide is described. The azide solution in CH_2Cl_2 (or DMF) is added to the amino component dissolved in DMF. Methylene chloride (if used) can then be evaporated in vacuo at low temperatures. The excess azide is subsequently hydrolyzed at 0 °C by treatment of the DMF solution with $KHCO_3/H_2O$ *in a homogenous phase*. The procedure permits isolation of analytically pure peptides in high yields. Syntheses of several dipeptides and Z-Gly-Gly-Gly-OEt are reported.

Summary

Analytically pure peptides were synthesized in excellent yields by a procedure employing a large excess of the amino acid azide component. Two equivalents of the protected amino acid azides were reacted with the amino group of a carboxyl protected amino acid or peptide in DMF. Consistent 85–90% yields of the coupled peptide were obtained. The excess azide components were eliminated during product isolation by rapid hydrolysis in a homogeneous potassium bicarbonate/H_2O/DMF solution. The large excess may reduce side reactions by permitting a low reaction temperature (0 °C or lower) during a relatively short time period (27 h). The relative freedom from racemization, an outstanding feature of azide couplings, was retained in this procedure. Additional purification steps were generally not required after the simple isolation of the product. Hydroxyl protective groups for serine and threonine were not needed, but side reactions occurred with the unprotected phenolic group of tyrosine. This procedure offers a convenient approach to the stepwise synthesis of many peptide sequences and may be of help in optimizing the yields of longer peptides.

Discussion

There have been recent successful applications of the excess mixed anhydride method[1,2] for the synthesis of peptides, such as secretin.[3] This has encouraged us to extend the advantages of the excess amino acid derivative concept to the development of new peptide synthesis procedures. The acid azide method of peptide synthesis has proven to be adaptable to procedural modifications, similar to the excess mixed anhydride method.

The azide method of peptide synthesis, in use for over 70 years, is held in high regard by peptide chemists due to several advantages. The starting materials (hydrazides) are easy to

prepare, racemization during coupling is minimal, and in many cases side chain protection is not required. The relative freedom from racemization, which is so important in peptide synthesis, has been repeatedly confirmed[4,5,10] during azide couplings. Some disadvantages of the azide method include frequent low yields, amide formation during the conversion of hydrazides to azides, and isocyanate formation via the Curtius rearrangement.[6] The excess azide method described below employs a significant excess of the acid azide to ensure a relatively quick, quantitative coupling. Azide preparations, reaction conditions, and other procedural details are designed to reduce potential side reactions to a minimum. This azide procedure, somewhat analogous to the excess mixed anhydride method, affords excellent yields of analytically pure peptides and can obviously be applied repetitively to the synthesis of larger peptides.

The carbobenzyloxy amino acid hydrazides were prepared from the methyl esters by the procedure of Zahn and Schnabel.[7] However, we found that good yields (72–94%) of the analytically pure hydrazides could be obtained by using 3 molar equiv of 85% hydrazine hydrate in the reaction.

Of key importance to the success of the excess azide procedure is the rapid and total elimination of the large excess of N-protected amino acid azide used in the reaction. This is demonstrated to be successful by treatment of the reaction mixture with a homogeneous $KHCO_3/DMF/H_2O$ solution. A solution of Z-Ala-N_3 dissolved in chloroform/methanol was treated for 45 min at 0 °C with a 50% saturated $KHCO_3/H_2O$ solution and water. As a control, an identical sample was treated with a 50% saturated $NaCl/H_2O$ solution and water. The azide remaining in these homogeneous solutions was extracted into chloroform. The infrared spectra of these extracts showed that the azide band (2140 cm^{-1}) was eliminated by the $KHCO_3$ treatment, but it was retained when treated with $NaCl/H_2O$. The azide hydrolysis product was further identified as the potassium salt of Z-Ala, which could be eliminated from coupling products by washing with water.

To enable incorporation of this alkaline hydrolysis into a generalized peptide synthesis procedure, solvent changes were required. DMF was chosen as a reaction solvent, since it would easily dissolve the acid azides and amino components, yet would be fully miscible with the $KHCO_3/H_2O$ treatment and so permit precipitation and easy isolation of the product. Methylene chloride (CH_2Cl_2) was chosen as the Z-amino acid azide extraction solvent because it is a good solvent for azides[8,9,13,14] and has a low boiling point. Even in the presence of DMF, CH_2Cl_2 can be quickly and totally removed (in vacuo) at temperatures lower than 0 °C.

Use of 1 equiv excess of the azide enables this sluggish reaction to proceed to completion in only 27 h at temperatures of 0 °C or lower. Further experimentation using the coupling of Z-Thr-N_3 to Phe-OMe as a model indicated that both 0.75 and 0.50 molar equiv excesses of the azide under identical reaction conditions gave complete reactions, but the time involved was considerably longer (51 and 96 h, respectively). To reduce possible side reactions in azide couplings we maintained the low temperature, the large 2:1 azide excess, and short reaction times in the remaining peptide syntheses.

To ensure complete conversion of the hydrazide to azide and eliminate amide formation,[11] excesses of both $NaNO_2$ and HCl at low temperatures were employed. Azide extracts in CH_2Cl_2 were washed extensively with water and the usual $NaHCO_3/H_2O$ washes were omitted to minimize possible racemization.[12]

The product isolated following the coupling of Z-Ser-N_3 and Phe-OMe using the excess azide method described below was examined for the presence of 4-carbobenzoxyaminooxazolidone-2, a cyclic urethane formed from the isocyanate of Z-Ser-N_3.[15] The cyclic urethane carbonyl stretch (1770 cm^{-1}) of this compound could not be detected during infrared analysis of this product. A parallel synthesis using excess Z-Ser-N_3 in an alternate azide procedure[16] gave a product in which IR, NMR, and other analyses showed a significant quantity of the cyclic urethane. Hence, the precise reaction conditions and rapid hydrolysis of the excess azide in the method described here seem to be significant in reducing side reactions and affording analytically pure peptides.

The excess azide method, however, does appear to preclude the use of tyrosine derivatives without phenolic protection. Dipeptide syntheses using unprotected tyrosine either as Z-Tyr-N_3 in excess or as the amino component, TyrOMe, all resulted in colored impurities in the products. The excess nitrite/HCl during azide formation as well as excess azide during the coupling itself may be leading to the nitration of the tyrosine aromatic ring or to other side reactions previously reported.[17] There were no problems, however, associated with synthesis of the hydrazide, Z-Tyr-NH-NH_2, in the usual manner.

The following examples outline the procedural details of the excess azide method of peptide synthesis. The only real variations involved are the occasional use of acetic acid in dissolving the N-protected amino acid hydrazide and the methods of isolating the product. Small, simple peptides appear to be synthesized in consistently good yields and purity. The excess azide method could also conceivably be used in a repetitive manner for the stepwise synthesis of larger peptides.

Experimental Section

All melting points were determined in a Thomas-Hoover melting point apparatus and are uncorrected. The compounds reported in this paper in most cases were precipitated by the addition of water to the DMF solutions of the compounds. This method of precipitation, while providing analytically pure peptides, yields amorphous substances whose melting points differ appreciably from those reported previously[18] in crystalline form by crystallization. Elemental analyses were performed on a Perkin-Elmer 240 analyzer. Thin layer chromatographic (TLC) results were obtained on 5 × 20 cm plates of silica gel F-254 (E. Merck, Darmstadt, Germany). The four different solvent systems used were A, 93:7:10 THF/cyclohexane/H_2O; B, 75:24:1 ether/MeOH/H_2O; C, 75:24:1 $CHCl_3$/MeOH/H_2O; D, 75:24:1 $CHCl_3$/BuOH/H_2O. Amino acid analyses were performed in a Beckman Model 120 C automatic amino acid analyzer. Optical rotations were measured in 5-cm tubes with a Perkin-Elmer Model 241 polarimeter. All the amino acids used were of the L configuration. The following abbreviations were employed: Z, benzyloxycarbonyl; DMF, *N,N*-dimethylformamide

Z-Thr-Phe-OMe.[18] A solution of Z-Thr-NH-NH_2 (2.1383 g, 8.0 mmol) dissolved in 32 mL of 1 N HCl and 30 mL of H_2O was cooled to 0 °C. $NaNO_2$ (0.828 g, 12 mmol), dissolved in 20 mL of H_2O and chilled, was added. After stirring for 50 min in an ice bath, the azide was extracted into 100 mL of CH_2Cl_2. The dichloromethane solution was washed six times with cold H_2O, dried over Na_2SO_4, and cooled to −15 °C. It was then added to a −15 °C solution of Phe-OMe, prepared by neutralizing HCl·Phe-OMe (0.863 g, 4 mmol) in 25 mL of DMF with *N*-methylmorpholine (0.49 mL, 4 mmol). The final reaction mixture was stirred for 3.5 h at −15 °C under strong vacuum, collecting the CH_2Cl_2 in a dry ice/methanol trap. The solution was stirred under vacuum at 0 °C for an additional 23.5 h.

A saturated $KHCO_3/H_2O$ solution of 25 mL at 0 °C was added. The homogeneous, pH 8 solution was stirred for 45 min at 0 °C under vacuum; 50 mL of a chilled 50% saturated $NaCl/H_2O$ solution was added. Stirring at 0 °C under vacuum continued for an additional 70 min. The precipitate was filtered, washed thoroughly with H_2O, and dried in vacuo. This procedure gave 1.6071 g (97%) of product, mp 99–101 °C.

Anal. Calcd for $C_{22}H_{26}N_2O_6$: C, 63.76; H, 6.32, N, 6.76, O, 23.16. Found: C, 63.72; H, 6.35; N, 6.76; O, 23.08.

TLC: one spot in all four solvent systems.

$[\alpha]^{25}_D$ +5.2° (*c* 10 mg/mL, DMF), $[\alpha]^{25}_D$ −8.2° (*c* 10 mg/mL, MeOH).

Amino Acid Anal. Thr, 0.97; Phe, 1.03.

Z-Ser-Phe-OMe.[18] This compound was prepared in 95% yield by the exact method described for the synthesis of Z-Thr-Phe-OMe; 1.5182 g of product was obtained, mp 75–76 °C.

Anal. Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.84; H, 6.14; N, 6.80.

TLC: one spot in all four solvent systems.

$[\alpha]^{25}_D$ +3.8° (c 3.5 mg/mL, DMF), $[\alpha]^{25}_D$ −5.1° (c 3.7 mg/mL, MeOH).

Amino Acid Anal. Ser, 0.83; Phe, 1.00.

Z-Phe-Phe-OMe.[18] Z-Phe-NH-NH_2 (2.5069 g, 8 mmol) was dissolved in a solution of 32 mL of 1 N HCl and 20 mL of acetic acid; 30 mL of H_2O was added and the solution cooled to 0 °C. $NaNO_2$ (0.828 g, 12 mmol), dissolved in 20 mL of H_2O and chilled, was added. The reaction proceeded for 25 min while cooled in an ice bath. Added was 100 mL of CH_2Cl_2. After mixing, the organic layer was washed six times with cold water, dried over Na_2SO_4, and cooled to −15 °C. The organic layer was then added to a −15 °C solution of Phe-OMe, prepared by neutralizing HCl·Phe-OMe (0.863 g, 4 mmol) in 15 mL of DMF with *N*-methylmorpholine (0.49 mL, 4 mmol). After stirring for 6 h at −15 °C under vacuum, the reaction proceeded for an additional 21 h at 0 °C.

Approximately 10–15 mL of a saturated $KHCO_3/H_2O$ solution at 0 °C was added. The homogeneous solution remained at pH 8 through 45 min of stirring at 0 °C under vacuum. A solution of 50% saturated NaCl/H_2O (50 mL) at 0 °C was added. Stirring at 0 °C under vacuum continued for an additional 70 min, during which time chilled water (25 mL or less) was periodically added. The solution was then filtered and the precipitate washed well with copious amounts of H_2O. After drying in vacuo, the entire sample was reprecipitated from EtOH with water to give 1.7241 g (93.6%) of product, mp 138–140 °C.

Anal. Calcd for $C_{27}H_{28}N_2O_5$: C, 70.42; H, 6.13; N, 6.08. Found: C, 70.39; H, 6.25; N, 6.35.

TLC: one spot in all four solvent systems.

$[\alpha]^{25}_D$ −20.0° (c 2.5 mg/mL, DMF), $[\alpha]^{25}_D$ −20.0° (c 2.6 mg/mL, MeOH).

Z-Phe-Ala-OMe.[18] This peptide was synthesized in a manner identical with the preparation of Z-Phe-Phe-OMe described above. An additional reprecipitation from MeOH with water yielded 1.4356 g (93.5%) of product, mp 118–120 °C.

Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.40; H, 6.08; N, 7.40.

TLC: one spot in all four solvent systems. $[\alpha]^{25}_D$ −11.5° (c 3.2 mg/mL, DMF), $[\alpha]^{25}_D$ −19.6° (c 3.4 mg/mL, MeOH).

Amino Acid Anal. Phe, 1.04; Ala, 0.96.

Z-Gly-Gly-OEt. A solution of Z-Gly-NH-NH_2 (4.4647 g, 20 mmol) dissolved in 80 mL of 1 N HCl was cooled to 0 °C. A chilled solution of $NaNO_2$ (2.066 g, 30 mmol) in 50 mL of H_2O was added. Stirring the solution for 30 min at 0 °C led to the appearance of a white precipitate (Z-Gly-N_3). The azide was extracted into 200 mL of CH_2Cl_2, washed six times with cold H_2O, dried over Na_2SO_4, and cooled to −15 °C. The CH_2Cl_2 extract was then added to a −15 °C solution of Gly-OEt, prepared by neutralizing HCl·Gly-OEt (1.3959 g, 10 mmol) in 100 mL of DMF with *N*-methylmorpholine (1.25 mL, 10 mmol). The reaction mixture was stirred at −15 °C under strong vacuum for 6 h plus an additional 21 h at 0 °C.

A chilled, saturated $KHCO_3/H_2O$ solution of 60 mL was slowly added. After stirring for 45 min under vacuum in an ice bath, 100 mL of a 50% saturated NaCl/H_2O solution at 0 °C was added. Cold water was occasionally added during 90 min of additional stirring at 0 °C under vacuum. The solution was then filtered and the precipitated material washed extensively with water and dried in vacuo.

Due to the expected partial solubility of the peptide in a homogeneous DMF/H_2O solution, examination of the above filtrate for additional product was undertaken. The dry residue resulting from lyophilization of the filtrate was triturated well in absolute ethyl acetate and the insoluble salts were removed by filtration. After drying over Na_2SO_4, the EtoAc solution was treated with decolorizing carbon. The carbon was filtered off and the solution was evaporated to dryness on a Büchi rotary evaporator. Following trituration of the residue with water, the crystalline product was filtered, washed well with water, and dried in vacuo. Both identical fractions were combined to give 2.7051 g (92%) of Z-Gly-Gly-OEt, mp 78–80 °C.

Anal. Calcd for $C_{14}H_{18}N_2O_5$: C, 57.14; H, 6.16; N, 9.52. Found: C, 57-04; H, 6.01; N, 9.30.

TLC: one spot in all four solvent systems.

Z-Gly-Gly-Gly-OEt. The amino component for this reaction, HCl·Gly-Gly-OEt, was prepared by dissolving Z-Gly-Gly-OEt (1.1772 g, 4 mmol) in 50 mL of absolute methanol. Palladium, 5% on activated carbon and wetted with acetic acid, was added, followed by 4 mL of 1 N HCl and 5 mL of DMF. After flushing with nitrogen, hydrogen gas was bubbled through the vigorously shaking solution for 4.5 h at room temperature. The carbon was filtered off and washed well with methanol. The filtrate was evaporated to an oily residue on a Büchi and redissolved in 20 mL of DMF. The compound Gly-Gly-OEt (4 mmol) was prepared by neutralizing this solution with *N*-methylmorpholine (0.49 mL, 4 mmol).

The remainder of the tripeptide synthesis was completed in a manner identical with the preparation of Z-Gly-Gly-OEt already described to yield 1.2128 g (87%) of product, mp 164–166 °C.

Anal. Calcd for $C_{16}H_{21}N_3O_6$: C, 54.70; H, 6.02; N, 11.96; O, 27.32. Found: C, 54.55; H, 5.94; N, 11.71; O, 27.71.

TLC: one spot in all four solvent systems.

Registry No.—Z-Thr-Phe-OMe, 19649-01-5; Z-Thr-Phe-NH-NH_2, 49706-30-1; Z-Thr-Phe-N_3, 41446-42-8; Phe-OMe, 2577-90-4; HCl·Phe-OMe, 7524-50-7; Z-Ser-Phe-OMe, 40290-58-2; Z-Ser-NH-NH_2, 26582-86-5; Z-Ser-N_3, 41446-15-5; Z-Phe-Phe-OMe, 4892-10-8; Z-Phe-NH-NH_2, 21887-86-5; Z-Phe-N_3, 62067-14-5; Z-Phe-Ala-OMe, 25422-44-0; Ala-OMe, 10065-72-2; HCl·Ala-OMe, 2491-20-5; Z-Gly-Gly-OEt, 3005-87-6; Z-Gly-NH-NH_2, 5680-83-1; Z-Gly-N_3, 50622-95-2; Gly-OEt, 459-73-4; HCl·Gly-OEt, 623-33-6; Z-Gly-Gly-Gly-OEt, 2503-35-7; HCl·Gly-Gly-OEt, 2087-41-4; Gly-Gly-OEt, 627-74-7.

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