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SYNTHESIS OF LARGE HAPTENIC COMPOUNDS HAVING A COMMON FUNCTIONAL GROUP THAT PERMITS COVALENT LINKAGE TO PROTEINS, CELL SURFACES, AND ADSORBENTS

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Abstract—Details are given for the synthesis and characterization of two series of tripeptide derivatives that were prepared by joining various, aromatic-ring-based structures to an N-terminal, N-acetyl-L-tyrosyl or β-alanyl residue through an azo or amide linkage, respectively. The remainder of the tripeptide derivative was, in all cases, glycylglycine tertiary-butyloxycarbonyl hydrazide. The latter, C-terminal feature allows these compounds to be stored in a stable form and to be converted readily to reactive acyl azides by methods commonly employed in peptide synthesis. The azides provide a suitable means for covalently linking the derivatized tripeptides to amino groups on carriers and cell surfaces under mild conditions in aqueous media (see Inman *et al.*, 1973). The linked groups serve very effectively as enlarged or spaced-out haptens in studies of immunological specificity. The design and advantageous features of these compounds and their corresponding haptenic groups are discussed.

Hapten–carrier conjugates have been widely used in many areas of immunological research since the time of Landsteiner's (1945) classical work. Progress in understanding the structural basis of immunological specificity has depended in large measure on the extent to which the structures of antigenic determinants have been known. There is now considerable evidence indicating that antibody combining sites can specifically accomodate determinants as large as or larger than tetrasaccharides or tripeptides (Goodman *et al.*, 1968; Kabat, 1968; Murphey and Sage, 1970; Schechter *et al.*, 1971; Van Vunakis *et al.*, 1966). Most, traditionally employed haptens fall below this range in size and therefore may be associated, in some cases, with parts of the carrier structure in forming actual determinants. This situation may arise, for example, with the widely used, substituted phenylazo haptens that are bound to proteins by direct reaction of diazonium salts with different side chain groups of the protein (e.g. those of tyrosyl, histidyl and lysyl residues). The validity of studies with such relatively small haptens rests largely upon the fact that they tend to play a dominant role in the specific binding process. Nevertheless, the unknown involvement of subjacent carrier structures in the determinants of immunogens or test antigens may lead to difficulties in interpreting results from studies of structure-specificity relationships where highly sensitive, discerning techniques are employed (Rouques *et al.*, 1972). Furthermore, the small haptens, even though being immunodominant features, may frequently rest in sterically unfavorable microenvironments on carrier surfaces. The effect of steric hindrance could be quite significant where simultaneous, multiple-site interactions are required for effector function. Such a circumstance may prevail in precursor cell activation, in various test systems employing multiply conjugated reagents (e.g. hapten-conjugated red cells or phage), and even in immune precipitation.

A large hapten, one more nearly approaching combining site size, can provide the following potential advantages in studies of immunological specificity: (1) determinants that are more likely to be comprised mainly of known structures, (2) a built in 'spacing-out' function which allows greater accessibility and orientational flexibility of the distal, immunodominant feature, and (3) the possibility of varying, at will, selected portions of the actual determinant in studies of structure-specificity relationships. We report here details for the synthesis of two series of tripeptide derivatives (Figs. 1 and 2) that have been designed especially for the purpose of covalently linking a variety of large haptens to primary amino groups on other compounds, proteins, cells, particles or adsorbent supports. The structures of the compounds prepared were chosen to offer the following features: (1) a selection of similar and dissimilar aromatic-ring-based structures on one (immunodominant) end of the molecule, (2) a stable group on the opposite end which can be readily converted to an amino-specific, acylating function (acyl azide group), (3) a common link (glycylglycyl) of low immunogenicity which spaces out the azide group so that its reactivity is not greatly influenced by structural variations at the opposite end of the

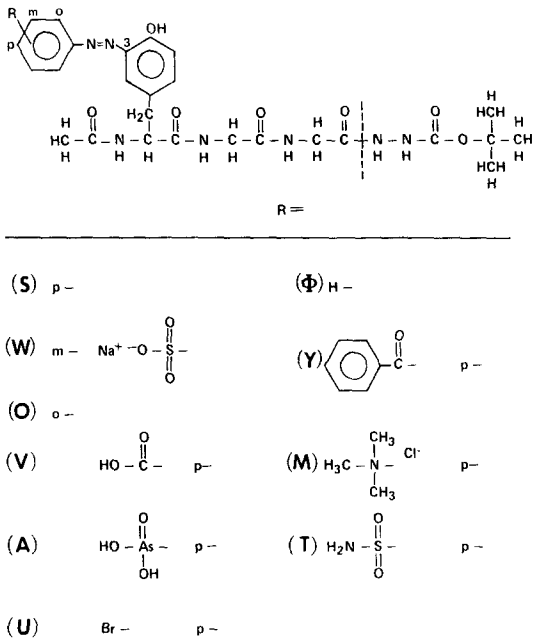

Fig. 1. Covalent structures of compounds of the type, 3-(R-phenylazo)-N-acetyl-L-tyrosylglycylglycine tertiary-butyloxycarbonyl hydrazide. The portion of structure to the left of the dashed line represents the haptenic groups which form amide bonds with amino groups on carriers, cell surfaces etc.

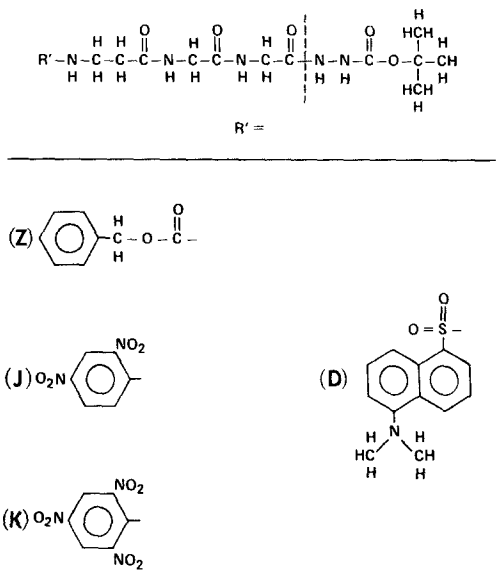

Fig. 2. Covalent structures of compounds of the type, N-R'-β-alanyl-glycylglycine tertiary-butyloxycarbonyl hydrazide. The portion of structure to the left of the dashed line represents the haptenic groups as explained in Fig. 1 legend.

molecule, (4) modular links (amino acid residues) which allow selective extensions and/or replacements for future analogs, (5) a reasonable degree of water solubility, and (6) the three potential advantages of large haptens listed above. Epsilon amino groups of lysyl residues in protein carriers are the almost exclusive loci of attachment. Thus, the lysyl side chain may be considered, in effect, a part of the haptenic structure. Other aspects of the design of these compounds that relate to their synthesis and use are included in the Discussion section.

Procedures for converting stable, tertiary-butyloxycarbonyl (Boc) hydrazides to acyl azides, and the coupling of these reactive intermediates to carrier proteins and red cells are described in an accompanying paper (Inman *et al.*, 1973). These compounds, when used in studies of immune responses by means of hapten-specific hemolytic plaque assays, have a number of distinct advantages over similar smaller haptens. These include important increases in (1) test red cell stability, (2) reproducibility of results, (3) plaquing efficiency, and (4) plaque quality. Immunizing conjugates of the large haptens also elicited exceptionally high numbers of antihapten antibody secreting cells in rabbits and inbred mice as compared with results from immunizations with smaller analogs bound to the same carriers.

*Abbreviations commonly used: Boc for tertiary-butyloxycarbonyl; tert. for tertiary; DMF for N,N-dimethylformamide.

MATERIALS AND METHODS

Materials and general procedure

The sources of chemicals employed are indicated in the procedures; otherwise, they were commonly available reagents or reagent grade solvents. Low temperature reactions were carried out under magnetic stirring in glass-stoppered flasks placed in alcohol baths cooled with limited amounts of dry ice. Flash evaporations, (i.e. rotary vacuum distillations) were carried out with an apparatus built by Buchler Instruments. Silica gel columns were prepared from silica gel powder, 60–200 mesh (J. T. Baker Chemical Co., #3405) which had extra-fine particles removed by repeated sedimentation in water followed by decantation. The powder was recovered by drying in air at 110–120°C and allowing to stand in room air for several days. Hydrogenations and separations were routinely followed by thin layer chromatography on commercially available, pre-coated silica on glass plates. Plates were usually developed in mixtures of chloroform and methanol. The silica layers, containing a fluorescent indicator, were visualized by white and u.v. light and after exposure to I_2 vapor.

Benzyloxycarbonylglycylglycine Boc hydrazide (I)*

To a suspension of 32 g (0·12 mole) of benzyloxycarbonylglycylglycine (N-CBZ-glycylglycine, Cyclo Chemical Co., Los Angeles, Calif.) in 450 ml N,N-dimethylformamide (DMF, Matheson, 'Spectroquality') was

added 13·4 ml (0·12 mole) of N-methylmorpholine (Aldrich Chemical Co., Milwaukee, Wis.). The mixture was cooled to −15°C, and 15·7 ml (0·12 mole) of iso-butyl chloroformate (Eastman) was added. After the mixture was stirred magnetically at −15°C for 40 min, a solution of 15·84 g (0·12 mole) of tert.-butyl carbazate (Aldrich Chemical Co.), dissolved in 60 ml of N,N-dimethylformamide, was added. The mixture was then stirred 1 hr at −5°C, 1 hr at 0°C, and overnight at room temperature. The solvent was removed at 45°C by flash evaporation. The residue was transferred to a large glass mortar with 350 ml of water, ground to fine particles, then collected on a suction filter. The grinding–filtering cycle was repeated once with 350 ml of 1*N* NH_4OH and twice more with 350 ml portions of water. Filtrates were discarded.

The crude product was dried *in vacuo* over $CaCl_2$ and dissolved in 750 ml of hot ethyl acetate. An insoluble residue was removed by filtration while hot. To the filtrate at 55°C was added 315 ml of hexane (55°C). After allowing the mixture to cool slowly and stand at +3°C overnight, the crystalline solid was collected by filtration, redissolved in 600 ml of hot ethyl acetate, and recrystallized by addition of about 250 ml of hexane (55°C). After the mixture had cooled to +3°C (overnight), the product was collected, and dried in air and under vacuum. The yield was 36·95 g or 81% of theory. After standing in room air, the product had a melting range of 103–109°C (with decomposition), and the composition of a monohydrate: % Found: C 51·3, H 6·58, N 14·1. % calculated (monohydrate, M.W. 398·42): C 51·2, H 6·58, N 14·1.

N-Acetyl-L-tyrosylglycylglycine Boc hydrazide (G)

A suspension of 1·6 g of 10% palladium on charcoal, catalyst (Matheson Scientific Co.) in 100 ml of cold methanol was added to a solution of 18·26 g (0·046 mole) of compound I (see above) in 144 ml of methanol. The mixture was stirred and equilibrated with hydrogen gas at 1 atm at room temperature for 60 min. The catalyst was removed by filtration, and the filtrate was flash evaporated to remove methanol. The residue was dissolved in 210 ml of N,N-dimethylformamide (DMF). The resulting solution of glycylglycine Boc hydrazide was held at 0°C.

In a separate flask, 11·39 g (0·048 mole) of N-acetyl-L-tyrosine hydrazide (Nutritional Biochemicals) was dissolved in 770 ml of DMF by stirring for 30 min at room temperature. The solution was cooled to −25°C and 48 ml of 4 *N* HCl (gas) in dioxane was added. While maintaining the temperature at −25°C, 6·4 ml (0·0534 mole) of tert.-butyl nitrite (J.T. Baker Chemical Co.) in 58 ml DMF was added, and the mixture was stirred for 35 min at that temperature. The temperature was then lowered to −50°C and 19·2 g (0·144 mole) of ethyldiethanolamine (Aldrich) plus 24·5 ml (0·175 mole) of triethylamine were added. The previously prepared, cold, solution of glycylglycine Boc hydrazide was added immediately. The mixture was stirred for 24 hr at 0°C and overnight at room temperature. The mixture was filtered and the filtrate was flash evaporated to a heavy oil (45°C bath). After vacuum treatment at room temperature overnight, the residue was suspended in (and ground with) 300 ml 1 *N* aqueous acetic acid. The solid residue was collected by suction filtration, washed with 300 ml of water in several portions, and dried under vacuum.

The crude product was dissolved in 140 ml of absolute ethanol at 55°C and stirred for 15 min with 0·75 g of powdered charcoal (Darco G60, Fisher Scientific Co.); the mixture was filtered (with a 10 ml ethanol rinse), and 510 ml of water at 55°C was mixed with the filtrate. The crystalline solid was collected after an overnight stand at +3°C, dried for several hours under vacuum, and redissolved in 155 ml of ethanol at 55°C; 465 ml of water at 55°C was added, and the mixture was allowed to cool slowly and stand in the refrigerator overnight. Crystals were collected and dried under vacuum. The yield was 19·75 g or 83 per cent of theory. Percentages Found: C 48·3, H 7·45 N 13·4. Percentages calculated for a solvate with 2·5 mole H_2O and 0·5 mole C_2H_5OH: having mol. wt of 519·56: C 48·6, H 7·18, N 13·5. An earlier preparation yielded a 2·5 H_2O hydrate (with apparently no ethanol) which melted at 113–115°C (with decomposition) and gave a molar ratio of glycine to tyrosine of 2·01 by amino acid analysis (Beckman 120 C Analyzer) after hydrolysis (6 *N* HCl, 108°C, 22 hr under N_2). No nitrotyrosine was detected. Both preparations chromatographed identically as single spots on silica thin layers in $CHCl_3$–CH_3OH, 78:22 v/v, with an R_F of about 0·56 (I_2 vapor stain).

General coupling procedure for preparation of azotyrosyl peptide derivatives

Ten substituted monophenylazo derivatives of N-acetyl-L-tyrosylglycylglycine Boc hydrazide (G) were formed by coupling the appropriate diazonium salt with G under alkaline conditions by the following procedure: the diazonium salt solution was prepared by dissolving or suspending 1·5 m-mole of the appropriately substituted aniline in 20 ml of water, adding 5·4 ml of 1·0 *N* aqueous HCl, cooling the mixture to 0°C in an ice bath, next adding 1·8 ml of 1·0 *M* sodium nitrite, and stirring the mixture at 0°C for 45–75 min. At this point, excess nitrous acid was destroyed by adding 0·45 ml of 1·0 *M* sulfamic acid.

In the meantime, a solution of 779 mg (1·5 m-mole) of compound G was prepared in 15 ml of dimethyl*acetamide* (Matheson, DX 1545). When complete dissolution was obtained, 10 ml of water was added, and the solution was cooled to 0°C in an ice bath. Five minutes or more after addition of sulfamic acid to the diazonium salt solution, the latter was added to the solution of G, and 1·0 *N* NaOH was added until the pH (glass electrode) read 9·0. The pH was held at 9·0 for about 1 hr, raised to 9·5 for 1–2 hr, then adjusted between 9·5 and 10·0. The darkly colored mixture was left standing overnight in a refrigerator. A surrounding ice bath was maintained throughout the above sequence of operations. Recovery and purification of the desired products were handled differently for each of the derivatives as described in the following sections.

3-Phenylazo-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (Φ)

Aniline was diazotized and coupled with compound G as described above. The pH of the reaction mixture was adjusted to 4·4 with 1 *N* HCl. Sixty ml of water, 50 ml of ethyl acetate and 10 ml of 1-butanol were added, and the mixture was shaken in a separatory funnel. The upper layer (containing the product) was shaken twice with 25-ml portions of water and flash evaporated. The residue was dissolved in 15–20 ml of the upper phase from the equilibration of 800 ml ethyl acetate, 200 ml 1-butanol, plus 150 ml water. The solution was passed through a 4·0 × 75 cm column bed of silica gel powder (J. T. Baker, #3405) using the same upper phase mixture for packing and development. The major colored band was collected,

flash evaporated and dried under vacuum. The yield was 451 mg or 53 per cent of theory.

3-(Sodium, o-azobenzenesulfonate)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (O)

o-Aminobenzenesulfonic acid (Eastman, recrystallized twice from hot water) was diazotized and coupled with G (see above). The reaction mixture was adjusted to pH 6·0 with 1 *N* HCl, filtered, and passed through a 3·5 × 44 cm column bed of DEAE-cellulose (Whatman DE 52, H. Reeve Angel Inc., Clifton, N.J.). The column was equilibrated and developed with 0·20 *M* $NaHCO_3$. The major orange band was collected and flash evaporated to dryness (40°C). The crude product was extracted into 50 ml (in portions) of chloroform–methanol 3 : 2 v/v. The extract was run through a 3·5 × 27 cm silica gel column with the same solvent. The major red-orange band was collected, flash evaporated, taken up in a small volume of methanol and dried in air. The yield was 217 mg or 20 per cent of theory.

3-(Sodium, m-azobenzenesulfonate)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (W)

m-Aminobenzenesulfonic acid (Eastman recrystallized twice from hot water) was diazotized and coupled with G (see above). The reaction mixture was passed through a 4·0 × 42 cm column bed of DEAE-cellulose (Whatman DE 52) with 0·20 *M* $NaHCO_3$. The faster moving major orange-red band was collected and flash evaporated (42°C) until $NaHCO_3$ started forming a heavy precipitate. The filtrate from this suspension was treated with a total of 100 ml of methanol at 0°C in portions, removing the precipitate after each addition. The final filtrate was flash evaporated to dryness. The dry residue was extracted into 90 ml (in portions) of chloroform-methanol 3 : 2 v/v. The extract was passed through a 4·0 × 20·5 cm bed of silica gel powder with the above solvent. The major orange-red band was collected and flash evaporated. The residue was taken up in a small volume of methanol, dried in air and *in vacuo* over $CaCl_2$, then equilibrated with room air. The yield was 502 mg or 47 per cent of theory.

3-(Sodium, p-azobenzenesulfonate)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (S)

Sulfanilic acid (recrystallized from ethanol) was diazotized and coupled with G (see above). The reaction mixture from a double-sized (3 m-mole) preparation was adjusted to pH 7·4 with 1 *N* HCl and passed through a 5·0 × 47 cm column bed of DEAE-Sephadex, A-50 (Pharmacia Fine Chemicals) with 0·20 *M* $NaHCO_3$. The first major colored band was collected and flash evaporated to about 75 ml volume. Precipitated $NaHCO_3$ was filtered off and washed with cold water (30 ml). The filtrate plus washings were combined with 220 ml of methanol in several portions at 0°C, the precipitate ($NaHCO_3$) was removed after each addition of methanol. The final filtrate was flash evaporated to dryness, and the product was extracted from residual $NaHCO_3$ into 120 ml of chloroform–methanol 3 : 2 v/v. Solvent was removed by flash evaporation; the residue was dissolved in 50 ml of chloroform–methanol 3 : 2 v/v, filtered and passed through a 5·0 × 39 cm column bed of silica gel with the above solvent. The major, colored band was collected, dried in air and under vacuum. The yield was 420 mg or 20 per cent of theory. Two smaller preparations gave, however, 40 and 46 per cent yields.

3-(p-Carboxyphenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (V)

p-Aminobenzoic acid (Fisher Scientific Co.) was diazotized and coupled with G (see above) using 1·75 (instead of 1·50) m-moles of the former and 2·0 ml of 1 *M* $NaNO_2$. The reaction mixture was diluted with 120 ml of water and adjusted to pH 2·5 with 1 *N* HCl. The precipitated product was collected by filtration, washed with 0·001 *N* HCl, redissolved in 0·20 *M* $NaHCO_3$, and passed through a 5·0 × 30 cm column bed of DEAE-Sephadex, A-50 with 0·20 *M* $NaHCO_3$. The major red-orange band was collected and acidified to pH 2·5. The precipitate was recovered by filtration, washed with a small volume of water, and dried under vacuum. The yield was 360 mg or 39 per cent of theory.

3-(p-Arsonophenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (A)

Arsanilic acid (recrystallized from ethanol) was diazotized and coupled with G (see above). To the reaction mixture was added 1/10 of its volume of 0·10 *M* BisTris (2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol, Aldrich Chemical Co.). The solution was adjusted to pH 5·9 with 1 *N* HCl, filtered and loaded onto a DEAE-Sephadex, A-50 column packed with a 5·0 × 50 cm bed. The column had been equilibrated with 0·20 *M* NaCl, 0·01 *M* BisTris, pH 5·90 and was developed with the same buffer. The major orange-red band was collected and acidified to pH 2·0 with 1 *N* HCl. The resulting solution was shaken with four 25-ml portions of 1-butanol. The upper layers were pooled and shaken twice with 15-ml portions of 0·01 *N* HCl and three times with 15-ml portions of water. The final upper layer was filtered and flash evaporated to dryness. The residue was taken up in a small volume of methanol, evaporated in air, dried under vacuum, and equilibrated with room air. The yield was 408 mg or 38 per cent of the yield calculated on the basis of a monohydrate still containing 2·6 per cent butanol as found by analysis (see Table 1).

3-(p-Bromophenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (U)

p-Bromoaniline (Eastman) was diazotized and coupled with G (see above). The reaction mixture was acidified to pH 4·0 (a heavy precipitate formed), mixed with 90 ml of water, shaken with 90 ml of ethyl acetate, and allowed to stand. The lower layer was shaken with another 120 ml of ethyl acetate. The upper layers were combined, washed twice with 40 ml portions of water, and flash evaporated. The residue was dissolved in 30 ml of upper phase from the equilibration of 1000 ml ethyl acetate, 177 ml 1-butanol plus 300 ml water. The solution was passed through a bed of silica gel (4·0 × 74 cm) using the same upper phase solvent mixture. The major colored band was collected, flash evaporated to dryness, dried under vacuum, and equilibrated in room air. Yield was 298 mg or 30 per cent of theory.

3-(p-Benzoylphenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (Y)

4-Aminobenzophenone (Aldrich Chemical Co.) was diazotized and coupled with G (see above). The reaction mixture was shaken with 60 ml water plus 60 ml ethyl acetate. The upper layer was flash evaporated. The residue was dissolved in 15 ml of the upper phase from the equilibration of 950 ml ethyl acetate, 50 ml 1-butanol plus 100 ml water, and passed through a 4·0 × 75 cm bed of

silica gel with the same upper phase mixture. An unretarded dark band was discarded. The next major colored band was collected, and the solvent was removed by flash evaporation. The residue was taken up in a small volume of methanol, dried in air, dried under vacuum, and equilibrated with room air. The yield was 207·6 mg or 21 per cent of theory.

3-(p-Trimethylaminophenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide chloride (M)

Trimethyl-(p-aminophenyl)-ammonium chloride hydrochloride, prepared according to Pressman *et al.* (1946) was diazotized and coupled with G (see above). To the reaction mixture was added 0·65 ml of 1·0 *M* sodium acetate, and the pH was adjusted to 4·5. The resulting solution was passed thrugh a 5·0 × 47 cm column bed of CM-Sephadex C-50 (previously adjusted to pH 4·5 and equilibrated with buffer) using 0·10 *M* NaCl, 0·01 *M* NaOAc, 0·01 *M* HOAc (pH 4·5) buffer. A major orange band was collected. The collection pool was flash evaporated to a volume of 35 ml andstirred with 7·3 g of NaCl. The resulting solution was shaken with four 20-ml portions of 1-butanol. The lower phase was then discarded. Upper phases were combined and shaken with small volumes of water until two layers started to form. The upper layer was then shaken three times with 5-ml portions of water. Lower layers were discarded, and the final upper layer was flash evaporated to dryness. The residue was transferred with methanol to a small dish, dried in air, dried under vacuum, equilibrated briefly in room air (the solid was hygroscopic). The yield was 310 mg or 30 per cent of theory.

3-(p-Sulfamylphenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide (T)

Sulfanilamide (Eastman) was diazotized and coupled with G (see above). The reaction mixture was mixed with 60 ml of water, adjusted to pH 5·1 with 0·3 *N* HCl, and extracted with 1-butanol three times (60, 20 and 20 ml, respectively). Upper layers were combined, flash evaporated, and the residue was dissolved in 40 ml of upper phase from 1000 ml ethyl acetate, 177 ml 1-butanol plus 300 ml water. A red oil was removed and discarded after centrifugation, and the remaining solution was passed through a 4·0 × 75 cm column of silica gel with the above solvent mixture (upper phase). The major band was recovered and dried under vacuum. The yield was only 54·4 mg or 5·7 per cent of theory. Nitrogen content was 5·6 per cent low although the product had spectral and functional group properties expected in qualitative tests. This derivative was not used extensively in subsequent experimental work.

Benzyloxycarbonyl-β-alanylglycylglycine Boc hydrazide (Z)

A suspension of 1·2 g of 10 per cent palladium on charcoal, catalyst (Matheson) in 80 ml of cold methanol was added to a solution of 15·20 g (0·038 mole) of compound I in 120 ml of methanol. The mixture was stirred and equilibrated with hydrogen gas at 1 atm at room temperature for 65 min. The catalyst was separated by filtration, and the methanol was removed by flash evaporation. The residue was dissolved in 60 ml of dimethylformamide (DMF). The resulting solution of glycylglycine Boc hydrazide was held at 0°C.

In a separate flask, 8·93 g (0·040 mole) of benzyloxycarbonyl-β-alanine (N-CBZ-β-alanine, Cyclo Chemical Co.) was dissolved in 200 ml of DMF, and 4·48 ml (0·040 mole) of N-methylmorpholine was added. The temperature was lowered to −20°C, 5·24 ml (0·040 mole) of iso-butyl chloroformate (Eastman) was added, and the mixture was stirred at −20°C for 40 min. The cold, previously prepared solution of glycylglycine Boc hydrazide was then added. The mixture was stirred at −5°C for 1 hr, 0°C for 16 hr, and room temperature for 3 hr. During early stages of the above reaction small volumes of triethylamine were added as required to give a slightly alkaline reaction (pH 8) on moistened indicator paper.

The reaction mixture was flash evaporated to dryness (45°C). The residue was ground with 100 ml of water, filtered and washed with 100 ml of 1 *N* ammonium hydroxide and 200 ml of water (in three portions). The crude product was dried under vacuum yield, (13·96 g), dissolved in 82 ml ethanol plus 82 ml water at 55°C. The solution was filtered and mixed with 164 ml of water at 55°C. The resulting solution was allowed to cool slowly and to stand overnight in the refrigerator. The wet crystals were collected by suction filtration and dissolved in 70 ml ethanol plus 38 ml water at 55°C. The solution was stirred with 1 g of powdered charcoal (Darco G 60) for 15 minutes, filtered, mixed with 170 ml of water (55°C), allowed to cool slowly and to stand overnight in the refrigerator. The crystals were collected and dried *in vacuo* over $CaCl_2$. Yield was 9·94 g or 57 per cent of theory (as the hemihydrate (Table 2). An earlier preparation by this method gave a molar ratio of glycine to β-alanine of 2·00 by amino acid analysis after hydrolysis (6 *N* HCl, 108°C, 20 hr under N_2). Both preparations chromatographed identically as essentially single components on silica thin layers in chloroform–methanol, 86 : 14 v/v, with an R_f of about 0·66 (I_2 vapor stain).

N-(5-Dimethylaminonaphthalene-1-sulfonyl)-β-alanylglycylglycine Boc hydrazide (D or "dansyl' derivative)

One hundred milligrams of 10 per cent palladium on charcoal (Matheson) was added to a solution of 0·903 g (2·0 mmole) of compound Z in 30 ml of methanol. The mixture was stirred at room temperature and equilibrated with hydrogen gas at 1 atm (room temperature) for 30 min. The catalyst was filtered off, and methanol was removed from the filtrate by flash evaporation. The residue was redissolved in 9 ml of methanol. To this solution was added 6 ml of water and 756 mg (9 mmoles) of $NaHCO_3$. To the resulting suspension was added slowly, with stirring, a solution of 809 mg (3·0 mmoles) of 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride, Calbiochem.) in 9 ml of acetone (insoluble residue was removed by centrifugation). The mixture was stirred overnight at room temperature, then flash evaporated and dried *in vacuo* over $CaCl_2$. The residue was extracted into 20 ml of chloroform–methanol 9 : 1 v/v, filtered and applied to a 4·0 × 56 cm column bed of silica gel. The column was developed with the 9 : 1 solvent mixture. The major colored band with an R_f of approximately 0·4 was collected and flash evaporated; the residue was dried under vacuum and equilibrated with room air. The yield was 943 mg or 83 per cent of theory.

N-(2,4-Dinitrophenyl-β-alanylglycylglycine Boc hydrazide (J)

A suspension of 200 mg of 10 per cent palladium on charcoal in a solution of 0·903 g (2·0 m-mole) of compound Z in 25 ml of methanol was stirred and equilibrated with 1 atmosphere of hydrogen gas for 30 min at room tempera-

ture. The catalyst was filtered off, and 0·67 ml (5·0 m-mole) of 2-diethylaminoethanol (Eastman) was added to the filtrate. Three ml of 1 *M* 2,4-dinitrofluorobenzene (Pierce Chemical Co.) in absolute ethanol was added dropwise with stirring to the above solution. The mixture was stirred (away from bright light) for several days, then flash evaporated to dryness. The residue was equilibrated with 84 ml of ethyl acetate–water–gl. acetic acid 10 : 10 : 1 v/v/v. The upper phase was shaken with 25 ml of water and flash evaporated. Lower phases were discarded. The residue was dried *in vacuo* over $CaCl_2$, then dissolved in 30 ml of chloroform–methanol 85 : 15 v/v. The solution was passed through a 4·0 × 40 cm column bed of silica gel with the same solvent mixture. Collection tubes with major concentrations of yellow product were pooled. The product was recovered by flash evaporation. The yield was 818 mg or 82 per cent of theory.

N-(2,4,6-Trinitrophenyl)-β-alanylglycylglycine Boc hydrazide (K)

A suspension of 100 mg of 10 per cent palladium on charcoal in a solution of 0·903 g (2·0 mmole) of Z in 30 ml methanol was stirred and equilibrated with 1 atm of hydrogen gas at room temperature for 30 min. To the filtrate of this mixture was added 6 ml of water, 756 mg (9 m-mole) of $NaHCO_3$, and a solution of 880 mg (3·0 m-mole) of 2,4,6-trinitrobenzenesulfonic acid (Nutritional Biochemicals) in 9 ml of methanol. The mixture was stirred (away from bright light) overnight at room temperature, flash evaporated and dried under vacuum. The residue was extracted with 63 ml of ethyl acetate–water–gl. acetic acid 10 : 10 : 1 v/v/v. The resulting upper phase (containing the product) was separated and shaken with 20 ml of water, separated again, and flash evaporated. The dry material was extracted into 70 ml of chloroform–methanol 85 : 15 v/v and passed through a 4·0 × 40 cm silica gel bed with the same solvent mixture. Several colored bands passed through before the major, trailing yellow band emerged. The latter was collected and flash evaporated to dryness. The yield was 521 mg or 49 per cent of theory.

Functional group test for tert.-butyloxycarbonyl hydrazide (Boc)hydrazide

Approximately 0·2 mg of the compound to be tested was placed in each of two tubes. To one tube was added 0·1 ml of anhydrous trifluoroacetic acid (TFA). After mixing and standing for 10 min, the TFA was quickly evaporated with a stream of air. To each tube was then added 0·4 ml of methanol (to dissolve the residue), 2·5 ml of 0·5 *M* sodium acetate, adjusted to pH 5·8 with acetic acid, and 0·2 ml of 1% w/v trinitrobenzenesulfonic acid. Tubes were shaken and allowed to stand 30 min. A Boc hydrazide gave no change in color in the non-TFA-treated tube and gave a marked increase in orange color in the TFA-treated tube. DMF (~1 ml) was added to tubes having precipitated material.

*Compound I (benzyloxycarbonylglycylglycine Boc hydrazide) was synthesized by Schwyzer and Tun-kyi (1962) using dicyclohexylcarbodiimide as the coupling agent. Their product crystallized from methanol–water as a hemihydrate (m.p. 100–101°C) whereas our product, which was crystallized from ethyl acetate–hexane, dried and exposed to room air, took the form of a monohydrate (m.p. 103–109°C).

RESULTS

The synthesized compounds were characterized in three ways: (1) by elemental analysis, (2) a u.v. and visible absorption spectrum (or amino acid analysis in the case of compounds G and Z), and (3) a qualitative test for tert.-butyloxycarbonyl-hydrazide. Melting points were of little or no value because of the thermal lability of Boc hydrazides. The unblocked hydrazides are libearated slowly at around 70–80°C, and decomposition occurs rapidly above 100°C. This fact must be kept in mind during preparative procedures.

The weight percentages of carbon, hydrogen, nitrogen and occasionally halogen or sulfur are given in Tables 1 and 2. Satisfactory agreement was found between determined and calculated values when one took into account reasonable amounts of water of hydration and, in several cases, some other substance from the terminal stage of purification.* Most of the compounds gained weight rapidly in room air after being stored *in vacuo* over calcium chloride. Weight gain slowed considerably after several hours, and later no further weight gain occurred. Stable hydrates apparently were formed. The amount of weight gain was equal to or less than the assumed hydration figure used for the calculated elemental composition. Thus, vacuum treatment over calcium chloride at room temperature may not always have removed all water of hydration. Complete dehydration by heating under vacuum was not feasible because of the thermal lability mentioned above. Several days under vacuum did not remove all butanol from compound A. In the case of compound M, one more equilibration of the final butanol solution with a small volume of water should remove the remaining NaCl. Compound T (sulfamylphenylazo derivative) proved difficult to purify possibly because of a low yield of the desired product in the reaction of compound G with the diazonium salt. The product isolated contained 5·6 per cent (or 1·0 per cent of total weight) less nitrogen than the calculated value. Further characterization was not attempted.

The spectra of the monoazo derivatives of N-acetyl-L-tyrosylglycylglycine Boc hydrazide in 0·1 *N* NaOH were found to have the same shape as the spectra reported for mono-(p-arsonophenylazo)-N-chloroacetyl-L-tyrosine (Tabachnick and Sobotka, 1959) and mono-(p-carboxyphenylazo)-L-tyrosine (Phillips *et al.*, 1965) in the same solvent. Between wavelengths of 250 to 620 nm (mμ) there occur two distinct minima and maxima. The positions, relative heights and widths of absorption peaks for compounds φ–M (Table 3 list) agree closely with published spectra (Phillips *et al.*, 1965; Tabachnick and Sobotka, 1959) of compounds possessing the same chromophoric structures. Molar absorptivities (ϵ_M) of compounds A, S and V (Table 3) differ by 10 per cent or less from published absorptivities (Phillips *et al.*, 1965; Tabachnick *et*

Table 1. Composition of derivatives of the type 3-(R-phenylazo)-N-acetyl-L-tyrosylglycylglycine Boc hydrazide

Symbol	*R*	Solvation[a]	mol. wt of solvate	Composition[b] C	H	N	
ϕ	H^- (unsubstituted)	0·5 H_2O	564·60	55·3	6·1	17·4	Calc.
				55·3	6·2	17·1	Found
0	o-$Na^{+-}O_3S^-$ ortho sulfonate	3·0 H_2O	711·69	43·9	5·4	13·8	Calc.
				44·4	5·4	13·3	Found
W	m-$Na^{+-}O_3S$- meta sulfonate	3·0 H_2O	711·69	43·9	5·4	13·8	Calc.
				44·1	5·4	14·0	Found
S	p-$Na^{+-}O_3S$ para sulfonate	3·0 H_2O	711·69	43·9	5·4	13·8	Calc.
				43·8	5·5	13·3	Found
V	p-HOOC- p-carboxy	1·0 H_2O	617·62	52·5	5·7	15·9	Calc.
				52·4	5·8	15·5	Found
A	p-H_2O_3As p-arsono	1·0 H_2O	716·2[d]	45·3	5·4	13·7	Calc.
		0·25 BuOH[c]		45·5	5·5	13·8	Found
U	p-Br[e] p-bromo	1·0 H_2O	652·51	47·9	5·3	15·0	Calc.
				47·5	5·0	14·6	Found
Y	p-C_6H_5CO- p-benzoyl	0·5 H_2O	668·71	59·3	5·7	14·7	Calc.
				59·0	5·8	14·4	Found
M	p-$(CH_3)_3N^+$- p-trimethylamino	2·0 H_2O	695·6[g]	50·1	6·5	16·1	Calc.
		0·18 NaCl[f]		50·2	6·5	16·2	Found

[a]Numbers express moles of water or solvent per mole of compound in the room air-equilibrated sample.

[b]Values are weight percentages of the respective elements in the room air-equilibated compounds (solvates).

[c]Slight odor of butanol (BuOH) noted in the sample that was analyzed. The amount of BuOH shown was used in arriving at the calculated C, H, N values.

[d]mol. wt of the monohydrate without butanol is 697·54.

[e]Calculated Br content is 12·3%, that found was 12·8%.

[f]The amount of NaCl shown was used in arriving at the calculated C, H, N values. The calculated Cl content is, correspondingly, 6·00%; that found was 6·13%.

[g]mol. wt of the dihydrate without NaCl is 685·18.

Table 2. Composition of derivatives of the type, N-R′-β-alanylglycylglycine Boc hydrazide

Symbol	R′	Solvation[a]	l. wt solvate	Composition[b] C	H	N	
Z	$C_6H_5CH_2OCO$- benzyloxycarbonyl	none	451·48	53·2	6·5	15·5	Calc.
				53·2	6·6	15·7	Found
D	5-$(CH_3)_2NC_{10}H_6$-1-SO_2-[c] "dansyl"	1·0 H_2O	568·65	50·7	6·4	14·8[f]	Calc.
				51·0	6·5	14·8	Found
J	2,4-$(NO_2)_2C_6H_3$-[d] dinitrophenyl	1·0 H_2O	501·46	43·1	5·4	19·6	Calc.
				43·3	5·5	19·3	Found
K	2,4,6-$(NO_2)_3C_6H_2$-[e] trinitrophenyl	none	528·44	40·9	4·6	21·2	Calc.
				40·8	4·5	21·3	Found

[a]Numbers express moles of water or solvent per mole of compound in the room air-equilibrated sample.

[b]Values are weight percentages of the respective elements in the room air-equilibrated compounds (solvates).

[c]5-dimethylaminonaphthalene-1-sulfonyl group.

[d]'DNP' group.

[e]'TNP' group.

[f]Sulfur content calculated is 5·64%; that found was 5·54%.

al., 1959) of the corresponding monoazo analogs at the respective maxima. The absorption data (Table 3) and spectra thus serve to identify the mono-orthophenylazophenol structural feature in compounds ϕ–M.

The absorbance data of derivatives J and K of

Table 3. Molar absorptivities of the tripeptide derivatives at maxima and minima

Compound symbol[a]	Solvent	Molar absorptivity[b]	(Wavelength, nm)
ϕ	0·1*M* NaOH	min. 5360 (282)	3070 (373)
		max. 11,210 (321)	8520 (473)
O	0·1*M* NaOH	min. 4640 (284)	2970 (375)
		max. 10,310 (324)	9130 (458)
W	0·1*M* NaOH	min. 5510 (283)	2890 (377)
		max. 12,000 (324)	9860 (482)
S	0·1*M* NaOH	min. 5090 (282)	3025 (381)
		max. 12,890 (327)	10,350 (487)
V	0·1*M* NaOH	min. 5300 (282)	3760 (382)
		max. 14,060 (329)	10,910 (485)
A	0·1*M* NaOH	min. 5590 (281)	3330 (379)
		max. 13,810 (327)	10,580 (482)
U	0·1*M* NaOH	min. 5300 (280)	3790 (380)
		max. 13,850 (329)	10,730 (480)
Y	0·1*M* NaOH	min. 8550 (293)	3970 (396)
		max. 15,470 (336)	12,090 (506)
M	0·1*M* NaOH	min. 4550 (285)	2160 (379)
		max. 9930 (327)	8410 (484)
D	0·01*N* HCl	min. 1360 (247)	none
		max. 7520 (287)	none
J	0·05*M* phosphate pH 7·4	min. 2210 (298)	none
		max. 16,300 (361)	none
K	0·05*M* phosphate pH 7·4	min. 3120 (286)	none
		max. 15,000 (345)	6620 (410)

[a]Identity of compounds represented by these symbols is found in Materials and Methods text, Figs. 1 and 2, or in Tables 1 and 2.

[b]Molar extinction coefficient calculated for a 1·00 molar solution in a 1·00 cm light path at the corresponding wavelength (nm = mμ) given in the adjacent parentheses.

β-alanylglycylglycine Boc hydrazide are very similar to published data on analogous derivatives of amino acids. The dinitrophenyl compound (J) has a single broad maximum above 250 nm positioned at 361 nm with a peak molar absorptivity of 16,300 (pH 7·4, Table 3). This agrees closely with the reported value of 16,310 at 360 for (2,4-dinitrophenyl)-β-alanine in aq. HCl at pH 1·0 but is 9 per cent less than 17,940 measured in bicarbonate solution at pH 8·3 (Ramachandran and Sastry, 1962). The higher pH of 8·3 and the presence of a nearby carboxylate anion in the reported structure at that pH may account for the difference observed. The molar absorptivity of 2,4,6-trinitrophenyl-ϵ-aminocaproic acid has been given as 15,400 at 350 nm (max.) (Benacerraf and Levine, 1962) and 15,700 at 348 nm (Little and Eisen, 1966), both in phosphate buffer, pH 7·4. The value of 15,000 at 345 nm (max.) found for compound K agrees well with these reported values. Compound K has a poorly defined maximum (plateau) around 410 nm. The spectrum of the dansyl derivatives (D) was obtained in acidic solution because of greater solubility and diminished fluorescence of the positively charged form of the compound. Published absorption data for dansyl compounds in neutral buffers apply to the uncharged dansyl group.

All final compounds gave the expected result in the test for Boc hydrazide. The color intensities were of comparable magnitude. Finally, the compounds ran on silica thin layers in chloroform–methanol mixtures as single spots with little or no visible contamination by minor components.

DISCUSSION

Large haptens have occasionally been employed by other workers, notably in studies on the specificity of sequential and conformation-dependent determinants of protein antigens (Fearney *et al.*, 1971; Arnon and Sela, 1969, respectively) and of biologically active peptides (Agarwal, 1971; Dietrich *et al.*, 1968; Goodfriend *et al.*, 1964; Haber *et al.*, 1965; Schick and Singer, 1961; Spragg *et al.*, 1967; Stason *et al.*, 1967; Vallotton, 1971). Parker *et al.* (1966) conjugated human serum albumin with a dinitrophenyl-lysyl tetrapeptide. Hapten-derivatized synthetic polypeptides have been used as immunogens (Arnon *et al.*, 1967; Haber *et al.*, 1965; Sela, 1965; Spragg *et al.*, 1967; Ungar-Waron, 1967; Vallotton, 1971).

The polypeptides provide both carrier function and relatively simplified substructures for attachment of the haptens. Examples of other large haptens that have been studied include fluorescein (Lopatin and Voss, 1971; Portmann *et al.*, 1971), p-(p'-azophenylazo)benzene derivatives (Nisonoff and Pressman, 1957; Pressman and Siegel, 1953) p-azophenyl-lactoside (Karush, 1957), and phenyl (p-azobenzoylamino) acetate (Karush, 1956). In addition, haptens spaced out from a carrier by hydrocarbon chains have been employed by several investigators (Benacerraf and Levine, 1962; Brownstone *et al.*, 1966).

The repeated preparation of a group of conjugated reagents from the previously employed large haptens would require an inconvenient variety of different procedures. Many sets of conditions would have to be individually established to achieve a reasonably uniform degree of substitution. It was mainly this problem that initially led us to design and synthesize the compounds reported here. The schema for synthesizing the large hapten Boc hydrazides (see Fig. 3) was designed to minimize the number of steps required to obtain several groups of derivatives. Accordingly, common structural features were assembled first from commercially available intermediates that already possessed some of the desired linkages. One or the other of the two protected tripeptide hydrazides (G or Z) could then be linked, in a final stage, to smaller immunodominant structures. The latter could include most of the smaller haptens used by immunochemists in the past.

At the outset we selected a common reactive functional group for effecting covalent attachment of the large haptens to various macromolecular carriers. It was required that such a group react readily with certain known groups of the carrier in aqueous media under mild conditions of temperature and pH. In addition, it should not react with other parts of the hapten molecule (e.g. with carboxyl groups, phenolic OH etc.), and should either be stable or be easily generated from a stable precursor. The acyl azide group, $-(C:O)N_3$, seemed to fit best the above criteria. The usefulness of this group for binding haptens to proteins and polypeptides was established by Brownstone, Mitchison and Pitt-Rivers (1966) who compared its efficiency with coupling *via* water-soluble carbodiimides, mixed anhydrides, active esters and acid chlorides.

Acyl azides are unstable at room temperature and require deep freeze storage, but they can be derived readily from hydrazides immediately prior to use. By protecting the hydrazide with a Boc group it was possible to introduce this common feature at the first stage synthesis. Boc hydrazides have been employed successfully in fragment condensations in peptide synthesis (Boissonnas *et al.*, 1960; Goodman and Langsam, 1966; Guttmann, 1961; Schwyzer *et al.*, 1960 and 1962), and the Boc group can be removed conveniently with trifluoroacetic acid at room temperature (Schwyzer *et al.*, 1960). This procedure and the subsequent conversion to azide at a low temperature do not alter the remainder of the structure, including the various immunodominant features. The Boc group did not fully protect the hydrazide linkage in the coupling of compound G with diazonium salts. The latter have been known to react with N,N'-diacylhydrazides in alkaline media to form diacyltetrazenes which then cyclize to tetrazoles with the elimination of one of the acyl groups (Dimroth, 1910). Principal colored impurities resulting from several of the couplings were examined and found to lack a hydrazide or Boc hydrazide functional group. Chromatographic behavior further suggested one of these byproducts to be a mono-azophenyl-N-acetyl-L-tyrosylglycylglycine. Nevertheless, the yield of desired product was satisfactory in most cases. The colored impurities which sometimes included bis-azophenyl derivatives, were removed especially well by ion-exchange chromatography where applicable. Collotti and Leskowitz (1970) successfully applied DEAE-cellulose columns to the separation of mono- and bis-azobenzenearsonate-N-acetyltyrosine.

An alternative to Boc hydrazides would be methyl or ethyl esters which can be converted to hydrazides with hydrazine in a final stage of synthesis. This strategy, which was used in an earlier study with the azobenzenesulfonate-N-acetyl-L-tyrosyl hapten (Rouques, Inman and Merchant, 1972), proved very troublesome because of the difficulty in removing excess hydrazine and its concomitant reduction of the established azo linkage. As a result, variable and heavy losses occurred, and additional purification steps were required.

Mixed carbonic anhydrides (Boissonnas, 1951; Vaughan and Osato, 1952; Wieland and Bernard, 1951) via isobutyl chloroformate provided convenient and effective intermediates for preparing the Boc hydrazide and for forming the β-alanylglycyl peptide bond. Mild conditions were selected (Anderson *et al.*, 1967; Tilak, 1970) even though racemization was not a potential problem here. However, in forming the L-tyrosyl-glycyl bond, the azide coupling method was preferred since this eliminated the need for protection and deprotection of the phenolic OH group and obviated concern over racemization. The modification by Hofmann *et al.* (1965, 1969) of the azide coupling method of Honzl and Rudinger (1961) was followed. The removal of the benzyloxycarbonyl (carbobenzoxy) protecting group was accomplished cleanly and smoothly by catalytic hydrogenation over palladium–charcoal catalyst in methanol. Addition of acetic acid in one case resulted in formation of some diketopiperazine from the liberated glycylglycine Boc hydrazide during flash evaporation. In

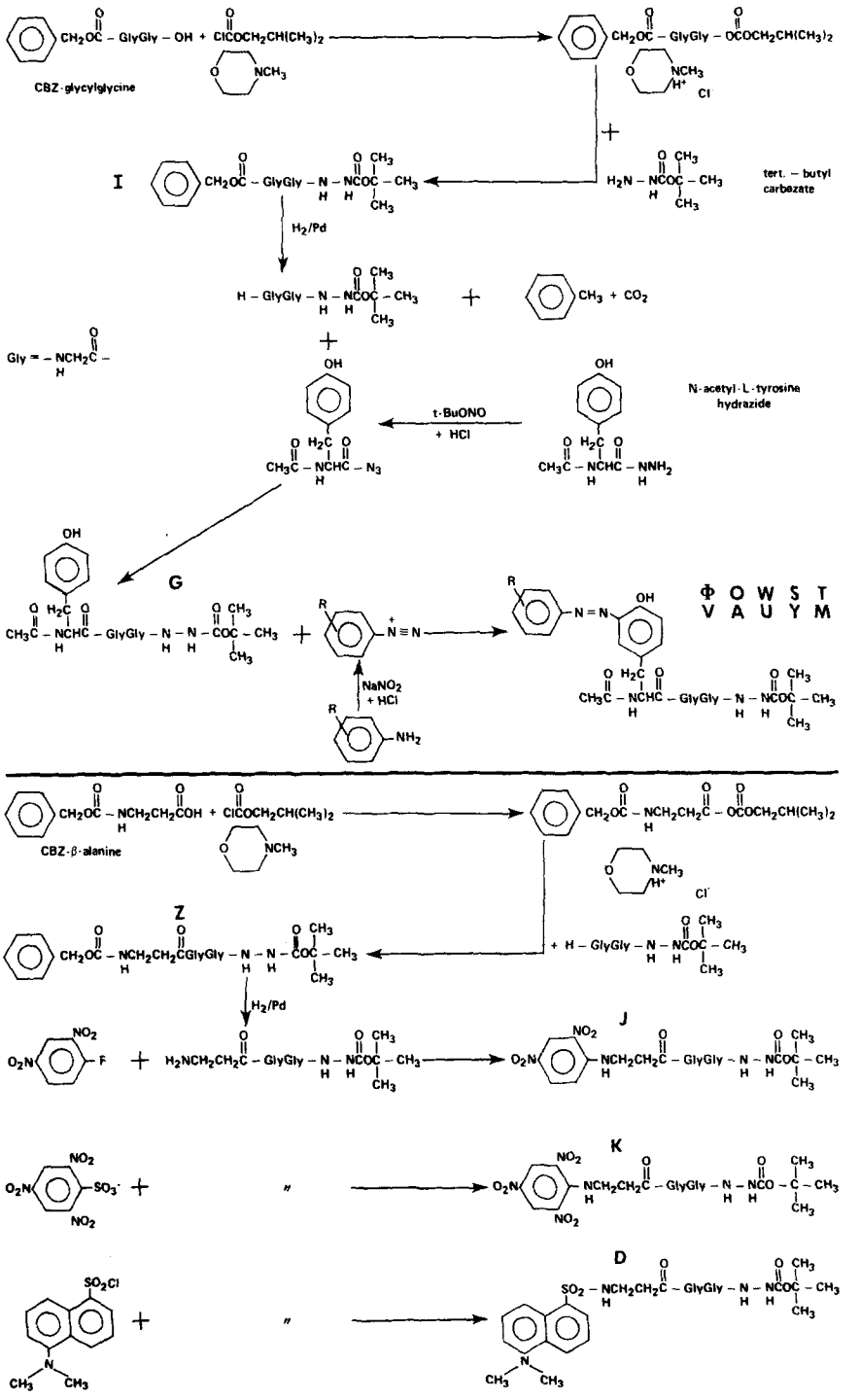

Fig. 3. Schema for the synthesis of both series of tripeptide derivatives. Bold-face capital letters (used as symbols for the individual derivatives) are identified in Tables 1 and 2, in the various subheadings of the Materials and Methods Section, and in Figs. 1 and 2. Gly = $-HNCH_2(C:O)-$, a glycyl residue.

neutral methanol the dipeptide Boc hydrazide remained intact.

The purified compounds have been stored in a refrigerator (+3°C), some of them for as long as 1½–2 yr, without evidence of decomposition.

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