COLLAGEN-LIKE POLYPEPTIDES: SYNTHESIS OF RADIOACTIVE POLYTRIPEPTIDES WITH THE INTERNAL SEQUENCE -GLY-PRO-PRO-

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SUMMARY

The synthesis of the tripeptide polymers $(Pro\text{-}Gly\text{-}Pro)_n$, $(Pro\text{-}Pro\text{-}Gly)_n$, and $(Gly\text{-}Pro\text{-}Pro)_n$ is described, with particular attention to procedures which favor the efficient insertion of radioactive proline in either of the two proline positions "X" or "Y" in the internal sequence, Gly-X-Y. By our methods, radioactive proline in the X-position is most efficiently obtained as $(Pro\text{-}Gly\text{-}{}^{*}Pro)_n$, while radioactive proline in the Y-position is most efficiently obtained as $(Gly\text{-}Pro\text{-}{}^{*}Pro)_n$. Some of the properties of these polymers and the stability of ^{3}H- and ^{14}C polymers are discussed. An additional finding relates to the synthesis of proline-containing tripeptides via the N-hydroxysuccinimide esters: our experience suggests the generalization that a peptide bond is not readily formed between pyrrolidine amino acids when a dipeptide is involved in such coupling reactions.

Key Words: Collagen, Polytripeptides, Tritium, Carbon-14

INTRODUCTION

Collagen-like synthetic polypeptides prepared as block polymers were originally reported from several laboratories (1-4) and the subject has been recently reviewed (5). The polymer $(Pro\text{-}Gly\text{-}Pro)_n$ (3) has probably been most widely used in biological studies (6-9).

Our interest in this subject is based on the use of such peptides, containing radioactive proline, in studying the specificity of prolyl hydroxylases (10). There have been few if any detailed studies of radioactive polymers of this type, although their synthesis has been referred to briefly (11,12).

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We have investigated alternative methods of synthesizing the familiar polymer $(Pro\text{-}Gly\text{-}Pro)_n$ so as to optimize the introduction of radioactive proline in either position. We also report our experience with the radioactive synthesis of two other polymers, $(Gly\text{-}Pro\text{-}Pro)_n$ and $(Pro\text{-}Pro\text{-}Gly)_n$, whose internal sequence is also (-Gly-Pro-Pro-).

MATERIALS AND METHODS

Commercial preparations included carbobenzoxy chloride, Z-Gly-OH*, Z-Pro-OH, and H-Gly-OEt,HCl, all from Sigma Chemical Company. Tetraethylpyrophosphite was a product of Aldrich Chemical Company. HBr in glacial acetic acid (45% w/v) was the Eastman-Kodak product. [3,4-^{3}H]-L-Proline and [U-^{14}C]-L-proline were purchased from New England Nuclear Corporation.

All samples of radioactive proline were purified by chromatography (after addition of small quantities of carrier proline) through a Dowex 50 H^+ column (0.9 x 30 cm), usually on a scale of 2-5 mCi. The radioactive proline was eluted with 0.5 M HCl; a small peak of counts in the region of hydroxyproline (after 4 to 5 column volumes and at least 5 column volumes before the beginning of the proline peak) was thereby removed.

Thin layer chromatography and amino acid analysis of peptides and intermediates were carried out by methods referred to earlier (13); designation of thin layer chromatographic solvents are those used previously (13). The method used for polymer molecular weight determination was a calibrated Sepharose column, equilibrated and eluted with Tris-buffered 1 M $CaCl_2$ (14). All melting points reported (Fisher-Johns hot stage) are uncorrected.

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*Abbreviations of amino acids and peptides and generally-used symbols are those listed in Biochem. J. 131: 1 (1973); abbreviations for N-blocking groups or esters of amino acids or peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (J. Biol. Chem. 247: 977 (1972). Other abbreviations: t.l.c. = thin layer chromatography; DCC = dicyclohexylcarbodiimide; TEA = triethylamine; HONSu = N-hydroxysuccinimide; HOAc = acetic acid; DMF = dimethylformamide; DMSO = dimethylsulfoxide; TEPP = tetraethylpyrophosphite.

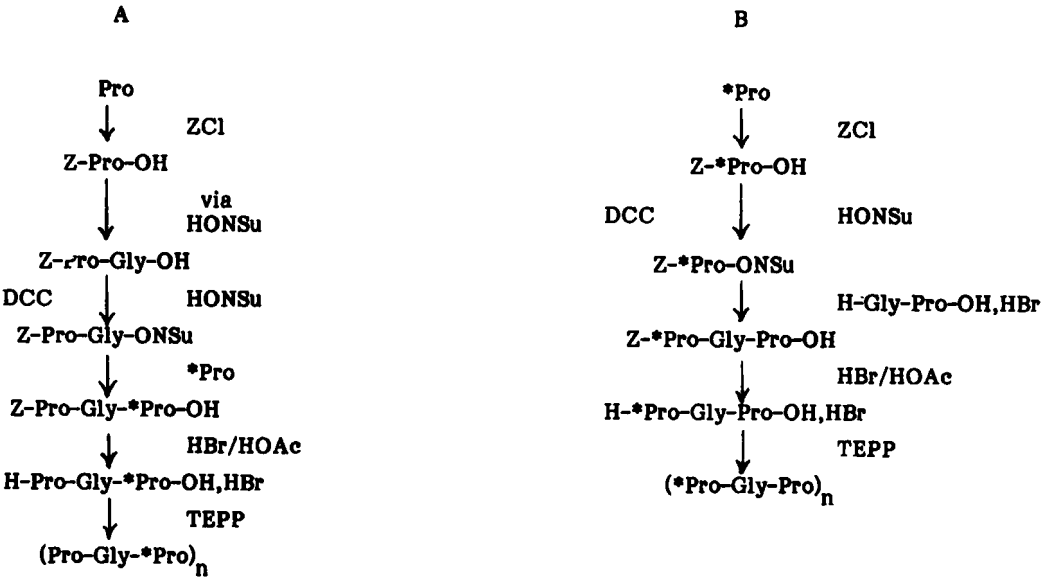
RESULTS AND DISCUSSION

Polymerization of $(Pro\text{-}Gly\text{-}Pro)_n$

Via Tetraethylpyrophosphite. The steps (Scheme 1) adopted for this synthesis are outlined below; for radioactive syntheses, radioactive proline is introduced where shown in order to label either Pro of the final product. This plan differs from that described earlier (3) in the use of the N-hydroxysuccinimide esters as intermediates in place of the acyl chlorides orginally utilized (3). In our hands the acyl chloride method gave poor yields.

For A (Scheme 1), Z-Pro-Gly-OH was made from Z-Pro-ONSu and Gly (15). The N-hydroxysuccinimide ester of Z-Pro-Gly-OH was prepared as reported for Z-Gly-ONSu (15); Z-Pro-Gly-ONSu was then coupled with free Pro following the general procedure for the synthesis of Z-Gly-Pro-OH by the succinimide ester route (15).

Similarly, in B (Scheme 1) Z-Pro-ONSu was coupled with H-Gly-Pro-OH,HBr, as in the coupling of Z-Pro-ONSu and Gly. Z-Pro-OH was made by a general procedure for carbobenzoxy amino acids (16) using excess $NaHCO_3$. H-Gly-Pro-OH,HBr was made by the HBr/HOAc treatment of Z-Gly-Pro-OH synthesized via Z-Gly-ONSu. Occasionally Z-Pro-Gly-Pro-OH was made by coupling Z-Pro-ONSu with free H-Gly-Pro-OH rather than with the hydrobromide.



SCHEME 1

Purity and Properties of H-Pro-Gly-Pro-OH,HBr. The best preparations of the tripeptide hydrobromide or free tripeptide, made by either branch of Scheme 1, appeared quite pure by chromatographic criteria. Thus, the free tripeptide (or hydrobromide) was eluted from the amino acid analyzer column as a single symmetrical peak at 180-190 min, using the 4-buffer system and the conditions noted earlier (17). This peak had an apparent color yield (absorbance at 570 nm) of 1.9 area units per μmole. This method separates H-Pro-Gly-Pro-OH slightly from H-Pro-Gly-OH (eluted 5-10 min earlier) but not from H-Gly-Pro-OH, which might therefore be undetected as a significant contaminant on the amino acid analyzer. On t.l.c. (Analtech silica gel G, phenol-water 80/20, 25°), both H-Gly-Pro-OH and H-Pro-Gly-OH migrated identically, but somewhat more slowly than H-Pro-Gly-Pro-OH (R_f 0.67 vs. R_f 0.71); by t.l.c., therefore, good preparations of H-Pro-Gly-Pro-OH,HBr were found to contain little or no H-Gly-Pro-OH or H-Pro-Gly-OH. Amino acid analysis of a hydrolyzate of the peak eluted from the analyzer gave a compatible Gly/Pro ratio of 0.34/0.62.

Polymerization of H-Pro-Gly-Pro-OH,HBr or of H-Pro-Gly-Pro-OH. This was carried out essentially as described by Engel et al (3).

Molecular Weights. Preparations of $(Pro\text{-}Gly\text{-}Pro)_n$, made as above by the tetraethylpyrophosphite method, gave peak molecular weights ranging from 1200-1800, averaging about 1500. Unfractionated preparations were of lower molecular weight than that first reported by Engel et al. (3), but it is of interest that several commercial preparations (Yeda-Miles, Ltd.), made by the method of Engel et al., agreed closely in molecular weight with our preparations.

Our preparations were consistently eluted from Sephadex G-50 (2.5 x 92 cm) as two distinct peaks. The smaller peak (one-quarter or less the area of the major peak) appeared at the column's void volume; these fractions, when run on the analytical Sepharose column (14), had a number average molecular weight of 3300, about twice that of the unfractionated polymer. A well-separated major peak was eluted next. Fractions from the central peak region gave a number-average molecular weight of about 1200.

Failure to Polymerize H-Pro-Gly-Pro-OH. An unexpected finding encountered

in making radioactive preparations was the failure of free H-Pro-Gly-Pro-OH (not the hydrobromide) to polymerize. We found repeatedly that H-Pro-Gly-Pro-OH, obtained by hydrogenation rather than hydrobromination of Z-Pro-Gly-Pro-OH, while seemingly pure by the criteria above and soluble in the pyridine solvent, failed to solidify or become viscous on standing in pyridine-tetraethylpyrophosphite. Such preparations were ether-precipitable from the polymerization medium, but gave only low molecular weights, compatible with the hexapeptide or smaller. Such preparations were largely unretarded by Dowex-50 H^+. Both observations suggest that cyclization of the tripeptide, or perhaps hexapeptide, had predominated over polymerization. It is of interest that somewhat similar non-cationic behavior was reported for polymers of H-Gly-Pro-Hyp-OH, made by the tetraethylpyrophosphite method (2), while Cowell and Jones (18) reported that when Z-Pro-Ala-2-benzyloxphenyl ester was hydrogenated to remove the Z-group only low molecular weight products were obtained from polymerization trials in TEA/DMSO, in contrast to the product (weight average mol. wt 12,000) obtained following Z-removal by HBr/HOAc.

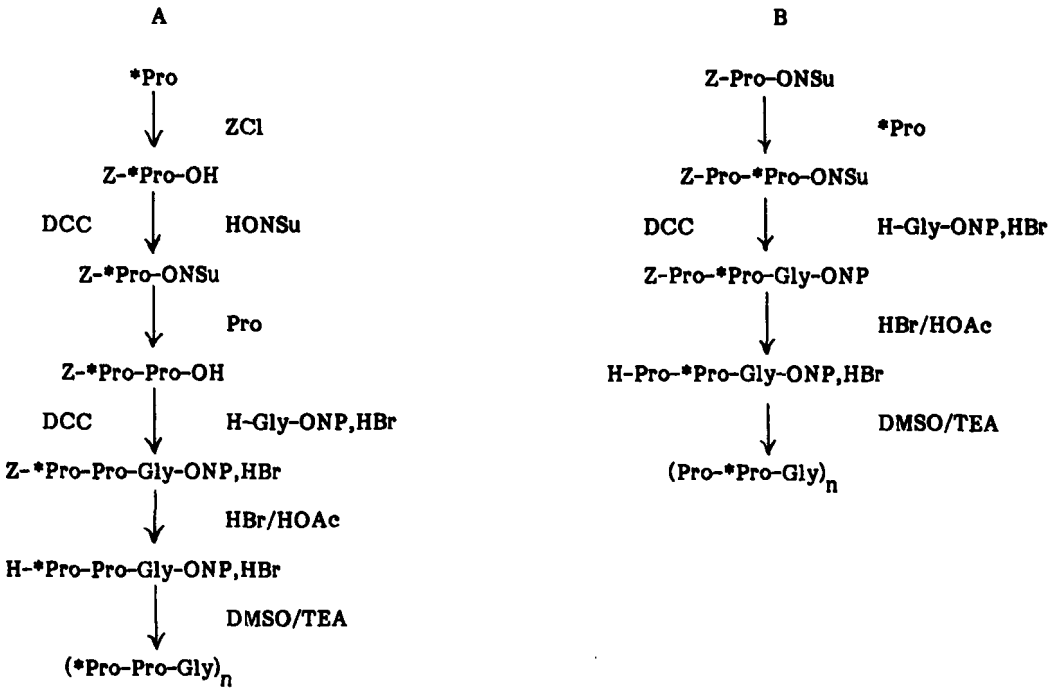
Attempts to Polymerize H-Pro-Gly-Pro-OH by Other Methods. Efforts were unsuccessful to make Z-Pro-Gly-Pro-ONSu from Z-Pro-Gly-Pro-OH by the method of Segal and Traub (19), or to make Z-Pro-Gly-Pro-ONP following the procedure of Bloom et al (4) for the synthesis of Z-Gly-Pro-Ala-ONP from Z-Gly-Pro-Ala-OH. An attempt to polymerize H-Pro-Gly-Pro-OH using DCC and HONSu in DMF at reduced temperature (20) was unsuccessful.

Radioactive $(*Pro\text{-}Gly\text{-}Pro)_n$ or $(Pro\text{-}Gly\text{-}*Pro)_n$. These labeled polymers were made as described above, Branch A (Scheme 1) being used for $(Pro\text{-}Gly\text{-}*Pro)_n$ and Branch B for $(*Pro\text{-}Gly\text{-}Pro)_n$. Most preparations utilized a scale of 4-5 mmoles of free proline (starting compound in B, final intermediate in A) and 2-4 mCi of ^{3}H-proline; final yields of the monomer, H-Pro-Gly-Pro-OH,HBr, ranged from 0.5-1.0 g and gave several hundred mg of radioactive $(Pro\text{-}Gly\text{-}Pro)_n$ of specific activity 4-5 x 10^6 dpm per mg. Preparations with ^{14}C-proline were made on a smaller scale, using about 2 mmoles of proline and 0.2 mCi of ^{14}C-proline and yielding polymer with specific activity approximately one-fifth that of the tritiated polymers.

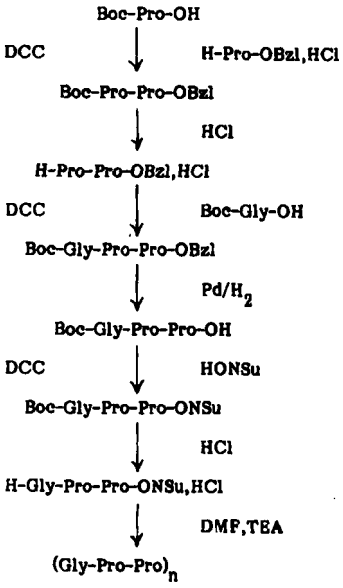
Polymerization of Pro-Pro-Gly

Steps in synthesizing the tripeptide p-nitrophenyl ester are outlined in Scheme 2.

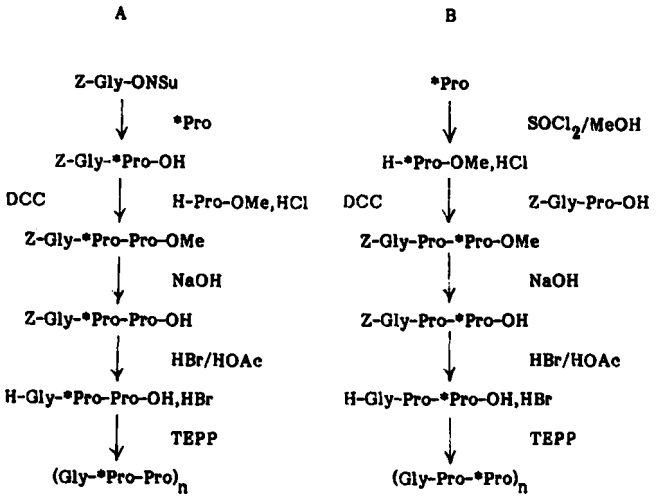
Synthesis of H-Pro-Pro-Gly-ONP,HBr. Z-Pro-OH was made as noted above, as was Z-Pro-ONSu. Z-Pro-Pro-OH has been previously made by a variety of methods (21-24). Our route via Z-Pro-ONSu, previously undescribed to our knowledge, appeared the simplest and utilized the availability of Z-Pro-ONSu used in our other syntheses. Z-Pro-Pro-OH was coupled with H-Gly-ONP,HBr (25) essentially as described by DeTar et al (26) for Z-Pro-Gly-ONP and Z-Hyp-Gly-ONP. The synthesis of Z-Pro-Pro-Gly-ONP has also been described by mixed anhydride coupling of Z-Pro-Pro-OH to H-Gly-ONP,HBr (27). The tripeptide hydrobromide was then made as usual by treatment with HBr/HOAc.



SCHEME 2



SCHEME 3



SCHEME 4

Table 1. Selectivity in Coupling Pyrrolidine Residues by the Succinimide Ester Method. The footnote or reference indicates the source of each observation

SUCCINIMIDE ESTER	COUPLES WITH	FAILS TO COUPLE WITH
Z-Pro-ONSu	Pro[a] Hyp (13) H-Gly-Pro-OH[a]	H-Pro-Gly-OH[a] H-Hyp-Gly-OH (13)
Z-Pro-Gly-ONSu	Pro[a]	
Z-Gly-Pro-ONSu	Ala[b]	Pro[a] H-Pro-OMe,HCl[a]
Z-Gly-Hyp-ONSu	Ala (13) Leu (13)	Hyp (13)

[a]Present paper

[b]Unpublished

Polymerization of H-Pro-Pro-Gly-ONP,HBr. This was carried out at room temperature in DMSO, on addition of an equivalent of TEA. Yields of the final ether-washed polymer approximated 50% by weight of the starting monomer. The product was largely insoluble in water and only partly soluble on heating in the Tris-buffered 1 M $CaCl_2$ used for molecular weight estimates (14). When the soluble supernatant phase of a suspension (40 mg in 5 ml of 1 M $CaCl_2$) was run on the Sepharose column, a single peak, slightly skewed toward the leading edge, was eluted; its peak corresponded to a number-average molecular weight of 3000; the small fraction of polymer directly soluble in aqueous solvents (e.g., 0.1 M HOAc) had a molecular weight less than 1000.

We are aware of only one reference to the block polymerization of Pro-Pro-Gly in solution, a preparation of low molecular weight (1100, by sedimentation) made via the N-hydroxysuccinimide ester: no further details of the synthesis or properties of this material were presented (28). Otherwise, $(Pro-Pro-Gly)_n$ has been made by the solid phase method which produces homogeneous polymers (29,30).

Radioactive $(Pro-Pro-Gly)_n$. These preparations were made by introducing tritiated proline into either position as shown in Scheme 2. The scale of such syntheses and the specific activities were similar to those noted for radioactive $(Pro-Gly-Pro)_n$.

Polymerization of Gly-Pro-Pro

The tripeptide Gly-Pro-Pro has previously been polymerized by several methods: as the free tripeptide with tetraethylpyrophosphite (31), giving a reported polymer of 5000 molecular weight by sedimentation; as the pentachlorophenyl ester (32), yielding an insoluble fraction (10%) of reported molecular weight 95,00-100,000; and as the ONP-ester (33), with an estimated molecular weight of 15,000.

In our case, two schemes have been tested for this type of synthesis, one (Scheme 3) employing the N-hydroxysuccinimide ester, and the other (Scheme 4) the polymerization of H-Gly-Pro-Pro-OH,HBr by tetraethylpyrophosphite. Radioactive syntheses were confined to the latter scheme.

Attempts to couple Boc-Pro-ONSu (15) with free Pro failed. As an alternative, Boc-Pro-OH was coupled to H-Pro-OBzl,HCl, using DCC and yielding an oil. Removal

of Boc in HCl/dioxane yielded crystalline H-Pro-Pro-OBzl,HCl, which was coupled with Boc-Gly-OH (DCC) to give an oil. Catalytic hydrogenation yielded Boc-Gly-Pro-Pro-OH, and from this the N-hydroxysuccinimide ester was made with DCC. Boc was again removed by HCl, and the active ester hydrochloride was obtained as a solid by precipitation with ether and dissolved in a small volume of DMF. The solution solidified within 24 hours after neutralization of the HCl with TEA. Addition of further DMF and filtration yielded two fractions, one insoluble and containing TEA,HCl plus polymer, and a DMF-soluble fraction which was treated with ether to yield a flocculent precipitate.

By gel chromatography (14) the DMF-soluble fraction gave a skewed peak of apparent low molecular weight (at peak height less than 1000). The much smaller quantity of DMF-insoluble material gave fractions approximating 2500 molecular weight. Because of the low yield of higher molecular weight polymer and the rather involved procedure, this Scheme was not used for radioactive syntheses.

Scheme 4. Of several approaches to Z-Gly-Pro-Pro-OH, only the one shown succeeded. Efforts failed to couple Z-Gly-Pro-ONSu (see EXPERIMENTAL) either with free Pro or with H-Pro-OMe,HCl. This is in contrast to coupling of Z-Pro-ONSu either with free Pro (see above) or free Hyp (13).

The failure of certain of these Pro- or Hyp-containing dipeptides (either free or as the Z-blocked N-hydroxysuccinimide ester) to couple with pyrrolidine amino acids suggests a generalization of interest. The observations summarized in Table 1 all deal with the synthesis of tripeptides by the N-hydroxysuccinimide ester method of coupling a dipeptide and amino acid. Under these conditions, we conclude tentatively that steric constraints probably interfere with the formation of the peptide bond between pyrrolidine amino acids. These constraints do not seem to apply to a 1 + 1 coupling of pyrrolidine amino acids by the N-hydroxysuccinimide ester method.

Z-Gly-ONSu was coupled with Pro (15) and the resulting Z-dipeptide coupled with H-Pro-OMe,HCl (34) via a conventional DCC procedure. Z-Gly-Pro-Pro-OMe has been reported earlier (35) by a mixed anhydride synthesis. It was saponified by a modification of a previous method (35). The resulting Z-tripeptide was converted

to the hydrobromide in the usual manner and treated in pyridine-tetraethylpyrophosphite exactly as for $(Pro\text{-}Gly\text{-}Pro)_n$(3). Crude polymer was obtained in good yield and gave an elution pattern from the Sepharose gel column indicating a number-average molecular weight of 2800. The somewhat asymmetric peak, skewed toward the leading edge, suggested that the earliest eluted fractions would yield polymer of considerably higher molecular weight.

For efficiency in introducing radioactive Pro, the synthesis of $(Pro\text{-}Gly\text{-}{}^{*}Pro)_n$, following Branch A of Scheme 1, is a convenient way of labeling the Pro following Gly (so-called X-position Pro in the sequence Gly-X-Y); to label Pro in the Y-position, Branch B of Scheme 4 would seem best. In each case radioactive Pro is introduced late in the sequence of synthetic steps.

Stability of Radioactive Polypeptides. The instability of ^{3}H-proline-containing $(Pro\text{-}Gly\text{-}Pro)_n$ was first noted by Hutton et al. (11) who reported loss of substrate activity for a prolyl hydroxylase on storage of the polymer at -15°. Our own observations on the substrate stability of such tritiated polymers (at specific activity comparable to or higher than that used by Hutton et al.) have yielded inconsistent results. Some preparations appeared to lose activity after storage of only a few months, either at -15° or at -90°. Other preparations, in particular one which was stored in the dry state at room temperature, appeared to retain substrate activity for more than two years. With present data, we are unable to relate apparent stability to the specific radioactivity of the polymer or the conditions of storage. We have found, however, that tritiated $(Pro\text{-}Gly\text{-}Pro)_n$, at specific activities of 1-3 μCi per mg, does not show fragmentation on storage for 6 months to 2 years: this conclusion is based on repeating the determination of molecular weight (14) and finding the same elution behavior of the radioactive peak as with the freshly-prepared polymer. In addition we have found that, after long storage, the specific activity of the proline in hydrolysates of these polymers remained unchanged, indicating no radiolytic destruction of the tritiated proline residues.

We have observed that ^{14}C-proline polymers $(Pro\text{-}Gly\text{-}[^{14}C]Pro)_n$ and $(Gly\text{-}Pro\text{-}[^{14}C]Pro)_n$, at specific activity about 0.4 μCi per mg, have retained their substrate

activity for almost a year. Because of the questionable stability of tritiated polymers, we should recommend the use of ^{14}C-polypeptides where storage for more than a few weeks is anticipated.

EXPERIMENTAL

These detailed descriptions are limited to new compounds or familiar compounds prepared by new methods or with new information or properties.

Z-Pro-Gly-ONSu. Z-Pro-Gly-OH (8 g, 0.026 mole) was dissolved in 80 ml of dioxane, treated with HONSu (3.01 g, 0.026 mole) and chilled. DCC (5.44 g, 0.026 mole) was added and the reaction mixture kept at 5°. After removal of dicyclohexylurea by filtration, the filtrate was concentrated to a dense oil which crystallized after addition of ether, yielding 7 g (70%), mp 137-141°. The product was recrystallized from about 30 ml of boiling ethyl acetate, mp 148-151°. Calculated for $C_{19}H_{21}N_3O_7$: C, 56.6%, H, 5.3%, N, 10.4%; Found: C, 56.6%, H, 5.2%, N, 10.6%. $[\alpha]_D^{25}$ (c= 1.2, MeOH) -58.4°. After this work was completed we became aware of an independent report of Z-Pro-Gly-ONSu, made by the same method as ours, and with similar mp and optical rotation (36).

Z-Pro-Gly-Pro-OH (from Z-Pro-Gly-ONSu + Pro). Z-Pro-Gly-ONSu (12.1 g, 0.03 mole) was dissolved in a solution of 180 ml of dimethoxyethane and 60 ml of H_2O. To this was added 3.48 g (0.03 mole) of proline and 2.52 g (0.03 mole) of $NaHCO_3$. The reaction mixture, after remaining overnight at room temperature, was reduced to small volume in a flash evaporator, acidified with 6 N HCl, and extracted three times with 100 ml vols of ethyl acetate. The product failed to crystallize but was dried to a powder under high vacuum. Yields were close to quantitative and the product appeared homogeneous (both by UV absorption and charring) on t.l.c. (Solvent H, R_f 0.12; Solvent L, R_f 0.03; Solvent B, R_f 0.66).

Z-Pro-Gly-Pro-OH (From Z-Pro-ONSu + H-Gly-Pro-OH or H-Gly-Pro-OH,HBr). In most syntheses, free H-Gly-Pro-OH rather than the hydrobromide was used, but the procedure was the same, except that 3 equivalents of $NaHCO_3$ were added for coupling with H-Gly-Pro-OH,HBr. Z-Pro-ONSu (11.6 g, 0.034 mole) was suspended in 150 ml of ethanol and dissolved with gentle heating. After cooling to room temperature, 6 g of H-Gly-Pro-OH (0.038 mole) and 6 g (0.071 mole) of $NaHCO_3$ were added. The

reaction mixture was kept at room temperature and treated exactly as in the previous preparation from Z-Pro-Gly-ONSu and proline.

Z-Pro-Pro-OH. Z-Pro-ONSu (10.4 g, 0.03 mole) was suspended in 100 ml of ethanol and heated with magnetic stirring. A solution of proline (3.46 g, 0.03 mole) and $NaHCO_3$ (5.0 g, 0.034 mole) in 60 ml of water was added. The suspension was heated and stirred to boiling, yielding a clear solution with much CO_2 production. The reaction mixture was kept at room temperature overnight, reduced to one-fourth its volume, and acidified with 6 N HCl. The thick oil that formed crystallized, yielding 7.6 g (73%), mp 172-178°. This was recrystallized (50% yield) from 35 ml of boiling ethanol; mp 186-189°. Similar melting points were reported by the other methods used earlier (see above).

Z-Pro-Pro-Gly-ONP. This preparation followed the general procedures of DeTar et al (26) in similar syntheses. Z-Pro-Pro-OH (1.79 g, 4.3 mmole) was suspended in 5 ml of acetonitrile and treated with 0.62 ml of TEA. This solution was added slowly at 5° to a chilled and stirred solution of H-Gly-ONP,HBr (1.17 g, 4.4 mmole) and DCC (1.0 g, 4.9 mmole) in 14 ml of acetonitrile. Addition required 30 to 60 min, and the solution was stirred at 5° for another two hours, then at room temperature overnight. The dicyclohexylurea was removed by filtration and the crystals washed with acetonitrile. The combined filtrate and washings were concentrated to about 8 ml, transferred to 50 ml of cold 0.01 N HCl and magnetic stirring continued at 5°. The yellow oil which formed sometimes hardened to filtrable crystals; if not, the aqueous phase was carefully removed, and the oil was dissolved in a few ml of ethyl acetate. On cooling, the product crystallized. In a number of small scale preparations, yields varied from 77% to 50%, the yield being greatest on direct crystallization from dilute HCl. mp 145-150°; lit.(mixed anhydride synthesis (27), mp 143-145°). The product was not recrystallized but was converted directly to the hydrobromide.

H-Pro-Pro-Gly-ONP,HBr. Z-Pro-Pro-Gly-ONP (570 mg, 1.09 mmoles) was dissolved with gentle heating in 1 ml of HOAc in a 25 ml round bottomed flask, fitted with a $CaCl_2$ drying tube. After addition of 6 ml HBr in HOAc, the solution was kept one hour at room temperature, then added dropwise to 50 ml of rapidly-stirred,

sodium-dried ether. The tan precipitate was filtered rapidly through sintered glass, washed with ether, and, before final drying, transferred to a pre-dried dessicator containing KOH, Drierite, and P_2O_5. Because of its extreme hygroscopicity, the preparation was kept under oil-pump vacuum until use. Yield, 335 mg (65%). It was not further characterized but used directly for polymerization.

(Pro-Pro-Gly)$_n$. Polymerization was usually carried out with 200 mg samples (mostly radioactive preparations) of H-Pro-Pro-Gly. This was dissolved in 0.3 ml of DMSO, treated with 0.06 ml of TEA and, after several days, the resultant polymer was precipitated with ether. Yields were about 90 mg (78%). Details on the solubility and size of the product are presented above under "Polymerization of Pro-Pro-Gly."

Boc-Pro-Pro-OBzl. An initial attempt to make Z-Pro-Pro-OBzl by coupling Z-Pro-OH with H-Pro-OBzl,HCl (DCC) yielded a crystalline product. Since subsequent attempts to remove Z selectively with HBr/HOAc failed, the preparation presented below was adopted. Boc-Pro-OH (10.75 g, 0.05 mole) was dissolved in 40 ml of $CHCl_3$ and added to a solution of H-Pro-OBzl,HCl (37) (12.1 g, 0.05 mole) and TEA (7 ml) in 50 ml of $CHCl_3$. The combined solution was chilled and treated with 11.33 g (0.055 mole) of DCC. Crystals formed at 0°, and the solution was then kept at room temperature. After 18 hours, the dicyclohexylurea was removed by filtration and discarded. The filtrate was washed with water, 10% citric acid, 1 M $NaHCO_3$, and dried over Na_2SO_4, yielding a thin oil that did not crystallize. The oil was homogeneous by t.l.c. (Solvent B, R_f 0.62; Solvent D, R_f 0.64) and was not further characterized. We learned later that its synthesis, as an oil, had earlier been reported (38) via a mixed anhydride method.

H-Pro-Pro-OBzl,HCl. The above oil was treated for 20 min at room temperature with 4-5 vol of 4 N HCl in dioxane. Addition of ether produced 13.3 g of crystals (79% relative to the starting reagents for Boc-Pro-Pro-OBzl), mp, 174-175°; neutralization equivalent, 341 (theory, 338). This compound has also been previously reported (38), but only as an oil.

Boc-Gly-Pro-Pro-OBzl. Mixture of H-Pro-Pro-OBzl,HCl (1.0 g, 0.003 moles)

and 0.45 ml TEA with Boc-Gly-ONSu (0.82 g, 0.003 mole) in 20 ml of $CHCl_3$, resulted in a suspension. Dioxane (20 ml) was added plus more $CHCl_3$ as required for solubilization. The reaction mixture was kept at room temperature overnight; a precipitate which had formed was redissolved by addition of more $CHCl_3$ and the solution was washed successively with water, 10% citric acid, 1 M $NaHCO_3$, water, and dried over Na_2SO_4. Concentration yielded a colorless oil that failed to crystallize but seemed homogeneous by t.l.c. (Solvent G, R_f 0.78; Solvent B, R_f 0.80). Yield 1.2 g (87%).

Boc-Gly-Pro-Pro-OH. Attempts to remove the -OBzl group by saponification in dilute NaOH yielded impure products; therefore, catalytic hydrogenation was used instead. Boc-Gly-Pro-Pro-OBzl from the above step (1 g, 2.2 mmoles) was hydrogenated for two hours at 1 atm in 10 ml of MeOH containing 0.5 ml HOAc and 100 mg of Pd-C catalyst. The reaction mixture, which showed a single streak-like spot (t.l.c., Solvent D, R_f centered at 0.2), was filtered to remove catalyst and evaporated to an oil that failed to crystallize. The yield was not determined.

Boc-Gly-Pro-Pro-ONSu. Boc-Gly-Pro-Pro-OH (3.3 g, 9.0 mmoles) was dissolved in 10 ml of ethyl acetate, treated with HONSu (1.15 g, 10.0 mmoles) and chilled. To the solution was added DCC (2.06 g, 10 mmoles) and the reaction mixture kept overnight at 5°. Dicyclohexylurea was removed by filtration; after evaporation of ethyl acetate, the residual oil was freed of several smaller batches of dicyclohexylurea crystals by treatment with more ethyl acetate. The ethyl acetate solution was treated with ether to form a viscous oil that solidified on drying to an extremely hygroscopic solid. (t.l.c., Solvent D, R_f 0.73). Yield 3.0 g (72%).

H-Gly-Pro-Pro-ONSu,HCl. The entire product from above was treated for 20 min at room temperature with 5 vols of 4 N HCl/dioxane. Addition of ether resulted in a dense white oil which soon changed to hygroscopic crystals. Yield 1.9 g (74%).

$(Gly\text{-}Pro\text{-}Pro)_n$. H-Gly-Pro-Pro-ONSu,HCl (1.5 g) was dissolved in 5 ml of freshly distilled DMSO and treated with 0.5 ml of TEA. Crystals of presumptive TEA·HCl formed immediately. The reaction mixture was kept at room temperature and appeared to solidify overnight. The reaction mixture was filtered to remove

DMF-insoluble material. The handling and characterization of the DMF-insoluble and DMF-soluble fractions of polymer are described under RESULTS AND DISCUSSION.

Z-Gly-Pro-ONSu. Z-Gly-Pro-OH (3.13 g, 0.010 mole) was dissolved in 60 ml of dioxane, treated with 1.17 g (0.010 mole) of HONSu, and chilled in ice. To the chilled solution was added 2.3 g of DCC (0.011 mole) and the mixture was kept overnight at 5°. Dicyclohexylurea was removed by filtration. The dioxane was removed by evaporation and the resultant oil was triturated with heptane to form a viscous oil. Crystals formed on treatment with ethanol/heptane, yielding 4.3 g (105%). The product was impure by t.l.c. and was recrystallized from isopropanol (50 ml/4 g), mp 95-96°; t.l.c. (Solvent D) R_f 0.73. Calculated for $C_{19}H_{20}N_3O_7 \cdot \frac{1}{2} H_2O$: C, 55.4%, H, 5.4%, N, 10.2%; Found: C, 55.8%, H, 5.2%, N, 10.3%. $[\alpha]_D^{25}$ (c = 1.2, MeOH) -56.6°.

Z-Gly-Pro-Pro-OMe. Z-Gly-Pro-OH (1.5 g, 4.9 mmoles) was dissolved in 14.8 ml of $CHCl_3$, and treated with 0.81 g (4.9 mmoles) of H-Pro-OMe,HCl and 0.69 ml of TEA in 10 ml of $CHCl_3$. The solution was chilled, treated with 1.03 g (5 mmoles) of DCC, and kept overnight at 5°. The dicyclohexylurea was removed by filtration, the $CHCl_3$ solution was washed successively with 50 ml each of 1 N HCl, 1 N $NaHCO_3$ and water, and dried over Na_2SO_4. After concentration and scratching in ethyl acetate, the product crystallized. Yield, 1.31 g (64%), mp 143-144°(lit. (35) 148-149°), t.l.c. (Solvent B) R_f 0.76; (Solvent D), R_f 0.63.

Z-Gly-Pro-Pro-OH. Z-Gly-Pro-Pro-OMe (1 g, 2.4 mmoles) was dissolved in 26 ml of acetone and treated with 26 ml of 0.1 N NaOH for 30 min at room temperature. The solution was extracted with 50 ml of $CHCl_3$ to remove unsaponified starting material, the acetone-water layer was acidified and again extracted twice with 50 ml of $CHCl_3$. The $CHCl_3$ solution was washed with water, dried over Na_2SO_4 and placed in a vacuum dessicator to yield a glassy solid. Yield 0.89 g (42%), t.l.c. (Solvent B) R_f 0.54; (Solvent D) streak centered at R_f 0.17.

H-Gly-Pro-Pro-OH,HBr. Z-Gly-Pro-Pro-OH (0.38 g, 0.88 mmoles) was dissolved in 0.86 ml of HOAc and treated with 3.8 ml of HBr/HOAc. After 45 min of vigorous stirring, the solution was poured into 23 ml of sodium-dried ether and the solid material was washed with ether. It was dissolved quickly in 6 ml of hot isopropanol,

reprecipitated with 4 vols of ether, filtered and washed further, then stored in a dessicator over Drierite, P_2O_5 and KOH under high vacuum. Yield 262 mg (80%) $(Gly\text{-}Pro\text{-}Pro)_n$. H-Gly-Pro-Pro-OH,HBr was polymerized in pyridine with tetraethylpyrophosphite, as noted under RESULTS AND DISCUSSION.

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