This material was identical with authentic[14] 3,5-pyrazoledicarboxylic acid (mp 289–295°, mmp 289–295°, lit.[14] mp 289°).

2H-Cyclododecapyrazole (12). To a solution of 2-hydroxymethylenecyclododecanone (6.46 g, 0.03 mole), prepared in 43% yield as previously described,[15] dissolved in ethanol (15 ml) was added dropwise to a solution of hydrazine (95%, 1.44 g, 0.04 mole) dissolved in ethanol (5 ml). The clear solution was heated at the reflux temperature for 12 hr, then cooled, and poured into water (50 ml). The resulting mixture was extracted with five 50-ml portions of ether. The combined ether extracts were dried ($MgSO_4$), and the solvents were removed on a rotary evaporator to give 12.73 g of a green oil, $n^{27}D$ 1.4309. Distillation of the oil gave 2H-cyclododecapyrazole (2.00 g, 32%) as a clear viscous oil, bp 160° (0.025 mm), $n^{28}D$ 1.5305. The oil crystallized upon standing overnight to give a white solid melting at 81.5–83.5°. Recrystallization of this product from petroleum ether (bp 60–68°) gave essentially a quantitative recovery of 2H-cyclododecapyrazole, mp 88.5–89.0°. The mixture melting point of 2H-cyclododecapyrazole and 3,5-[10]-pyrazolophane (mp 92.5–93.0°) was depressed (mp 49–63°).

Anal. Calcd for $C_{13}H_{22}N_2$: C, 75.67; H, 10.75; N, 13.58. Found: 75.72; H, 10.47; N, 13.42.

2H-Cyclododecapyrazole showed: infrared (NH at ν 3180–3110 cm^{-1}, C=C stretch at ν 1690 and 1620 cm^{-1}); nmr (CH_2, broad, τ 0.95, CH_2—C=CH, complex multiplet, τ 2.58, =C(H)—N, singlet, τ 2.78, N—H, broad, τ 3.40).

(14) L. Knorr, *Ann.*, **279**, 218 (1888).

(15) L. I. Zakharkin and U. V. Korneva, *Izv. Akad. Nauk. SSSR, Ser. Khim*, **12**, 2206 (1964).

Sequence Peptide Polymers. I. Polymers Based on Aspartic Acid and Glycine[1,2]

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Abstract: This is the first of a series of papers giving particulars of the "active" ester method of making sequence peptide polymers. Polymerization of HBr-H-Asp(OCH_3)-Gly-Gly-ONP and TosOH-H-Asp(Im)-Gly-Gly-ONP give the respective sequence peptide polymers poly Asp(OCH_3)-Gly-Gly and poly Asp(Im)-Gly-Gly. Polymerization was carried out in dimethyl sulfoxide or in dimethylformamide solutions by mixing the salt with triethylamine. Number-average molecular weights are 5000–10,000 and the aspartic acid residue is entirely L. Improved methods have been developed for characterizing the optical purity of aspartic acid peptides. The problems of synthesizing sequence peptide polymers are principally those of making the requisite highly reactive "monomers" in chemically and optically pure form. Efficient syntheses are described as are important limitations of certain possible routes.

The development of efficient methods of preparing homopolymers and of random copolymers of amino acids has led to extensive studies of these polypeptides as protein models.[3-5] The next step, the preparation of random peptide polymers with known repeating sequences, has also been studied for many years. Early examples have been reviewed by Bamford, Elliott, and Hanby[4] and more comprehensively by Katchalski.[3] In most cases the approaches used were suitable only for sequences without reactive side chains. More recently poly Gly-Pro-Hypro(H)[6] was prepared by polymerization of the free tripeptide with tetraethylpyrophosphite[7] and poly Gly-Ser(H)-Ala was prepared by action of N-bromosuccinimide on the acid hydrazide.[8] The use of dicyclohexylcarbodiimide as polymerization reagent has also been studied.[3,4,9]

Some time ago we reported several examples of the "active" ester synthesis of sequence peptide polymers.[2] The present paper is the first of a series which provides the details. The generality of the method has been further illustrated by recent publications.[10-12] The

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(2) For preliminary account see D. F. DeTar, W. Honsberg, U. Honsberg, A. Wieland, M. Gouge, H. Bach, A. Tahara, W. S. Brinigar, and F. F. Rogers, Jr., *J. Am. Chem. Soc.*, 85, 2873 (1963).

(3) This work has been admirably reviewed: (a) E. Katchalski, M. Sela, H. I. Silman, and E. Berger, *Proteins*, **2**, 405 (1964); (b) E. Katchalski and M. Sela, *Advan. Protein Chem.*, **13**, 224 (1958).

(4) C. H. Bamford, A. Elliott, and W. E. Hanby, "Synthetic Polypeptides," Academic Press Inc., New York, N. Y., 1956.

(5) R. E. Nylund and W. G. Miller, *J. Am. Chem. Soc.*, 87, 3537 (1965); W. G. Miller and R. E. Nylund, *ibid.*, 87, 3542 (1965); R. L. Snipp, W. G. Miller, and R. E. Nylund, *ibid.*, 87, 3547 (1965).

(6) The conventional amino acid abbreviations are used in a special way: the symbols stand for the stem alone. Glycine is H-Gly-OH; aspartic acid is H-Asp(OH)-OH; the β-methyl ester of aspartic acid is H-Asp(OCH_3)-OH. This procedure permits the precise description of all derivatives. In addition **Z** is used for benzoyloxycarbonyl, Bz always stands for benzoyl, Bl stands for benzyl, HONP for *p*-nitrophenol, HOPCP for pentachlorophenol, DCC for dicyclohexylcarbodiimide, DCU for dicyclohexylurea, DPC is diisopropylcarbodiimide, DPU is the urea, and CMC is cyclohexyl(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. See M. Goodman and G. W. Kenner, *Advan. Protein Chem.*, **12**, 488 (1957); and R. Schwyzer, J. Rudinger, E. Wünsch, and G. T. Young, "Peptides," G. T. Young, Ed., Pergamon Press Ltd., London, 1963, p 261.

(7) (a) N. S. Andreeva, V. A. Debabov, M. N. Millionova, V. A. Shibnev, and Y. N. Chirgadze, *Biophysics* (USSR), **6**, 272 (1961); (b) A. D. Morozkin, *ibid.*, 8, 465 (1963). The initial molecular weight of 25,000 was later corrected to 4000 when Archibald measurements were carried out.

(8) Y. Wolman, P. M. Gallop, A. Patchornik, and A. Berger, *J. Am. Chem. Soc.*, 84, 1889 (1962).

(9) T. Vajda, *Acta Chim. Hung.*, **46**, 221 (1965); *Chem. Ind.* (London), 785 (1963).

(10) F. H. C. Stewart, *Australian J. Chem.*, **18**, 887 (1965).

(11) R. D. B. Fraser, B. S. Harrap, T. P. MacRae, F. H. C. Stewart, and E. Suzuki, *J. Mol. Biol.*, **12**, 482 (1965).

(12) J. Kovacs and A. Kapoor, *J. Am. Chem. Soc.*, 87, 118 (1965); J. Kovacs, R. Giannotti, and A. Kapoor, *ibid.*, 88, 2282 (1966).

method has been applied to polymers containing serine, aspartic acid, glutamic acid, hydroxyproline, and histidine as well as to single amino acid *p*-nitrophenyl esters and to dipeptide *p*-nitrophenyl esters where cyclization to a diketopiperazine might have been expected instead. Under certain conditions it is possible to use an optically active residue in the C-terminal position without loss of optical activity.[13b]

At the time we began this investigation there were no general routes to the requisite monomers such as **18**.[14] Methods used previously for somewhat similar nitrophenyl esters all involved hydrolysis steps which would cause loss of the aspartyl ester group,[15,16] *e.g.*

HBr-H-Asp(OCH_3)-Gly-Gly-ONP **18** H-Asp(OCH_3)-Gly-Gly-OH **15b**

Z-Asp(OCH_3)-Gly-Gly-OH **15a** Z-Asp(OCH_3)-Gly-Gly-ONP **19**

We therefore carried out a number of exploratory studies directed both toward the "active" esters and also toward simple tripeptides. The goal was to develop methods useful for synthesis on a molar scale or larger. This goal has been achieved.

The best route we have found to **19** and hence to **18** is based on the principle that with care the *p*-nitrophenyl ester group can be used as a blocking group in peptide syntheses (eq 1).[17] However, even more important to the synthesis of sequence polymers was the discovery in our laboratories that this principle can be extended to dipeptide nitrophenyl esters (eq 1).[18] This is quite a remarkable reaction, for competing cyclization of **13** to a diketopiperazine might have been expected to predominate. It is not surprising that the choice of reaction conditions is rather restricted.

Z-Asp(OCH_3)-OH (**2**) + HBr-H-Gly-Gly-ONP (**13**) ⟶ **19** (1)

Certain other routes to "monomer" were found to be deficient for reasons which are important in planning a new approach. Synthesis of Z-Gly-Asp(OCH_3)-ONP (**12**) according to eq 2 and 3[19,20] gave racemic *p*-nitrophenyl ester **12**,[22] although **10** was analytically and optically pure. Preliminary studies on the partly racemic hydrobromide **11** showed that the dipeptide nitrophenyl ester hydrobromide **11** forms polymer as well as the cyclic diketopiperazine.

Z-Gly-ONP (**5**) + HCl-H-Asp(OCH_3)-OH ⟶ Z-Gly-Asp(OCH_3)-OH (**10**) (2)

10 + DCC + HONP ⟶ Z-Gly-Asp(OCH_3)-ONP (**12**) (3)

12 + HBr $\xrightarrow{HOAc}$ HBr-H-Gly-Asp(OCH_3)-ONP (**11**) (4)

11 + TEA ⟶ poly Gly-Asp(OCH_3) + cyclic product (5)

Attempts to convert the dipeptide acid **10** directly to peptide by reaction with H-Gly-ONP (*cf.* eq 1) gave Z-Gly-Asp(OCH_3)-Gly-ONP (**21**) with considerable racemization. In other quite similar cases racemization has been minimized or avoided by proper conditions;[25] however, a study of variations in solvent, proportion, and order of mixing of reagents, and temperature gave at best a product consisting or 90% L- and 10% DL-**21**, in unsatisfactory yield. The alternate route to **21** (eq 9) was examined briefly and found to give an optically pure product in about 10% yield.[26]

21 + HBr $\xrightarrow{HOAc}$ HBr-H-Gly-Asp(OCH_3)-Gly-ONP (**20**) (6)

20 + TEA ⟶ poly Gly-Asp(OCH_3)-Gly + cyclic product (7)

Z-Gly-OH + HBr-H-Asp(OCH_3)-Gly-ONP (**7**) ⟶ **21** (8)

Attempted synthesis of Z-Asp(OCH_3)-Gly-Gly-OH **15a** *via* eq 9 gave considerable imide **15**; and in fact **15a** titrates to pH 9 as a dibasic acid because of facile imide formation and hydrolysis. The possibility of imide formation was well known,[27] but the mildness of conditions was unexpected at the time of the experiments. Detailed studies have since been reported.[28] The presence of imide **15** was established first by analyses for methoxyl and later by nmr. The sharp methoxyl singlet at δ 3.65 (trifluoroacetic acid solution) shows up very clearly.

Z-Asp(OCH_3)-ONP (**3**) + H-Gly-Gly-OH ⟶ Z-Asp(OCH_3)-Gly-Gly-OH (**15a**) (9)

15a ⟶ Z-Asp(Im)-Gly-Gly-OH (**15**) (10)

Z-Asp(OCH_3)-ONP (**3**) + H-Gly-OH ⟶ Z-Asp(OCH_3)-Gly-OH (**6a**) (11)

6a + HBr $\xrightarrow{HOAc}$ HBr-H-Asp(OCH_3)-Gly-OH (**6**) (12)

It turns out that the closely related reaction with glycine, eq 11, gives a reasonable yield of methyl ester **6a**; the nmr curve for the hydrobromide **6** gives excellent agreement with theory for one methoxyl group. Imide formation seems to be negligible. However, in spite of a lengthy series of experiments including careful pH control, no useful recipe for preparing pure methyl ester

(13) (a) D. F. DeTar, H. C. Bach, and F. F. Rogers, Jr., in preparation; (b) D. F. DeTar and N. F. Estrin, to be published.

(14) Compounds have been assigned serial numbers which are in an indexing order to facilitate cross references with the Experimental Section.

(15) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2186, 2190 (1958).

(16) R. Schwyzer and B. Gorup, *ibid.*, **41**, 2199 (1958).

(17) M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).

(18) This approach has also been developed by Stewart.[10,11]

(19) M. Bodanszky, *Nature*, **175**, 685 (1965).

(20) For reviews of active ester syntheses see N. A. Albertson, *Org. Reactions*, **12**, 157 (1962); M. Goodman and G. W. Kenner, *Advan. Protein Chem.*, **12**, 465 (1957); ref 21, p 1016; K. Hofmann and G. Katsoyannis, *Proteins*, **1**, 94 (1963).

(21) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961.

(22) The reasons for this have since been demonstrated experimentally.[23,24]

(23) D. F. DeTar, R. Silverstein, and F. F. Rogers, Jr., *J. Am. Chem. Soc.*, 88, 1024 (1966).

(24) M. Goodman and L. Levine, *ibid.*, 86, 2918 (1964).

(25) *Cf.* M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963); G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, 80, 2902 (1958).

(26) Results of later work indicate that much higher yields should be obtainable under proper conditions.

(27) J. E. H. Hancock and R. P. Linstead, *J. Chem. Soc.*, 3490 (1953); E. Sondheimer and R. J. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954); **79**, 3767 (1957); A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).

(28) S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Am. Chem. Soc.*, 84, 2421 (1962); A. J. Adler, G. D. Fasman, and E. R. Blout, *ibid.*, 85, 90 (1963).

15a *via* the direct route of eq 9 could be found. Apparently the amount of base required to bring glycylglycine into solution is enough to cause imide formation to compete extensively with condensation. The reaction is, however, suitable for purposes of forming pure imide **15**.[29]

Conversion of the imide acid **15** to the *p*-nitrophenyl ester **17** (eq 13) was accomplished in reasonable yield.

$$\text{Z-Asp(Im)-Gly-Gly-OH} + \text{HONP} + \text{DCC} \longrightarrow \underset{\mathbf{15}}{\phantom{\text{Z-Asp(Im)-Gly-Gly-OH}}} \quad \underset{\mathbf{17}}{\text{Z-Asp(Im)-Gly-Gly-ONP}} \quad (13)$$

Preparation of "Monomer" Hydrobromides. Tripeptide nitrophenyl esters were converted to the respective hydrobromides with a solution of hydrogen bromide in acetic acid. Difficulty was experienced with HBr-H-Asp(OCH_3)-Gly-Gly-ONP (**18**). Initial samples were very hygroscopic, gave high bromide titrations, and were unpredictably unstable toward liquefaction. Apparently both monohydrobromide and dihydrobromide (with the central peptide group as the second basic group) were present. It was ultimately discovered that recrystallization from a small amount of water gave analytically pure samples which were relatively nonhygroscopic. The hydrobromide of the imide proved noncrystalline, but the toluenesulfonate, TosOH-H-Asp(Im)-Gly-Gly-ONP (**16**), was readily prepared in analytically pure form.

Criteria for Identity and Purity. Nmr spectra in trifluoroacetic acid have proved especially valuable. In most cases every hydrogen atom can be located unambiguously. Exceptions are the OH hydrogens which exchange with solvent[30] and the ZNH hydrogen which is probably very broad but which may exchange; it seldom shows up. The nmr curves can be used to establish the composition of a peptide within about 5–10% relative for each structural feature.

Optical purity of the various intermediate peptides was established by finding that independently prepared samples give identical rotation values, usually at five wavelengths and often in several solvents. In addition, a hydrolysis procedure has been worked out which is very successful with aspartic acid derivatives; it is capable of giving the optical purity of an aspartic acid peptide to within 1 or 2%. The methods are described in the Experimental Section. Extensive rotation data for the peptides are presented in Table II.

Poly Asp(OCH_3)-Gly-Gly. Early runs used the HBr-H-Asp(OCH_3)-Gly-Gly-ONP containing excess hydrogen bromide but still gave polymer of moderate molecular weight. These are summarized briefly in the Experimental Section. Table I summarizes later runs carried out with analytically pure "monomer." A number of solvents and a number of bases were tried, as mentioned in the Experimental Section. The use of dimethyl sulfoxide and triethylamine proved both convenient and quite satisfactory.

(29) It may be noted at this point that other aspartic acid derivatives also differ in their tendency to imide formation: HBr-H-Asp(OCH_3)-Gly-Gly-ONP posed serious difficulties, while HBr-H-Asp(OCH_3)-Ser(H)-Gly-ONP gave only incomplete imide formation with excess of triethylamine. The solvents were anhydrous dimethyl sulfoxide or dimethylformamide.

(30) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p 417.

Table I. Polymerization of HBr-H-Asp(OCH_3)-Gly-Gly-ONP[a]

Solvent[b]	Yield, %[c]	$[\eta]$[d]	N[e,f]	$\overline{DP}$[g]
13.5 g DMF	30	0.14	15.6	(8)
3.2 g DMF	60	0.17	16.3	(14)
5.4 g DMSO	40	0.20	16.4[h]	(21)
4.3 g DMSO	45	0.24	15.5	(33)
2.3 g DMSO	70	0.22	15.4	(26)
1.5 g DMSO	..	0.23	16.4	(30)
20 ml DMSO[j]	65	0.21	15.8[i]	(23)
2 ml MP	65	0.24	16.6	(33)
2 ml HMP	50	0.18	16.0	(17)
4 ml DMSO	Purified	0.258	k	40

[a] See Experimental Section for further details, including "fractionation" of the polymer. The analytically pure hydrobromide "monomer" (1.6–1.7 g) was suspended in the specified solvent and the theoretical amount of triethylamine added. Polymerization was allowed to proceed at room temperature overnight. The mixture was then diluted with solvent, usually methylene chloride; the polymer was filtered and dried. [b] Abbreviations: DMF, dimethylformamide; DMSO, dimethyl sulfoxide; MP, N-methylpyrrolidone; HMP, hexamethylphosphoramide. For the latter, DMSO was added because of insolubility in HMP. The solvents were all redistilled and dried over Al_2O_3. [c] There were also obtained soluble fractions which account for the rest of the starting material. [d] Intrinsic viscosity in dichloroacetic acid at 30°. [e] *Anal.* Calcd for $C_9H_{13}N_3O_5$: C, 44.4; H, 5.39; N, 17.28. [f] All samples estimated to have 95–100% CH_3O based on nmr peak area. [g] Degree of polymerization (tripeptide monomer units). See text. [h] C, 41.5; H, 5.38. [i] C, 41.5; H, 5.41. [j] Using 17 g of "monomer." [k] Repurified sample described in Experimental Section; OCH_3 100% by Zeisel, $\overline{DP}$ by dinitrophenylation. The number-average molecular weight is 9700.

Elemental analyses have been performed on a large number of polymer samples after a variety of purification treatments. In general the ratios of C:N:OCH_3 are very good, but absolute values tend to run 5–10% low. In a few cases ash has been present, presumably silica from overzealous recovery of precious materials, but in most cases the discrepancy is ascribed to water.

It has been found that various small molecules are held tenaciously by peptide polymers. An example in which chloroform was held even after extensive heating at very low pressures is reported with poly Asp(OCH_3)-Ser(H)-Gly.[13] Some solvents such as dimethylformamide and dimethyl sulfoxide can be removed by adequate washing. These solvents, as well as chloroform, ether, acetonitrile, and ethyl acetate and several others, are readily detectable in the nmr spectra. Water, however, is not directly observable. Some work has been done on the use of gas chromatography to assay for water, but the procedures are not yet completely satisfactory.

Nmr spectra of the polymers in trifluoroacetic acid gave rather broad peaks: 483 (13), NH; 345 (15), CH; 261 (10), Gly-CH_2; 233 (3), OCH_3; 190 (12), Asp-CH_2. The figures refer respectively to peak position in cycles per second (at 60 Mc) from tetramethylsilane and to the half-width in cycles per second (in parentheses). The areas were in the theoretical ratio 3:1:4:3:2 within an experimental error of 5–10% for all curves.

It was possible to estimate the amount of N-terminal aspartyl residues or an upper possible limit from the nmr spectra since low polymers showed a second methoxyl peak at 236. This downfield shift is also observed with peptides and with the "monomer" itself. On this basis the polymers listed in Table I had a degree of polymerization greater than about 20 (mol wt 4900).

There was no detectable spur, and it is estimated that 5% or more of terminal aspartyl would be readily observable.

Definitive estimation of molecular weights has proved to be a most difficult problem. Solubility in noncorrosive solvents is too low to make it possible to do ultracentrifuge measurements, and work with such solvents as trifluoroacetic acid has progressed only slowly.[31]

The procedure adopted was therefore indirect. A combined sample of the polymers was subjected to extraction with chloroform (which removed little material) and with dimethyl sulfoxide which removed about 30%. The soluble material is expected to be rich in low molecular weight polymer and in cyclic products. The resulting sample was then assayed for free amino end groups by the modified DNP (dinitrophenylation) procedure to give the degree of polymerization on the last line of Table I.[13,32] Another reference point was available from work with monomer and with low polymer which suggested an intrinsic viscosity of about 0.08 for a $\overline{DP}$ of 2. These points were then used on a log–log plot to provide a graph from which were read DP estimates in parentheses.[33]

Relatively few viscosity–molecular weight curves have been reported for peptide polymers. Extensive measurements on poly Asp(OCH_3)-Ser(H)-Gly have established a relationship in which a given intrinsic corresponds to somewhat less than half the above degree of polymerization; *i.e.*, for $[\eta] = 0.240$, $\overline{DP} = 12$ (number-average basis).[13] Measurements reported for poly Glu(OBl) in this range do not define a straight line;[34] $[\eta] = 0.26$ corresponds to a weight-average molecular weight of 42,000 while our $[\eta] = 0.26$ corresponds to a number-average molecular weight of 9700 or a weight-average of 19,000.

Errors in end-group determinations of the molecular weight usually operate to give overestimates; the most likely source of error with poly Asp(OCH_3)-Gly-Gly and with poly Asp(Im)-Gly-Gly is the presence of cyclic peptides. Kenner[35] and Schwyzer[15,16] have used the active ester approach to prepare cyclic hexapeptides, and hence these are certain to be present. The possibility for larger rings remains unknown at present.

The reproducibility of the DNP end-group assays is good, about 5%. Based on correlations with other polymers, we estimate that the values cited in Table I are within about 25–30% of the correct values. For polymers of this type the weight-average molecular weights should be about twice the number-average.[33b]

The optical rotation of poly Asp(OCH_3)-Gly-Gly is reported in Table II. Hydrolysis showed that the aspartyl residue is unracemized with the estimated accuracy of the procedure; 97 ± 2% L.

The following evidence supports the structures assigned to the sequence polymers. (1) The "monomers" have been prepared repeatedly in an analytically and optically pure form. (2) The polymerization reaction is of a type which is well understood and has been widely used in synthesizing both simple and cyclic peptides.[16,20] (3) There are two principal side reactions, imide formation and racemization. Reversible imide formation would, of course, lead to β linkages. Independent studies show, however, that imide formation occurs considerably more slowly than polymerization even with an excess of base, although care is necessary to prepare a polymer with more than 90% of methoxyl. The extent of imide formation is negligible under conditions used for the polymers reported in Table I. Racemization may accompany imide formation but seems to be considerably less than half as fast. (4) The nmr spectra of the polymers show all protons and are in close accord with data obtained from a wide variety of peptides.

It is not to be expected that poly Asp(OCH_3)-Gly-Gly nor poly Asp(Im)-Gly-Gly will form an α helix,[36] and indeed the rotatory dispersion curves are accurately correlated by the Drude equation.[37] Attempts to obtain oriented films have met with only partial success so far.

Inspection of Table I shows a correlation between the concentration of the "monomer" and the polymer yield and perhaps with the degree of polymerization. The degree of polymerization will be determined by the following factors. (1) End-grouping reactions owing to (a) the presence of end-grouping impurities such as Z-X-X-X-ONP or HBr-H-X-X-X-OH, to (b) the occurrence of side reactions such as hydrolysis, and to (c) the presence of unreacted H_3N^+X owing to insufficient base. One mole of base is required per mole of hydrobromide. (2) Cyclization reactions; these are favored by low reactant concentrations. The polymerizations place a heavy demand on working at the maximum concentration attainable. (3) Solubility factors. If the polymer is not very soluble, it may precipitate and cause chain growth to cease. This factor is somewhat imponderable, since the possibility of further reaction will depend on whether the reactive ends are accessible on the surface or whether they are buried. The degree of swelling will also be important, and an insoluble but swollen polymer may continue to grow.

Poly Asp(Im)-Gly-Gly. The polymerization of Tos-OH-H-Asp(Im)-Gly-Gly-ONP was carried out several times as described for the ester. One sample had $[\eta] = 0.23$ and a molecular weight of 5000 (DP = 24) by the modified DNP method.[13] These results show a viscosity–molecular weight relationship fairly close to that obtained for the ester.

A second method of preparing the polymer was to treat HBr-H-Asp(OCH_3)-Gly-Gly-ONP with somewhat more than 2 equiv of base in a dimethyl sulfoxide solution. The resulting polymer was entirely the imide and was about 45% DL. Examination of the infrared curves of the reaction mixture as reaction proceeded showed that polymerization was mostly complete within an hour or two and that imide formation was much slower, requiring a day or two for completion.

Conversion of polymeric ester to polymeric imide proceeded slowly over a period of several days when a slurry of the polymer was stirred with a solution of triethylamine and dimethyl sulfoxide.

(31) Very good ultracentrifuge results have been obtained with many other sequence peptide polymers: D. F. DeTar and R. Albers, in preparation.

(32) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(33) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953: (a) p 310; (b) p 325.

(34) J. C. Mitchell, A. E. Woodward, and P. Doty, *J. Am. Chem. Soc.*, **79**, 3955 (1957).

(35) G. W. Kenner and J. M. Turner, *Chem. Ind.* (London), 602 (1955).

(36) For a review see J. A. Schellman and C. Shellman, *Proteins*, **2**, 45 (1964).

(37) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).

Table II. Rotations of Aspartic Acid Derivatives[a]

Compound	Mol wt	Opt. purity[b]	Solvent[c]	No. of samples[d]	$a' \times 10^{-6}$[e]	λ_0[e]	589[f]	546[f]	Error[g]
H-Asp(OH)-OH	133.1		2 5 *N* HCl	3, 9	9.9612 ± 0.15	212.91 ± 5	32.97	39.36	0.2
Z-Asp(OH)-OH	267.2	9.54 (3)	2 H_2O	2, 2	−3.8259 ± 0.25	275.66 ± 9	−14.1	−17.2	3.5
H-Asp(OCH_3)-OH	147.1	9.73 (1)	2 H_2O	1, 2	0.54663 ± 0.1	314.27 ± 11	2.20	2.74	9.0
HCl-H-Asp(OCH_3)-OH	183.6	9.68 (1)	2 5 *N* HCl	2, 2	9.8085 ± 0.55	219.47 ± 13	32.8	39.2	2.0
Z-Asp(OCH_3)-OH	281.3	9.49 (5)	2 DMF	2, 2	−24.569 ± 0.3	222.20 ± 3	−82.6	−98.8	0.3
Z-Asp(OCH_3)-OH	281.3	9.49 (5)	2 EtO–Ac	1, 1	*h*		27.4	31.5	...
Z-Asp(OCH_3)-OH	281.3	9.49 (5)	2 95% EtOH	1, 1	−2.3231[i] ± 0.1	317.39 ± 6	−9.4	−11.8	...
Z-Asp(OCH_3)-ONP	402.3	9.43 (4)	2 DMF	3, 3	−50.957[j] ± 0.4	247.00 ± 3	−178.2	−214.9	0.3
Z-Asp(OCH_3)-ONP	402.3	9.43 (4)	2 EtO–Ac	4, 4	−34.554[j] ± 0.9	258.11 ± 7	−123.3	−149.3	0.5
Z-Asp(OCH_3)-ONP	402.3	9.43 (4)	2 Acetone	1, 1	−42.591[j] + 0.3	253.91 ± 2	−151.0	−182.0	...
HBr-H-Asp(OCH_3)-Gly-ONP	406.2	9.45 (2)	2 H_2O	1, 1	22.087[j] + 0.4	218.64 ± 7	73.8	88.2	...
HBr-H-Asp(OCH_3)-Gly-ONP	406.2	9.45 (2)	2 DMF	1, 1	15.930[j] ± 0.3	238.34 ± 7	54.9	66.0	...
HBr-H-Asp(OCH_3)-Gly-ONP	406.2	9.45 (2)	0.6 AcOH	1, 1	7.7020[j] ± 1.2	225.53 ± 60	26.0[k]	31.0[k]	...
Z-Asp(OCH_3)-Gly-ONP	459.4	9.18 (2)[l]	2 DMF	2, 2	−28.134[j] ± 0.2	234.00 ± 42	−96.3	−115.6	0.5
Z-Asp(OCH_3)-Gly-ONP	459.4	9.18 (2)[l]	2 DMSO	1, 2	−20.180[j] ± 1	207.41 ± 20	−66.4	−79.1	2.0
Z-Gly-Asp(OCH_3)-OH	338.3	9.47 (1)	2 Acetone	2, 2	*n*		34.9	40.6	3.0
Z-Gly-Asp(OCH_3)-OH	338.3	9.47 (1)	DMF	1, 1	−11.158 ± 0.2	259.36 ± 2	−39.9	−48.3	...
Z-Asp(Im)-Gly-Gly-OH	363.3	9.31 (2)	2 DMF	2, 4	−22.506 ± 0.6	273.33 ± 3	−82.7	−100.7	0.6
TosOH-H-Asp(Im)-Gly-Gly-ONP	522.5	9.35 (2)	2 DMSO	2, 4	−25.632[i] ± 3	255.09 ± 30	−90.9	−110.0	...
Z-Asp(Im)-Gly-Gly-ONP	484.4	9.31 (8)	2 DMF	2, 2	−20.909[j] ± 0.2	293.08 ± 42	−80.0	−99.0	...
Z-Asp(Im)-Gly-Gly-ONP	484.4	9.31 (8)	2 CH_3CN	1, 1	−40.669[i] ± 5	256.71 ± 10	−144.0	−175.0	...
Z-Asp(Im)-Gly-Gly-ONP	484.4	9.31 (8)	2 Acetone	1, 2	−32.092 ± 1.7	263.77 ± 14	−115.7	−140.4	...
HBr-H-Asp(OCH_3)-Gly-Gly-ONP	463.2	*m*	2 H_2O	2, 3	24.797[i] ± 0.6	218.73 ± 9	82.9	99.1	0.6
Z-Asp(OCH_3)-Gly-Gly-ONP	516.4	9.29 (7)[o]	2 CH_3CN	2, 3	−7.0478[i] ± 0.7	195.35 ± 44	−22.8	−27.1	2.0
Z-Asp(OCH_3)-Gly-Gly-ONP	516.4	9.29 (7)[o]	2 DMF	2, 2	−19.907[i] ± 0.6	193.30 ± 16	−64.3	−76.3	1.0
Z-Asp(OCH_3)-Gly-Gly-ONP	516.4	9.29 (7)[o]	2 Acetone	1, 1	−8.3609[i] ± 0.4	194.32 ± 21	−27.0	−32.0	...
HBr-H-Gly-Asp-(OCH_3)-Gly-ONP	463.0	8.391 (1)[p]	2 H_2O	1, 1	−29.230[i] ± 0.2	248.87 ± 10	−113.0[q]	−137.0[q]	...
Z-Gly-Asp(OCH_3)-Gly-ONP	516.4	9.27 (2)	2 DMF	1, 2	−36.541[j] ± 0.2	262.99 ± 40	−132.0	−160.0	...
Poly Asp(OCH_3)-Gly-Gly	243.2	*r*	0.1 DMSO	1, 1	−20.863[i]	232.36	−71.0	−85.0	...
Poly Asp(OCH_3)-Gly-Gly	243.2	*r*	2 DCA	1, 1	−15.649[i]	292.72	−60.0	−74.0	...
Poly Asp(Im)-Gly-Gly	211.2	*s*	2 DMSO	1, 1	−16.565	302.85	−65.0	−80.0	...

[a] See text for description of procedures and for definitions of terms. [b] Tabulated value is Drude $a' \times 10^{-6}$ calculated for fixed $\lambda_0 = 214.0$ for hydrolyzed samples. The number in parentheses is the number of runs averaged to give the tabulated a'. For 100% L-aspartic acid $a' = 9.555 \pm 0.04 \times 10^6$ (standard deviation of the average). Acceptable error limits for 100% L are ±0.36 for one determination, ±0.26 for the average of two, ±0.21 for three, ±0.18 for four, ±0.16 for five. See text. Unless noted, all tabulated samples are considered to be 100% L. [c] The first number is the concentration in wt %. DMF is dimethylformamide. DMSO is dimethyl sulfoxide. DCA is dichloroacetic acid. [d] First number is number of independent preparations measured, second is total number of rotation sets. [e] Drude equation parameters. These can be used to provide calculated molar rotations to better than 1 part in 1000. [f] Molar rotations. [g] Per cent standard deviation of calculated molar rotations $= s/\sqrt{n}$; s = standard deviation of observed values from calculation, n = number of sets. Readings covered the range from 589 to 365 mμ, except as noted. Where only one preparation was examined, the error is left blank since verification is lacking. It would be misleading to report the accuracy with which the Drude equation represents one set of data, for this is usually 0.5% or better. [h] Does not fit Drude. Moffitt parameters: $a_0 = 445.58 \pm 460$, $b_0 = 767.87 \pm 1500$, $\lambda_0 = 150.60 \pm 74$. [i] 589–435. [j] 589–546. [k] Observed rotations low (under 0.1). [l] Possibly some DL. Lower limit for average of two is 9.29. [m] 8.62(2) or 90% L after 16 hr at 100°; 55% L after 4 hr; 65% after 8 hr. The trend is general but more extreme than usual. It is presumably due to contributions to rotation by partly hydrolyzed peptides. The rotation values reach a maximum at ~15 hr hydrolysis; longer times do not give higher values. Three independent preparations all having correct analyses for N, ONP, and Br gave identical rotations at four wavelengths in water. Hence the material is considered to be 100% L. [n] Does not follow Drude: Moffitt $a_0 = 298.38 \pm 11$, $b_0 = 236.21 \pm 31$, $\lambda_0 = 200.00$ (fixed arbitrarily). [o] Lower statistical limit (95% confidence interval) is about 9.32 for average of 7. Since this result is actually at the 97% level it is still border line. Other data suggests that the sample is pure L: two entirely independent preparations several months apart, each with correct analyses for N and ONP and one of which was analyzed also for C, H, O, and OCH_3 with correct values, gave concordant rotations in acetonitrile and in DMF in four wavelengths. This is regarded as highly unlikely unless the two samples were both pure L isomers. One of these samples was a precursor of one of the samples of HBr-H-Asp(OCH_3)-Gly-Gly-ONP. [p] 87% L. [q] Drude a' reported for observed values. Molar rotations corrected to pure L on basis that sample is 90% L, 10% DL. [r] 8.64 (1) assuming 100% purity. However, the N analysis on this sample and C, H, N on similar samples indicate the presence of about 7–8% water. The Drude a' values and the reported rotations have not been corrected. If 8.64 is corrected for 92.5% purity, the corrected value is 9.35, and this is well within the range for 100% L. Similar results were obtained with an entirely different polymer sample. [s] 9.14 (2) corresponds to a sample of about 95% purity; hence sample is pure L; a' and rotations are not corrected.

Structural Features of Sequence Peptide Polymers. With the availability of a good method for making sequence polymers it is worthwhile to consider briefly the structural patterns that result from various choices of "monomer."

If an α helix is formed, then amino acid residues which are in a 1, 5, 9 sequence have the side chain of amino acid 5 adjacent to those of 4 and 6 along the sequence and to those of 1 and 9 on adjacent turns of the helix. The patterns attainable with di-, tri-, tetra-, and pentapeptides are shown in Figure 1. Repeating dipeptide and tetrapeptide units give rows of side chains lined up as kernels on an ear of corn, while repeating tripeptide units cause a given amino acid such as B to be surrounded to A and C.

Other peptide conformations or packing patterns can also provide interesting juxtapositions. For example, the β structures (pleated sheet arrangements)

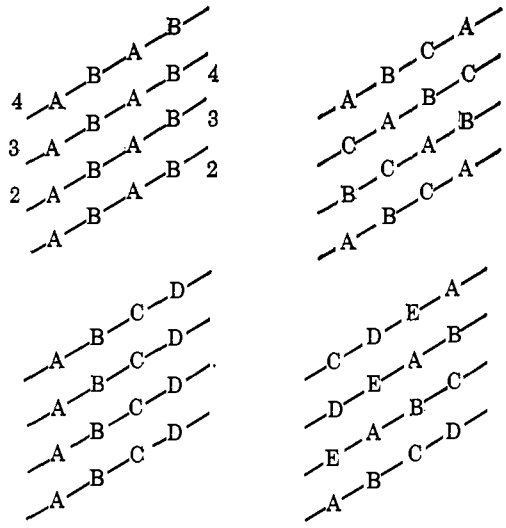

Figure 1. Patterns of side chains along an α helix composed of repeating peptide sequences. The helix has been slit open and unrolled: line segment 2 at the right coincides with line segment 2 at the left in the intact helix.

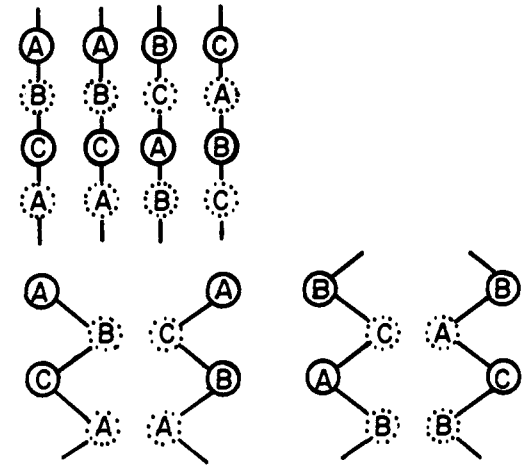

Figure 2. Schematic diagram of parallel (above) and antiparallel (below) pleated sheet structures of a peptide polymer. The solid circles are above the general plane of the sheet, the dotted circles are below.

can bring various side chains together depending upon the phase. The results are pretty much independent of sequential order and are illustrated in Figure 2.[38]

There is a growing body of evidence that secondary structures are determined exclusively or at least principally by the primary structures or sequences. Thus myoglobin and hemoglobin assumed their regular crystalline structure even though there are no cross-links. Furthermore there is evidence that cross-links will reform in a unique pattern, as in ribonuclease. Hence it may be expected that many interesting structural consequences may be exhibited by peptides with repeating sequences.

Experimental Section[39]

Determination of *p*-Nitrophenol. The following procedure gives results with a standard deviation of about one part in one hundred. A sample of *p*-nitrophenyl ester containing 1.2 mg of *p*-nitrophenol is dissolved in 1 ml of redistilled (or reagent grade) dimethyl sulfoxide in a 25-ml, glass-stoppered erlenmeyer flask. The sample need not be completely soluble. Next is added 1 ml of 0.1 *N* sodium hydroxide solution (accurately measured amount), and the mixture is swirled and warmed at 50° in a water bath for about 30 min. The flask is cooled to room temperature and 10 ml of aqueous Tris buffer added. The resulting solution is designated as solution S. (The Tris buffer contains 0.5 mole of tris(hydroxymethyl)-aminomethane and 0.027 mole of hydrochloric acid per liter of water.)

The total amount of solution S is determined by weight. This method was chosen in preference to diluting solution S to volume because of convenience; a wide-mouth flask can be used for the hydrolysis step without necessity for subsequent transfer to a volumetric flask. A 1-ml aliquot of solution S is then diluted to 25 ml in a volumetric flask and the absorbance measured at 400 mμ. Recrystallized *p*-nitrophenol and pure *p*-nitrophenyl acetate are suitable reference standards.

The weight of *p*-nitrophenoxy groups = weight of sample × fraction of *p*-nitrophenoxy groups in sample = β × weight of solution S × absorbance/volume of aliquot. The calibration constant β = 25 ml × mol wt of the *p*-nitrophenoxy group/(cell length × effective molar extinction coefficient of *p*-nitrophenol × density of solution S). It is determined empirically by incorporating a standard sample for each three or four unknown samples. Values reported are for the *p*-nitrophenoxy group, $O_2NC_5H_4O^-$.

Optical Rotations. These were measured with a Rudolph Model 80 high-precision polarimeter equipped with interchangeable sodium and mercury lamps, with a photoelectric detector, and with a mechanically oscillating polarizer. The sodium and the mercury lines were isolated by means of filters supplied by the manufacturer. The lamp–filter combination was tested by using it as a source for the Beckman DU. Except for the 435 line which had about 1% contamination by a line at 467, all lines showed undetectable cross contamination from any other lines in the sources (less than 0.5%). The polarimeter tubes were either sealed quartz cells (American Instrument Co) or were of the center filling type. The windows were not disturbed between readings of solvents and of solutions. The cell compartment was thermostated.

The reproducibility of the technique was evaluated by valid statistical procedures based on (1) repeatability of blanks, (2) a series of runs designed for application of variance analyses, and (3) from the application of the Drude equation calculations described below. Instrument repeatability is 0.0015° (standard deviation per reading), the deviation of points from a randomly selected set of 100 Drude curves was 0.0035 (standard deviation for one point), and over-all reproducibility including small variations in sample purity, sample preparation, and all instrumental errors were 0.8% relative (standard deviation of single reading). Readings on quartz plates calibrated at the Bureau of Standards checked within acceptable limits at 589 and at 546. Calibration data are not available at the other wavelengths.

Since the 405 reading frequently deviated markedly from the value calculated by the Drude equation, although rotations at other wavelengths showed very close agreement, these values have been omitted. The energy at this wavelength is rather low.

Treatment of Optical Rotation Data. Observed rotation values were routinely measured at 589 mμ (sodium D-line) and at the mercury lines 578, 546, 435, and 365. The temperature was 25° throughout. A few samples gave too little signal for measurement at 365 or at 435. The data were processed by OPTROT in order to find the best values of the Drude parameters.[40,41]

In order to present a very large series of numbers as conveniently as possible, tables have been prepared with the Drude (or Moffitt) parameters and with the values of the molar rotations at 589 and at 546. The use of molar rotations permits direct comparison of the numbers from one derivative to another. The molecular weights are tabulated to facilitate calculation of specific rotations if these are required. The values of the parameters are given to sufficient places so that they reproduce the calculated rotations to at least

(38) See, *e.g.*, for pictures of the various peptide arrangements, R. E. Dickerson, *Proteins*, **2**, 603 (1964).

(39) Nitrogen analyses (semiautomatic Dumas), *p*-nitrophenol determinations, and the optical rotation measurements were carried out by Mrs. L. Ross. Analyses for other elements were by F. Pascher (Bonn) or by A. Bernhardt (Mühlheim, Ruhr).

(40) D. F. DeTar, *Biophys. J.*, **6**, 505 (1966).

(41) OPTROT was written by D. F. DeTar. It consists of about 1500 source cards in FORTRAN II for the IBM 709. A users manual is available.

one part in one thousand. While the standard deviations of the parameters are fairly large, this merely reflects the fact that many pairs of λ_0 and a' values will adequately reproduce the molar rotations.[40] The molar rotations which are readily calculated from the data reported may be regarded as representing, at any given wavelength, the average of the observed rotations. The last column gives the standard deviation of this average.

The defining equations are as follows where α is the observed

$$M = \alpha \times \mathrm{MW}/(\mathrm{length} \times \mathrm{concn} \times 10) \quad \text{observed}$$

$$M = a'/(\lambda^2 - \lambda_0^2) \quad \text{Drude}$$

$$M = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2 \quad \text{Moffitt}$$

rotation in degrees, M is the molar rotation, MW is the molecular weight, length is the cell length in cm, concentration is in g/ml, and λ is the wavelength in mμ.

It may be noted that no refractive index correction has been applied.[37] This was done for two reasons. First, the refractive index data are not generally available for the solvents used for measuring the optical rotations. Second, the main purpose of presenting the Drude parameters at all is to make it convenient for the reader to estimate on a desk calculator the molar and specific rotations at any desired wavelength within the range of validity specified. To include refractive index corrections would not add appreciably to the theoretical significance of the Drude values, but it would make them almost useless for reproducing molar rotations.

Aspartic Acid. Commercial aspartic acid (Nutritional Biochemicals) appears to be very pure. Samples of three lots, dried over phosphorus pentoxide *in vacuo* at 60°, gave the theoretical nitrogen assay (within the limits of our method, 0.5% relative) and the rotation values summarized in Table II. The specific rotations are within 0.5% of those reported by Greenstein and Winitz.[42]

Determination of Optical Purity of Aspartic Acid Derivatives. Acid hydrolysis with 5 *N* HCl at 100° for 15 hr in a sealed tube worked well with all of the peptides tabulated here. Periods of 8 hr or less gave erratic results in some cases, suggesting incomplete hydrolysis, and 15 hr at 120° resulted in about 4% loss of activity of aspartic acid itself. With some derivatives an oily layer (benzyl alcohol, *e.g.*) was present. In such cases the solution was extracted with chloroform.

To obtain reference values for hydrolyzed aspartic acid, data for the following runs were combined: eight runs on three samples of H-Asp(OH)-OH, five runs on four samples of Z-Asp(OCH_3)-OH, four runs on three samples of Z-Asp(OCH_3)-ONP, one run on H-Asp(OCH_3)-OH, one run on HCl-H-Asp(OCH_3)-OH, and three runs on three samples of Z-Asp(OH)-OH. To average the results, Drude a' calculations were made at a fixed λ_0 value of 214; use of other λ_0 values, *e.g.*, 210, gave equally satisfactory results as long as the same λ_0 was used for all samples. Comparisons were made on the Drude a' terms. The average a' from the 22 hydrolyzed samples was 96.2% of the a' value for pure aspartic acid. The standard deviation of one sample is 1.9% and the standard deviation of the average is 0.4%. Simple flooding of the hydrolysis mixture with nitrogen before sealing did not affect the results.

It may also be noted that rotation measurements for various preparations of a given peptide or derivative (unhydrolyzed) showed close agreement.

Preparations. The peptides are arranged in an indexing order. To index all cases, a fairly elaborate set of rules is required. For present purposes these may be abbreviated to the following: monopeptides come first, then dipeptides, tripeptides, and polymers. Within these classes the order is alphabetical beginning with the amino acid at the amino end. Two amino acids (*e.g.*, Asp) with different substituents on the side chain are regarded as representing distinct amino acids, and these are arranged in order of their formulas.

Infrared data are given in cm^{-1}; 137 signifies the Perkin-Elmer Infracord (± 10 cm^{-1}), 21 the Perkin-Elmer Model 21 (± 3 cm^{-1}), and 221 the Perkin-Elmer Model 221 (± 3 cm^{-1}). Spectra were taken in mineral oil, in a potassium bromide pellet, and occasionally in a solvent as specified.

Nmr spectra were run on the Varian A-60 spectrometer at 60 Mc/sec. Peak positions are given in cycles per second from tetramethylsilane as internal standard. The accuracy is about ± 2 cps. The instrument calibration was checked with $CHCl_3$–$(CH_3)_4Si$ in $CDCl_3$ with the correct chloroform peak taken as 437 cps. Solutions were 15–20%. Abbreviations used: b, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. The number preceding the parentheses usually represents the relative area. For a simple multiplet the center is specified with a coupling constant. In the case of widely separated multiplets the peak positions are given.

All aspartic acid residues are pure L isomer unless noted in those few cases where racemization has occurred.

Z-Asp(OH)-OH (1). This was prepared from aspartic acid and benzyl chloroformate in the presence of either magnesium oxide or sodium bicarbonate, mp 122–114° after recrystallization from an ethyl acetate–hexane mixture (lit.[43] 116°). The infrared spectrum (21, oil or KBr) showed: 1708 and 1531.

Z-Asp(OCH_3)-OH (2). Aspartic acid (1.5 moles) was converted to HCl-H-Asp-(OCH_3)-OH by the methanol–thionyl chloride procedure[44] in 80% yield; mp 194–195° (lit.[45] mp 187–190°). The infrared spectrum (137, KBr or oil) showed: 1740 (COOH, $COOCH_3$), 1645, 1620, 1570, 1505, 1450, 1425, and 1390. The nmr spectrum (TFA) showed: 465 (20 cps at half-height), 3.2 (NH_3^+); 293, 288, 282, 277, 1.1 (CH); 236, 3.0 (OCH_3); 207 d, $J = 5$ cps, 2.1 (Asp-CH_2).

The HCl-H-Asp(OCH_3)-OH was converted to Z-Asp(OCH_3)-OH; mp 94.5° (lit.[45] mp 96–98°).

Anal. Calcd for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98; CH_3O, 11.03; mol wt, 281.3. Found: C, 55.60; H, 5.35; N, 5.01; CH_3O, 11.13, mol wt (neut equiv), 283 ± 5.

The infrared spectrum (21, KBr) showed: 1750 ($COOCH_3$), 1697 (Z + COOH), 1543, 1471, 1456, 1441, and 1429. The nmr spectrum (TFA) showed: 443, 5 (C_6H_5), 317, 1.9 (benzyl CH_2); 298 b (CH); 231, 3.1 (OCH_3); 190 d, $J = 5.5$ cps, 1.9 (Asp-CH_2).

Z-Asp(OCH_3)-ONP (3).[46] A mixture of 265 g of Z-Asp(OCH_3)-OH and 139 g of *p*-nitrophenol in 1.1 l. of ethyl acetate was cooled to 5°. A solution of 202 g of DCC in 400 ml of ethyl acetate was added in one portion. After 3 hr, filtration gave 206 g of dicyclohexylurea. When reduced to 300 ml volume on the rotary evaporator 245 g (65%) of product separated, mp 90–95°. Further evaporation gave a second crop of 79 g, mp 70–90°. First crop material was usually used without further purification. Recrystallization from a 7:5 benzene:hexane mixture gave an 80% recovery of material with mp 101–102° (lit.[46] 105–106°), $[\alpha]D$ −43.7 (*c* 2, DMF). (The value from Table II is −44.3.) Hence two different methods of preparation give concordant results.

Anal. Calcd for $C_{19}H_{18}N_2O_5$: C, 56.71; H, 4.51; N, 6.96; ONP, 34.32. Found: C, 57.0; H, 4.48; N, 6.88–7.04 (several samples); ONP, 33.6–34.0 (several samples).

The material is 100% L enantiomer according to the specifications in Table II.

The infrared spectrum (21, KBr) showed: 1763 (COONP), 1735 ($COOCH_3$), 1702 (Z), 1620 w, 1596 w, and 1527 (NO_2). The nmr spectra showed: ($CDCl_3$), 496, 505 (*ortho* to NO_2); 436 (446 hidden) (*meta* to NO_2); 445 (Bl-C_6H_5); 313 (Bl-CH_2); 295 m (CH); 225 (OCH_3); 187 m (Asp-CH_2); and (TFA), 507, 498, 1.8 (*ortho* to NO_2); 445 (Bl-C_6H_5). For the TFA readings, the other nitrophenyl peaks are not resolved and appear only as the toe of the 445 peak (reproducible on several curves); total area 390–455 is 7.4 for 7 H(Bl-C_6H_5, *meta* to NO_2). ZNH seldom appears on nmr curves in TFA; it is probably very broad but may be washed out by rapid exchange; 318 (6 cps at half-height) (Bl-CH_2); 305 b (CH); total area 290–325 is 3.1 for 3 H(Bl-CH_2, CH); 235, 3.0 (OCH_3) shifts to 239 as the benzyloxycarbonyl group solvolyzes (60% gone after 24 hr at room temperature); 200 d, $J = 5$ cps, 1.9 (Asp-CH_2). Bl-CH_2 sharpens (3 cps) and shifts to 327 (Bl-$OCOCF_3$) as the group solvolyzes.

HBr-H-Gly-ONP (4).[47] Z-Gly-ONP (66 g) was mixed with 240 ml of 20% HBr in acetic acid; solution occurred within about 6 min, and the product crystallized. Dry ether (500 ml) was added after 45 min to give 54 g of crude product, mp 213°. This was dissolved in 150 ml of methanol (55°), which was cooled and diluted with 100 ml of ether to give 51.5 g of product, mp 217–218°; reported 213° dec. Analysis of many different samples reproducibly showed 49.8–50.3% ONP (theory 49.9), 9.96–10.13% N (theory 10.12), and 28.3–29.0% Br (theory 28.8). The infrared spectrum

(42) Reference 21, p 116.

(43) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(44) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(45) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

(46) M. Goodman and F. Boardman, *ibid.*, **85**, 2483 (1963).

(47) M. Goodman and K. C. Stueben, *ibid.*, **81**, 3980 (1959).

(21, KBr) showed: 1770 (COONP), 1625, 1593, 1560 b, 1531 (NO_2), 1510, and 1346 (NO_2). The nmr spectrum (TFA, not soluble enough to give a good curve) showed: 273, 268, plus others (Gly-CH_2).

Z-Gly-ONP (5).[48-50] To a stirred mixture of 209 g of Z-Gly-OH (mp 120–121°), 800 ml of methylene chloride, and 153 g of *p*-nitrophenol at 20° was added a solution of 210 g of DCC in 500 ml of methylene chloride over a period of 20 min. The solution was filtered after another hour, to remove 212 g of DCU. The filtrate was concentrated to 500 ml and diluted with 1 l. of ether. The precipitated products, 290 g (88%) in the first crop, 23 g in the second, both had mp 127–128° (lit. mp 128°,[48] 124–125°[49]). Yields in acetone were 82%, in ethyl acetate 88%. The product reproducibly showed 41.7% ONP (theory 41.8) and 8.50% N (theory 8.48). The infrared spectrum (21, KBr) showed: 1770 (COONP), 1703 (Z), 1620 w, 1598 w, and 1527.

HBr-H-Asp(OCH_3)-Gly-OH (6). A solution of 2.8 g of glycine and 3.1 g of triethylamine in 25 ml of water was poured into a solution of 10 g of Z-Asp(OCH_3)-ONP in 50 ml of acetonitrile. Acidification after 2 days gave 5.5 g of Z-Asp(OCH_3)-Gly-OH. This was treated with a 20% solution of HBr in acetic acid to give an 88% yield of hydrobromide, mp 178°. It is interesting to note that this is the ester, not the imide.

Anal. Calcd for $C_7H_{13}N_2O_5Br$: N, 9.83. Found: N, 9.53.

The infrared spectrum (21, KBr) showed: 1727 ($COOCH_3$), 1711 (COOH), 1660 (CONH), 1587, and 1493. The nmr spectrum (TFA) showed: 460 and 480, 4.2 (NH_3^+, NH); 302 m, 1.3 (CH); 259 d, J = 5.5 cps, 1.9 (Asp-CH_2); 233, 3.0 (OCH_3); 203 d, J = 5.5 cps, 1.9 (Gly-CH_2).

HBr-H-Asp(OCH_3)-Gly-ONP (7). This was obtained in 80% yield by action of 20% HBr in acetic acid on Z-Asp(OCH_3)-Gly-ONP followed by precipitation with ether and recrystallization from methanol–ether; mp 183–186°.

Anal. Calcd for $C_{13}H_{16}N_3O_7Br$: N, 10.35; Br, 19.63; ONP, 34.02. Found: N, 9.90, 10.00; Br, 19.28; ONP, 32.6.

The infrared spectrum (21, KBr) showed: 1757 (COONP), 1738 ($COOCH_3$), 1715 sh, 1662 (CONH), 1617 w, 1593, 1560, and 1510. The nmr spectrum (TFA) showed: 493, 502 (*ortho* to NO_2); 436, 445 (*meta* to NO_2); 460 b (NH_3^+, NH); 300 b, 1.2 (CH); 273 d, J = 6 cps, 1.9 (Asp-CH_2); 232, 2.7 (OCH_3); 204 d, J = 7 cps, 2.1 (Gly-CH_2); 410–505 area, 8.14 for 8 H (4 H of ONP, NH_3^+, NH).

Z-Asp(OCH_3)-Gly-ONP (8). To a stirred mixture of 14.1 g of Z-Asp(OCH_3)-OH, 14 g of HBr-H-Gly-ONP, 25 g of CMC,[6] and 200 ml of methylene chloride at 5° was added 5.2 g of triethylamine. After reaction for another half-hour with the ice bath removed, the solvent was evaporated and the residue triturated with 200 ml of water to give 16.3 g (70%) of product, mp 166–167°. Recrystallization from ethyl acetate gave material with mp 167–168°.

Anal. Calcd for $C_{21}H_{21}N_3O_9$: C, 54.90; H, 4.61; N, 9.15; ONP, 30.06. Found: C, 55.2; H, 4.72; N, 9.31, 9.10; ONP, 29.4, 29.7, 30.2. (This is pure L within the limits specified in Table II.)

The infrared spectrum (21, KBr) showed: 1760 (COONP), 1741 ($COOCH_3$), 1697 (Z), 1654 (CONH), 1635 w, 1622 w, 1598 w, 1555, and 1527 (NO_2). The nmr spectrum (TFA) showed: 503, 494 (*ortho* to NO_2); 445, 436 (*meta* to NO_2); 439 (Bl-C_6H_5); 315, 2.2 (Bl-CH_2); 300 b (CH); 269 d, J = 6 cps, 2.3 (Gly-CH_2); 228, 3.1 (OCH_3); 189 d, J = 6 cps, 2.1 (Asp-CH_2); NH's around 480, total area 440–510 is 11 for 11 H (4 ONP, 5 Bl, 2 NH).

Gly-Asp(OCH_3)-Cyclic (1) (DL) (9). To a suspension of HBr-H-Gly-Asp(OCH_3)-ONP (DL) in 25 ml of chloroform was added 1.01 g of triethylamine. The diketopiperazine precipitated, 1.55 g, mp 197–198°. Recrystallization from dimethyl sulfoxide gave a 55% recovery of material, mp 203°. Alternatively the crude diketopiperazine could be purified by washing with alcohol and crystallizing from water; mp 206°.

Anal. Calcd for $C_7H_{10}N_2O_4$: N, 15.05. Found: N, 15.05, 15.26 (two samples).

The infrared spectrum (137, KBr) showed: 1730 ($COOCH_3$), 1680, 1460, 1430, 1410, 1370, 1335, 1315, and 1265. The nmr spectrum (TFA) showed: 521, 504, 210 (2 NH) (each 8 cps at half-height); 295 (11 cps), 1.1 (CH); 276 (5 cps), 1.9 (Gly-CH_3); 240, 2.9 (OCH_3); 201 d, J = 5 cps, 2.0 (Asp-CH_2).

Z-Gly-Asp(OCH_3)-OH (10). A mixture of 33 g of Z-Gly-ONP, 184 g of HCl-H-Asp(OCH_3)-OH, 20.2 g of triethylamine, and 200 ml of ethyl acetate was allowed to react at 25–30° for 5 hr. Filtration gave 13.3 g of triethylamine hydrobromide, and the filtrate was extracted with water and acidified with concentrated hydrochloric acid to a pH of 1.5. The crystalline product weighed 30.1 g, mp 112°. Recrystallization from 150 ml of ethyl acetate gave 26 g (78%) of material, mp 120°.

Anal. Calcd for $C_{15}H_{18}N_2O_7$: neut equiv, 338.3; N, 8.28. Found: neut equiv, 335 (to pH 7.5); N, 8.31, 8.32 (two samples). (Hydrolysis with 5 *N* hydrochloric acid showed this to be 100% L. The yield in methylene chloride was nil, in methanol mediocre.)

The infrared spectrum (21, KBr) showed: 1750 ($COOCH_3$), 1732 (COOCH), 1707 (Z), 1628, 1550, and 1523. The nmr spectra showed: (137, CH_2Cl_2), 1720 ($COOCH_3$), (COOH), 1680 (Z), 1505; and (TFA), 477, 468, 1.0 (peptide NH); 436, 5.2 (Bl-C_6H_5); 312 (Bl-Cl_2); 303 b (CH); total 290–320, 3.0 for (Bl-CH_2 + CH); 250, 2.0 (Gly-CH_2); 227, 2.8 (OCH_3); 185 d, J = 5 cps, 2.2 (Asp-CH_2).

Polymerization of HBr-H-Gly-Asp(OCH_3)-ONP. To 4.1 g of hydrobromide in 2 ml of dimethyl sulfoxide was added 1.06 g of triethylamine. Heat was evolved, the material partly dissolved, and a new solid was formed. After 5 days, 50 ml of methanol was added to give 1.25 g of crude product isolated by filtration. Extraction with hot water gave 0.8 g of a solid, mp 199–203°, the diketopiperazine. The insoluble residue, 0.35 g, was polymeric material which dissolved in dimethyl sulfoxide and was precipitated by addition of water or ethanol.

HBr-H-Gly-Asp(OCH_3)-ONP(DL) (11). This was obtained in 90% yield by action of 20% HBr in acetic acid on Z-Gly-Asp-(OCH_3)-ONP (DL) followed by precipitation with ether and recrystallization from methanol; mp 173°.

Anal. Calcd for $C_{13}H_{16}N_3O_7Br$: C, 38.46; H, 3.97; N, 10.35; Br, 19.67; ONP, 34.02. Found: C, 38.6; H, 4.10; N, 10.49; Br, 19.6, 18.7, 19.3; ONP, 32.5, 30.7, 33.5, 33.2 (different preparations).

The infrared spectrum (21, KBr) showed: 1752 (COONP), 1733 ($COOCH_3$), 1671 (NH), 1618 w, 1591 w, 1543, and 1517. The nmr spectrum (TFA) showed: 509, 500 (*ortho* to NO_2); 449, 440 (*meta* to NO_2); 330 b, 1.1 (CH); 268 d, J = 6 cps, 2.0 (Gly-CH_2); 236, 3.0 (OCH_3); 206 b, s, 1.9 (Asp-CH_2); NH about 450; total area 410–520 is 8.0 for 8 H (NH_3^+, NH, ONP).

Z-Gly-Asp(OCH_3)-ONP (mostly DL) (12). A mixture of 0.338 g of Z-Gly-Asp(OCH_3)-OH (L), 0.139 g of *p*-nitrophenol, 0.206 g of DCC, and 5 ml of ethyl acetate was stirred for 15 min at room temperature and 5 mg of triethylamine was added. After 4 hr, filtration gave 97% of theoretical DCU and evaporation of the solvent gave 0.435 g (94%) of colorless product, mp 114–115°.

Anal. Calcd for $C_{21}H_{21}N_3O_9$: C, 54.90; H, 4.61; N, 9.15; ONP, 30.06. Found (two different samples): C, 54.69, 54.84; H, 4.55, 4.56; N, 9.16, 9.13; ONP, 29.9, 29.0.

One sample was 25% L, 75% DL the other 15% L, 85% DL. The azlactone has been shown to be an intermediate (infrared spectra of reaction mixtures). Without the small amount of triethylamine the yield is very poor.

The infrared spectra showed: (221, KBr), 1766 (COONP), 1730 ($COOCH_3$), 1695 (Z), 1642, 1613, 1590, 1540, 1515, and (137, CH_2Cl_2), 1780 (COONP), 1730 ($COOCH_3$), 1690, 1530, 1500, 1350 (NO_2). The nmr spectrum (TFA) showed: 510, 501 (*ortho* to NO_2); 446 (Bl-C_6H_5); 449, 440 (*meta* to NO_2); 318 (Bl-CH_2); 258, 2.1 (Gly-CH_2); 234, 2.9 (OCH_3); 193 d, J = 5 cps, 1.9 (Asp-CH_2); NH is about 585, total area 430–520 is 10.0 (Bl-C_6H_5), ONP, NH = 10); CH is under Bl-CH_2 at about 238, total area here 3.1 (Bl-CH_2, CH).

HBr-H-Gly-Gly-ONP (13).[51] To 92 g of Z-Gly-Gly-ONP was added, with stirring, 180 ml of a 30% solution of dry HBr in anhydrous acetic acid. After about 30-min reaction at room temperature, carbon dioxide evolution ceased and the product precipitated. It was filtered and washed with dry ether; yield 69 g (85%), mp 192–199° dec. The hydrobromide is not particularly hygroscopic.

Anal. Calcd for $C_{10}H_{12}N_3O_5Br$: N, 12.58; Br, 23.87; ONP, 41.36. Found (three different preparations): N, 12.38, 12.49, 12.25; Br, 23.4, 23.1, 23.5; ONP, 41.3, 41.0, 40.0.

The purity of the sample can be checked by either paper chromatography with *n*-butyl alcohol, acetic acid, water, or with thin layer

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(51) Procedure developed by Dr. R. Albers and Mr. E. Heimer.

chromatography. The infrared spectrum (21, KBr) showed: 1789 (COONP), 1694, 1622 w, 1592, 1570 b, and 151.1 b. The nmr spectrum (TFA showed: 512, 503 (*ortho* to NO_2); 454, 445 (*meta* to NO_2); 283, 278, 274, 268, 262 (doublet 281, J = 5.5 cps, plus quartet 271 J = 6 cps); NH about 490, NH_3^+ about 455 both under the ONP peaks.

Z-Gly-Gly-ONP (14).[52] Preparation of this compound in a state of high purity requires care.[51] Glycylglycine (133 g) was converted to Z-Gly-Gly-OH in 97% yield, mp 174–178°, by reaction in a suspension of 252 g of sodium bicrbonate in 1 l. of water with 200 ml of benzyl chloroformate at room temperature. Recrystallization from ethanol gave a product melting at 176–178°. The infrared spectrum (137, KBr) showed: 1740, 1660, 1560, and 1430.

Conversion of Z-Gly-Gly-ONP was accomplished with 80 g of *p*-nitrophenol, 116 g of DCC, and 1.7 l. of acetonitrile to which was added 150 g of solid Z-Gly-Gly-OH. The ensuing paste was stirred for 4 hr, heated to reflux, and filtered. The product was extracted from the solid by extracting it four times with hot mother liquor. This gave a total yield of 171 g (80%) of product, mp 155–160°. Recrystallization from acetone gave material with mp 159–161°. Best results are obtained upon recrystallization from methanol which gives a product with mp 162–163°, with a single spot on tlc, and with 35.1% ONP (theory 35.6). The infrared spectrum (21, KBr) showed: 1770, 1669 b, 1620 w, 1597, 1560 b, 1524; (137, CH_3CN) 1780, 1730, and 1690. The nmr spectrum (TFA) showed: 509, 500 (*ortho* to NO_2); 478 b (peptide NH); 450, 441 (*meta* to NO_2); 443 (Bl-C_6H_5); 318 (Bl-CH_2); 272 d, J = 5 cps (C-terminal Gly-CH_2), 258 (N-terminal Gly-CH_2).

Z-Asp(Im)-Gly-Gly-OH (15). A solution of 39.6 g of glycylglycine and 30 g of triethylamine in 200 ml of water was filtered to remove a small amount of insoluble material. This was poured into a vigorously stirred solution of Z-Asp(OCH_3)-ONP in 400 ml of acetonitrile. The mixture was stirred for 3 hr at room temperature and concentrated to 300 ml under reduced pressure. The residue was diluted with water to 750 ml and extracted with ethyl acetate to give 28 g of *p*-nitrophenol. The aqueous layer was then diluted to 1.5 l. and acidified to pH 1.5 with concentrated HCl. The product separated as an oil which crystallized. It was washed with water and dried; yield 44 g (55%), mp 199°.

Anal. Calcd for $C_{16}H_{17}N_3O_7$: C, 52.9; H, 4.72; N, 11.57; CH_3O, 0. Found: C, 52.7; H, 4.65; N, 11.75; CH_3O, 0.3.

The infrared spectrum (137, oil or KBr) showed: 1760 (imide), 1705, 1680 (Z), 1670 (amide I), 1570 w, and 1540 (amide II). The nmr spectrum (TFA) showed: 468 b (NH); 445 (Bl-C_6H_5); 316 (Bl-CH_2); 297 b (CH); 283 (255) (central Gly-CH_2); 260 d, J = 6 cps (C-terminal Gly-CH_2); 207, 198, 192 b (Asp-CH_2). The presence of two peaks for the central glycine is based on broadening of the 257 peak, on area measurements, and on results obtained with 17. The origin is ascribed to N protonation of the imide. ZNH appears to be present as a very broad peak at about 425. There is no peak in the CH_3O region (232).

A very large number of runs was made in an effort to develop a suitable procedure for obtaining the ester, Z-Asp(OCH_3)-Gly-Gly-OH, by this route. Particular attention was paid to maintaining the pH at 6–7.5 by adjusting the rate of addition of triethylamine. It was not possible to get a crystalline product at this stage although conversion of the glass product to HBr-H-Asp(OCH_3)-Gly-Gly-OH was sometimes fairly successful. The main problem arises from the incompatible solubilities of the Z-Asp(OCH_3)ONP (only in organic solvents) and the glycylglycine (only in water).

TosOH-H-Asp(Im)-Gly-Gly-ONP (16). A mixture of 10.3 g of Z-Asp(Im)-Gly-Gly-ONP and 40 ml of 20% HBr in acetic acid gave 80% or more of the expected carbon dioxide in 1 hr at room temperature. After another half hour 200 ml of anhydrous ether was added to precipitate the product. The solid was taken up in 50 ml of methanol and filtered, and a solution of 5.79 g of *p*-toluenesulfonic acid and 3.1 g of triethylamine in 25 ml of methanol was added. The toluenesulfonate of the peptide separated shortly; yield 9.25 g (83%), mp 216–218°. Recrystallization from 92 ml of hot water gave 6.7 g, mp 228–230°.

Anal. Calcd for $C_{21}H_{22}N_4O_{10}S$: C, 48.28; H, 4.24; N, 10.72; S, 6.13; ONP, 26.43. Found: C, 49.18, 49.38; H, 4.32, 4.33; N, 10.48, 10.52, 10.68, 10.63; S, 5.82, 6.19; ONP, 26.2, 25.4, 25.9.

The infrared spectrum (21, KBr) showed: 1782 (COONP), 1735 m, 1708, 1680 m, 1632 w, 1622 w, 1595 m, 1572 m, and 1525. The nmr spectrum (TFA) showed: 507, 498 (*ortho* to NO_2); 479 (NH_3^+); 472, 463 (*ortho* to SO_3^-); 449, (440) (*meta* to NO_2); 446, (437) (*meta* to SO_3^-, the 440 peak and the 437 peaks are not resolved, but area measurements show the expected 4 H's in the 450–435-region); 301 very b (CH); 282 s (central Gly-CH_2); 276 d, J = 6 cps (C-terminal Gly-CH_2); 221, 214, 211, 208 (Asp-CH_2); 141 (tosyl-CH_3).

The peak areas or grouped areas give satisfactory agreement with the assignments. The assignments were verified by comparison with the spectra of Z-Asp(Im)-Gly-Gly-ONP in TFA taken after 7 days when solvolysis was complete. There is no peak in the CH_3O region (236).

Z-Asp(Im)-Gly-Gly-ONP (17). To a mixture of 4.0 g of Z-Asp-(Im)-Gly-Gly-OH, 1.4 g of *p*-nitrophenol, 70 mg of triethylamine, and 50 ml of methylene chloride at room temperature, was added 4.5 g of CMC.[6] After 1 hr, the solvent was removed by evaporation under reduced pressure and 100 ml of water was added to the residue. The crystals were filtered and dried; yield 5.1 g, mp 111–115°. The crude product was heated briefly with 30 ml of methanol, then cooled; 4.0 g of product was obtained (75%), mp 156–158°.

Anal. Calcd for $C_{22}H_{20}N_4O_9$: C, 54.6; H, 4.16; N, 11.6. Found: C, 55.3; H, 4.53; N, 11.4.

The infrared spectrum (21, KBr) showed: 1754 (*p*-nitrophenyl ester), 1720 (imide), 1691 (ZN); 1657 (amide I), 1619 w, 1596 w, 1548 (amide II), and 1522.

Direct comparison with the infrared curve of the ester shows several differences in intensities and in positions of peaks; *e.g.*, the amide band at 1650 is the most intense peak in the methyl ester, while the imide has moderately strong peaks at 920, 875, 750, 740, which are absent in the ester. The nmr spectrum (TFA) showed: 508, 499 (*ortho* to NO_2); 475 (NH); 449, (440) (*meta* to NO_2); 449 (Bl-C_6H_5); 316 (Bl-CH_2); 285, 256 (central Gly-CH_2); 274 d, J = 6 cps (C-terminal Gly-CH_2); 208, 200, 193 m (Asp-CH_2). The presence of two peaks for the central glycine is believed due to N protonation of the imide. There is no peak in the CH_3O region (232).

HBr-H-Asp(OCH_3)-Gly-Gly-ONP (18). A mixture of 10 g of Z-Asp(OCH_3)-Gly-Gly-ONP and 15 ml of a 40% solution of anhydrous hydrogen bromide in glacial acetic acid was stirred vigorously at room temperature. The theoretical amount of CO_2 was evolved in 15–30 min and the solution became clear. In some runs the product crystallized spontaneously, in others it separated upon addition of 50 ml of absolute ether. The very hygroscopic crude material was filtered and washed with dry ether (drybox: caution, benzyl bromide). The dry product (10 g) gave a high analysis for bromide ion (22%) and a low analysis for *p*-nitrophenol (22–24%). It is unpredictably unstable toward liquefaction (presumably hydrolytic decomposition). Paradoxically the material can be stabilized and rendered nonhygroscopic by adding 3 ml of cold water. The material dissolved and then crystallized out. Trituration with 100 ml of absolute ethanol gave 6.6 g of product (73%), mp 176–178°, after recrystallization from hot ethanol. To minimize exposure to alcohol and to ensuing alcoholysis, the solid was dissolved in preheated ethanol and the solution cooled rapidly.

Anal. Calcd for $C_{15}H_{19}BrN_4O_8$: C, 38.9; H, 4.14; N, 12.1; Br, 17.22; CH_3O, 6.69; ONP, 29.8. Found: C, 38.9; H, 4.20; N, 11.8; 12.0; Br, 17.0; CH_3O, 6.69; ONP, 30.0. Analyses of another six samples gave Br 16.8–17.5 (average 17.1), ONP, 29.0–30.2 (average 29.5), N, 11.92–12.06 (average 12.0).

The infrared spectrum (21 KBr) showed: 1777 (COONP), 1748 ($COOCH_3$), 1697 (amide I), 1652 (amide I), 1620 w, 1596 w, 1550 b (amide II), and 1527. The nmr spectrum (TFA) showed: 511, 502 (*cis* to NO_2); 498 b (NH); 468 b (NH_3^+); 453, 444 (*meta* to NO_2); 305 b (CH); 279, 273 (273 larger, Gly-CH_2); 237, 2.9 (OCH_3); 208, 203, 2.0 (Asp-CH_2); 430–252 total area, 8.9 (ONP, NH_3^+, 2 NH = 9); 255–320 total area, 5.2 (2 Gly, CH = 5).

Z-Asp(OCH_3)-Gly-Gly-ONP (19). A solution of 62 g of dicyclohexylcarbodiimide in 1 l. of acetonitrile was cooled to −15°. With stirring, 93 g of HBr-H-Gly-Gly-ONP was added rapidly as a solid, and immediately thereafter addition was begun of a solution of 85 g of Z-Asp(OCH_3)-OH and 37 ml of triethylamine in 200 ml of acetonitrile. The addition required about 30 min, the reaction temperature being maintained at −10°. Reaction was allowed to proceed for an additional 1.5 hr at −10° and then for 2–3 hr after removal of the cooling bath. Filtration gave 66 g of dicyclohexylurea. Two-thirds of the solvent was removed by distillation under reduced pressure (water bath at 40°) and the product precipitated by pouring the residue into 1.2 l. of cold water. The dried

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crude product was triturated with 500 ml of absolute ethanol, and the trituration was repeated. A total of 100 g (65%) of material was obtained, mp 159°, including that recovered from the solvent. It is possible to recrystallize the material from ethanol with 50% recovery by preheating the ethanol to minimize exposure. After several recrystallizations from 1:1 methanol–ether, the melting point was 163–165°. The aspartic acid group is unracemized (Table II).

Anal. Calcd for $C_{23}H_{24}N_4O_{10}$: C, 53.5; H, 4.68; N, 10.85; O, 30.98; CH_3O, 6.01; ONP, 26.7. Found (recrystallized sample): C, 53.5; H, 4.79; N, 10.91; O, 31.2; CH_3O, 6.08; ONP, 26.7. Found (triturated samples): C, 53.5, 53.7; H, 4.89, 5.08; N, 10.85, 10.59; CH_3O, 6.24, 6.42; ONP, 26.2, 25.3.

The infrared spectrum (21, KBr) showed: 1774 (COONP), 1748 ($COOCH_3$), 1697 (Z), 1633 b (amide I), 1598, 1540 b. The nmr spectrum (TFA) showed: 507, 498 (*ortho* to $-NO_2$); 448, 439 (*meta* to $-NO_2$); 443 (Bl-C_6H_5); 316 (Bl-CH_2); 300 m (CH); 270 d, J = 5 cps (C-terminal Gly-CH_2); 260 d, J = 5 cps (Gly-CH_2); total Gly area 3.9; 229, 3.1 (OCH_3); 189 d, J = 5.5 cps, 1.9 (Asp-CH_2).

HBr-H-Gly-Asp(OCH_3)-Gly-ONP (20). The Z-Gly-Asp(OCH_3)-Gly-ONP reacted with 20% HBr in acetic acid at room temperature and the product was precipitated with ether and recrystallized from methanol.

Anal. Calcd for $C_{15}H_{19}N_4O_8Br$: C, 38.91; H, 4.14; N, 12.10; Br, 17.22; ONP, 29.82. Found (samples of various melting points): C, 38.63, 38.69, 37.02; H, 4.12, 4.17, 4.39; N, 11.99, 11.9, 12.1; Br, 17.25, 17.32, 17.7; ONP, 28.4, 29.8, 29.0. DL material had mp 195–196°, and material about 80% L had mp 183–186°.

The infrared spectrum (21, KBr) showed: 1774 (COONP), 1739 ($COOCH_3$), 1663 (amide I); 1618 w, 1599, 1580, 1548, and 1511. The nmr spectrum (TFA) showed: 510, 501 (*ortho* to $-NO_2$); 452, 443 (*meta* to $-NO_2$); 315, 308, 1.0 (CH); 279, 273, 264, 4.2 (both Gly-CH_2, perhaps d + q); 234, 2.8 (OCH_3); 195 d, J = 5.5 cps, 2.0 (Asp-CH_2); NH is about 495, NH_3^+ is about 450 (under the ONP peaks), total area 420–510 is 9.0 (4 aryl + NH_3 + 2NH).

Z-Gly-Asp(OCH_3)-Gly-ONP (mostly DL) (21). To a stirred mixture of 34 g of Z-Gly-Asp(OCH_3)-OH, 28 g of HBr-H-Gly-ONP, 56.3 g of CMC, and 150 ml of methylene chloride at 0° was added over a period of 20 min 10.1 g of triethylamine in 100 ml of methylene chloride. After 1 hr, the solvent was removed and 300 ml of water added with vigorous stirring. The product was separated by filtration and washed with water and with methanol; 43.69 g; mp 169–173°. Recrystallization from 500 ml of acetonitrile gave 36.5 g (70%) of material, mp 171–173°. The same yield was obtained in another run with 46 g of CMC (cyclohexyl(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate).

Anal. Calcd for $C_{23}H_{24}N_4O_{10}$: C, 53.49; H, 4.68; N, 10.85; ONP, 26.74. Found (three different preparations): C, 53.11, 53.41, 53.43; H, 4.75, 4.54, 4.82; N, 11.08, 11.06, 11.09; ONP, 26.2, 26.4.

The material on hydrolysis in 5 *N* hydrochloric acid had a rotation about 25% of that expected for an optically pure sample.

Several alternate preparations were examined using other diimides and acetonitrile or methanol as solvents but yields were lower. It was, however, possible to obtain a product with very little racemization (but in low yield) by the following procedure. To a solution of 1.01 g of triethylamine, 3.38 g of Z-Gly-Asp(OCH_3)-OH, and 2.27 g of HBr-H-Gly-ONP in 40 ml of methylene chloride was added over a period of 30 min a solution of 5 g of CMC in 10 ml of methylene chloride. After 5 hr about 75% of the diimide had reacted (infrared). The solvent was removed, the oily residue washed with water and dried *in vacuo*. Trituration with ethyl acetate gave 1.35 g of solid which upon further washing with water gave 0.55 g (11%) of product, mp 176–177°. Analyses were comparable to those reported above.

The infrared spectrum (21, KBr) showed: 1758 (COONP), 1739 ($COOCH_3$), 1697 (Z), 1661 s, 1640, 1623 s, 1595 w, 1541, 1513. The nmr spectrum (TFA) showed: 505, 496 (*ortho*- to $-NO_2$); 483, 476, 471 (NH); 446, 437 (*meta* to $-NO_2$); 441 (Bl-C_6H_5); 315, 3.3 (Bl-CH_2 + CH); 268 d, J = 5 cps (C-terminal Gly-CH_2); 254 (N-terminal Gly-CH_2, changes as benzyloxycarbonyl group solvolyzes); area 3.8 for both Gly; 229, 3.0 (OCH_3); 183 d, J = 6 cps, 1.7 (Asp-CH_2); total area 440–510 is 11.3 (9 aryl, 2 NH) (ZNH does not show up).

Z-Gly-Asp(OCH_3)-Gly-ONP (L). To 5 mmoles of HBr-H-Asp(OCH_3)-Gly-ONP was added 50 ml of methylene chloride, 5 mmoles of Z-Gly-OH, 5 mmoles of CMC, and then 5 mmoles of triethylamine; temperature, 5°. The product was worked up as described above for the alternate preparation above except that the product was recrystallized from methanol and then from acetonitrile; yield of pure product, mp 169–171°, was 0.52 g (20%). Analyses for nitrogen and for ONP were within 1% relative of theory, *i.e.*, comparable to those reported above. This material is 100% L within the limits described in Table II.

Polymerization of HBr-H-Asp(OCH_3)-Gly-Gly-ONP. The samples of hydrobromide (before the trick of recrystallization from water was discovered) had varying excesses of hydrogen bromide. However, polymer was obtained using a variety of solvents (dimethylformamide, dimethyl sulfoxide, N-methylpyrrolidone, and chloroform) and a variety of bases (triethylamine, pyridine, N-methylpyrrolidine, imidazole, N,N′-dimethylpiperazine, and dimethylaminoacetonitrile). Later work showed that 1 mole of triethylamine per mole of hydrobromide is adequate, and that sodium *p*-nitrophenoxide can be used as base. An excess of any base gave some imide formation.

These early polymerization studies provided low molecular weight polymers and polymers with some loss of the methoxyl group. It was discovered that nmr spectra of some samples showed a doubling of the OCH_3 peak. The normal methoxyl peak was at 232 and the extra peak (N-terminal aspartyl) was at 235 cps from tetramethylsilane in trifluoroacetic acid. Comparison with peptides confirmed this shift of the methoxyl peak. Up to a $\overline{DP}$ of 15–20 the presence of the N-terminal peak can be used to give an estimate of molecular weight using relative peak heights as a rough count of end groups.

Purification of Asp(OCH_3)-Gly-Gly-Poly (22).[53] The remaining polymer from all runs in Table I except the large scale run was combined (1.79 g). A portion of this, 1.12 g, was extracted with chloroform for 36 hr in a Soxhlet (fritted glass) extractor and after drying had lost 4% of weight. A second extraction caused another 1% loss. The material was somewhat hygroscopic; in the air it slowly gained weight and over P_2O_5 it lost weight (4% change).

A portion of the extracted polymer, 948 mg, was stirred with 25 ml of dimethyl sulfoxide for 24 hr and centrifuged. The solvent was removed from the supernatant portion on the vacuum train at 0.001 mm to give 210 g of material. The insoluble portion was extracted with chloroform for 18 hr and then dried over P_2O_5 at 100° (0.1 mm) to give 677 mg; $[\eta]$ in dichloroacetic acid at 25° is 0.258; mol wt by DNP end-group assay, 9950 and 9600 ($\overline{DP}$ 25 of tripeptide units).[13]

Anal. Calcd for $C_9H_{13}N_3O_5$: C, 44.44; H, 5.39; N, 17.28; O, 32.89; OCH_3, 12.75. Found (on portion insoluble in DMSO): C, 41.35; H, 5.58; N, 16.37; O, 32.21; OCH_3, 11.44; Cl, 0.0. The sum of the elements found is 95.5. The analysis corresponds closely with a polymer containing 95% ester and 5% imide with 4% of ash (silica) and 2% of water. Calcd: C, 41.8; H, 5.23; N, 16.1; O, 32.2; OCH_3, 11.4. This polymer does not retain chloroform.

The infrared spectrum (21, KBr) showed: 3320, 3090, 2950, 1739, 1664, 1550, 1441, 1414, 1373, 1336, 1282, 1238 b, 1179, and 1029, peaks generally rather broad. The nmr spectrum (TFA) showed: 482 (15 cps at half-height), 3.2 (NH); 313, 1.1 (CH); 262 (12 cps), 3.9 (both Gly-CH_2); 233 (2.5 cps), 3.2 (OCH_3); 190 (12 cps), 1.9 (Asp-CH_2); all are somewhat broadened singlets. There is no detectable solvent present, particularly no $CHCl_3$ and no DMSO.

Poly Asp(Im)-Gly-Gly (23). To a solution of 1.1 g of TosOH-H-Asp(Im)-Gly-Gly-ONP in 2 ml of dimethyl sulfoxide at room temperature was added 0.32 ml of triethylamine. A yellow homogeneous solution was gradually formed. After 3 days 20 ml of methanol was added, and the mixture was stirred to give a granular product, filtered, and washed with four 25-ml portions of methanol to give 0.47 g of polymer, mp >260°.

The polymer is insoluble in chloroform, acetonitrile, acetic acid, acetone, and methanol, but soluble in dimethylformamide, dimethyl sulfoxide, and dichloroacetic acid. It is swollen by water but does not dissolve appreciably. Intrinsic viscosity in dichloroacetic acid at 30° was 0.49. Another preparation had an intrinsic viscosity of 0.31.

Anal. Calcd for $C_8H_9N_3O_4$: C, 45.5; H, 4.30; N, 19.9; OCH_3, 0. Found: C, 41.5; H, 5.17; N, 17.5; OCH_3, 0.6. This corresponds to about 10% of water. A reprecipitated sample had C, 43.1; H, 4.69; N, 18.5.

A different preparation started with HBr-H-Asp(OCH_3)-Gly-Gly-ONP (2.32 g) which was dissolved in 3.1 ml of dimethyl

(53) This was carried out by F. F. Rogers, Jr.

sulfoxide and treated with 2.2 moles of triethylamine (1.57 ml).[53] After a 50-hr reaction time, the mixture was worked up as above and the polymer dried over P_2O_5 at 100° (0.1 mm) to give a 55% yield. The material showed less than 2% methoxyl group (nmr); 17.5% N found; [η] 0.23 in dichloroacetic acid at 30°; DNP molecular weight 5000. This material was 55% L and 45% DL.

The infrared spectrum (21, KBr) showed: 3400, 3090, 2960, 1760 (weak), 1730–1667 b, 1540 b, 1430, 1410 (shoulder), 1330, 1250, 1190 (shoulder), 1170, 1025, 970, and 920.

It is possible to distinguish grossly the methyl ester from the imide using the infrared spectra, but the sensitivity is poor. The imide has a characteristic weak peak at 1760, and the peak at 1730 is strong with a shoulder at 1660 while the methyl ester has a strong peak at 1664 and the satellite 1740 peak due to the methyl ester group is only moderately strong.

The nmr spectrum (TFA) showed: 478, 2.0 (NH); 193 b, 1.2 (CH); 278, 1.7 (central Gly-CH_2); 259, 2.2 (C-terminal Gly-CH_2); 195, b m, 1.9 (Asp-CH_2), no peak in the 230 region (no OCH_3).

Sequence Peptide Polymers. II. Poly Glu(OH)⁻Gly, Poly Glu(OH)-Ser(H)-Gly, and Their Benzyl Esters[1,2]

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Abstract: Poly Glu(OBl), poly Glu(OBl)-Gly, poly Glu(OBl)-Ser(H)-Gly, and the corresponding free acids (all L configuration) have been prepared from the corresponding *p*-nitrophenyl ester salts. These polymerizations are in competition with pyrrolidone ring closure, an end-grouping reaction which is serious with poly Glu(OBl) and minor with poly Glu(OBl)-Ser(H)-Gly. The problems of stereochemical integrity have been studied, although there are experimental difficulties. There is no evidence of racemization of poly Glu(OBl)-Gly nor of poly Glu(OBl) where a C-terminal *p*-nitrophenyl ester is involved. Rotations of samples of poly Glu(OBl)-Ser(H)-Gly have been evaluated with a technique capable of showing about 5% racemization of Glu or 15% racemization of Ser and are optically pure within these limits. The infrared spectra of polymer films suggest that poly Glu(OBl)-Gly and poly Glu(OH)-Gly have the α-helical conformation while poly Glu(OBl)-Ser(H)-Gly has the pleated sheet (β) structure. Optical rotatory dispersion data in dichloroacetic acid–chloroform mixtures are consistent; poly Glu(OBl)-Gly shows a transformation at 15–20% dichloroacetic acid which is similar to the helix–random coil change reported for various homopolymers, while poly Glu-(OBl)-Ser(H)-Gly appears to undergo a change from random coil to β aggregate instead. In aqueous buffers both poly Glu(OH)-Gly and poly Glu(OH)-Ser(H)-Gly appear to be random coils at all pH values from 2 to 9. The tripeptide sequence Glu(OH)-Ser(H)-Gly is similar to the sequence at the active site of certain hydrolytic enzymes, and the corresponding polymer consists entirely of such "active sites." However, poly Glu(OH)-Ser(H)-Gly at 0.003 *M* concentration of tripeptide units showed no detectable catalytic activity toward the hydrolysis of *p*-nitrophenyl acetate. This result is in accord with hypotheses that the "active sites" of enzymes involve a larger number of residues, probably located rather remotely on the chain but brought together by folding.

Poly Glu(OH)-Ser(H)-Gly is of interest for many reasons. Since the sequence Gly-Glu(OH)-Ser-(H)-Ala has been found at the active site of hydrolytic enzymes,[4] a detailed investigation of the properties of a closely similar grouping is desirable in formulating theories of enzymic activity. The sequence also has two reactive functional groups, the mutual interactions of either or both of which may be studied theoretically and experimentally.[5] There is also interest in polymer properties and their relation to chain conformations, and for this the extensive studies on poly Glu(OBl) and on random copolymers containing glutamyl residues provide an unusually good background.[6]

In the present work the synthesis and characterization of several sequence polymers containing glutamic acid was undertaken. Because of the reactivity of side chains, the intermediates and the polymers were obtained at only a moderate level of purity, but the results nevertheless are of interest and significant.

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