A New Stepwise Synthesis of an Octapeptide Corresponding to a Sequence around the "Reactive" Serine of Chymotrypsin[1]

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Abstract: The occurrence of a single reactive serine residue in several proteolytic enzymes has indicated that the peptide sequences containing this reactive serine are close to, or at, the active site. Thus, as part of investigations of the mechanism of proteolytic enzyme catalysis, the synthesis of the residue 193–200 octapeptide segment of bovine chymotrypsinogen A, H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OMe, was carried out. A new, rapid synthesis was performed by repeated aminolysis of side-chain-protected *t*-butyloxycarbonyl amino acid N-hydroxysuccinimide esters by the amino-terminal end of the appropriate peptide. The reaction mixtures were worked up by conventional extractions leading to chromatographically pure residual BOC-peptide methyl esters for each step. Removal of the *t*-butyloxycarbonyl group from amino-protected peptides was carried out with anhydrous HCl–dimethoxyethane. Upon degassing and trituration with ether, chromatographically pure amino peptide esters were isolated in yields of >95%. The octapeptide methyl ester was obtained, after removal of the side-chain blocking groups, in 29% over-all yield, mp 158–160°, $[\alpha]^{27}D$ −106°. Elemental composition, amino acid analysis, and enzymic hydrolysis indicated that the octapeptide ester was chemically and optically pure.

Several syntheses of peptides which are components of proteins have been reported.[3] For the practical synthesis of peptides containing many amino acid residues, it is apparent that as the number of amino acids to be coupled in a predetermined sequence increases, high yields and fast reactions become more important. A number of procedures aimed at providing rapid peptide synthetic methods have been recently published.[4] As another general approach to a fast synthetic method we report here the stepwise synthesis of an octapeptide methyl ester, IX, corresponding to the residue 193–200 segment containing the "reactive" serine of bovine chymotrypsinogen A.[5] This synthesis makes use of *t*-butyloxycarbonyl amino acid N-hydroxysuccinimide esters as intermediates.[6]

The preparation of peptides which correspond to sites containing the reactive serine in proteolytic enzymes has been undertaken to help explain some aspects of the mechanism of enzyme action. Since aspartylseryl sequences constitute part of active sites in chymotrypsin,[7] trypsin,[8] thrombin,[9] and elastase,[10] the synthesis of related peptides may be helpful in explaining some of the common features of these enzymes. In addition, they may serve as models in further studies of facile $\alpha \rightarrow \beta$ rearrangements typical of aspartylseryl peptides.[11] Some peptides of this type may also show esteratic activity[12] dependent on the amino acid sequence and size of the peptide.

A protected derivative of octapeptide IX, Form-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3, was previously synthesized in this laboratory[3c] by a combination of stepwise elongation and fragment condensation methods using benzyloxycarbonyl as an amino protecting group and N-ethyl-5-phenylisoxazolium-3′-sulfonate[13] as a coupling reagent. It was subsequently discovered that the formyl amino blocking group[14] could not be removed from the amino end of this peptide without extensive side-product formation. This difficulty is presumably due to the facile rearrangement of blocked or unblocked aspartylseryl sequences *via* succinimide intermediates under relatively mild acidic or basic conditions.[11] The synthesis reported here was undertaken to (a) provide an alternate route to the free amino octapeptide which avoids rearrangements of this type and (b) to apply and test an approach to rapid methods of peptide synthesis.

Of the several amino acid coupling methods which have been available to peptide chemists in recent years, *p*-nitrophenyl active esters[15] have proved very useful in stepwise syntheses of peptides during the past decade.[16] This method has been shown to give high

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(2) National Institutes of Health Postdoctoral Fellow, 1965–1966.

(3) (a) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966); (b) K. Poduska, V. Gut, H. Zimmermannova, J. Rudinger, and F. Sorm, "Proceedings of the 7th European Symposium on Peptides," Budapest, 1964; *Acta Chim. Acad. Sci. Hung.*, **44**, 165 (1965); (c) H. T. Cheung, T. S. Murthy, and E. R. Blout, *J. Am. Chem. Soc.*, **86**, 4200 (1964); (d) P. Cruickshank and J. C. Sheehan, *ibid.*, **86**, 2070 (1964); (e) G. Losse and H. Weddige, *Ann. Chem.*, **678**, 148 (1964); (j) S. Bernhard, Z. Grdinic, H. Noller, and N. Shaltiel, *Proc. Natl. Acad. Sci. U. S.*, **52**, 1489 (1964); (g) S. Sano, K. Ikeda, and S. Sakakibara, *Biochem. Biophys. Res. Commun.*, **15**, 284 (1964); (h) S. Inouye, S. Sakakibara, and S. Akabori, *Bull. Chem. Soc. Japan*, **37**, 713 (1964).

(4) (a) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Weber, E. F. Schoenwaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschman, *J. Am. Chem. Soc.*, **88**, 3163 (1966); (b) J. C. Sheehan, J. Preston, and P. A. Cruickshank, *ibid.*, **87**, 2492 (1965); (c) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

(5) B. S. Hartley, *Nature*, **201**, 1284 (1964).

(6) (a) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964); (b) *ibid.*, **85**, 3039 (1963).

(7) M. L. Bender and F. Kezdy, *ibid.*, **86**, 3704 (1964), and references cited therein.

(8) G. H. Dixon, D. L. Kauffmann, and H. Neurath, *J. Biol. Chem.*, **233**, 1373 (1958).

(9) J. A. Gladner and K. Laki, *J. Am. Chem. Soc.*, **80**, 1263 (1958).

(10) M. A. Naughton, F. Sanger, B. S. Hartley, and D. C. Shaw, *Biochem. J.*, **77**, 149 (1960).

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

(12) J. C. Sheehan, G. B. Bennett, and J. A. Schneider, *ibid.*, **88**, 3455 (1966).

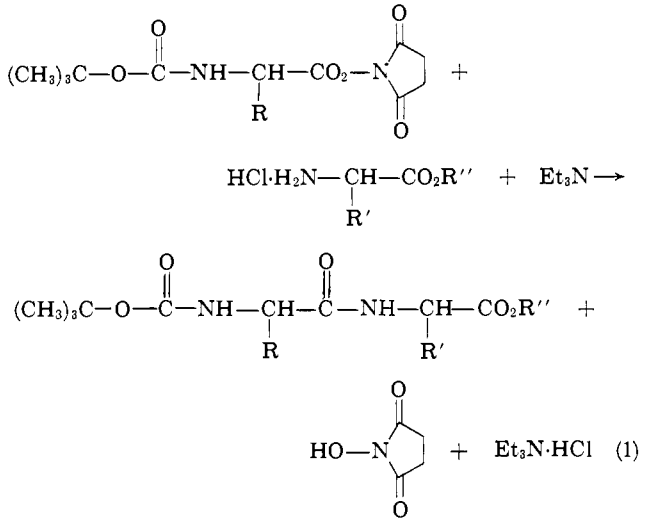
(13) R. B. Woodward, R. A. Olofson, and H. Meyer, *ibid.*, **83**, 1010 (1961).

(14) J. C. Sheehan and D. H. Yang, *ibid.*, **80**, 1154 (1958).

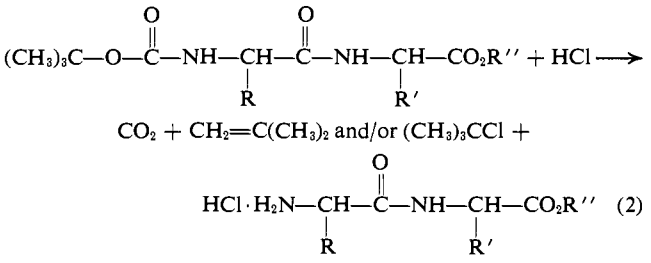
(15) M. Bodanszky, *Nature*, **175**, 685 (1955).

(16) (a) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **49**, 134 (1966); (b) P. G. Katsoyannis and K. Suzuki, *J. Am. Chem. Soc.*, **84**, 1420 (1962); (c) M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *ibid.*, **82**, 3195 (1960).

yields and to proceed with no racemization under favorable conditions.[17] Other recently reported active esters include pentachlorophenyl[18] and N-hydroxysuccinimide (NHS) derivatives. The use of NHS esters was introduced to peptide synthesis by Anderson, *et al.*,[6] in 1963 and examples of their application have since been reported by other investigators.[3b,19] Amino acid NHS esters appeared to be particularly suitable for a rapid synthesis in view of their easy preparation, crystallinity, high reactivity, and the presence of easily removable by-products during the coupling stage (eq 1).



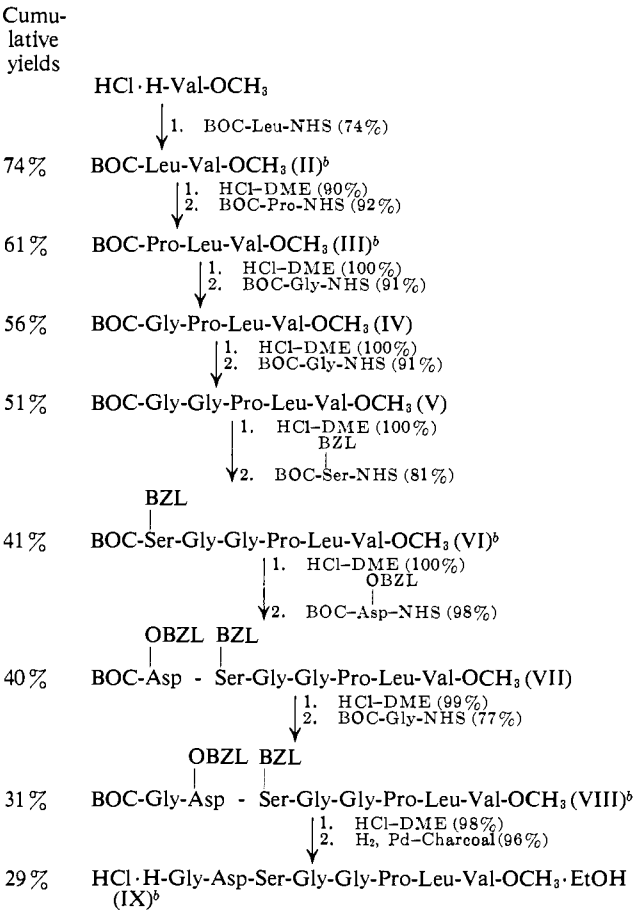
The amino protecting group chosen for use with the NHS ester was the *t*-butyloxycarbonyl (BOC) function,[20] placed on the amino moiety *via* *t*-butyloxycarbazide,[20b] and quantitatively removable from it under relatively mild acidic conditions[21] leading to the ammonium salt and volatile by-products (eq 2).



As shown in Chart I the peptide was extended from its amino end by aminolysis of the BOC-amino acid NHS ester with the aminopeptide methyl ester, followed by removal of the BOC protecting group with HCl in dimethoxyethane (DME). The coupling was routinely carried out in anhydrous DME at room temperature for 2 hr, although the reaction may have been complete in less time. The BOC-aminopeptide methyl ester products, usually obtained in yields of 90% or better, were chromatographically pure (silica gel H thin layer plates). The di-, tri-, hexa-, and octapeptides were readily obtained in crystalline form.

(17) E. Schroder and K. Lubke, *Peptides*, **1**, 325 (1965).
(18) J. Kovacs, R. Giannotti, and A. Kapoor, *J. Am. Chem. Soc.*, **88**, 2282 (1966).
(19) (a) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *ibid.*, **87**, 631 (1965); (b) M. Low and L. Kisfaludy, "Proceedings of the 7th European Symposium on Peptides," Budapest, 1964; *Acta Chim. Acad. Sci. Hung.*, **44**, 165 (1965).
(20) (a) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957); (b) L. A. Carpino, *ibid.*, **79**, 98 (1957).
(21) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **43**, 1910 (1960).

Chart I[a]



[a] Individual reaction yields, next to corresponding steps. [b] Crystalline sample. Reaction yield refers to once-crystallized product.

Removal of the BOC group from amino-protected intermediates was carried out using anhydrous HCl in dimethoxyethane at room temperature. The aminopeptide methyl ester hydrochlorides, obtained in yields of 95% or better, were chromatographically pure (silica gel H thin layer plates). The procedures described in the Experimental Section were routinely followed in the preparation of 14 intermediate peptides, four of which were further characterized by elemental analysis.

The octapeptide VIII, in which the side chains and both terminal residues were protected, was obtained in crystalline form in 31% over-all yield (based on HCl·H-Val-OCH_3). Its N-terminal BOC-amino protecting group was removed in the usual manner, and the benzyl blocking functions of the aspartylseryl sequence were hydrogenolyzed. The deblocked product, HCl·H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3 (IX), crystallized readily. The results of elemental analysis are included in the Experimental Section, and amino acid ratios of 6 *N* HCl and enzymically hydrolyzed samples of IX are shown in Table I. The data indicate that octapeptide ester IX was chemically pure, and that the optical purity of its constituent amino acids was preserved throughout the synthesis.

The synthesis of the octapeptide corresponding to the 193–200 sequence around the reactive serine of chymotrypsin illustrates the adaptability of reactions involving aminolysis of NHS esters and removal of BOC protecting groups to rapid work-up procedures. Conditions of reaction and purification are sufficiently

Table I. Amino Acid Ratios of Hydrolyzed Samples of HCl·H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3

Mode of hydrolysis	Amino acid ratios[a] Gly	Asp	Ser	Pro	Leu	Val
6 *N* HCl, 110°, 24 hr	3.17	1.05	0.92	0.95	0.95	0.96
Prolidase + leucine aminopeptidase,[b] 37°, 69 hr[c]	3.10	1.05	0.83	0.99	1.01	1.01

[a] Beckman-Spinco amino acid analyzer. [b] K. Hofmann, H. Yajima, T. Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *J. Am. Chem. Soc.*, **84**, 4481 (1962); R. L. Hill and W. R. Schmidt, *J. Biol. Chem.*, **237**, 389 (1962); K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961). [c] The digest was free of peptides as indicated by thin layer chromatography.

mild to preclude facile rearrangements such as those involving aspartylseryl sequences. The coupling and deblocking steps proceed in high yields, with no observed side reactions, and the accompanying by-products are either volatile or conveniently removed by conventional extraction techniques. It appears that the rapid synthetic methods described herein may prove generally applicable in the synthesis of high molecular weight peptides with predetermined sequences of different amino acids. The octapeptide ester VIII serves as an intermediate for more extensive syntheses, including its incorporation into macromolecular polypeptide systems containing other amino acids involved in proteolytic enzyme action.

Experimental Section

General Procedures. A. Materials. Dimethoxyethane (DME) was dried by distillation from $LiAlH_4$. HCl-saturated DME was prepared by bubbling dry HCl (98% H_2SO_4 and Dry Ice traps) through stirred anhydrous DME in a Grignard flask at 0° for 1.5 hr. Equilibration to 26° usually led to HCl-DME (7.5 *N*). The solution was tightly capped, stored in the cold, and titrated before use.

BOC-Gly-OH, BOC-Pro-OH, and BOC-Leu-OH were prepared as previously described.[22] BOC-Asp(OBZL)-OH and BOC-Ser(BZL)-OH were synthesized using the method of Wunsch.[23] BOC-Ser(BZL)-OH was obtained as a colorless oil in 82% yield, R_f (S-IV) 0.45, characterized as its NHS ester. BOC-Asp(OBZL)-OH was isolated in crystalline form, 37% yield, mp 102–103°, characterized as its NHS ester.

BOC-Gly-NHS, BOC-Pro-NHS, and BOC-Ser(BZL)-NHS were prepared by previously described methods.[6a]

BOC-Asp(OBZL)-NHS was obtained in 70% yield, mp 103–104°, $[\alpha]^{26}D$ −20.0° (*c* 0.55, dioxane). *Anal.* Calcd for $C_{20}H_{24}O_8N_2$: C, 57.13; H, 5.75; N, 6.66. Found: C, 57.11; H, 5.85; N, 6.75. BOC-Ser(BZL)-NHS was obtained in 52% yield, mp 112–113°, $[\alpha]^{26}D$ +6.5° (*c* 0.523, dioxane). *Anal.* Calcd for $C_{19}H_{26}O_7N_2$: C, 58.15; H, 6.17; N, 7.14. Found: C, 58.3; H, 6.3; N, 7.1.

B. The Coupling Step. The aminopeptide methyl ester hydrochloride (1 equiv) and BOC-amino acid NHS active ester (1 equiv) were dissolved or suspended in anhydrous DME (*ca.* 7 ml/mequiv of either reactant). The hydrochloride was neutralized with Et_3N (1 equiv), and the suspension was stirred at room temperature for 2 hr. The system was reduced to one-half volume, diluted with H_2O (four volumes), and extracted four times with EtOAc (one-quarter volume). The combined EtOAc phases were washed with H_2O (one-quarter volume), twice with ice cold 1 *N* NaOH (one-quarter volume) and brine (one-quarter volume), filtered through anhydrous Na_2SO_4, and flash evaporated *in vacuo* to dryness.

C. Removal of BOC-Amino Protecting Group. A solution of BOC-aminopeptide methyl ester (1 equiv) in DME (*ca.* 1 ml/mequiv) was treated with 5–8 *N* anhydrous HCl-DME (3–10 equiv) at 0°, and stirred at 26° for 0.5 to 4 hr. The solvent was flash evaporated *in vacuo*, and the residue was freed of HCl by two cycles of addition and evaporation of DME. Traces of unreacted material were eliminated by triturating the residue with ether and decanting the solvent.

D. Analysis and Characterization of Intermediate Peptides. Products were isolated by flash evaporation at 30–45° and dried over P_2O_5 and NaOH at 0.2–0.5 mm, 26–35°, overnight. Reactions were followed and products were characterized by thin layer chromatography (tlc) on silica gel H, solvent system S-I, *n*-BuOH-HOAc-H_2O (10:1:3); solvent systems S-IV, S-V, and S-VI are outlined in Table II. The purity of protected peptide intermediates was checked by tlc in at least two solvent systems (R_f values herein reported were read off a single plate per solvent) and by infrared spectra. Crystalline BOC-peptides were also characterized by melting point, $[\alpha]D$, and C, H, N analysis. All melting points (Kofler block, polarizing filters) were uncorrected.

Table II. Solvent Systems for Use in Thin Layer Chromatography

System no.	CCl_4, ml	*n*-BuOH ml	HOAc, ml
S-IV	85	10	5
S-V	70	20	10
S-VI	55	30	15

Specific Peptide Syntheses. A. BOC-Leu-Val-OCH_3 (II). HCl·H-Val-OCH_3 (Sigma Chemicals Co.) (9.97 g, 59.5 mmoles) and BOC-Leu-NHS (19.2 g, 58.4 mmoles) were suspended in DME (180 ml) and treated with Et_3N (8.25 ml, 59.0 mmoles). The suspension was stirred at 25° for 2 hr, concentrated to one-half volume, poured into chilled H_2O (400 ml), and extracted three times with EtOAc (250 ml). The EtOAc phases were filtered through Na_2SO_4 and evaporated. The BOC-dipeptide (II) residue crystallized from hexane-EtOAc, 14.9 g, 74% yield, mp 144–147°, R_f (S-I) 0.92, R_f (S-IV) 0.50, $[\alpha]^{26}D$ −41.1° (*c* 0.527, methanol). *Anal.* Calcd for $C_{17}H_{32}O_5N_2$ (recrystallized sample, mp 150.0–151.5°): C, 59.28; H, 9.37; N, 8.13. Found: C, 59.00; H, 9.44; N, 8.26.

B. BOC-Pro-Leu-Val-OCH_3 (III). BOC-dipeptide (II) (13.9 g, 40.4 mmoles) was treated with 5.5 *N* HCl-DME (73 ml, 404 mmoles) and stirred at 26° for 0.5 hr. The solvent was evaporated, and the residual oil was dried over KOH, triturated overnight with excess ether, and transformed to a crystalline form. Additional product was obtained from the mother liquor by evaporation and retrituration, total yield HCl·H-Leu-Val-OCH_3 (IIa) (a denotes the corresponding hydrochloride of amino-protected peptides II–VIII) 10.2 g, 90%, R_f (S-I) 0.45. Compound IIa and BOC-Pro-NHS were coupled as described in part A, leading to BOC-Pro-Leu-Val-OCH_3 (III) which crystallized from hot hexane-EtOAc, 14.7 g, 92%, mp 109.5–111.0°, R_f (S-I) 0.94, R_f (S-IV) 0.26, $[\alpha]^{26}D$ −83.9° (*c* 0.404, methanol). *Anal.* Calcd for $C_{22}H_{39}O_6N_3$ (recrystallized sample, mp 115.5–116.5°): C, 59.84; H, 8.90; N, 9.52. Found: C, 60.2; H, 9.1; N, 9.5.

C. BOC-Ser-Gly-Gly-Pro-Leu-Val-OCH_3 (VI). BOC-tripeptide (III) was treated with 7.95 *N* HCl-DME as reported in part B. The hygroscopic product HCl·H-Pro-Leu-Val-OCH_3 (IIIa) was isolated in 100% yield, R_f (S-I) 0.35. IIIa (2.50 g, 6.63 mmoles) and BOC-Gly-NHS (1.80 g, 6.63 mmoles) were suspended in DME (47 ml) and treated with Et_3N (0.93 ml, 6.63 mmoles). The system was stirred for 2 hr at 26°, concentrated to one-half volume, diluted with H_2O (100 ml), and extracted four times with EtOAc (25 ml). The combined EtOAc phases were extracted with H_2O (25 ml), twice with chilled 1 *N* NaOH (25 ml) and brine (25 ml), filtered through Na_2SO_4, and evaporated, yielding BOC-Gly-Pro-Leu-Val-OCH_3 (IV) in 91% yield, R_f (S-V) 0.45. This work-up procedure was carried out in subsequent coupling stages. A solution of IV (1.30 g, 2.61 mmoles) in DME (3 ml) was treated with 4.4 *N* HCl-DME (1.78 ml, 7.83 mmoles) at 0°, and stirred at 26° for 4 hr. The solvent was evaporated, and the residue was freed of HCl by two cycles of addition and evaporation of DME. Trituration with ether afforded HCl·H-Gly-Pro-Leu-Val-OCH_3 (IVa), 1.1 g, 100% yield, R_f (S-I) 0.41. This work-up method was carried out in all subsequent removals of the BOC protecting group. IVa was coupled with BOC-Gly-NHS as previously described, yielding BOC-Gly-Gly-Pro-Leu-Val-OCH_3 (V), 1.32 g, 91%, R_f (S-I) 0.65, R_f (S-IV) 0.33. Removal of the BOC protecting group from V was performed in the usual manner, affording HCl·H-Gly-Gly-Pro-Leu-Val-OCH_3 (Va), 1.16 g, 100%, R_f (S-I) 0.32. Coupling

(22) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

(23) E. Wunsch and A. Zwick, *Chem. Ber.*, **97**, 2500 (1964).

of Va with BOC-Ser-NHS and crystallization of the product afforded BOC-Ser(BZL)-Gly-Gly-Pro-Leu-Val-$OCH_3 \cdot H_2O$ (VI), 1.4 g, 81%, mp 85–90°, R_f (S-I) 0.75, R_f (S-VI) 0.32, $[\alpha]^{26}D$ −70.7° (*c* 0.308, methanol). *Anal.* Calcd for $C_{36}H_{56}O_{10}N_6 \cdot 0.5H_2O$ (recrystallized sample, mp 85–88°): C, 58.58; H, 7.78; N, 11.19; H_2O, 1.2. Found: C, 58.32; H, 7.83; N, 11.31. A 1.1% weight loss was found upon drying to constant weight at 80° (0.2 mm).

D. BOC-Gly-Asp(OBZL)-Ser(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VIII). The BOC protecting group was removed from VI, affording HCl·H-Ser(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VIa), 100%, R_f (S-I) 0.45. VIa was coupled with BOC-Asp(OBZL)-NHS in the usual way, except for omission of alkali washing during the work-up procedure (in view of the known[11] base-catalyzed rearrangement of Asp-Ser sequences), yielding BOC-Asp(OBZL)-Ser-(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VII), 98%, R_f (S-I) 0.80, R_f (S-VI) 0.56. A solution of VII (1.58 g, 1.69 mmoles) in DME (4.0 ml) was treated with 6.1 *N* HCl–DME (1.24 ml, 7.6 mmoles) at 0° for 4 hr and worked up as previously indicated, leading to HCl·H-Asp-(OBZL)-Ser(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VIIa), 1.47 g, 99% yield, R_f (S-I) 0.50. VIIa was coupled with BOC-Gly-NHS as above, and the product, BOC-Gly-Asp(OBZL)-Ser(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VIII) crystallized from hexane–ethanol, 1.20 g, 77%, mp 122–130°, R_f (S-I) 0.74, R_f (S-VI) 0.16, $[\alpha]^{26}D$ −62.5° (*c* 0.320, methanol). *Anal.* Calcd for $C_{49}H_{70}O_{14}N_8$ (recrystallized sample, mp 130–133°: C, 59.14; H, 7.09; N, 11.26. Found: C, 58.90; H, 7.06; N, 10.99.

E. HCl·H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3 (IX). Removal of the BOC group from VIII was carried out as described in part D, leading to HCl·H-Gly-Asp(OBZL)-Ser(BZL)-Gly-Gly-Pro-Leu-Val-OCH_3 (VIIIa) in 98% yield, R_f (S-I) 0.41. A solution of VIIIa (0.233 g, 0.25 mmole) in EtOH (45 ml) was hydrogenated over 10% palladium–charcoal (0.11 g) at 23° for 5.5 hr, consuming an equivalent volume of H_2. The suspension was filtered through Celite, the filtrate was evaporated, and the residue was dried over P_2O_5. The product, HCl·H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3 (IX), 0.18 g, 96% yield, mp 151–156°, R_f (S-I) 0.11, was devoid of ultraviolet benzylic absorption at 257 mμ. Crystallization from EtOH–EtOAc led to granular crystals, mp 158–160°, $[\alpha]^{27}D$ −106° (*c* 0.057, H_2O). *Anal.* Calcd for $C_{30}H_{51}O_{12}N_{18}Cl_1 \cdot C_2H_5OH$: C, 48.20; H, 7.21; N, 14.05; Cl, 4.45. Found: C, 48.35; H, 6.98; N, 13.94; Cl, 4.48.

Compound IX (2.25 mg) was digested with a mixture of preincubated solutions of 0.1% leucine aminopeptidase and prolidase[24] (0.5 ml of each, 1 mg/ml of $MnCl_2 \cdot 4H_2O$, 0.025 *M* Tris buffer, pH 8.5, 37°, 69 hr). Thin layer chromatography (silica gel H; *n*-BuOH–HOAc–H_2O, 2:1:1, in milliliters; C_6H_5OH–H_2O, 3:1, in grams) of the digest accounted for all constituent amino acids and did not detect peptides with chlorine–tolidine. Samples of enzymic and acid (6 *N* HCl, 110°, 24 hr) digests were analyzed with the Beckman-Spinco analyzer. The amino acid ratios are given in Table I.

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(24) See footnote *b*, Table I.

The Crystal and Molecular Structure of 2,4-Dithiouracil[1]

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Abstract: The molecular structure of 2,4-dithiouracil has been determined by X-ray diffraction. The crystals are monoclinic, space group $P2_1/c$, $a = 4.247$ A, $b = 14.586$ A, $c = 9.907$ A, $\beta = 98.77°$, and $Z = 4$. Evidence is presented that dithiouracil contains highly polarized thionamide groups, the polarization of these groups being position dependent. The C–S bond lengths were found to be 1.645 and 1.685 (±0.006) A. The packing of the molecules in the crystal lattice is dominated by S···(H)–N hydrogen bonds. The lengths of the hydrogen bonds appear to be a function of the degree of polarization about the sulfur atoms.

Numerous sulfur-substituted pyrimidines and purines have found applications as clinically useful drugs. In many of these it was noted that the position in which sulfur was introduced was crucial to biological activity. For instance, 2-thiouracil, but not 4-thiouracil, has useful antithyroid activity,[2] while 6-thioguanine and 6-mercaptopurine, but not the corresponding 2-thio compounds, exert antineoplastic action.[3] 4-Thiopteridines and -pyrimidines and 6-thiopurines, but not the 2-substituted isomers, bind to dihydrofolic acid reductase.[4] These differences in biological activity are paralleled by differences in the chemical reactivities of such compounds. Thus, the reaction of uracil with phosphorus pentasulfide yields only 4-thiouracil and 2,4-dithiouracil, but no 2-thiouracil,[5] while the reaction of 2,4-dithiouracil with ammonia yields only 2-thiocytosine, none of the 2-amino compound being formed.[6]

It has also been noted that the replacement of oxygen by sulfur[5] or by selenium[7] induced much greater bathochromic shifts in the ultraviolet spectra of 2,4-disubstituted pyrimidines (or the corresponding 2,6-disubstituted purines) when it took place in the 4 position of the pyrimidines (6 position of the purines) than when it

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(2) E. B. Astwood, A. Bissell, and A. M. Hughes, *Endocrinology*, 37, 456 (1945).

(3) R. K. Robins, *J. Med. Chem.*, 7, 186 (1964).

(4) J. R. Bertino and H. G. Mautner, unpublished data.

(5) G. B. Elion, *et al.*, *J. Am. Chem. Soc.*, **68**, 2137 (1946).

(6) G. H. Hitchings, *et al.*, *J. Biol. Chem.*, **177**, 357 (1949).

(7) H. G. Mautner, *J. Am. Chem. Soc.*, **78**, 5292 (1956).