from the trachea into an infrared carbon dioxide analyzer (P. K. Morgan Ltd.). The arterial $pCO_2$ was kept within the limits of 34–42 mmHg and the arterial pH between 7.35 and 7.43 by either the adjustment of respiration or the iv injection of a 1.0 M $NaHCO_3$ solution. Esophageal temperature was recorded from a thermister probe (Yellow Springs Instrument Co., Inc.) and maintained at 37 ± 1 °C by heating lamps above and below the animal.

In each dog, response curves relating iv injections of racemic isoproterenol (1) in the range 0.05 to 10 μg/kg to peak changes in heart rate (HR) and hind limb perfusion pressure (HLPP) were obtained. About 45 min after the final injection of 1 when HR and HLPP had returned to control values, cumulative dose–response curves relating changes in HR and HLPP to the dose of a test compound were obtained. The compounds were administered as solutions in physiological saline.

**Acknowledgment.** The authors thank B. J. Ball and I. J. Edwards for their expert technical assistance in synthesizing the compounds, A. Nuttall and Mrs. J. Moors in testing them, and C. J. Howarth for providing analytical data.

# Fibrin Polymerization. 1. Alkylating Peptide Inhibitors of Fibrin Polymerization[1]

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A series of analogues relating to the $NH_2$-terminal region of the fibrin α chain, i.e., Gly-Pro-Arg-Pro, were prepared by stepwise solid-phase synthesis, and their abilities to inhibit fibrin polymerization and to prolong thrombin-initiated clotting time were evaluated. Among the analogues systematically modified at different positions, replacement of the $NH_2$-terminal three residues of Gly-Pro-Arg-Pro by either chlorambucil, *p*-nitrophenyl-L-alanine, or *p*-aminophenyl-L-alanine gave inactive compounds in the thrombin time assay, whereas similar substitution or extension of the COOH terminus produced the highly active analogues Gly-Pro-Arg-Phe(4-$NH_2$), 22%; Gly-Pro-Arg-Pro-Phe(4-$NO_2$), 88%; and Gly-Pro-Arg-Pro-Phe(4-$NH_2$), 105%; relative to Gly-Pro-Arg-Pro = 100% in the fibrin polymerization inhibitory assay. As potential photoaffinity labeling probes, analogues containing a nitrophenylalanine residue in position 4 or 5 underwent photolysis under the experimental photoactivation conditions. As a potential alkylating probe, Chl-Pro-Arg-Pro was selectively effective in inhibiting thrombin amidolysis and fibrin polymerization. In the latter assay, Chl-Pro-Arg-Pro was approximately 20 times more potent than Gly-Pro-Arg-Pro in inhibiting fibrin aggregation.

The enzymatic conversion of the soluble plasma glycoprotein fibrinogen to its spontaneously aggregating polymer fibrin by thrombin, leading to clot formation, is central to normal hemostasis.[2] This event[3,4] has been proposed to initially involve binding of the newly released fibrin $NH_2$ termini (E domain) with the COOH termini (D domain) of an adjacent unit, resulting in an end to end aggregate of the type I fibrin, which is subsequently reinforced by lateral association forming the more compact type II fibrin.

In an attempt to localize these two sets of polymerization sites, Laudano and Doolittle[5] synthesized the tetrapeptides Gly-Pro-Arg-Pro and Gly-His-Arg-Pro corresponding to the $NH_2$ termini of human fibrin α and β chains and observed that both peptides selectively bound to fragment D (80 000 daltons) of fibrinogen in a reversible manner but not to fragment E.[6] Furthermore, the more tightly bound Gly-Pro-Arg-Pro ($K_a = 5 \times 10^4\ M^{-1}$) could prevent fibrin polymerization in vitro. Although these studies localized part of the "E" binding sites, precise localization of the complementary "D" domain polymerization sites, as well as further characterization of the "E" sites, is not feasible due to the reversible nature of their binding. One approach to circumvent this limitation is to develop probes which may label these binding sites selectively and irreversibly. Such affinity-labeling probes can lead to better understanding of the detailed mechanism of fibrin polymerization, as well as its causal relationship in thrombotic and other disorders, and are also useful as potential therapeutic anticoagulants.

Among the active-site-directed affinity labels,[7] the chemical alkylating agents, such as the nitrogen mustards,[8] have the advantage of being effective in vivo[9] but require appropriate steric alignment of the label with a suitable recipient on the receptor in order for covalent linking to occur. On the other hand, the photoaffinity labeling agents have the advantage of being unusually reactive upon irradiation,[7,10] such that alkylation of the receptor can occur

(1) (a) The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1972); *Biochemistry*, 14, 449 (1975)]. Other abbreviations include: Phe(4-$NO_2$), 4-nitrophenylalanine; Phe(4-$NH_2$), 4-aminophenylalanine; Chl, chlorambucil [4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyric acid]; X, photolysis products of 4-nitrophenylalanine. (b) Peptides prepared for this series are also referred by their compound number shown in Table II. (c) This report was presented in part; see "Abstracts of Papers", Second Chemical Congress of the North American Continent, San Francisco, CA, Aug 1980, American Chemical Society, Washington, D.C., 1980, Abstr MEDI 111.

(2) For background information, see (a) "Haemostasis: Biochemistry, Physiology, and Pathology", D. Ogston and B. Bennett, Eds., Wiley, New York, 1977; (b) "Plasma Proteins", B. Blombäck, and L. A. Hanson, Eds., Wiley, New York, 1979; (c) "Chemistry and Biology of Thrombin", R. L. Lundblad, J. W. Fenton II, and K. G. Mann, Eds., Ann Arbor Science Publishers, Ann Arbor, Mich., 1977; (d) E. W. Davie and K. Fujikawa, *Annu. Rev. Biochem.*, 44, 902 (1975).

(3) B. Blombäck, B. Hessel, D. Hogg, and L. Therkildsen, *Nature (London)*, **275**, 501 (1978).

(4) R. F. Doolittle, in ref 2a, p 169.

(5) A. P. Laudano and R. F. Doolittle, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3085 (1978).

(6) A. P. Laudano and R. F. Doolittle, *Fed. Proc., Fed. Am. Soc. Exp. Biol. Abstr.: Biochem.*, **38**, 2968 (1979).

(7) For a review, see (a) F. Wold, *Methods Enzymol.*, **46**, 3 (1977); (b) S. J. Singer, *Adv. Protein Chem.*, **22**, 1 (1967); (c) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, 1967.

(8) J. A. Stock, in "Drug Design", Vol. 2, E. J. Ariens, Ed., Academic Press, New York, 1971, p 532.

(9) For a review, see (a) *Handb. Exp. Pharmacol.*, **38** (2), section C (1975); (b) R. F. Dorge, in "Textbook of Organic Medicinal and Pharmaceutical Chemistry", C. O. Wilson, O. Gisvold, and R. F. Dorge, Eds., Lippincott, Philadelphia, 1977, p 170.

(10) J. R. Knowles, *Acc. Chem. Res.*, **5**, 155 (1972).

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Table I. Synthetic and Analytical Data of Anticoagulant Peptide Analogues

| no. | peptide sequence | yield, % | TLE, $E_p$[a] 1.9 | TLE, $E_p$[a] 8.0 | TLC, $R_f$[b] I | II | III | IV | amino acid anal. 1 | 2 | 3 | 4 | 5 | peptide content, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Gly-Pro-Arg-Pro | 40 | 0.83 | 2.50 | 0.04 | 0.04 | 0.06 | 0.16 | 1.02 | 0.99 | 1.00 | 0.99 | | 72 |
| 2 | Chl-Gly-Pro-Arg-Pro | 28 | 0.53 | 0.75 | 0.28 | 0.37 | 0.34 | 0.46 | 0.98 | 1.00 | 1.04 | 1.00 | | 62 |
| 3 | Chl-Pro-Arg-Pro | 21 | 0.56 | 0.75 | 0.32 | 0.41 | 0.38 | 0.46 | | 1.00 | 0.90 | 1.00 | | 58 |
| 4 | Chl-Arg-Pro | 20 | 0.66 | 0.75 | 0.35 | 0.43 | 0.41 | 0.47 | | | 0.98 | 1.02 | | 66 |
| 5 | Ac-Gly-Pro-Arg-Pro | 28 | 0.55 | 1.67 | 0.09[c] | 0.10 | 0.11 | 0.23 | 0.95 | 0.99 | 1.08 | 0.99 | | 65 |
| 6 | Phe(4-$NO_2$)-Pro-Arg-Pro | 23 | 0.75 | 1.50 | 0.13 | 0.10 | 0.18 | 0.28 | 1.00 | 0.98 | 1.03 | 0.98 | | 91 |
| 7 | Gly-Phe(4-$NO_2$)-Arg-Pro | 25 | 0.77 | 1.42 | 0.14 | 0.11 | 0.17 | 0.29 | 0.93 | 1.06 | 1.03 | 0.98 | | 69 |
| 8 | Gly-Pro-Phe(4-$NO_2$)-Pro | 34 | 0.47 | 1.33 | 0.19 | 0.23 | 0.21 | 0.35 | 1.03 | 0.97 | 1.04 | 0.97 | | 95 |
| 9 | Gly-Pro-Arg-Phe(4-$NO_2$) | 94 | 0.78 | 2.42 | 0.12 | 0.10 | 0.16 | 0.31 | 1.02 | 1.00 | 0.94 | 1.04 | | 100 |
| 10 | Gly-Pro-Arg-Pro-Phe(4-$NO_2$) | 86 | 0.72 | 2.50 | 0.11 | 0.07 | 0.15 | 0.29 | 0.98 | 0.94 | 1.03 | 0.94 | 1.15 | 83 |
| 11 | Phe(4-$NH_2$)-Pro-Arg-Pro | 64 | 1.03 | 1.92 | 0.02[c] | 0.02 | 0.06 | 0.17 | 0.60 + 0.41[d] | 1.00 | 1.04 | 1.00 | | 73 |
| 12 | Gly-Phe(4-$NH_2$)-Arg-Pro | 13 | 1.09 | 2.25 | 0.03[c] | 0.04 | 0.05 | 0.17 | 0.97 | 0.99 | 1.04 | 1.01 | | 100 |
| 13 | Gly-Pro-Phe(4-$NH_2$)-Pro | 23 | 0.92 | 1.00 | 0.05[c] | 0.06 | 0.09 | 0.25 | 0.97 | 1.00 | 1.03 | 1.00 | | 81 |
| 14 | Gly-Pro-Arg-Phe(4-$NH_2$) | 9[e] | 1.11 | 2.17 | 0.02 | 0.03 | 0.03 | 0.15 | 1.01 | 0.95 | 1.01 | 1.03 | | 71 |
| 15 | Gly-Pro-Arg-Pro-Phe(4-$NH_2$) | 6[f] | 0.97 | 2.33 | 0.02 | 0.03 | 0.04 | 0.18 | 0.99 | 0.98 | 1.01 | 0.98 | 1.03 | 76 |

[a] $E_p$ indicates electrophoretic mobility relative to Phe(4-$NH_2$) = 1.00 at pH 1.9 and 8.0. [b] $R_f$ values in: I, 4:1:5 of butanol-acetic acid-water (upper phase); II, 3:1:1 of butanol-acetic acid-water; III, 8:1:2:9 of butanol-pyridine-acetic acid-water (upper phase); IV, 15:10:3:12 of butanol-pyridine-acetic acid-water. [c] Not homogeneous. [d] Substantial amount of unreduced material. [e] A large percentage (75%) of the product was isolated from the catalytic hydrogenation mixture of the precursor nitrophenylalanine compound. This side product showed an equimolar ratio of each amino acid in the sequence except aminophenylalanine, in place of which two new amino acids appeared. The nature of these peptides are being studied.[33] [f] Similar side product was isolated in 34% yield.

without the presence of especially reactive receptor nucleophiles. Since a method for photoactivation in vivo is currently unavailable, the photoaffinity labels are ideally suited for in vitro systems, and both the azides (nitrene precursor) and the diazo compounds (carbene precursor) have been used successfully to label a variety of proteins and to localize hormonal receptors.[11,12] More recently, the nitrophenyl group was shown to be a useful photoaffinity label[13,14] with remarkable chemical stability which can be converted by hydrogenation to the aminophenyl group, an intermediate for the synthesis of the aryl azide photoaffinity label. Because the nitrophenyl group is more stable than other types of photoaffinity labels toward laboratory light and the strongly oxidizing conditions of radioiodination, its ease of handling and the possibility of introducing high levels of radioactivity concomitant with affinity labeling make the nitrophenyl group an attractive probe for active-site localization.

For this study, we incorporated the chlorambucil mustard,[15] the nitrophenylalanine, and the aminophenylalanine into different positions of Gly-Pro-Arg-Pro, in order to examine the structure–activity relationship of this anticoagulant peptide and to design an affinity label which may inhibit fibrin polymerization irreversibly and specifically. A list of these peptides is presented in Table I.

## Results and Discussion

**Photoactivation Experiments.** Since Escher and Guillemette[13] observed that nitrophenylalanine was photolyzed by continuous irradiation with mercury vapor lamps at 365 nm but not by flash photolysis at the same wavelength, it became important to test whether analogues **6** to **10** containing nitrophenylalanine in various positions could be suitably activated under the experimental conditions. When the UV spectra of these peptides before and after irradiation were compared, the irradiated products (**16**, **19**, and **20**) were very different from the starting compounds (**6**, **9**, and **10**) at the region of 250–300 nm. In addition, amino acid analysis of a hydrolysate of the photolyzed products indicated complete retention of every amino acid except nitrophenylalanine, which was eliminated almost entirely from the hydrolysates. Similar results were obtained from TLC and TLE, in which multiple components different from their corresponding nitrophenylalanine-containing or aminophenylalanine-containing analogues were observed. These findings indicate that irradiation at 350 nm for 30 min is sufficient to photolyze the nitrophenyl group, although the photolysis rate appears to be greater for nitrophenyl groups at the terminal region of the peptide than at the less accessible central region of the sequence, which could, nevertheless, be photolyzed completely upon more prolonged irradiation.

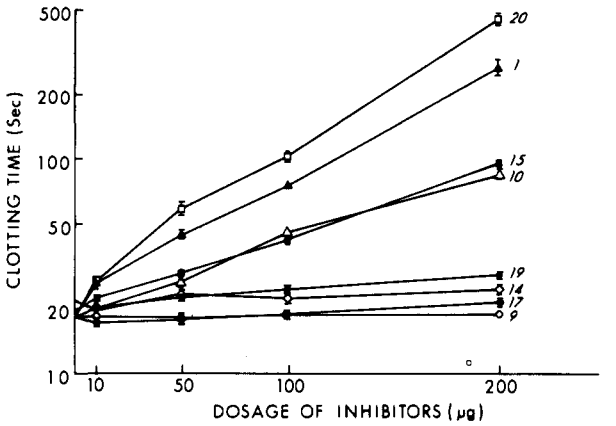


**Figure 1.** Thrombin/plasma clotting time in the presence of anticoagulant analogues.

**Anticoagulant Activities of the Analogues.** The analogues including the photolysis products were evaluated for their ability to prolong the thrombin-initiated clotting time of citrated human plasma,[16] and the results were

(11) H. Bayley and J. R. Knowles, *Methods Enzymol.*, **46**, 69 (1977).
(12) V. Chowdhry and F. H. Westheimer, *Annu. Rev. Biochem.*, **48**, 293 (1979).
(13) E. H. Escher and G. Guillemette, *J. Med. Chem.*, **22**, 1047 (1979).
(14) E. Escher and R. Schwyzer, *FEBS Lett.*, **46**, 347 (1974).
(15) W. C. Ross, *Handb. Exp. Pharmacol.*, **38** (1), 33 (1974).
(16) E. J. W. Bowie, J. H. Thompson, P. Didisheim, and C. A. Owen, "Mayo Clinic Laboratory Manual of Hemostasis", Saunders, Philadelphia, 1971, p 75.

Table II. Relative Potency of Synthetic Analogues to Inhibit Coagulation

| no. | compd | (a) inhibn of thrombin time, % | (b) inhibn of thrombin-catalyzed amidolysis | (c) inhibn of fibrin polymerization, % | (d) rel potency of a/c |
|---|---|---|---|---|---|
| 1 | Gly-Pro-Arg-Pro | 100 | 0 | 100 | 1 |
| 2 | Chl-Gly-Pro-Arg-Pro | 0 | + | + | |
| 3 | Chl-Pro-Arg-Pro | 0 | + | + | |
| 4 | Chl-Arg-Pro | 0 | + | + | |
| 9 | Gly-Pro-Arg-Phe(4-$NO_2$) | 2 ± 0.1 | 0 | 0 | |
| 10 | Gly-Pro-Arg-Pro-Phe(4-$NO_2$) | 73 ± 8 | 0 | 88 | 0.83 |
| 14 | Gly-Pro-Arg-Phe(4-$NH_2$)[a] | 5 ± 1 | 0 | 22 | 0.22 |
| 15 | Gly-Pro-Arg-Pro-Phe(4-$NH_2$)[b] | 81 ± 8 | 0 | 105 | 0.77 |
| | Photoactivated Products of Nitrophenylalanine-Containing Analogues (350 nm, 30 min) | | | | |
| 16 | X-Pro-Arg-Pro | 0 | 0 | | |
| 17 | Gly-X-Arg-Pro | 10 ± 3 | 0 | | |
| 18 | Gly-Pro-X-Pro | 0 | 0 | | |
| 19 | Gly-Pro-Arg-X | 14 ± 0.8 | 0 | | |
| 20 | Gly-Pro-Arg-Pro-X | 156 ± 4 | 0 | 167 | 0.93 |

[a] The isolated side product had 52% activity in the thrombin time inhibitory assay and 57% activity in the fibrin polymerization inhibitory assay but did not inhibit thrombin-catalyzed amidolysis. [b] The isolated side product had 100% activity in the thrombin time inhibitory assay and 127% activity in the fibrin polymerization inhibitory assay but did not inhibit thrombin-catalyzed amidolysis.

compared with the reference Gly-Pro-Arg-Pro and expressed on a molar basis (Table II, column a). Although analogues modified at the N-terminal three residues (2–8 and 11–13) were inactive, significant prolongation of thrombin time was observed for **10**, **15**, **17**, and **19**, with the most active compound **20**, the photolysis product of Gly-Pro-Arg-Pro-Phe(4-$NO_2$), inducing even more prolonged clotting time than Gly-Pro-Arg-Pro (Figure 1). Since a large variety of peptide,[17,18] chloromethyl ketone,[19] and aldehyde[20,21] derivatives related to the sequence of Gly-Pro-Arg-Pro were shown to inhibit thrombin-catalyzed proteolytic reactions, it is conceivable that the anticoagulant activities of the above analogues could derive from either inhibition of thrombin generation of fibrin, the subsequent polymerization of fibrin, or a combination of both. In order to differentiate these possibilities, the analogues were further examined for their ability to inhibit thrombin-catalyzed proteolysis independent of blockade of fibrin polymerization. This is especially interesting in that a closely related chloromethyl ketone, D-Phe-Pro-Arg-$CH_2Cl$, was recently shown to irreversibly inactivate thrombin in the clotting and esterase assays to a significant and comparable degree.[22]

When the effect of compounds 1 to **20** on the thrombin-catalyzed amidolysis of a chromogenic substrate (Tos-Gly-Pro-Arg-nitroanilide) was evaluated, none of the analogues previously shown to prolong the thrombin clotting time of plasma affected the amidolytic activity of thrombin. However, the chlorambucil-containing peptides **2–4**, which were inactive in the thrombin-clotting time assay, inhibited thrombin amidolysis to different degrees (Figure 2). Preincubation of thrombin with these chlorambucil-containing peptides (15 min, 4 °C) did not alter its procoagulant activity in the fibrinogen-containing plasma but further inhibited amidolysis of the chromogenic substrate to a much greater degree. Since Chl-Pro-Arg-Pro (**3**) was much more active than Chl-Arg-Pro (**4**) and Chl-Gly-Pro-Arg-Pro (**2**), this selectivity suggests that inhibition of thrombin is initiated by the specific peptide structure of the inhibitors, followed by alkylation of the enzyme through the chlorambucil group.

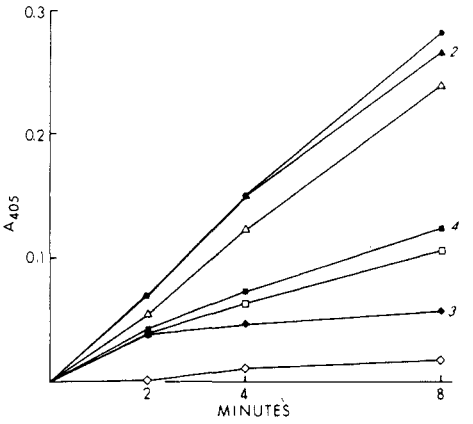


**Figure 2.** Inhibition of thrombin amidolysis (●–●) by Chl-Gly-Pro-Arg-Pro (**2**), Chl-Pro-Arg-Pro (**3**), and Chl-Arg-Pro (**4**) with (in open symbols) and without (in solid symbols) preincubation.

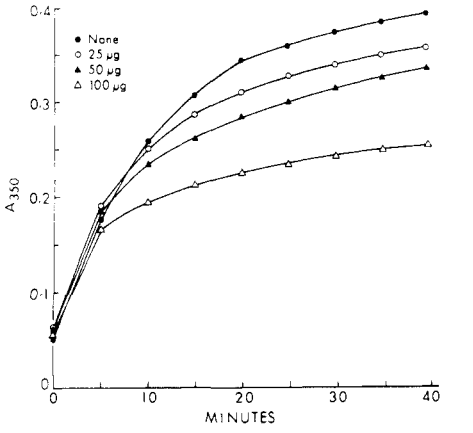


**Figure 3.** Reaggregation of fibrin monomers (0.207 μg) in the absence and presence of Gly-Pro-Arg-Pro (**1**).

(17) B. Blombäck, M. Blombäck, P. Olsson, L. Svendsen, and G. Aberg, *Scand. J. Clin. Invest.*, **24** (Suppl 107), 59 ((1968).
(18) L. C. Dorman, R. C. Chow, and F. N. Marshall, in "Chemistry and Biology of Peptides", J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 455.
(19) C. Kittner and E. Shaw, ref 18, p 129.
(20) S. Bajusz, E. Barabas, E. Szell, and D. Bagdy, in "Peptides: Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Eds., Ann Arbor Science Publishers, Ann Arbor, Mich., 1975, p 603.
(21) S. Bajusz, E. Barabas, P. Tolnay, E. Szell, and D. Bagdy, *Int. J. Pept. Protein Res.*, **12**, 217 (1978).
(22) C. Kettner and E. Shaw, *Thrombosis Res.*, **14**, 969 (1979).

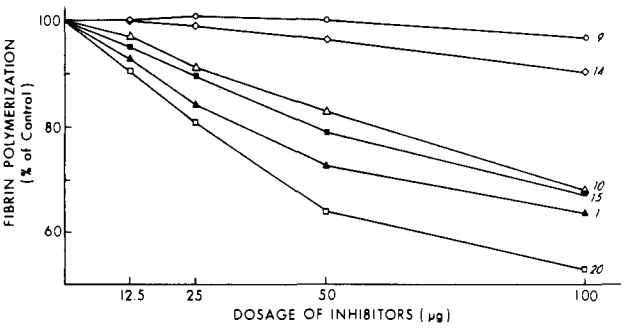


**Figure 4.** Inhibition of fibrin polymerization by anticoagulant analogues.

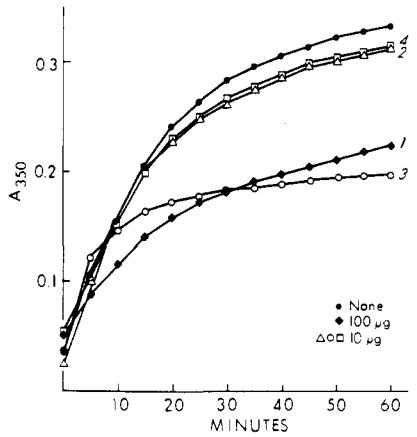


**Figure 5.** Reaggregation of fibrin monomers (0.215 μg) in the absence and presence of Gly-Pro-Arg-Pro (1), Chl-Gly-Pro-Arg-Pro (2), Chl-Pro-Arg-Pro (3), and Chl-Arg-Pro (4).

When a solution of preformed fibrin clot dissolved in a sodium bromide solution was diluted by an appropriate buffer, rapid reaggregation of fibrin monomer occurred, which leveled off after 20 min. In the presence of a polymerization inhibitor of the reversible type, such as Gly-Pro-Arg-Pro, fibrin aggregation was reduced in a dose-dependant manner, and the percentage of reduction at each dosage of the inhibitor was essentially the same between the time interval of 20 to 40 min (Figure 3). Thus, this value was used to evaluate the degree of inhibition of fibrin polymerization by the inhibitor. When tested in this system, compounds **5** to **9** and **11** to **13**, which did not prolong clotting, did not inhibit polymerization either, whereas all analogues causing prolonged clotting also inhibited polymerization of fibrin (Figure 4). Although these findings suggest that the anticoagulant effect of the inhibitors derives from their ability to prevent fibrin polymerization, potential action on other plasma components by Gly-Pro-Arg-Pro (**1**) and by the photolysis product **20** is indicated in that analogues **10**, **14**, and **15** showed significantly less anticoagulant effect than would be anticipated from their ability to inhibit fibrin aggregation (Table II, column d).

When the effect of the alkylating analogue Chl-Pro-Arg-Pro was evaluated, fibrin aggregation appeared normal initially but was followed by significant blockade of further polymerization, resulting in aggregation curves different in slope from those of the control and of the reversible inhibitor Gly-Pro-Arg-Pro (Figure 5). Although elimination of aggregation by the chlorambucil peptide was difficult to demonstrate due to the multiple sites involved in fibrin polymerization, preincubation of fibrin with this peptide reduced polymerization and produced a shallow aggregation curve (Figure 6). Since chlorambucil alone did not inhibit polymerization, whereas Chl-Pro-Arg-Pro

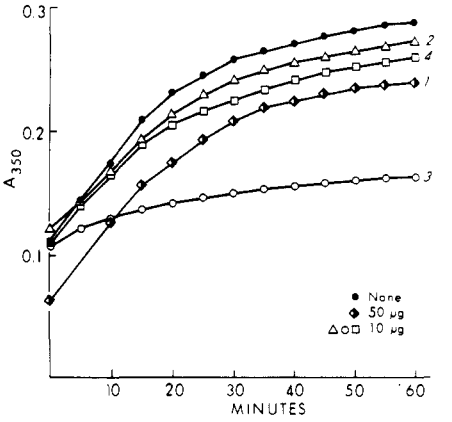


**Figure 6.** Reaggregation of fibrin monomers (0.212 μg) preincubated with buffer (●–●), Gly-Pro-Arg-Pro (1), Chl-Gly-Pro-Arg-Pro (2), Chl-Pro-Arg-Pro (3), and Chl-Arg-Pro (4).

was approximately 20 times more effective than Gly-Pro-Arg-Pro (Figures 5 and 6), the results suggest specific inhibition of fibrin polymerization.

## Conclusion

With the limited number of analogues prepared for this study, our results suggest that the structural requirements for the peptide alkylating probes to bind to fibrin and inhibit polymerization involve the $NH_2$-terminal Gly-Pro-Arg sequence of the fibrin $\alpha$ chain. A basic nitrogen with a sterically unhindered peptide backbone in the L side appeared to be important for position 1, as N-terminal acetylation or substitution by other L-amino acids produced inactive analogues. In addition, position 2 proline and position 3 arginine also had a unique function. The finding that their structural modifications resulted in loss of anticoagulant activity is in agreement with the observation that mutation of arginine in this region led to the incoagulable fibrinogen Detroit,[23] as well as with a recent report that similarly modified analogues did not bind to fibrinogen nor affect its clotting.[24]

On the other hand, our results indicate that residues following the Gly-Pro-Arg sequence are less critical for binding with fibrin, and structural modification in this region provided several active analogues, the most active of which was derived from C-terminal extension. These include the potential photoaffinity label Gly-Pro-Arg-Pro-Phe(4-$NO_2$), which was stable to laboratory light as well as to the rigorous conditions of solid-phase peptide synthesis, yet was readily photolyzable within 15 min of irradiation at 350 nm. The major action of the active analogues appears to be reversible inhibition of fibrin aggregation, which could be saturated but not overcome by large excess ($10^2$–$10^3$ molar excess over fibrin) of the inhibitors, possibly due to multiple binding sites involved in fibrin polymerization, as well as multiple effects of the inhibitors on clotting.

The potential for diverse actions of the inhibitors is shown in the chlorambucil-containing alkylating analogues, which inhibited thrombin-mediated amidolysis but not thrombin-initiated clotting. Furthermore, Chl-Pro-Arg-Pro (**3**) was a very potent inhibitor of fibrin polymerization. Whether the same sites normally bound to Gly-Pro-Arg-Pro are affected by this chlorambucil-containing analogue is difficult to evaluate by the anticoagulant screening tests

(23) M. Blombäck, B. Blombäck, E. F. Mammen, and A. S. Prasad, *Nature (London)*, **218**, 134 (1968).
(24) A. P. Laudano and R. F. Doolittle, *Biochemistry*, **19**, 1013 (1980).

used in this study. Nevertheless, the information derived from such structure–activity relationship analysis is of particular relevance to the further development of an affinity label in conjunction with high radioactivity that may permit the unambiguous identification of fibrin polymerization sites.

## Experimental Section

All chemicals were of reagent grade. Chlorambucil was obtained from Sigma Co., St. Louis, MO. $N^{\alpha}$-(*tert*-Butyloxycarbonyl)-*p*-nitrophenyl-L-α-alanine, Boc-Phe(4-$NO_2$), and other Boc amino acids were supplied by Bachem, Inc., Torrance, CA. The arginine side chain was protected with $N^{g}$-nitro group for compounds 1–7 and with $N^{g}$-tosyl group in compounds 9 and 10. SP-Sephadex C-25 was obtained from Pharmacia, Piscataway, NJ, and silica gel G (10–40 μm) was obtained from Merck AG. Homogeneity of the synthetic peptides was evaluated by thin-layer chromatography (TLC) on Merck precoated silica gel glass plates (type G60-F254) in different solvent systems and by thin-layer electrophoresis (TLE) on Eastman cellulose chromatogram sheets at 1000 V, pH 1.9 and 8.0. The products were detected by a combination of UV, fluorescamine and chlorox–tolidine sprays. Peptide samples were hydrolyzed for 24 h at 110 °C in 6 N HCl + 0.2% phenol containing norleucine as an internal standard in sealed tubes. Amino acid analyses were performed on a Beckman Model 119 analyzer equipped with a Beckman system AA computing integrator using the 4-h methodology.[25] Peptide content was calculated in terms of free peptide.

**Peptide synthesis** was carried out by using Merrifield's stepwise solid-phase method[26] as described previously.[27] The first Boc amino acid was attached to the chloromethylated polystyrene–1% divinylbenzene resin (1.04 mmol of Cl/g) using the triethylamine procedure. Deprotection of the amino group was accomplished with 25% trifluoroacetic acid in methylene chloride. Neutralization was carried out twice by using 5% diisopropylethylamine or triethylamine in methylene chloride. Coupling was performed using a threefold excess of Boc amino acid and dicyclohexylcarbodiimide (DCC). Reverse addition of DCC followed by Boc amino acid[28] was carried out for incorporation of the third residue, and 1-hydroxybenzotriazole was added during coupling of Boc-Gly. Coupling steps were monitored for completion by the Kaiser test,[29] and coupling was repeated when indicated. Termination of the remaining incomplete sequence was carried out with *N*-acetylimidazole[30] following the coupling step. The peptide was simultaneously removed from the resin and from the side-chain protecting group by liquid HF–anisole treatment at 0 °C for 1 h in a Kel-F/Teflon apparatus. The mixture was dried and washed with anhydrous ether, and the chlorambucil-containing peptides (2–4) were extracted from the resin with dimethylformamide (DMF) and vacuum evaporated to dryness, whereas other peptides were extracted from the resin with aqueous acetic acid and lyophilized. Except for the purification of the chlorambucil-containing peptides which was carried out by ascending dry column chromatography,[31] the compounds were purified on a column (1.2 × 67 cm) of SP Sephadex C-25 (40–120 μm) in 0.1 M AcOH and eluted with a two-component or a four-component gradient containing 0.1 M AcOH adjusted to different pH with $NH_4OH$. Eluate fractions were analyzed by UV, TLC, and TLE, and the appropriate fractions were combined and lyophilized. Catalytic hydrogenation of about 50 mg of the purified products (6–8) in 5 mL of DMF with 30 mg of 5% Pd/$BaSO_4$ under 1 atm of $H_2$ for 2 h[32] gave the unreacted material only. Further hydrogenation of these mixtures with 30 mg of 10% Pd/C in DMF under 2 atm of $H_2$ overnight produced the corresponding aminophenylalanine-containing peptides. When the crude products (500 mg) of 9 and 10 following HF cleavage and lyophilization were hydrogenated in 10 mL of 10% AcOH with 250 mg of 10% Pd/C under 2 atm of $H_2$ overnight, aminophenylalanine-containing peptides, as well as a mixture of two side products, were isolated, one of which was further identified by mass spectroscopy to contain a residue having a molecular weight corresponding to that of aminocyclohexylalanine (M + H of 313 vs. M + H of 307 for aminophenylalanine upon conversion into their respective diacetyl and isopropyl ester derivatives).[33] Identity of the peptides, including the aminophenylalanine analogues, was confirmed by amino acid analysis following acid hydrolysis of a sample. Dry column chromatography of the chlorambucil-containing peptides was performed on a vacuum-packed column (1 × 30 cm) of silica gel G (10–40 μm, containing 13% $CaSO_4$) and eluted with the upper phase of a mixture of 8:1:2:9 1-butanol–pyridine–acetic acid–water at a flow rate of 2–10 mL/h. Fractions (0.1 mL) were collected and analyzed by TLC. After the appropriate fractions were combined and evaporated to dryness, the peptides were extracted from $CaSO_4$ with DMF and filtered. The filtrate was evaporated to dryness, and the residue was recrystallized from a mixture of DMF–$CHCl_3$–$Et_2O$, giving the desired peptides with traces of contaminants.

**Irradiation experiments** were performed with eight lamps (RUL-3500 Å) in a Rayonet Type RS preparative photochemical reactor (RPR-208, South New England Ultraviolet Co., Middletown, Conn.) emitting light of wavelength around 350 nm. Further filtration through glass or fluid to narrow the bandwidth of the light was not attempted. In a typical experiment, the nitrophenylalanine-containing peptide in a glass test tube was dissolved in 1–2 mL of normal saline for UV measurement and for thrombin clotting and amidolytic assays. For fibrin reaggregation assays, the peptides were dissolved in 1–2 mL of phosphate buffer (80 mM, pH 6.3). Simultaneous irradiation of the peptide solutions in the test tubes for various intervals produced the test samples. For bioassays, freshly irradiated samples were prepared for each experiment, and the results from different experiments were highly reproducible when normalized against results from the reference compound, Gly-Pro-Arg-Pro.

For spectroscopic evaluation, the photolyzed solutions (2 mg/mL of saline) were diluted with normal saline to 0.02 mg/mL and analyzed by a Perkin-Elmer scanning double-beam spectrophotomer (Model 124, Coleman Instruments Division, Maywood, IL) equipped with a Perkin-Elmer recorder (Model 165). In a representative experiment, Gly-Pro-Arg-Pro-Phe(4-$NO_2$) in saline prior to photolysis exhibited the absorption maxima and minima ($\lambda_{max}$ or $\lambda_{min}$ in nanometer wavelength, with the molar extinction coefficient value, $\epsilon$, in parentheses) as following: $\lambda_{max}$ 205 (6210), $\lambda_{min}$ 231 (1290); $\lambda_{max}$ 242 (1610), $\lambda_{min}$ 256 (1340); $\lambda_{max}$ 282 (2010). Irradiation of this solution for 30 min reduced the absorption peak at 282 nm to a shoulder and depressed the trough at 265 nm further to $\lambda_{max}$ 205 (5680), $\lambda_{min}$ 232 (1610); $\lambda_{max}$ 240 (1710), $\lambda_{min}$ 262 (890); $\lambda_{max}$ 285 (1260).

**Thrombin time assays** were according to established procedure,[16] except that thrombin (bovine, Parke-Davis, Detroit, MI) was dispensed in saline or in imidazole-buffered saline so that a 100-μL solution produced a clotting time of 20 s upon mixing with 200 μL of citrated human plasma and 100 μL of saline at 37 °C. In the presence of peptide additives (in 100 μL of saline), thrombin time was evaluated as the time interval between addition of thrombin to the first appearance of the clot. Inhibitory activities were calculated on a molar basis by comparing the slopes of the dose–response curve for the compounds with the slope of the reference Gly-Pro-Arg-Pro, while the molarities of the peptide solutions, including the reference standard, were based on peptide content found from amino acid analysis of the peptide hydrolysates.

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For the chlorambucil peptides, the stock solutions (10 mg/mL of dimethyl sulfoxide) were diluted with normal saline immediately before the experiment in order to avoid potential undesirable interaction between chlorambucil and the buffer.[34] Preincubation experiments were carried out by mixing citrated human plasma or thrombin with the chlorambucil peptides for 15 min at 4 °C, and thrombin time for these analogues was evaluated as usual.

**Thrombin amidolytic assays** were carried out as described previously[35] by measuring at 405 nm (Gilford Model 240 spectrophotomer) the amount of chromophore generated by 0.5 unit of thrombin at 37 °C from the mixture of 0.25 mL of 1 mM Tos-Gly-Pro-Arg-*p*-nitroanilide acetate (Boehringer Mannheim), 0.25 mL of saline containing 2 mg/mL of peptide, and 1.5 mL of normal saline.

When preincubation was indicated, saline solutions containing the chlorambucil peptides were incubated with thrombin at 4 °C for 15 min, and the mixtures were added to the remaining buffer and chromogenic substrate solutions as usual.

**Fibrin reaggregation assays** were performed by diluting 25 μL of fibrin monomer solution (8.3 mg of protein/mL) derived from human fibrinogen (grade L, Kabi, Stockholm, Sweden) in 1 mL of phosphosphate buffer containing the tested peptide, and the resultant reaggregation was monitored at 350 nm (Gilford Model 240 spectrophotometer) as described by Laudano and Doolittle.[5] Different preparations of fibrin or different sets of experiments gave similar results when the concentrations of fibrin and inhibitors were held constant. Averaged values were used, and inhibitory activities were calculated on a molar basis by comparing the slopes of the linear part of the dose–response curve for the compounds with the slope of the reference.

When preincubation was indicated, 10 μL of the chlorambucil peptide (1 mg/mL of $Me_2SO$) and of Gly-Pro-Arg-Pro (5 mg/mL of $Me_2SO$) were diluted in 10 μL of 1 M NaBr/0.05 M NaOAc (pH 5.3) and mixed with 20 μL of fibrin monomers (10.6 mg of protein/mL) for 15 min at room temperature, followed by the addition of the phosphate buffer (1 mL) as usual.

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# Salicylamide Derivatives Related to Medroxalol with α- and β-Adrenergic Antagonist and Antihypertensive Activity[1]

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Analogues of medroxalol (1) were prepared in which the carboxamide function, the phenolic hydroxy group, and the aralkylamine side chain were modified. *N*-Alkyl-substituted amide analogues of 1 showed diminishing β-blocking activity with increasing steric bulk of the alkyl group. This allowed the conclusion that deactivation of the phenolic hydroxy group of 1 by the carbonyl group of the amide function is responsible for the β-adrenergic antagonistic properties of 1. This conclusion was strengthened by the finding that the phenolic *O*-methyl analogue 5-[2-[[3-(1,3-benzodioxol-5-yl)-1-methylpropyl]amino]-1-hydroxyethyl]-2-methoxybenzamide (**13**) was found to have enhanced β-adrenergic blocking activity. The finding that **13** also had decreased α-blocking activity compared to 1 indicated that the phenolic hydroxy group of 1 enhances α-adrenergic antagonism. The finding that 1 and **13** showed such a large difference in relative α- to β-blocking potency while exhibiting approximately equal antihypertensive activity in spontaneously hypertensive rats was surprising. It indicated that pharmacologic properties other than α- and β-adrenergic blockade may contribute to the antihypertensive activity of medroxalol. One of the analogues in which the aralkylamine side chain of 1 was replaced by a fragment of a known α-adrenergic receptor blocker, 2-hydroxy-5-[1-hydroxy-2-[4-(2-methylphenyl)-1-piperazinyl]ethyl]benzamide (**22**), showed an interesting pharmacologic profile of potential therapeutic usefulness.

Adrenergic α-receptor antagonists were reported years ago to be useful in the treatment of hypertension.[2-4] However, side effects, such as postural hypertension, palpitations, and failure of sexual function, discouraged their use.[5] More recently, β-adrenergic receptor antagonists have been shown to be effective in the treatment of hypertension and they are now widely used for this indication.[6,7] Their potential to depress myocardial function or to precipitate bronchial asthma, however, limits their use.[7] Attempts to improve antihypertensive therapy by combining an α-adrenergic antagonist with a β-adrenergic antagonist have resulted in good control of blood pressure with few side effects in some studies[8,9] but with a discouraging incidence of side effects in others.[10,11] The

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