Registry No. 1, 69256-17-3; 2, 114652-73-2; 3, 114674-41-8; 4, 114652-74-3; 5, 114652-75-4; 6, 114652-76-5; 7, 114652-77-6; 8, 104904-83-8; 9, 104904-85-0; 10, 69123-98-4; 11, 114652-78-7; n12, 114652-79-8; 13, 114652-80-1; 14, 114652-81-2; 15, 24523-96-4; 17, 114652-82-3; 18, 83374-60-1; 19, 114652-83-4; 20, 114674-42-9; 21, 114652-84-5; 22, 114652-85-6; 23, 114652-86-7; 24, 114652-87-8; TS, 9031-61-2; 3-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-D-arabinofuranosyl bromide, 92283-83-5.

Substrate Analogue Inhibitors of the IgA1 Proteinases from *Neisseria gonorrhoeae*

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Substrate analogues based on the amino acid sequence of the hinge region of human IgA1 around the cleavage site of the IgA1 proteinases secreted by *Neisseria gonorrhoeae* are competitive inhibitors of these enzymes. The octapeptide Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser, which occurs between residues 233 and 240, has an IC_{50} value of 0.26 mM for the type 1 proteinase and 0.50 mM for the type 2 enzyme. Acetylation of the octapeptide N-terminal amino group lowers affinity for the type 1 proteinase sixfold but does not change binding to the type 2 enzyme. Amidation of the C-terminal carboxyl group does not change binding to the type 1 proteinase but improves IC_{50} for the type 2 enzyme. Simultaneous blockade of both the N- and C-termini drastically lowers affinity of the octapeptide for both proteinases. Sequential replacement of the hydroxy amino acids in the blocked octapeptide with cysteine yields a series of inhibitors that generally bind to the neisserial IgA1 proteinases as well as or better than the unblocked octapeptide. The most effective inhibitor contains a cysteine residue at position 6 (P_3') and has an IC_{50} value for the type 2 IgA1 proteinase of 50 μM. Dimerization of the cysteine-containing octapeptides significantly diminishes inhibitory properties. The substrate analogues described here are the first synthetic inhibitors of the neisserial IgA1 proteinases to be reported.

Pathogenic members of the genera *Neisseria*, *Hemophilus*, and *Streptococci* secrete highly specific proteolytic enzymes (EC 3.4.24.13), which inactivate human IgA1 by cleavage of hinge-region peptide bonds on the C-terminal side of specific prolyl residues. Cleavage occurs within a 16-residue sequence formed by duplication of the octapeptide Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser.[1] The only known substrate for these proteinases is IgA1 from humans and great apes,[2] although isolated human IgA1 heavy chain (α-chain) is reported to be slowly cleaved by an IgA1 proteinase from *Hemophilus*.[3]

Strains of *N. gonorrhoeae* produce one of two related proteinases (type 1 or type 2) that cleave IgA1 at slightly different positions (Figure 1). The IgA1 proteinase secreted by *S. sanguis* is inhibited by EDTA;[4] while an IgA1 proteinase isolated from *B. melaninogenicus* is blocked by reagents that inhibit cysteine proteases.[5]

Previous research[6,7] indicated that synthetic peptides homologous with the amino acid sequence of IgA1 between residues 225 and 240 could inhibit the type 2 proteinase from *N. gonorrhoeae*. IC_{50} values of a more complete set of substrate analogue inhibitors for both the type 2 and type 1 proteinases are reported here.

Results

The amino acid sequence and IC_{50} values of the substrate analogue inhibitors for the neisserial proteinases are given in Table I. These values are different from those given in a preliminary report[7] and reflect development of a more consistent assay. Previously reported IC_{50} values from this laboratory should be viewed with this in mind.

Both the hexadecapeptide (HRP-1) and the octapeptide (HRP-2) inhibit the IgA1 proteinases from *N. gonorrhoeae* in the high micromolar range. Amidation of the C-terminal carboxyl group in the octapeptide (HRP-75) slightly decreases binding to the type 1 enzyme while increasing affinity for the type 2 proteinase. Acetylation of the N-terminus does not markedly change affinity for the type 2 proteinase but increases the IC_{50} value for the type 1 enzyme sixfold. Simultaneous blockade of both the N- and C-terminal residues (HRP-25) increases the IC_{50} value

Table I. Inhibition of the Neisserial IgA1 Proteinases

		IC_{50}, mM	
		type 1	type 2
HRP-1	Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser	0.31	[a]
HRP-2	Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser	0.26	0.50
HRP-25	Ac———NH_2	9.35	6.68
HRP-59	Ac———	1.57	0.51
HRP-75	———NH_2	0.40	0.18
HRP-18	(Ac———Cys-NH_2)$_2$	[b]	3.30
HRP-19	(Ac——— Cys ——NH_2)$_2$	5.15	[b]
HRP-20	(Ac——— Cys ———NH_2)$_2$	0.20[c]	0.17[c]
HRP-21	(Ac-Cys ———NH_2)$_2$	1.81	3.45
HRP-61	Ac———Cys-NH_2	1.03	0.49
HRP-62	Ac——— Cys ——NH_2	0.20	0.05
HRP-63	Ac——— Cys ———NH_2	ND[d]	ND[d]
HRP-64	Ac-Cys———NH_2	0.31	0.12

[a] 19% inhibition at 0.30 mM. [b] 0% inhibition at >2 mM. [c] Assayed in 40% TFE. [d] ND: not determined because of poor solubility. Addition of adequate TFE to solubilize HRP-63 denatures the IgA1 proteinases.

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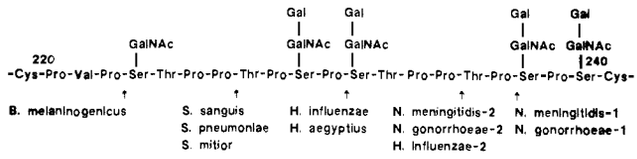

Figure 1. Sequence of human IgA1 with the known cleavage sites of the IgA1 proteinases.

40-fold for the type 1 proteinase and 13-fold for the type 2 enzyme.

The peptide inhibitors do not appear to be substrates for the IgA1 proteinases. Three lines of evidence support this contention. First, the rate of production of the IgA1 F_{AB} remains constant throughout the assay period for all inhibitors. If the inhibitor were degraded, the rate of F_{AB} production should increase.

Second, all radioactivity is recovered at the elution time for the intact starting material when a mixture of the type 1 IgA1 proteinase and HRP-1, HRP-2, or HRP-59 are subjected to HPLC after 1-h digestion. Under these conditions IgA1 is almost completely cleaved.

Last, Edman degradation of a mixture of HRP-59 and the type 1 proteinase does not show any free amino acid during the first cycle. If cleavage of the acetylated octapeptide had occurred, a free N-terminal amino acid should have been generated. At cycles 2–3 small amounts of proline were observed, but these were less than 1% of the peptide subjected to sequence analysis.[8,9]

Sequential replacement of each hydroxy amino acid in the blocked octapeptide with cysteine yields a series of monomers (HRP-61–HRP-64), which inhibit both neisserial IgA1 proteinases much more effectively than does HRP-25. IC_{50} values for inhibition of the type 1 proteinase are between 0.2 and 1 mM, which is similar to that observed for unblocked octapeptide (HRP-2). Three of the cysteine-containing monomers inhibit the type 2 proteinase as well as or better than HRP-2. HRP-62, which has a sulfhydryl group at P_3',[10] has an IC_{50} value of 50 μM. One of the octapeptides, HRP-63, is too insoluble to be assayed under physiologic conditions.

Dimerization of the cysteine-containing octapeptides drastically lowers affinity for the IgA1 proteinases from *N. gonorrhoeae*. The best inhibition by a dimeric substrate analogue is observed when the disulfide linkage occurs through residues adjacent to the N-terminus or C-terminus. Inhibitors that have the disulfide linkage at the P_3' site do not inhibit the appropriate proteinases.

One of the dimeric octapeptides, HRP-20, is too insoluble for assay in completely aqueous buffers. The inhibitor is soluble in buffers containing 40% trifluoroethanol (TFE), and IC_{50} values of 0.20 and 0.17 mM were measured for the type 1 and type 2 enzymes, respectively.

The substrate analogs presented here do not inhibit the IgA1 proteinases from *Streptococcus sanguis* or *Hemophilus influenzeae* at submillimolar levels.

Magnetic Resonance Studies. The temperature dependence of the chemical shift of the four amide protons in both HRP-25 and HRP-2 in aqueous solution (pH 7.4) is 6–7 ppb/deg (Figure 3). This is within the normal range for solvent-exposed protons.[11] None of the amide protons

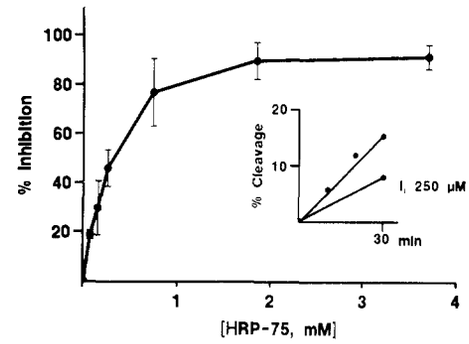

Figure 2. Inhibition of the type 1 IgA1 proteinase from *N. gonorrhoeae* by HRP-75. Inset shows the rates of cleavage of IgA1 in the presence and absence of inhibitor.

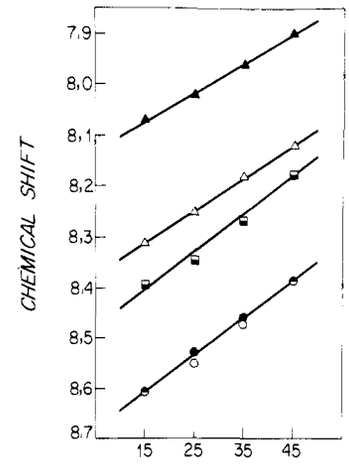

Figure 3. Chemical shift of the amide protons of HRP-2 (filled symbols) and HRP-25 (open symbols) as a function of temperature, 0.01 M Na phosphate buffer, 0.15 M NaCl, pH 7.40.

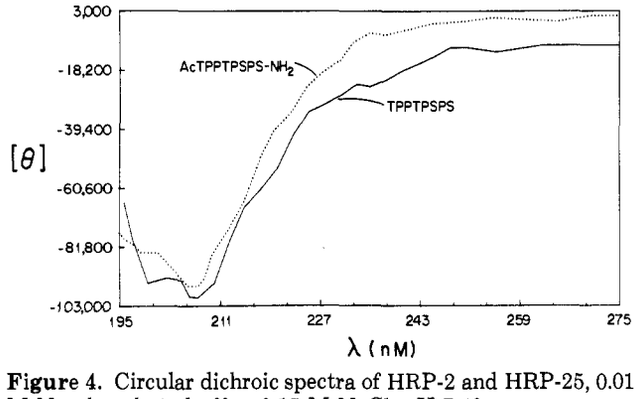

Figure 4. Circular dichroic spectra of HRP-2 and HRP-25, 0.01 M Na phosphate buffer, 0.15 M NaCl, pH 7.40.

appear to be involved in H bonds that could stabilize a solution conformation.

Circular Dichroism Studies. The circular dichroic spectra of HRP-2 and HRP-25 (Figure 4) in aqueous solution (pH 7.4) are characteristic of random coils.[12]

Discussion

IgA1 proteinases produced by *N. gonorrhoeae* are not blocked by conventional protease inhibitors such as EDTA, diazonorleucine ethyl ester, or proteinase inhibitors derived from fungi.[2] The substrate analogues reported here are the first compounds, other than antibodies,[13] to inhibit the neisserial IgA1 proteinases.

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Similar IC_{50} values were obtained for the hexadecapeptide (HRP-1) and the octapeptide (HRP-2) (Table I). Doubling the length of the peptide chain to include the entire hinge region sequence does not improve inhibitory properties.

Blockade of the N-terminal amino group of the octapeptide lowers affinity for the type 1 proteinase without changing binding to the type 2 enzyme. Conversion of the C-terminal carboxyl to the carboxamide does not affect affinity for the type 1 proteinase but improves inhibition of the type 2 enzyme.

Simultaneous blockade of both the N- and C-terminal groups yields much poorer inhibitors of both types of IgA1 proteinase. These results are unexpected since the IgA1 proteinases are endopeptidases and should not recognize a charged group at the peptide termini. In addition, neither CD studies, which yield superimposable spectra for HRP-2 and HRP-25 (Figure 3), nor temperature-shift NMR experiments, which do not show the presence of intramolecular H bonds (Figure 4), indicate the existance of a solution conformation that could explain the marked loss of affinity for the niesserial proteinases.

Replacement of the hydroxy amino acids in the blocked octapeptide with cysteine markedly increases affinity for both the type 1 and type 2 IgA1 proteinases. IC_{50} values of the cysteine-containing monomers for the type 1 proteinase are similar to those of HRP-2 and about 30 times better than the parent blocked octapeptide (HRP-25). A similar, but more marked, increase in inhibitory properties is seen for the type 2 proteinase. The best inhibitor (HRP-62) binds about 150 times better than HRP-25. The improvement in binding may be due to interaction between the cysteine residue and the S_3' binding site.

Dimerization of the cysteine-containing octapeptides yields, at best, only marginal inhibitors of both types of IgA1 proteinase. The lack of improvement of inhibitory properties on dimerization is consistent with published models showing that the neisserial IgA1 proteinases bind to only one of the hinge-region peptide chains at a time.[14] Loss of affinity is most marked when the amino acid residues involved in the disulfide link appear at the P_3' site. HRP-18, for example, does not inhibit the type 1 proteinase, and HRP-19 does not block the type 2 enzyme. This may indicate that S_3' in both IgA1 proteinases is restricted and that the accompanying peptide chain in the dimeric inhibitors prevents binding to the enzyme.

Experimental Section

IgA1 Preparation. Monoclonal, monomeric human serum IgA1 was purified[15] and extrinsically labeled with ^{125}I by the chloramine-T method[16] (8 Ci/μg).

***N. gonorrhoeae* Type 1 and Type 2 IgA1 Proteinase.** IgA1 proteinases were partially purified from a cell-free cultures of *N. gonorrhoeae* grown in defined medium[17] for 16 h at 37 °C, as previously described.[13] Enzyme preparations are stored at −70 °C until use.

Inhibition of IgA1 Proteinase. Solutions of lyophilized peptides were dissolved in 0.05 M Tris buffer (pH 7.5), the pH was adjusted to 7.5 with 1 N NaOH, and the solution was diluted with Tris-HCl buffer (0.05 M, pH 7.5).

To quantitate cleavage of IgA1, 25 μL of inhibitor solution was first incubated for 30 min at 37 °C with the proteinase. Twenty-five microliters of [^{125}I]IgA1 mixed with 50 μg of unlabeled IgA1 was added, and at 10-min intervals, 10 μL of the reaction mixture was removed and added to 100/μL of solution containing SDS (1.25%) and 2-mercaptoethanol (0.0125%). This was boiled for 5 min and electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. After electrophoresis, the gels were fixed and stained with 0.5% Coomassie Blue in 25% 2-propanol–10% acetic acid, dried, and autoradiographed for 2 h at −70 °C with use of Kodak Xomat XAR-5 film, with screen. The autoradiograph was used as a guide to cut the gels into segments containing Fd, Fc, and uncleaved α-chain. Counts in each segment were converted into the percent IgA1 cleaved as described.[13]

The percent cleavage of IgA1 samples taken at 10-min intervals over a period of 30 min was plotted as a function of time, and the rate of hydrolysis (v) was calculated from the slope of this line (Figure 2). The ratio of the rates of hydrolysis in the presence and absence of the inhibitors multiplied by 100 is used as the percent inhibition of the IgA1 proteinase. At least two separate determinations of v were made with each concentration of inhibitor.

Peptide Synthesis. *tert*-Butyloxycarbonyl amino acids were purchased from Peninsula Laboratories (San Mateo, CA). Side-chain protecting groups are cysteine, 4-methylbenzyl; serine, benzyl; and threonine, benzyl. Tritiated proline was prepared by reaction of labeled proline (New England Nuclear, Boston, MA), diluted to approximately 0.1 Ci/mol with unlabeled proline (Eastman, Rochester, NY), with di-*tert*-butyl dicarbonate (Tridom, Hauppage, NY). Et_3N was purchased from Pierce Chemical Co. (Rockford, IL). Both CF_3COOH and DCC (dicyclohexylcarbodiimide) were obtained from Aldrich (Milwaukee, WI). Dichloromethane (Dow, Midland, MI) was redistilled before use. The support used for solid-phase synthesis was either LS-601 Merrifield Resin containing 0.75 mM/Cl^- per g (Laboratory Systems, San Mateo, CA) or *p*-methylbenzhydrylamine resin containing 0.35 mmol of NH_2/g (Geneva, Hygiene, CO). Dioxane (Fisher, Medford, MA) was redistilled from Na^0; 6 N HCl–dioxane was prepared by bubbling electronic grade HCl gas (Matheson, Gloucester, MA) through dioxane cooled in an ice bath. Once a saturated solution was obtained, the reagent was diluted to 6 N and stored in a closed container. Other reagents were of analytical grade.

Synthetic reactions were performed in 60-mL polypropylene syringes fitted with a frit (70 μm porosity, Bolab, Derry, NH) by using apparatus and techniques previously described.[18] Completeness of coupling was judged with the ninhydrin test[19] by viewing the beads on filter paper at 40X under a dissecting microscope.

Peptides were hydrolyzed at 105 °C for 24 h under vacuum in 6 N HCl. Amino acid analyses were performed with a Beckman D-6000 analyzer.

Free sulfhydryl groups were determined with Ellman's reagent.[20]

UV spectra were obtained on an HP8450A spectrophotometer equipped with an HP7445A plotter (Hewlett Packard, Palo Alto, CA). High-pressure liquid chromatography was done with a 410 system (Beckman Instruments, Palo Alto, CA), eluted through a 178.32 flow cell (Helma, Jamaica, NY). Data was collected and converted into an elution profile with an HP-216 computer. HPLC purifications were done on a Beckman ODS column (1 × 25 cm) with a gradient of CH_3CN–0.2% CF_3COOH in 0.2% CF_3COOH solution.

Threonylprolylprolylthreonylprolylserylprolylserylthreonylprolylprolylthreonylprolylserylprolylserine (HRP-1). The chemical synthesis of HRP-1 has been described previously.[6] Physical properties of the hexadecapeptide are given in Table II.

Threonylprolylprolylthreonyl[^3H]prolylserylprolylserine (HRP-2). Three grams of the polymer containing 0.21 mM serine was used for the solid-phase synthesis of HRP-2. The C-terminal seryl residue was attached with use of a twofold excess of the protected amino acid and a 1.8-fold excess of Et_3N. The remainder

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Table II. Physical Properties of the IgA1 Substrate Analogue Inhibitors

compd	amino acid composition	R_f T2	R_f T4	R_f T6	specific activity, Ci/mol	ϵ_{220} (M AcOH)	t_R,[a] min
HRP-1	Ser, 4.04; Thr, 3.74; Pro, 8.21				0.238	30480	18.3
HRP-2	Ser, 2.38; Thr, 1.95; Pro, 3.78	0.12	0.39	0.00	0.087	13100	16.7[a]
HRP-18	Ser, 1.11; Thr, 2.02; Pro, 3.98; Cys, 0.89	0.15	0.52	0.00	0.058	18600	16.8
HRP-19	Ser, 1.09; Thr, 2.00; Pro, 3.94; Cys, 0.96	0.08	0.50	0.00	0.064	18600	16.1
HRP-20	Ser, 2.01; Thr, 1.00; Pro, 4.00; Cys, 1.00	0.08	0.50	0.00	0.042	18700	17.3
HRP-21	Ser, 1.97; Thr, 1.01; Pro, 4.04; Cys, 0.97	0.09	0.51	0.00	0.068	18700	17.7
HRP-25	Ser, 1.79; Thr, 1.89; Pro, 4.14	0.10	0.55	0.00	0.044	13100	22.5[a]
HRP-59	Ser, 2.03; Thr, 1.92; Pro, 4.04	0.10	0.49	0.00	0.077	20910	14.5
HRP-61							15.8
HRP-62							15.7
HRP-63							15.8
HRP-64							16.0
HRP-75	Ser, 2.17; Thr, 2.00; Pro, 3.83	0.15	0.51	0.00	0.040	15330	13.5

[a] 0–100% CH_3CN–0.2% CF_3COOH–0.2% CF_3COOH over 30 min. 1 × 25 cm ODS column.

of the amino acid residues were added sequentially by using the standard coupling cycle with a fivefold excess of protected amino acid and DCC. Each coupling was repeated before testing with ninhydrin; 6 N HCl–dioxane was used to remove the Boc (*tert*-butyloxycarbonyl) group. The dried peptidyl-resin (3.28 g) was treated with 33 mL of HF–10% (v/v) anisole for 1 h at 0 °C. After evaporation of the HF and anisole, the cleaved resin was transferred to a porosity D sintered-glass funnel (Ace, Vineland, NJ) with 30 mL of cold AcOEt, and the resin was sequentially extracted with 100-mL aliquots of 1%, 5%, 10%, and 25% CH_3COOH solution. Radioactivity (98.8%) was found in the 1% extract, which was lyophilized (82%). Next, 134.9 mg of crude peptide was dissolved in 12 mL of 10% CH_3COOH solution and gel filtered on Sephadex G-25 (2.5 × 110 cm, 76 mL/h). Fractions eluting between 396 and 456 mL were pooled and lyophilized to yield 120.2 mg (89.1%) of homogeneous HRP-2.

Acetylthreonylprolylprolylthreonylprolylseryl[^{3}H]prolylserinamide (HRP-25). Three grams of *p*-methylbenzhydrylamine polymer containing 1.0 mmol of serine was used in the solid phase synthesis of HRP-25. After addition of the N-terminal threonyl residue, the peptide was acetylated (Ac_2O–Et_3N). On completion of the synthesis, 3.00 g of the peptidyl resin was cleaved and extracted as described for HRP-2. Radioactivity (99.7%) was found in the 1% CH_3COOH extract; 775.3 mg of the crude peptide was gel filtered on Sephadex G-25, and fractions eluting between 396 and 455 mL pooled and lyophilized to yield 567.2 mg (73.2%) of homogeneous HRP-25.

Acetylthreonylprolylprolylthreonylprolylseryl[^{3}H]prolylserine (HRP-59). A 3.11-g sample of the polymer containing 0.21 mmol of serine was used in the solid-phase synthesis of HRP-59. On completion of the synthesis and cleavage, radioactivity (94.3%) was found in the 1% extract, which was lyophilized. The crude peptide was gel filtered on Sephadex G-25 (1.1 × 110 cm, 13 mL/h). Fractions eluting between 56 and 65 mL were pooled and lyophilized to yield the homogeneous compound.

Threonylprolylprolylthreonylprolylseryl[^{3}H]prolylserinamide (HRP-75). Three grams of the *p*-methylbenzhydrylamine polymer containing 1.35 mmol of serine was used in the solid-phase synthesis of HRP-75. On completion of the synthesis and cleavage, radioactivity (86.6%) was found in the 1% CH_3COOH extract, which was lyophilized. The crude peptide was gel filtered on Sephadex G-25, and fractions eluting between 43 and 55 mL was pooled and lyophilized to yield the homogeneous compound.

Bis(acetylthreonylprolylprolylthreonylprolylseryl[^{3}H]prolylcysteinamide) (HRP-18). The solid-phase synthesis of HRP-18 and HRP-61 was begun with 2.30 g of *p*-methylbenzhydrylamine polymer containing 0.75 mmol of cysteine. After cleavage with HF–10% anisole–1% 2-mercaptoethanol, radioactivity (97.8%) was found in the 1% acetic acid extract, which was made 0.01 M in phosphate. The pH of the solution was raised to 8.0 with 10 N NaOH, and the solution was stirred for 72 h and lyophilized; 407.6 mg (49.1%) of the crude extract was dissolved in 10% CH_3COOH solution and gel filtered on Sephadex G-25 (2.5 × 110 cm, 68.0 mL/h). Fractions eluting between 359 and 411 and 443 and 502 mL were pooled independently and lyophilized. Material in the first peak was shown to be the dimer (159.1 mg, 39.0%) while that in the second peak was monomer (HRP-61) (120.3 mg, 29.5%).

Acetylthreonylprolylprolylthreonylprolylseryl[^{3}H]prolylcysteinamide (HRP-61). HRP-61 was prepared by reducing HRP-18 dissolved in 50 mM Tris buffer (pH 8.5) with a fivefold excess of DTT under N_2. After 1 h at room temperature, the reaction mixture was acidified with concentrated HCl (pH 2) and DTT extracted with AcOEt, and the aqueous phase was subjected to HPLC. CH_3CN was removed from fractions containing peptide by rotary evaporation, and the material was lyophilized. HPLC was used to show that the lyophilized monomer had not reconverted into the dimer before assay.

Bis(acetylthreonylprolylprolylthreonylprolylcysteinyl-[^{3}H]prolylserinamide) (HRP-19). Three grams of the *p*-methylbenzhydrylamine polymer containing 1.0 mmol of serine was used for the synthesis of HRP-62 and HRP-19. Synthesis and acetylation were completed as described previously, and, after HF cleavage, the peptide was extracted into 1% CH_3COOH (98.5%), neutralized (pH 8.0), air-oxidized, and lyophilized to yield 490.8 (59.1%) mg of crude material. This was dissolved in 10% acetic acid solution and gel filtered on Sephadex G-25. Fractions eluting between 338 and 402 mL were pooled and lyophilized to yield 553.3 mg (112.7%) of the homogeneous dimer.

Acetylthreonylprolylprolylthreonylprolylcysteinyl[^{3}H]-prolylserinamide (HRP-62). HRP-62 was prepared by reducing HRP-19 dissolved in 50 mM Tris buffer (pH 8.5) with a fivefold excess of DTT under N_2. After 1 h at room temperature, the reaction mixture was acidified with concentrated HCl (pH 2) and extracted with AcOEt, and the aqueous phase was subjected to HPLC to yield the homogeneous monomer as described for HRP-61.

Bis(acetylthreonylprolylprolylcysteinylprolylseryl[^{3}H]-prolylserinamide) (HRP-20). The solid-phase synthesis of HRP-20 and HRP-63 was begun with 3.00 g of the *p*-methylbenzhydrylamine polymer containing 1.0 mmol of serine. On completion of synthesis, the peptidyl resin was cleaved with HF and extracted; 87.4% of the radioactivity was found in the 1% extract, which was neutralized and converted to the dimer by air oxidation. A 541.5-mg portion (66.4%) of the crude peptide was purified by gel filtration on Sephadex G-25 (2.5 × 100 cm, 72 mL/h). Fractions eluting between 336 and 390 mL were pooled and lyophilized to yield 411.4 mg (76.0%) of homogeneous dimer.

Acetylthreonylprolylprolylcysteinylprolylseryl[^{3}H]prolylserinamide (HRP-63). HRP-63 was prepared by reducing HRP-20 dissolved in 50 mM Tris buffer (pH 8.5) with a fivefold excess of DTT under N_2. After 1 h at room temperature, the reaction mixture was acidified with concentrated HCl (pH 2) and extracted with AcOEt, and the aqueous phase was subjected to HPLC to yield the homogeneous monomer as described for HRP-61).

Bis(acetylcysteinylprolylprolylthreonylprolylseryl[^{3}H]-prolylserinamide) (HRP-21). Synthesis of HRP-21 was begun with 3.00 g of the *p*-methylbenzhydrylamine polymer containing 1.0 mmol of serine. Synthesis, acetylation, global deprotection, and extraction were completed by using methods described for preparation of other inhibitors; 74.4% of the radioactivity was

found in the 1% CH_3COOH extract, which was neutralized, oxidized, and lyophilized. A 338.9-mg portion (41.5%) of the crude extract was purified by gel filtration on Sephadex G-25, and fractions eluting between 342 and 396 mL pooled and lyophilized to yield 312.4 mg (92.2%) of homogeneous dimer.

Acetylcysteinylprolylprolylthreonylprolylseryl[^{3}H]prolylserinamide (HRP-64). HRP-64 was prepared by reducing HRP-21 dissolved in 50 mM Tris buffer (pH 8.5) with a fivefold excess of DTT under N_2. After 1 h at room temperature, the reaction mixture was acidified with concentrated HCl (pH 2) and extracted with AcOEt, and the aqueous phase was subjected to HPLC as described for HRP-61.

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Registry No. HRP-1, 114791-17-2; HRP-2, 91417-20-8; HRP-2 (tritium labeled), 114819-71-5; HRP-18, 91417-25-3; HRP-19, 91417-24-2; HRP-20, 91417-23-1; HRP-21, 91417-22-0; HRP-25, 91417-21-9; HRP-59, 114819-68-0; HRP-61, 114819-70-4; HRP-62, 114791-18-3; HRP-63, 114791-19-4; HRP-64, 114791-20-7; HRP∞75, 114819-69-1; HRP-75 (tritium labeled), 114791-21-8.

Soft Drugs. 7. Soft β-Blockers for Systemic and Ophthalmic Use

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The "inactive metabolite approach" was used to design a series of "soft" drugs derived from the acidic metabolite of metoprolol. Pharmacokinetic and pharmacodynamic properties of these novel "soft" β-adrenoceptor antagonists were determined: half-lives in human blood ranged from 5 to 754 min. The rates of in vivo disappearance of representative slow, medium, and fast hydrolyzing esters were determined in rats. In each case rapid and quantitative conversion to the corresponding free acid was observed. This suggests a facile, one-step degradation to the predicted major metabolite. The compounds were tested for their ability to decrease intraocular pressure in a rabbit model. Five of the new compounds exerted an ocular hypotensive action comparable to or greater than that of the reference compound, timolol maleate, and with a prolonged duration of action in some cases. In contrast the new compounds showed reduced and shorter duration systemic activity. The adamantylethyl ester emerges as a potentially effective antiglaucoma agent with significantly improved site-specific activity.

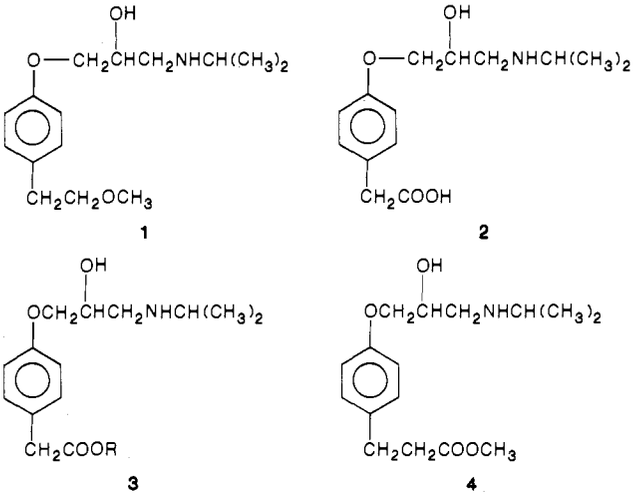
We have recently reported[3] the application of the "inactive metabolite approach"[4-6] for the design of a new class of soft β-adrenoceptor antagonists derived from metoprolol (1). According to this approach, a set of esters of the acidic metabolite **2** of metoprolol were synthesized and tested as short-acting β-blockers. The principles of the design process were previously described;[3-5] an inactive hydrophilic metabolite is converted to active derivatives which will be deactivated in a predictable and controllable way by a one-step metabolic conversion to the known inactive metabolite.

Earlier results[3] suggested that the alcohol used to derivatize **2** has a significant effect on the extent and duration of activity of the esters, whereas the rates of hydrolytic (esterase) deactivation did not seem to affect these important parameters. Thus, the more lipophilic cyclohexyl and 3,3,5,5-tetramethylcyclohexyl esters showed the highest β-blocking potency in dogs and rats, despite the fact that these compounds are rapidly hydrolyzed ($t_{1/2}$ = 1 min) in plasma.

In the present study we have further concentrated our efforts on the ester functions: a set of lipophilic alcohols were incorporated in the molecule as indicated by the general formula **3**.

The potential importance of the soft drug concept was recognized by others: a short-acting β-blocker (the methyl ester of the homologue of **2**, Esmolol, **4**) was developed.[7] This compound **4** was also included in our studies for comparison.

All of these compounds **3a–g** were tested first for their in vitro rates of hydrolysis in human blood and for their in vivo rates of disappearance (esterase hydrolysis) in rats. Pharmacological activity studies on **3a–g** showed the effects of these compounds on resting heart rate and on



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