employed by dissolving 1–3 in 10 mM aqueous NaOH solution.

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References and Notes

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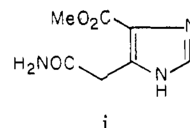
(4) 7*H*-Pyrrolo[2,3-*d*]pyrimidines (7-deazapurines); see, for example, J. Davoll, *J. Chem. Soc.*, 131 (1960), and R. L. Tolman, R. K. Robins, and L. B. Townsend, *J. Am. Chem. Soc.*, **91**, 2102 (1969).
(5) 1*H*-Imidazo[4,5-c]pyridines (3-deazapurines); see, for example, P. D. Cook, R. J. Rousseau, A. M. Mian, P. Dea, R. B. Meyer, Jr., and R. K. Robins, *J. Am. Chem. Soc.*, **98**, 1492 (1976).
(6) 1*H*-Imidazo[4,5-*b*]pyridines (1-deazapurines); see, for example, T. Itoh, J. Inaba, and Y. Mizuno, *Heterocycles*, **8**, 433 (1977).
(7) For the clinical effectiveness of 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol) as a xanthine oxidase inhibitor (which can also serve as a standard of comparison for the therapeutic usefulness of other potential inhibitors), see D. M. Woodbury and E. Fingl in "The Pharmacological Basis of Therapeutics", L. S. Goodman and A. Gilman, Ed., 5th ed, Macmillan, New York, N.Y., 1975, pp 352–355.
(8) A preliminary account of the preparation of 1 has been reported: S. W. Schneller and R. S. Hosmane, *J. Heterocycl. Chem.*, 14, 1291 (1977).
(9) G. A. Swan and A. Waggott, *J. Chem. Soc. C*, 285 (1970).
(10) (a) R. K. Robins, J. K. Horner, C. V. Greco, C. W. Noell, and C. G. Beames, Jr., *J. Org. Chem.*, **28**, 3041 (1963); (b) ref 10a employed extensive spectroscopic analysis to assign i as the correct structure for an analogous imidazole system.



i

(11) E. Benary, *Ber.*, 44, 493 (1911).
(12) Q. N. Porter and J. Baldas, "Mass Spectrometry of Heterocyclic Compounds", Wiley-Interscience, New York, N.Y., 1971, pp 477–478.
(13) B. R. Baker and J. L. Hendrickson, *J. Pharm. Sci.*, **56**, 955 (1967).
(14) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
(15) (a) R. H. Springer, M. K. Dimmitt, T. Novinson, D. E. O'Brien, R. K. Robins, L. N. Simon, and J. P. Miller, *J. Med. Chem.*, **19**, 291 (1976); (b) W. W. Cleland, "The Enzymes", Vol. II, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1970, p 19.
(16) I. H. Segel, "Enzyme Kinetics", Wiley-Interscience, New York, N.Y., 1975, p 133.
(17) Our calculations of the K_{is} values at $100K_m$ were obtained by the Plowman method: K. M. Plowman, "Enzyme Kinetics", McGraw-Hill, New York, N.Y., 1972, p 60.
(18) These values are comparable to those obtained by the Segel method[16] and from Dixon plots.[19]
(19) See ref 23 cited in ref 15a above and P. J. Butterworth, *Biochim. Biophys. Acta*, **289**, 251 (1972).
(20) F. Arndt in "Organic Syntheses", Collect. Vol. II, A. H. Blatt, Ed., Wiley, New York, N.Y., 1943, p 165.
(21) Numerous attempts at removing water from 2 were unsuccessful. This characteristic seems general for this series of compounds as rigorous dehydrative procedures were necessary to obtain 1 free of associated water.

Cyclic Analogues of Luteinizing Hormone-Releasing Hormone with Significant Biological Activities

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There is evidence that, in its receptor-binding conformation, the N and C terminus of LH-RH may be in close proximity and two cyclic analogues of the hormone were synthesized to test the hypothesis. Cyclic [β-Ala[1],D-Ala[6],Gly[10]]- and [6-aminohexanoic acid[1],D-Ala[6],Gly[10]]-LH-RH were prepared by treatment of their linear precursor peptides with dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole in dilute dimethylformamide solution. Although the linear peptides possessed no detectable LH-releasing activity in ovariectomized rats, the cyclic β-Ala analogue had 1.2% the activity of LH-RH, whereas the longer chain cyclic 6-aminohexanoic acid analogue had 0.65% activity. These results support the concept of an important interaction between the ends of the LH-RH molecule possibly involving hydrogen-bond formation between the pyrrolidone carbonyl group of pyroglutamic acid and the glycinamide group.

It is now reasonably well established by analogue studies and free-energy analysis[2,3] that the active conformation of LH-RH contains a type II β bend hinged around glycine in position 6. A notable consequence of this is that the substitution of D-amino acids,[4] particularly those with bulky side chains,[5] results in large increases in gonadotropin-releasing activity presumably due to the stabilization of the β bend. With this configuration at the center of the chain, the LH-RH molecule assumes a "U" shape in which the <Glu residue in position 1 and glycinamide in position 10 are in quite close proximity.

Structure–activity studies[6] on <Glu strongly suggest that the pyrrolidone carbonyl group contributes to full biological activity by taking part in hydrogen-bond formation either with a complimentary group on the receptor or with some part of the LH-RH chain itself. Slight alterations to Momany's CC conformer[3] for LH-RH, which was derived from computer minimum free-energy calculations and a consideration of analogue activities, readily enable <Glu and glycinamide to approach close enough for hydrogen-bond formation between the γ-carbonyl group and the glycine NH_2 group.

If there is such an interaction between the termini of LH-RH, then it should be possible to covalently link the ends of the peptide chain and at least retain appreciable biological activity. At best, if the optimum stereochemical

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requirements for such a linkage were determined, it might be possible to obtain analogues with increased biological activity due to irreversible stabilization of the active conformation. In this study, two cyclic analogues were synthesized with linking groups of different lengths. They were formed by the cyclization of linear peptides with free amino and carboxyl terminals since LH-RH is blocked at both ends. Both peptides contained D-alanine in position 6 as a possible conformational aid in the cyclization reactions.

Experimental Section

Unless otherwise stated, amino acids were of the L configuration. Amino acid derivatives were purchased from Bachem, Inc., Torrance, Calif.

All amino acids were protected at their α-amino groups by *tert*-butyloxycarbonylation. Reactive side chains were protected as follows: Arg, tosyl; Ser, benzyl; Tyr, 2-bromocarbobenzoxy; His, tosyl. The protected amino acids (3 mmol) were coupled in a Beckman Model 990 automatic peptide synthesizer to a 1% cross-linked, divinylbenzene–polystyrene Merrifield glycyl resin (1 mmol, 0.5 mmol of amino acid per gram of resin) in the presence of dicyclohexylcarbodimide (3 mmol), and *tert*-butyloxycarbonyl protection was removed at each step by treatment with 33% TFA in CH_2Cl_2. The details of the synthetic cycle have been previously described.[7]

The completed peptides were cleaved from the resin and deprotected simultaneously in hydrogen fluoride containing 10% anisole (45 min, 0 °C) and purified as described below. The homogeneity of final products was demonstrated by TLC on SiO_2 plates in the following solvent systems: (A) 1-BuOH–AcOH–H_2O (4:1:5, upper phase); (B) 2-PrOH–1 M AcOH (2:1); (C) 1-BuOH–AcOH–H_2O–EtOAc (1:1:1:1); (D) EtOAc–pyridine–AcOH–H_2O (5:5:1:3). Solvent fronts were allowed to travel 10–12 cm and spots were visualized with ninhydrin (cyclic peptides negative) followed by Cl–starch–KI reagents. Amino acid analyses were carried out on samples which were hydrolyzed (110 °C, 18 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[8] using a Beckman 119 amino acid analyzer.

β-Ala-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly·HCl (I). After its solid-phase synthesis and HF cleavage, the crude peptide was purified on a column (2.5 × 95 cm) of Sephadex G-25 (fine) by elution with 0.2 M AcOH followed by partition chromatography[9] on a column (2.5 × 95 cm) of Sephadex G-25 (fine) using 1-BuOH-1-PrOH–AcOH–H_2O (7:1:2:10) as the biphasic medium. The major peak obtained from elution volumes 530–770 mL yielded pure peptide acetate salt. This material was converted to its hydrochloride by stirring with Dowex IRA 400 resin (Cl form) to give peptide I in 24% yield: R_f(A) 0.1, R_f(B) 0.3, R_f(C) 0.25, R_f(D) 0.4; $[\alpha]_D$ −7.5° (c 0.53 in 0.2 M AcOH; *T*, 26 °C). Amino acid analysis gave Ser, 0.92; Pro, 1.04; Gly, 1.00; Ala, 1.01; Leu, 1.01; Tyr, 1.00; β-Ala, 0.98; His, 0.93; Trp, 0.97; peptide content, 87%.

***cyclo*-(β-Ala-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly) (II).** A solution of peptide I (120 mg, 0.1 mmol) in 50 mL of DMF was dropped into a stirred solution of DCC (20 mg, 0.1 mmol) and 1-hydroxybenzotriazole (31 mg, 0.2 mmol) in 50 mL of DMF at room temperature over a period of 12 h. Two days later, the solvent was removed and the crude residue was purified by gel filtration on a column (2.5 × 95 cm) of Sephadex G-25 (fine) by elution with 0.2 M AcOH followed by partition chromatography on a column (2.5 × 95 cm) of Sephadex G-25 (fine) in the solvent system 1-BuOH–1-PrOH–AcOH–H_2O (7:1:2:10). The major peak obtained from elution volumes 180–300 mL resulted in 20 mg of pure peptide II: R_f(A) 0.2, R_f(B) 0.45, R_f(C) 0.5, R_f(D) 0.7; $[\alpha]_D$ −25 °C (c 0.7 in 0.2 M AcOH; *T*, 26 °C). Amino acid analysis gave Ser, 0.92; Pro, 1.05; Gly, 1.00; Ala, 1.00; Leu, 1.02; β-Ala, 1.01; His, 0.94; Trp, 0.89; Arg, 0.97; peptide content, 71%.

6-Aminohexanoyl-His-Trp-Ser-Tyr-D-Leu-Arg-Pro-Gly·HCl (III). The crude peptide was purified in a similar manner to peptide I. A major peak from elution volumes 400–560 mL off the partition column yielded peptide acetate which was converted to peptide III in 23% overall yield: R_f(A) 0.12, R_f(B) 0.25, R_f(C) 0.25, R_f(D) 0.47; $[\alpha]_D$ −26 °C (c 0.77 in 0.2 M AcOH; *T*, 26 °C). Amino acid analysis gave Ser, 0.90; Pro, 1.00; Gly, 1.00; Ala, 1.00; Leu, 0.87; Tyr, 1.00; His, 0.96; Trp, 0.87; Arg, 1.00; peptide content, 84%.

***cyclo*-(6-Aminohexanoyl-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly) (IV).** A solution of peptide III (88 mg 75 μmol) in dimethylformamide (50 mL) was dropped into a stirred solution of DCC (17 mg, 82 μmol) and 1-hydroxybenzotriazole (25 mg, 160 μmol) in dimethylformamide (80 mL) over a period of 10 h at room temperature. Two days later, the solvent was removed and the residue purified in a similar manner to peptide II. The major peak from elution volumes 200–270 mL gave pure peptide IV in 20% yield: R_f(A) 0.25, R_f(B) 0.5, R_f(C) 0.5, R_f(D) 0.7; $[\alpha]_D$ −5.7° (c 0.7 in 0.2 M AcOH; *T*, 26 °C). Amino acid analysis gave Ser, 0.96; Pro, 1.02; Gly, 1.00; Ala, 1.00; Leu, 0.95; Tyr, 0.99; 6-aminohexanoic acid, 1.01; His, 1.03; Trp, 0.89; Arg, 1.00; peptide content, 70%.

Evidence of Cyclic Nature of Peptides II and IV. It was considered necessary to prove that the purified products of the cyclization reactions were indeed cyclic. Linear precursors and cyclic products were, therefore, digested with gastricsin enzyme[10] which was found to have some specificity for the Trp-Ser peptide bond in LH-RH. The cleavage products were subjected to TLC (system C) and N-terminal amino acid determinations by the dansyl Edman procedure.[11] After digestion, both of the linear peptides gave predominantly two ninhydrin-positive spots by TLC. The N-terminal amino acids of these fragments were found to be β-Ala and Ser for peptide I and 6-aminohexanoic acid and Ser for peptide III. The cyclic analogues gave only one ninhydrin-positive spot and, in both instances, only N-terminal Ser was detected as expected. An indication of the correct molecular size of the cyclics was obtained during gel filtration on Sephadex G-25. Both peptides eluted slightly later than their linear precursor peptides, suggesting that polymeric materials were not present.

Bioassays. The LH-releasing activities of the peptides (Table I) were determined by the stimulation of LH release at two dose levels in ovariectomized rats pretreated[12,13] with estrogen and progesterone, followed by radioimmunoassay[14] for LH. Serum LH levels were compared with responses elicited by administration of saline alone and 2.5- and 10-ng doses of synthetic LH-RH.

Table I. LH-Releasing Activities of LH-RH Analogues in Steroid-Treated, Ovariectomized Rats[a]

peptide	dose, ng	serum LH levels, ng/mL ± SE	% act. with 95% confidence limits
saline		64.80 ± 7.21	
LH-RH	2.5	481.12 ± 21.61	
	10	831.31 ± 83.28	100
peptide I	250	80.74 ± 6.48	
	1000	65.69 ± 5.31	<0.01
peptide III	250	80.64 ± 7.79	
	1000	74.24 ± 4.65	<0.01
saline		51.72 ± 3.50	
LH-RH	2.5	206.80 ± 7.98	
	10	332.39 ± 22.67	100
peptide II	250	206.67 ± 13.51	
	1000	364.59 ± 7.07	1.20 (0.92–1.56)
peptide IV	250	151.02 ± 9.83	
	1000	315.81 ± 17.83	0.65 (0.48–0.89)

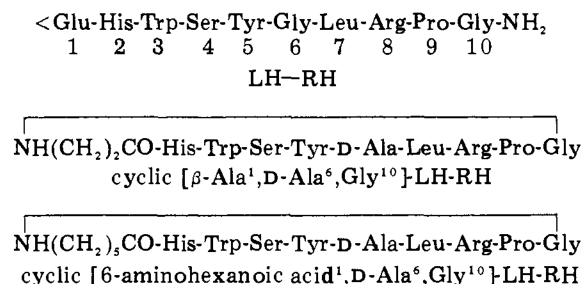
[a] Six per group.

Results and Discussion

Cyclization yields (in the region of 20%) from the reactions of the linear peptides with DCC in dilute DMF solutions were generally improved by the addition of 1-hydroxybenzotriazole[15] to the reaction mixture. Conversion of the linear peptides to their HCl salts prior to their cyclization also had favorable effects possibly due to the prevention of acetylation reactions caused by the presence of acetic acid.

Since <Glu and $GlyNH_2$ are very critical residues for the biological activity of LH-RH, the extremely low LH-releasing activities (<0.01%, Table I) of the two linear compounds, peptides I and III (Chart I), in which these amino acids are replaced, were to be expected. Upon

Chart I. Amino Acid Sequences of LH-RH and the Cyclic Analogues



cyclization, however, their activities increased enormously and the cyclic analogues II and IV (Chart I) were found to have 1.2 and 0.65% the LH-releasing activity of the standard LH-RH preparation, respectively. The best explanation for this is, we believe, that a close proximity exists between the ends of the LH-RH decapeptide chain in its receptor binding conformation, particularly since the shorter chain cyclic peptide was the most potent. It is possible that such an interaction, when worked out in detail, might explain many of the biological properties of some LH-RH agonists and antagonists, many of the more interesting of which contain critical modifications in their N- and C-terminal regions.

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References and Notes

(1) First Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary.
(2) F. A. Momany, *J. Am. Chem. Soc.*, **98**, 2990 (1976).
(3) F. A. Momany, *J. Am. Chem. Soc.*, **98**, 2996 (1976).
(4) M. Monahan, M. S. Amoss, H. A. Anderson, and W. Vale, *Biochemistry*, **12**, 4616 (1973).
(5) D. H. Coy, J. A. Vilchez-Martinez, E. J. Coy, and A. V. Schally, *J. Med. Chem.*, **19**, 423 (1976).
(6) K. Nikolics, D. H. Coy, J. A. Vilchez-Martinez, E. J. Coy, and A. V. Schally, *Int. J. Pept. Protein Res.*, **9**, 57 (1977).
(7) D. H. Coy, E. J. Coy, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **54**, 1267 (1973).
(8) S. Moore in "Chemistry and Biology of Peptides", J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 629.
(9) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).
(10) W-Y. Huang and J. Tang, *J. Biol. Chem.*, **244**, 1085 (1969).
(11) W. R. Gray, *Methods Enzymol.*, **11**, 139 (1964).
(12) V. D. Ramirez and S. M. McCann, *Endocrinology*, **73**, 193 (1963).
(13) A. V. Schally, R. M. G. Nair, T. W. Redding, and A. Arimura, *J. Biol. Chem.*, **246**, 7230 (1971).
(14) G. D. Niswender, A. R. Midgeley, S. E. Monroe, and L. E. Reichert, *Proc. Soc. Exp. Biol. Med.*, **128**, 863 (1972).
(15) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

Synthesis of 2-Substituted Primaquine Analogues as Potential Antimalarials

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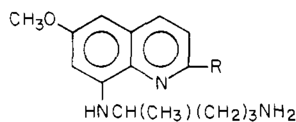
A series of 2-substituted primaquine analogues has been synthesized and evaluated against *Plasmodium berghei* in the mouse and *Leishmania donovani* in the hamster. Three members (**3a**,**d**,**e**) of the series were evaluated against *Plasmodium cynomolgi* in the rhesus monkey. One analogue (**3d**) was evaluated against *Trypanosoma rhodesiense* in the mouse, and two (**3b**,e) were evaluated against *Schistosoma mansoni* in the mouse. Several analogues possessed significant activity against *P. berghei* (3e,**f**) and *L. donovani* (**3a**,e).

Although quite toxic,[1] primaquine (1) is an important



1, R = H
2, R = $OCH_2C_6H_5$
3, R = OCH_3, NH_2, $N(CH_3)_2$, Cl, C_2H_5, $CH{=}CH_2$, $NHCOCH_3$

radical curative and causal prophylactic antimalarial agent. Our earlier studies[2] had indicated that 2-benzyloxy analogues, **2**, were less toxic than primaquine, as measured in the Rane mouse screen,[3] and this suggested the possibility that 2-substituted 8-aminoquinolines may offer a lead in the search for more effective and less toxic agents. We have now synthesized and evaluated a number of variously 2-substituted analogues, **3**. Selection of moieties for the 2 position was patterned after those which have shown interest when present at the 4 or 5 position.[4]

Chemistry. Compounds **5a**–**c** (Scheme I) were prepared by direct nucleophilic displacement of the halogen in 2-chloro-6-methoxy-8-nitroquinoline (**4**)[5] by an appropriate reagent (e.g., sodium methoxide, phenol-ammonia, or dimethylamine). The 2-amino analogue **5b** was converted to the 2-acetamido derivative **5g**, and the 8-nitro group of **5g** and **5c** was reduced with iron–acetic acid[6] to yield **6g** and **6c**. Compound **5f** (Scheme II) was prepared from 6-methoxy-2-methyl-8-nitroquinoline (**8**),[7] via the quaternary salt (**10**) of the Mannich base (**9**).[8] Reduction of **5f** with stannous chloride–hydrochloric acid gave **6f**, whereas reduction with Raney nickel–hydrazine hydrate[9] gave **6e**. Compounds **6a** and **6d**[10] (prepared directly from **4**) were also obtained by Raney nickel–hydrazine hydrate reduction. Alkylation of the amines **6** with 4-bromo-1-phthalimidopentane,[11] utilizing the triethylamine procedure,[12] gave the intermediate phthalimidoalkylaminoquinoline derivatives **7**. These were subjected directly to hydrazinolysis, and the resulting amines **3** were characterized as maleate or fumarate salts. (During hydrazinolysis, the acetamido group present in **7g** and unreacted **6g** was cleaved to give the desired **3b** and amine **6b**.[13]) Pertinent physical and analytical data for all new compounds are summarized in Tables I and II.

Biological Results. The antimalarial test results were provided by the Walter Reed Army Institute of Research. The suppressive activity was assessed against *Plasmodium berghei* in mice by the method of Rane and co-workers.[3,14] As noted in Table I, several **3** analogues were "active", but primaquine-like toxicity, as observed in the Rane test, was still present, except for **3e**. (No mouse data were available for **3b**.) Compounds **3a**,**d**,**e** were also tested for radical

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