Synthesis and Antifolate Activity of New Diaminopyrimidines and Diaminopurines in Enzymatic and Cellular Systems

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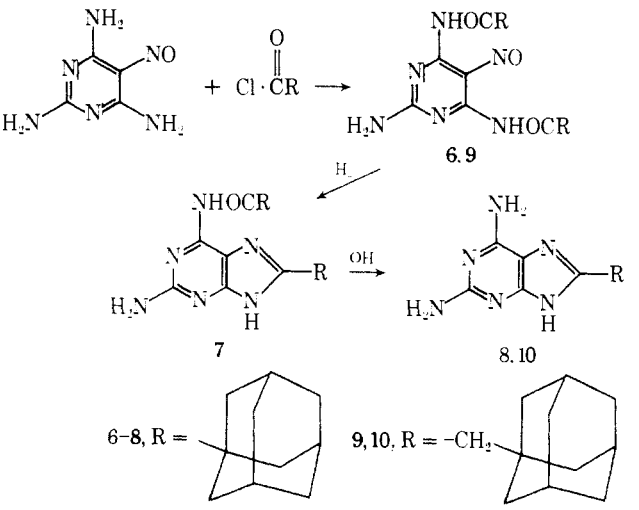
The following new 2,4-diamino-6-methylpyrimidines, 5-cyclohexylmethyl, 5-cyclohexylethyl, and 5-(2-naphthyl), as well as 2,6-diaminopurines, 8-adamantyl and 8-adamantylmethyl, were synthesized as potential antifolates. These, as well as three known compounds, 2,4-diamino-5-cyclohexyl-6-methylpyrimidine, 2,4-diamino-5-(1-naphthyl)-6-methylpyrimidine, and 2,6-diaminopurine, were compared with respect to the inhibition of growth of mammalian cells in culture (TA 3) and with respect to the inhibition of partially purified dihydrofolate reductase. All of the pyrimidines except for the 5-(1-naphthyl) derivative were competitive inhibitors of dihydrofolate reductase, with K_i values ranging from 0.07 to 0.4 μM. They were 2–5 times better as inhibitors of the isolated dihydrofolate reductase than of the cell growth. 2,4-Diamino-5-(1-naphthyl)-6-methylpyrimidine was a noncompetitive inhibitor of the enzyme with a K_i value of 56 μM. This compound was more potent in inhibiting cell growth than the isolated enzyme, indicating that its biological activity was not related to the inhibition of dihydrofolate reductase. All of the purine derivatives were poor growth inhibitors and although some of them inhibited isolated dihydrofolate reductase, their mode of action in cellular system did not seem to concern folate metabolism, as judged by the inability of hypoxanthine, thymidine, and glycine to provide protection. The implication of these findings as to the structural requirements for inhibition of dihydrofolate reductase is discussed. The pitfalls of the determination of ID_{50} values instead of a complete kinetic analysis in structure–activity studies are emphasized.

Earlier work from this laboratory indicated that the affinity of 2,4-diaminopyrimidines to the enzyme dihydrofolate reductase increased with the size of the hydrocarbon substituent at C-5.[1] Furthermore, hydrocarbon substituents with a bulky and rigid conformation, such as cyclohexane or adamantane, imparted a much stronger affinity to the enzyme than straight-chain hydrocarbons of similar size. The exploration of the biological activity of 2,4-diaminopyrimidines having at C-5 either a combination of cyclohexane with a short straight hydrocarbon or a bulky substituent other than adamantane or cyclohexane was undertaken to learn more about the structural requirements for inhibition of dihydrofolate reductase. Also, since in the case of diaminopyrimidines[1,2] and diaminopteridines[3] the presence of a lipophilic group adjacent to the heterocyclic ring increased considerably their biological activity, it was of interest to see if a similar effect would be observed in the case of lipophilic substituents at C-8 of 2,6-diaminopurines. The parent compound 2,6-diaminopurine has been reported to be a weak competitive inhibitor of dihydrofolate reductase from chicken liver[4] and was shown to protect this enzyme from mouse sarcoma cells against proteolytic inactivation.[5] A preliminary report on this work has been published.[6]

Syntheses. Two synthetic approaches were used for the preparation of diaminopyrimidines. 2,4-Diamino-5-cyclohexylmethyl-6-methylpyrimidine (**2**) and 2,4-diamino-5-cyclohexylethyl-6-methylpyrimidine (**4**) were synthesized by the condensation of guanidine with the appropriately substituted ethylacetoacetate to form the 2-amino-4-hydroxy-5-substituted 6-methylpyrimidines. These compounds were converted to the corresponding 2,4-diaminopyrimidines by chlorination followed by amination. 2,4-Diamino-5-(2-naphthyl)-6-methylpyrimidine (**5**) was prepared essentially as described for the preparation of the corresponding 5-(1-naphthyl) derivative **11**.[7] 2,5-Diamino-8-adamantylpurine (**8**) and 2,6-diamino-8-adamantylmethylpurine (10) were synthesized as described for other 8-substituted purines[8] by reacting 2,4,6-triamino-5-nitrosopyrimidine with either 1-adamantanecarboxylic acid chloride or 1-adamantaneacetic acid chloride to form corresponding 4,6-adamantoylamino or adamantylacetylamino pyrimidines, respectively (Scheme I). Reduction of the nitroso group led to closure of the purine ring and desired compounds were obtained after hydrolysis of 6-acyl groups.

Scheme I

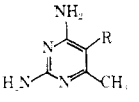
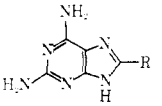


Inhibition of Dihydrofolate Reductase. All of the five newly synthesized compounds were tested as inhibitors of partially purified dihydrofolate reductase from an amethopterin-resistant subline (AT/3000) of sarcoma 180 cells. The purification of the enzyme, the assay procedure, and the use and advantages of a transformed Lineweaver–Burk equation at substrate concentrations much above the K_m value were reported and discussed previously.[10] The effects of the newly synthesized compounds on the dihydrofolate reductase are presented in Figure 1. 2,4-Diamino-5-(1-naphthyl)-6-methylpyrimidine (**11**) is a compound described previously[7] but it has not been tested for inhibition of dihydrofolate reductase and therefore was included for comparison in Figure 1.

Growth Inhibition of Mammalian Cells in Culture. The culture medium, conditions, and assay procedures used for mouse mammary adenocarcinoma cells (TA 3) were as described elsewhere.[11]

In order to determine whether the site of action of the inhibitors was related to folate metabolism, the TA 3 cells were grown under conditions in which folate metabolism was blocked, *i.e.*, in a medium supplemented with 1 μM amethopterin, 30 μM thymidine, 100 μM hypoxanthine.

Table I

Compd	Ring structure	R	ID_{50}/TA 3, μM FA-med[a]	ID_{50}/TA 3, μM HTG-med[b]	K_i (DFR),[c] μM
2		Methylcyclohexyl	0.74	10	0.41[e]
4		Ethylcyclohexyl	1.30	41	0.27[e]
5		2-Naphthyl	0.28	43	0.07[e]
11		1-Naphthyl	2.5		56[f]
12		Cyclohexyl	0.39	420	0.13[e]
8		Adamantyl	48	42	4.8[e]
10		Methyladamantyl	10[d]		13.3[e]
13		H	35	50	1200[e]

[a]Cells grown in the normal medium supplemented with folic acid. [b]Cells grown in the medium supplemented with 1 μM amethopterin, 100 μM hypoxanthine, 30 μM thymidine, and 100 μM glycine. [c]Dihydrofolate reductase. [d]Only 20% of growth inhibition was obtained at this concentration. [e]Competitive inhibitors. [f]Noncompetitive inhibitors.

and 30 μM glycine (HTG-medium).[12] If compounds are inhibitory under such conditions, their mode of action must be unrelated to folate metabolism. Table I summarizes the results of the biological testing. For comparison with the newly synthesized compounds three known compounds were included: 2,4-diamino-5-cyclohexyl-6-methylpyrimidine (**12**),[13] 2,4-diamino-5-(1-naphthyl)-6-methylpyrimidine (**11**),[7] and 2,6-diaminopurine (**13**).

Discussion

The purines included in this study were poor inhibitors of cell growth and although **8** and **10** inhibited dihydrofolate reductase to a certain extent, their mode of action in cellular system did not seem to concern folate metabolism. Thus in the case of **8** and **13** the hypoxanthine–thymidine–glycine mixture did not prevent the growth inhibition. In the case of **13** this was not surprising since 2,6-diaminopurine is known to be an analog of adenine. As such it is converted to nucleotides through phosphoribosyltransferase and thus it acts as an inhibitor in that form.[14] Whether **8** and **10** can be converted to nucleotides is not known.

In the pyrimidine series all compounds except **11** were moderately strong growth inhibitors. Their growth inhibitory potency was decreased by one to two orders of magnitude under conditions where folate metabolism is inoperative, indicating that their primary mode of action involves interference with folate metabolism. Indeed, all of these pyrimidines were even more potent (2–5 times) in inhibiting the enzyme than the cell growth. In each case the inhibition was competitive with dihydrofolate. Since in the case of amethopterin about 100–1000 times higher concentration is needed to inhibit the cell growth than the enzyme, it appears that the transport of these pyrimidines through the cell membrane is less hindered than that of amethopterin. The insertion of one or two methyl groups between cyclohexyl group and the pyrimidine ring decreased the biological activity relatively little. This is in contrast to the insertion of the NHCO group between adamantyl and the pyrimidine ring which lowered the biological activity of the analogs by three orders of magnitude.[10] The latter observation was originally interpreted that only lipophilic substituents adjacent to the pyrimidine ring fit into the hydrophobic area of the enzyme. The length of the $-CH_2 \cdot CH_2-$ linkage is not much different from that of –NHCO– linkage; however, the latter may impose specific conformations not necessarily encountered with the $-CH_2 \cdot CH_2-$ bridge. Thus it appears that the hydrophobic area of this dihydrofolate reductase is not limited to a strictly confined region,

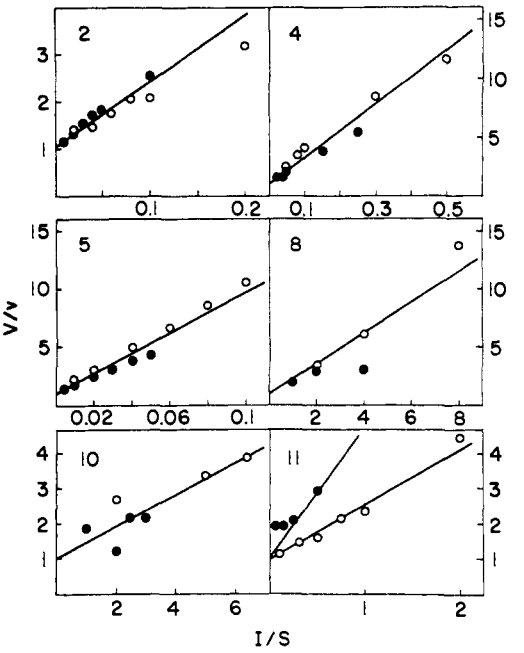


Figure 1. Inhibition of dihydrofolate reductase of sarcoma 180 AT/3000 cells by 2,4-diaminopyrimidines and 2,6-diaminopurines. Numbers refer to compounds designated in the text. For competitive inhibition $V/v = 1 + K_m/K_i \times [I]/[S]$, where V is maximal velocity; v is initial velocity; K_m and K_i are Michaelis and inhibition constants, respectively; and [I] and [S] are molar concentration of inhibitor and substrate, respectively. For noncompetitive inhibition $V/v = 1 + [S]/K_i \times [I]/[S]$. For calculation of K_i's the published K_m value was used, *i.e.*, 6 μM.[9] ○, substrate (dihydrofolate) concentration 1×10^{-4} M; ●, substrate concentration 2×10^{-4} M.

directly adjacent to the pyrimidine binding site. The location of hydrophobic area with respect to the active site of dihydrofolate reductase has been discussed at length by Baker.[2]

In agreement with the results of an earlier study,[1] replacement of the cyclohexyl group by 2-naphthyl increased the inhibitory activity of diaminopyrimidine only slightly. That study showed that a linear relationship existed between the molar volume of the 5-substituent of 2,4-diaminopyrimidines and the hydrophobicity, as determined from the water–heptane partition coefficient. Further, the affinity of the pyrimidines to dihydrofolate reductase increased with the hydrophobicity of the 5-substituent. Consequently, the larger the molar volume of the substituent the greater should be the affinity to the enzyme. The estimated

molar volumes of cyclohexane and naphthalene are very similar, namely 108 and 112 ml, respectively, and the free dissociation energies of enzyme–inhibitor complex as calculated from the K_i values were also found to be similar, +9.8 kcal for **12** and +10 kcal for **5**.

It is of interest that the 1-naphthyl derivative **11** differs from the 2-naphthyl isomer **5** by being a weak, noncompetitive inhibitor of dihydrofolate reductase. This suggests that the compound does not fit in the active site of the enzyme. Comparison of the molecular models of the two isomers reveals striking differences. In both cases the naphthyl residue can neither rotate freely nor assume a planar conformation with the pyrimidine ring. However, whereas the bulk of the naphthyl residue in the case of 2-naphthyl isomer deviates from the longitudinal axis of the molecule only by 30° or less, depending on conformation, this deviation reaches 90° in the case of 1-naphthyl isomer. It appears that such deviation does not allow a proper fit of the inhibitor into the substrate binding site of the enzyme. The fact that the 1-naphthyl isomer inhibits cell growth at substantially lower concentration than it does dihydrofolate reductase suggests that this enzyme is not the target of action of this compound.

The observation that one of two compounds, chemically as similar as 1-naphthyl and 2-naphthyl derivatives of diaminopyrimidines, is a noncompetitive whereas the other a competitive inhibitor of an enzyme points to the general necessity for determination of actual K_i values and of the type of inhibition, when structure–activity relationship is studied. The determination of ID_{50} values alone for enzyme inhibition in such studies is highly inadequate and can easily lead to erroneous conclusions. The other pitfalls in the use of ID_{50} values in enzyme inhibitor studies have been discussed.[15,16]

Experimental Section

All melting points were taken on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by G. I. Robertson, Florham Park, N.J. Those analyses in which the results are within 0.4% of the calculated values are denoted by the symbols for these elements. Tlc was carried out on Brinkman silica gel (F-254) plates on aluminum. Ir confirmed the assigned structure of all compounds discussed. Uv spectra were recorded on Cary 14 spectrophotometer and were performed in absolute ethanol unless stated otherwise. No attempt was made to optimize yields in the reactions described below.

2-Amino-4-hydroxy-5-cyclohexylmethyl-6-methylpyrimidine (1). A solution of sodium ethoxide (0.79 g of Na, 0.0034 g-atom) in 50 ml of absolute ethanol was cooled in an ice bath and guanidine hydrochloride (1.65 g, 17.2 mmol) was added. Ethyl 2-cyclohexylmethylacetoacetate[17] (3.89 g, 17.2 mmol) was added and the mixture was refluxed for 72 hr. The mixture was flash evaporated to a paste and neutralized with 5 *M* HCl to give a glassy tan precipitate, which melted on the filter as filtration was attempted. The gum was treated with ether and dissolved in hot ethanol. Upon cooling a white solid precipitated. This was collected by filtration and recrystallized from absolute ethanol to give the product (1.5 g, 39%): mp 272–273°. *Anal.* ($C_{12}H_{19}N_3O$) C, H, N.

2,4-Diamino-5-cyclohexylmethyl-6-methylpyrimidine (2). 1 (1.0 g, 4.5 mmol) was refluxed for 2 hr in a solution of 10 ml of $POCl_3$ and 1.0 g of PCl_5. The mixture was poured on crushed ice. The resulting white solid was filtered off and dried *in vacuo* over P_2O_5 for 4 days. The resulting gum was dissolved in 125 ml of absolute ethanol which was saturated with ammonia at 0° and heated in a bomb at 160° for 24 hr. After evaporation of the solvent the residue was stirred for 1 hr with 0.5 *M* NaOH. The solid was filtered off, washed with ether, and recrystallized twice from hot absolute ethanol: yield 1.0 g (100%); mp 209–210°. *Anal.* ($C_{12}H_{20}N_4$) C, H, N.

2-Amino-4-hydroxy-5-cyclohexylethyl-6-methylpyrimidine (3). Guanidine hydrochloride (10.1 g, 105 mmol) in a solution of sodium ethoxide–absolute ethanol (4.86 g, 0.211 g-atom of Na) and ethyl 2-cyclohexylethylacetoacetate[12] (23.9 g, 992 m*M*) were refluxed for 48 hr. The reaction mixture was cooled and evaporated to give a solid residue which was crystallized from aqueous ethanol; 10.9 g (46%, needles) was obtained, mp 226–229°. *Anal.* ($C_{13}H_{21}N_3O$) C, H, N.

2,4-Diamino-5-cyclohexylethyl-6-methylpyrimidine (4). This compound was prepared by chlorination and amination of 3 (2.0 g, 8.5 mmol) essentially as described for **2** except for the work-up of the final product which was as follows. After evaporation of ethanol–ammonia the solid was taken up in small amount of 0.5 *M* ethanolic sodium hydroxide, stirred for 1 hr, and filtered. The solid was dissolved in acetone and filtered. Evaporation of acetone gave 1.6 g of crude product which was recrystallized three times from absolute ethanol: yield 0.52 g (26%) of white needles; mp 157–159°; λ_{max} 232, 288 (ϵ_{max} 6.79). *Anal.* ($C_{13}H_{22}N_4$) C, H, N.

2,4-Diamino-5-(2-naphthyl)-6-methylpyrimidine (5). This compound was prepared as described for the 1-naphthyl derivative 11[7] except that β-(2-naphthyl)acetonitrile was the starting material. The yield was 57%: mp 223–224°; tlc in absolute ethanol, R_f 0.35. *Anal.* ($C_{15}H_{14}N_4$) C, H, N.

2-Amino-4,6-bis[(1-adamantoyl)amino]-5-nitrosopyrimidine (6). 2,4,6-Triamino-5-nitrosopyrimidine (6.16 g, 40 mmol) was added to a solution of 1-adamantanecarboxylic acid chloride (17.0 g, 85 mmol) in 600 ml of dry DMF at 0°. After the starting material dissolved the solution turned blue and a blue solid precipitated. This was filtered and washed with anhydrous ether, yield 10.4 g (54%), and recrystallized from hot mixture of absolute ethanol and chloroform. *Anal.* ($C_{26}H_{34}N_6 \cdot 2H_2O$) C, H, N, Cl.

2-Amino-6-(1-adamantoyl)amino-8-(1-adamantyl)purine (7). **6** (1 mmol, 478 mg) was placed in a mixture of 14 ml of ethanol, 2.3 ml of acetic acid, and 2.3 ml of water. To this was added portionwise 0.69 g of zinc dust while the mixture was heated at 60°. Thereafter the mixture was refluxed for 90 min. After filtering the remaining zinc the volume of the solution was reduced to 5 ml. A white solid crystallized: yield 500 mg (99%); mp 216–217°; tlc (ethoxyethanol–ethyl acetate–4% formic acid, 1:12:2) R_f 0.60; uv λ_{max} 320. *Anal.* ($C_{26}H_{34}N_6O \cdot AcOH$) C, H, N.

2,6-Diamino-8-(1-adamantyl)purine (8). **7** (200 mg, 0.395 mmol) was refluxed in 40 ml of ethanol while HCl gas was allowed to pass through the reaction mixture for 3 hr. The precipitate which formed was filtered and washed with acetone and ether. The cream-colored solid (150 mg) was dissolved in boiling water, filtered, and precipitated with concentrated ammonium hydroxide: yield of dry material, 60 mg (54%). A sample for analysis was obtained by recrystallization from aqueous ethanol: mp 359–361; tlc (ethoxyethanol–ethyl acetate–4% formic acid, 1:12:2) R_f 0.25; uv λ_{max} 253, 285, 321 (ϵ_{max} 33.5, 29.8, 23.6 × 10^3). *Anal.* ($C_{15}H_{20}N_6$) C, H, N.

2-Amino-4,6-bis(adamantaneacetylamino)-5-nitrosopyrimidine (9). To 15.5 g (73 mmol) of 1-adamantylacetic acid chloride in 70 ml of dry DMF at 0° was added quickly 5.6 g (36.6 mmol) of 2, 4, 6-triamino-5-nitrosopyrimidine. The mixture was stirred for 4 hr at 0° and for 2 hr at room temperature. A blue solid which formed was collected, washed with anhydrous ether, and dried: yield 10.3 g (86%); mp 263–264°. *Anal.* ($C_{28}H_{38}N_6O_3 \cdot 0.5HCl$) C, H, N.

2,6-Diamino-8-(1-adamantylmethyl)purine (10). **9** (1 mmol, 525 mg) was placed in a mixture of 14 ml of ethanol, 2.3 ml of acetic acid, and 2.3 ml of water, and 690 mg of zinc dust was added portionwise. The mixture was refluxed for 3 hr. After removal of the remaining zinc the solution was flash evaporated to a glassy mass. This was dissolved in 50 ml of absolute ethanol and refluxed for 3 hr while HCl gas was slowly bubbled through. The crystalline solid which formed was filtered: yield 260 mg (87%); mp 300–303°. An analytical sample was obtained by recrystallization from 50% ethanol. *Anal.* ($C_{16}H_{22}N_6 \cdot 0.5H_2O$) H, N; C: calcd, 62.51; found, 63.03.

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References

(1) Y. K. Ho, S. F. Zakrzewski, and L. H. Mead, *Biochemistry*, 12, 1003 (1973).
(2) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibition," Wiley, New York, N.Y., 1967.
(3) J. P. Jonak, S. F. Zakrzewski, L. H. Mead, and L. D. Allshouse, *J. Med. Chem.*, 15, 1331 (1972).
(4) S. F. Zakrzewski, *J. Biol. Chem.*, 238, 1485 (1963).

(5) M. T. Hakala and E-M. Suolina, *Mol. Pharmacol.*, 2, 465 (1966).
(6) I. Kavai, L. H. Mead, J. Drobniak, and S. F. Zakrzewski, Abstracts of 168th National Meeting of the American Chemical Society, Atlantic City, N.J., 1974, MEDI 066.
(7) P. B. Russel and G. H. Hitchings, *J. Amer. Chem. Soc.*, 73, 3763 (1951).
(8) F. E. Kemper, H. Rokos, and W. Pfleiderer, *Chem. Ber.*, 103, 885 (1970).
(9) S. F. Zakrzewski, M. T. Hakala, and C. A. Nichol, *Mol. Pharmacol.*, 2, 423 (1966).
(10) Y. K. Ho, M. T. Hakala, and S. F. Zakrzewski, *Cancer Res.*, 32, 1023 (1972).
(11) J. P. Jonak, S. F. Zakrzewski, and L. H. Mead, *J. Med. Chem.*, 14, 408 (1971).
(12) M. T. Hakala, *Science*, 126, 255 (1957).
(13) J. P. Jonak, S. F. Zakrzewski, and L. H. Mead, *J. Med. Chem.*, 15, 662 (1972).
(14) M. T. Hakala in "Drug Resistance and Selectivity, Biochemical and Cellular Bases," E. Mihich, Ed., Academic Press, New York, N.Y., 1973, p 263.
(15) Y-C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, 22, 3099 (1973).
(16) T. C. Chou, *Mol. Pharmacol.*, 10, 235 (1974).
(17) W. R. Vaughan and K. S. Andersen, *J. Amer. Chem. Soc.*, 77, 6702 (1955).

Synthesis and Biological Activity of LH-RH Analogs Modified at the Carboxyl Terminus

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Des(Pro^9,Gly^{10})-LH-RH ethylamide, des(Pro^9,Gly^{10})-LH-RH butylamide, desGly^{10}-LH-RH 2-aminoethylamide, and desGly^{10}-LH-RH hydrazide were synthesized by a solid-phase method involving cleavage of protected peptide intermediates from their resin support by reaction with ethylamine, butylamine, ethylenediamine, and hydrazine, respectively. In the assay utilizing steroid pretreated, ovariectomized rats, the peptides were found to have the following LH-releasing activities when compared with natural LH-RH: ethylamide, 0.2%; butylamide, 0.1%; 2-aminoethylamide, 2.4%; hydrazide, 12%. DesGly^{10}-LH-RH hydrazide was used as a precursor in the synthesis of desGly^{10}-LH-RH allylamide and desGly^{10}-LH-RH propargylamide by conversion to the azide and reaction with allylamine and propargylamine, respectively. LH and FSH levels were measured over a 4-hr period after subcutaneous injection of these two peptides into immature male rats in order to detect any prolongation of activity. The allylamide analog was quite active, releasing 1.7 times more LH and 1.3 times more FSH than the same dose of LH-RH. The propargylamide analog was considerably less active, exhibiting 50% LH-releasing activity and 64% FSH-releasing activity. Neither peptide appeared to be longer acting than LH-RH.

Since Fujino and coworkers found[1,2] that desGly^{10}-LH-RH ethylamide (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$NHCH_2CH_3$) and propylamide, nonapeptides in which the glycinamide moiety of LH-RH is replaced by ethylamide and propylamide groups, exhibited greatly enhanced gonadotropin-releasing and ovulation-inducing activities, much attention has been focused on the synthesis and biological evaluation of C terminally modified analogs. This has now led to the preparation of even more potent, related peptides such as desGly^{10}-LH-RH 2,2,2-trifluoroethylamide,[3] analogs containing the ethylamide modification in conjunction with D-alanine[3,4] and D-leucine[5] in position 6 of the chain which induce massive and prolonged release of gonadotropins, as well as peptides with improved antagonism toward LH-RH.[3,6]

In a continuing study of those structural requirements at the C terminus necessary for maintaining very high biological activity, we have synthesized four peptides containing new replacements, similar in size to the original ethyl and propyl groups, for the glycinamide residue in LH-RH. Two octapeptides were also prepared in which Pro-Gly-NH_2 was replaced by an ethylamide and a butylamide group in order to examine the effects that removal of proline would have on biological potency.

Synthesis. The automated procedure used for assembling the protected peptide intermediates, beginning with either an arginine or proline 1% divinylbenzene–cross-linked polystyrene resin, has been described in detail elsewhere.[7] The protected peptide intermediates for the ethylamide, butylamide, 2-aminoethylamide, and hydrazide analogs were removed from the resin by direct treatment (0°, 6 hr) with ethylamine, butylamine, ethylenediamine, and hydrazine, respectively. All these reactions were accompanied by simultaneous removal[8] of the dinitrophenyl group protecting the imidazole ring of histidine. The remaining protecting groups were then eliminated in the usual fashion with anhydrous hydrogen fluoride in anisole (20%). The free peptides were purified by gel filtration on Sephadex G-25 followed by partition chromatography on Sephadex G-25.

DesGly^{10}-LH-RH allylamide and propargylamide were made by a novel route from desGly^{10}-LH-RH hydrazide by its conversion to the azide followed by reaction with allylamine and propargylamine in good yield. In this manner, undesirable side reactions between HF and the olefinic and acetylenic groups were avoided. The two peptides were readily purified by partition chromatography alone.

Homogeneity of all peptides was demonstrated in several tlc solvent systems and by amino acid analysis.

Biological Assays. The LH-releasing activities of des-(Pro^9,Gly^{10})-LH-RH ethylamide and butylamide and desGly^{10}-LH-RH 2-aminoethylamide and hydrazide (Table I) were determined by stimulation of LH release at two appropriate dose levels after administration to ovariectomized, estrogen–progesterone pretreated rats. Serum LH levels were measured 30 min after injection by radioimmunoassay.[9] The LH levels were compared to those found after administration of two doses of natural LH-RH, allowing relative activities to be calculated with 95% confidence limits.

In view of the delayed peak gonadotropin responses[10] of desGly^{10}-LH-RH propylamide, it was considered advisable to test desGly^{10}-LH-RH allylamide and propargylamide in greater depth. An immature male rat system[10] was chosen