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Structure–Activity Relationships in Luteinizing Hormone-Releasing Hormone

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Three analogs of luteinizing hormone-releasing hormone (LH-RH) of the structure <Glu-His-Trp-Ser-Tyr-Gly-Gly-Leu-Arg-Pro-Gly-NH_2, involving substitutions in positions 1, 3, and 8 with nonprotein amino acids, have been synthesized by the solid-phase method. They are [pyro-L-α-(1-aminoadipic)]-LH-RH, [3-(2-naphthyl)-L-Ala^3]-LH-RH, and [δ-*N*-*i*-Pr-L-Orn^8]-LH-RH. Their LH-RH activities in vivo were 12.5, 51.8, and 3.7% that of LH-RH, respectively, in the assay using ovariectomized, estrogen- and progesterone-treated rats. In a test based upon subcutaneous injection into immature male rats, [3-(2-naphthyl)-Ala^3]-LH-RH released 1.2 times as much LH and 0.8 times as much FSH as synthetic LH-RH.

The isolation and structural elucidation of the decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, luteinizing hormone-releasing hormone,[1,2] has led to the synthesis and testing of many analogs for agonist and antagonist activity. Most of the analogs have involved the substitution of one of the residues by another of the amino acids that occurs in proteins or the deletion of one or more residues entirely. We report the synthesis and testing of three analogs having nonprotein amino acids: (1) pyro-L-aminoadipic acid (<Aad, pAad) in place of the N-terminal pyroglutamic (XI), (2) 3-(2-naphthyl)-L-alanine in place of tryptophan (XII), and (3) δ-*N*-isopropyl-L-ornithine in place of arginine (XIII).

Synthesis. Synthesis of the peptides was carried out essentially by the Merrifield method of solid-phase peptide synthesis[3] except for a few minor modifications. The following side-chain protecting groups were used in the synthesis: Arg, N^G-Tos, Tyr and Ser, *O*-Bzl, δ-*N*-*i*-Pr-Orn, N^δ-Tos; N^{im}-Bzl-His was used for synthesizing the peptides XI and XIII, and the N^{im}-Tos derivative was used in the synthesis of XII. When N^{im}-Bzl-His was used, the DCC coupling was carried out in the presence of *N*-1-hydroxybenzotriazole[4] in order to minimize racemization. <Aad and <Glu in the case of peptides XI and XIII were coupled to the peptide chain in the presence of DCC with DMF as the solvent, but pentachlorophenylpyroglutamate in DMF (reaction time 36 h) was used in the synthesis of peptide XII. To ensure the complete removal of the Boc group, the deblocking step was carried out twice, once with 1 N HCl–HOAc and secondly with 25% TFA in CH_2Cl_2.[5] The peptides were checked for their homogeneity by TLC in three different solvent systems, TLE, and amino acid analysis.

Bioassays. LH-RH activities were determined in vivo by stimulation of LH release at two dose levels in ovariectomized rats pretreated with estrogen and progesterone,[6] followed by radioimmunoassay for LH.[7] Serum LH levels after injection of samples are compared with those obtained after administration of saline and two doses of natural LH-RH. A four-point factorial assay[8] was used to calculate the LH-RH activity with 95% confidence limits. The LH/FSH-RH activities of [3-(2-naphthyl)-Ala^3]-LH-RH were also assayed against synthetic LH-RH by subcutaneous injection in immature male rats. Integrated levels of LH and FSH over a 6-h period after the injection of peptide were considered as a parameter of the LH/FSH-releasing activities.[9-11] Rat FSH was measured by a RIA.[12]

Biological Results and Discussion. The LH-releasing activities of the peptides are shown in Table I. The

Table I. LH-RH Activity of the Analogs Compared with That of Natural LH-RH in Ovariectomized, Estrogen-Progesterone-treated Rats

Sample	Dose, ng per rat	Mean serum LH ng/ml	Mean serum LH SE	p vs. control	Potencies vs. pure natural LH-RH,[a] %
Saline		13.6	1.2		
Natural LH-RH	1	40.6	3.5	0.01	
	5	67.0	6.6	0.01	
Analog XI[b]	100	86.1	12.8	0.01	12.5
[pAad^1]-LH-RH	500	126.9	23.5	0.01	(2.4-35.9)
Saline		6.8	0.7		
Natural LH-RH	1	39.6	0.5	0.01	
(AVS 77-33, no. 215-269)	5	83.2	2.6	0.01	
Analog XII	2	37.1	8.1	0.05	51.8
[3-(2-Naphthyl)-Ala^3]-LH-RH	10	87.8	3.3	0.01	(33.8-79.8)
Saline		5.2	0.9		
Natural LH-RH	1	15.2	1.5	0.01	
	5	67.7	8.6	0.01	
Analog XIII[c]	25	37.1	8.6	0.05	3.7
[δ-*N*-*i*-Pr-Orn^8]-LH-RH	125	43.6	2.9	0.01	(1.4-9.1)

[a] Pure natural LH-RH (AVS 77-33, no. 215-269) assumed 100%. [b] Although the values for analog XI fall outside those for the standards, we feel the assay is valid because the slopes of the standard and unknown determinations are parallel. Four rats were used per point. [c] The most probable reason why no dose response was observed was due to the fact that "the rats in the low dose 25 ng over-responded".

LH-RH and FSH-RH activities of [3-(2-naphthyl)-Ala^3]-LH-RH (XII) as determined after sc injection of immature male rats are shown in Figure 1. The integrated amounts of LH released after 100 ng of XII were approximately 1.2 times greater than those after 100 ng of LH-RH. The integrated amounts of FSH released after 100 ng of XII were approximately 0.8 times as great as those after 100 ng of LH-RH.

The activity of LH-RH is extremely sensitive to changes in the N-terminal pyroglutamic residue. A number of analogs, including [Pro^1]-LH-RH, which differs from the natural product only in lacking a carbonyl group, have negligible activity.[13-19] Okada et al.[17] suggested that the minimum structural requirement in the N-terminal residue is -CO-NH-CH-CO-. The present work indicates that even the size of the ring is important; substitution of <Aad for <Glu reduced activity to 12.5%.

When this work was undertaken, no substitutions of Trp^3 had resulted in retention of significant activity.[20,21] The substitution by 3-(2-naphthyl)-L-alanine, which occupies approximately the same space as Trp, was designed to test the importance of the hydrogen-bonding capacity of Trp and has shown that this is not important. In the meantime, [pentamethylphenyl-3-alanine]-LH-RH has been shown to have high activity,[22] ascribed to its ability to donate electrons in a charge-transfer complex with an electron acceptor in the receptor site. The present results neither support nor contradict the hypothesis, since there is no precise determination of the relative electron-donating properties of naphthalene and pentamethylbenzene.

The δ-*N*-isopropylornithine residue has almost the same steric and electrostatic properties as an arginine residue but lacks the potential to form four hydrogen bonds. This is apparently not essential for activity since [δ-*N*-*i*-Pr-Orn^8]-LH-RH has exhibited 3.7% of the activity of natural LH-RH. A number of other substitutions have been made in position 8;[13,16,23,24] in general, analogs with protonated residues in position 8 have considerable activity.

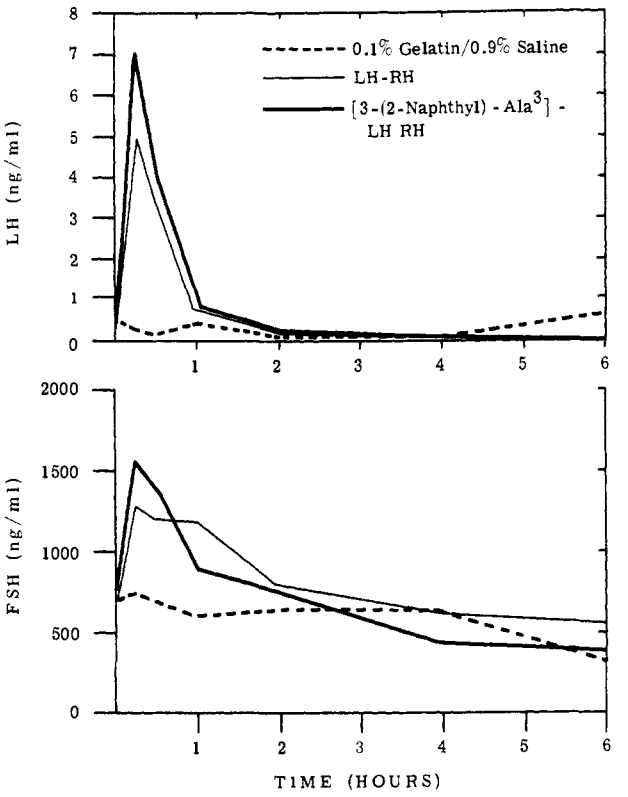

Figure 1. Mean serum LH and FSH levels at various time intervals after sc injection of LH-RH and [3-(2-naphthyl)-Ala^3]-LH-RH in 25-day-old male rats. LH expressed as NIH-LH-S17. FSH expressed as NIAMD-Rat-FSH-RP-1.

Experimental Section

Melting points were uncorrected. Elemental analyses were performed by Midwest Microlab Ltd., Indianapolis, Ind. Amino acid derivatives except for glycine were of L configuration and were either made in our laboratory or bought from Beckman Instruments, Inc., Palo Alto, Calif. Amino acid analyses were performed on samples which were hydrolyzed in evacuated sealed tubes in 6 M HCl at 100° for 22 h in the presence of 0.1% phenol. Solvent systems used for TLC were R_f^1, 1 N BuOH-AcOH-H_2O (4:1:5, upper phase); R_f^2, 1 N BuOH-AcOH-H_2O-EtOAc (1:1:1:1); R_f^3, 1 N BuOH-AcOH-H_2O-Pyr (30:6:24:20). Ascending TLC was conducted on silica gel and/or cellulose supported on glass plates (Brinkman Instruments, Des Plaines, Ill.). Thin-layer electrophoresis (TLE) (Camag, Inc., New Berlin, Wis.) was at 400 V, using HCO_2H-AcOH buffer prepared by diluting 60 ml of HCO_2H and 240 ml of AcOH to 2 l. with distilled water (pH 1.9). Glutamic acid was used as the reference compound and *E*(Glu) indicates the electrophoresis mobility relative to glutamic acid = 1.00. NMR spectra were obtained on a Varian T-60.

Procedure for Synthesis and Purification. *tert*-Butyloxycarbonylglycine was esterified[25] to chloromethyl polystyrene (1% cross-linked, purchased from Lab Systems, Inc., San Mateo, Calif.) containing 0.75 mmol of Cl/g of resin. Amino acid analysis indicated 0.37 mmol of glycine/g of resin. The following steps were taken to incorporate each amino acid residue: (1) three washings with glacial HOAc (10 ml/g of resin); (2) 1 N HCl-HOAc for 25 min; (3) three washings with HOAc and CH_2Cl; (4) 25% TFA in CH_2Cl_2 for 15 min; (5) three washings with CH_2Cl_2, EtOH, and DMF; (6) 10% TEA in DMF for 10 min; (7) three washings with DMF and CH_2Cl_2; (8) protected amino acid (2.5 equiv) in CH_2Cl_2 or CH_2Cl_2-DMF for 10 min; (9) DCCI (2.5 equiv) in CH_2Cl_2 and overnight reaction; (10) three washings with CH_2Cl_2 and EtOH. After step 10 of each cycle, the resin was tested for the presence of free amino groups by the method of Kaiser et al.[26] If the test was positive, the coupling step was either repeated or the free amino group was acylated with 3-nitrophthalic anhydride.[27] Derivatives of Arg, Tyr, Trp, and His were dissolved in

a minimum amount of DMF and the solution was made up with CH_2Cl_2. <Aad and <Glu were coupled in DMF. A 1% solution of 2-mercaptoethanol[28] was used during the deprotection step with 1 N HCl–HOAc as well as in acid washings after the introduction of Trp. The TFA deprotection step was eliminated after Trp incorporation.

The protected decapeptide resin was suspended in absolute MeOH (30 ml/g of resin) and stirred at 0° for 3 h and then saturated with dry ammonia freshly distilled from sodium. The flask was sealed and allowed to stand for 72 h with stirring. The contents were evaporated to dryness; the residue was triturated with DMF, filtered, and washed with DMF and MeOH. The combined filtrate was evaporated to dryness and the peptide precipitated from hot MeOH.

Deprotection. Procedure A. When the Na–NH_3[29] deprotection procedure was used, the protected peptide amide was dissolved in liquid ammonia (200 mg in ~250 ml of NH_3) and sodium from a glass tube was introduced intermittently into the boiling ammonia solution until a light blue color persisted for 30 s. The blue color was then discharged with a few crystals of NH_4Cl. The ammonia was allowed to evaporate to about 20 ml and lyophilized. The lyophilizate was first desalted through a Sephadex G-15 column (2.1 × 105 cm) with 50% HOAc as eluent, chromatographed on Sephadex G-15 (2.5 × 124 cm) using 0.2 N HOAc for elution, and finally subjected to partition chromatography on Sephadex G-25 (fine, 2.5 × 124 cm) using the upper phase of the solvent system BAW (4:1:5) as the moving phase.

Procedure B. When HF was used for removing the protecting groups, the protected peptide amide (200 mg) was allowed to react with 10 ml of liquid HF in the presence of anisole (1 ml) and methyl ethyl sulfide (1 ml) at 0° for 40 min. The HF was evaporated in vacuo; the residue was triturated with ether, filtered, washed well with ether, dried in vacuo for 5 min, taken up in 0.2 N HOAc, and lyophilized. The powder was dissolved in 5 ml of the upper phase of the BAW solvent system and subjected to partition chromatography on Sephadex G-25 (fine). The peptide was then subjected to gel filtration on Sephadex G-15, using 0.2 N HOAc.

L-Pyro-α-aminoadipic Acid (I). pAad was prepared by a modification of the method of Greenstein et al.[30] L-α-Aminoadipic acid (Calbiochem) (2.5 g, 15.5 mmol) in 75 ml of H_2O was refluxed for 20 h. The aqueous solution was evaporated to dryness and the residue extracted with hot EtOAc which upon cooling to room temperature gave I in 50% yield: mp 128°; $[\alpha]^{23}D$ +16° (*c* 2.0, H_2O) [lit.[30] D-6-oxopipecolic acid, $[\alpha]^{25}D$ −16.5° (*c* 2.0, H_2O)].

pAad-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (XI). The protected peptide amide [pAad-His(Bzl)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2] was prepared following the general procedure mentioned above. Deblocking of the protecting groups and purification of the peptide was carried out according to procedure A. The peptide was eluted from Sephadex G-15 (0.2 N HOAc) with R_f 0.46 and showed an R_f 0.23 when subjected to partition chromatography. Compound XI was obtained in an overall yield of 22.7% (based on the amount of *tert*-butyloxycarbonylglycine esterified to the resin): $[\alpha]^{23}D$ −46.9° (*c* 0.5, 1 M HOAc); TLC (silica gel) R_f^1 0.15, R_f^2 0.52, R_f^3 0.47; TLC (cellulose) R_f^1 0.70, R_f^2 0.87, R_f^3 0.88; TLE *E*(Glu), 0.37. Amino acid analysis gave Aad + Gly, 2.86; His, 0.98; Trp, 0.56; Ser, 0.86; Tyr, 1.00; Leu, 1.05; Arg, 0.98; Pro, 0.99; NH_3, 1.07. Anal. ($C_{56}H_{77}N_{17}O_{13}\cdot CH_3COOH\cdot 3H_2O$).

***N*-Acetyl-3-(2-naphthyl)-DL-alanine (II).** A solution of 5.0 g (0.023 mol) of 3-(2-naphthyl)-DL-alanine, prepared from 2-bromomethylnaphthalene and ethyl acetamidocyanoacetate,[31] in 100 ml of 1 N NaOH was treated with 5 ml of acetic anhydride over a period of 10 min. The solution was acidified to pH 2.0 and the product filtered and washed well with water. Recrystallization from ethyl acetate gave 3.6 g (86%): mp 170–173°; TLC [$CHCl_3$–CH_3OH–HOAc (90:30:5)] R_f 0.66.

3-(2-Naphthyl)-L-alanine (III). Method 1, Using Acylase. A suspension of 2.1 g (8.2 mmol) of compound II in 2.5 l. of deionized H_2O was stirred overnight while maintaining the pH at 8.2 by addition of 4 N NH_4OH to give a clear solution. The pH was lowered to 7.2 with 3 N HCl and 400 mg of hog kidney acylase I (Nutritional Biochemical Corp.) was added. The incubation was allowed to proceed at 38° for 70 h with monitoring by TLC. The pH was adjusted to 2.0 and the unreacted acetyl derivative was extracted with EtOAc (5 × 250 ml). The aqueous solution was filtered through Celite and concentrated in vacuo to about 500 ml. The pH was adjusted to 6.0 and the solution was allowed to stand at 5° for 2 days. The precipitated amino acid was filtered, washed with a little water, and dried over P_2O_5 in vacuo: 0.46 g (52%); mp 237–240° dec; $[\alpha]^{23}D$ −27° (*c* 0.51, HOAc); TLC (CMA) R_f 0.27; $[\alpha]^{23}D$ −71° (*c* 0.1, 5 N HCl) (lit.[32] −73.02°). The configuration of this isomer has been established.[32]

Method 2, Using Chymotrypsin. A suspension of 3.78 g (0.014 mol) of *N*-acetyl-3-(2-naphthyl)-DL-alanine methyl ester in 250 ml of 0.1 N aqueous KCl containing 40 mg of α-chymotrypsin (Worthington, 57 units/mg) was stirred at 37° while pH 7.6 was maintained by addition of 0.20 N NaOH with a pH-stat. Another 40 mg of α-chymotrypsin was added after 24 h. Uptake of NaOH (35.2 ml) stopped after 90 h. The suspension was filtered and the filtrate was extracted with ethyl acetate. The aqueous solution was acidified to pH 2.5 with HCl and extracted with ethyl acetate (3 × 200 ml). After evaporation of the ethyl acetate, the residue was refluxed with 2 N HCl for 3 h. Neutralization of the solution with NaOH gave a white precipitate, 763 mg (51%). The melting point and rotation were identical with the product obtained from the resolution with acylase.

α-*N*-*tert*-Butyloxycarbonyl-3-(2-naphthyl)-L-alanine (IV). Compound III (0.46 g, 2.1 mmol) was dissolved in a mixture of 50 ml of H_2O and 50 ml of THF and treated with *tert*-butyloxycarbonyl azide (0.46 ml, 3.2 mmol). The mixture was stirred for 18 h while maintaining the pH at 10.0 with pH-stat and washed with ether; the pH was adjusted to 3.0 and extracted with EtOAc (4 × 50 ml). The product was crystallized from EtOAc–petroleum ether: 0.45 g (67%); mp 93–95°; $[\alpha]^{23}D$ +42° (*c* 0.5, EtOH); TLC (CMA) R_f 0.75. Anal. ($C_{18}H_{21}NO_4$).

<Glu-His-3-(2-Naphthyl)-Ala-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (XII). The protected peptide amide synthesized following the procedure given above was deprotected and purified according to procedure B. The peptide was eluted at R_f 0.30 on partition chromatography and at R_f 0.49 on gel filtration with 0.2 N HOAc as eluent. The overall yield of the peptide was 37%: $[\alpha]^{23}D$ −43.9° (*c* 0.525, 1 M HOAc); TLC (silica) R_f^1 0.13, R_f^2 0.56, R_f^3 0.55; TLC (cellulose) R_f^1 0.60, R_f^2 0.95, R_f^3 0.92; TLE *E*(Glu), 0.22. Amino acid analysis gave Glu, 0.92; His, 1.1; 3-(2-naphthyl)-Ala, 0.76; Ser, 0.89; Tyr, 1.0; Gly, 2.1; Leu, 1.1; Arg, 0.90; Pro, 0.90; NH_3, 1.0. Anal. ($C_{57}H_{76}N_{16}O_{13}\cdot 3CH_3COOH\cdot 5H_2O$).

δ-*N*-*i*-Pr-α-*N*-Boc-Orn (IX). δ-*N*-Z-α-*N*-Boc-Orn (VIII) (18 g, 49 mmol) was dissolved in AR acetone (70 ml) and H_2O (50 ml). The pH was adjusted to 10.5 with 4 N NaOH and water added to bring the acetone–H_2O ratio to ~1.0. To this clear solution was added 0.18 g of 5% Pd/C catalyst and hydrogenated for 24 h at 40 psi (2.8 kg/cm^2). The catalyst was separated by filtration and washed well with water. The pH of the filtrate was brought to 7.0 and evaporated in vacuo (<40°). The residue was extracted with hot 2-propanol to give 15 g of IX (100%) (hygroscopic): mp 203–207° dec; $[\alpha]^{23}D$ +7.2° (*c* 1.7, H_2O); TLC (BAW, 4:1:1) R_f 0.35; NMR (D_2O + DSS) (CH_3)$_2$CH–NH (d, 6 H, J = 7 Hz, δ 1.36), (CH_3)$_3$C– (s, 9 H, δ 1.50). Anal. ($C_{13}H_{26}N_2O_4\cdot H_2O$).

δ-*N*-Tos-δ-*N*-*i*-Pr-α-*N*-Boc-L-Orn (X). A solution of 15 g (0.055 mol) of compound IX in 250 ml of H_2O was brought to pH 10.3 with 2.5 N NaOH. Tosyl chloride (25 g, 0.13 mol) in ether was added while maintaining the pH at 10.3 using pH-stat and stirred vigorously for 48 h at that pH. The solution was extracted with ether (4 × 50 ml); the aqueous solution was acidified to pH 2.0 and extracted with EtOAc (6 × 150 ml). The combined EtOAc extract was dried over $MgSO_4$ and evaporated in vacuo to give an oil. Chromatography over a silica gel column (60–200 mesh) gave 4.7 g (20%) of X being eluted with $CHCl_3$–C_6H_6 (50:50), which was recrystallized from C_6H_6–hexane: mp 100–105°; $[\alpha]^{23}D$ +1.8° (*c* 2, EtOH); TLC [EtOAc–HOAc–EtOH (9:1:1)] R_f 0.73; NMR ($CDCl_3$ + Me_4Si) –$SO_2NCH(CH_3)_2$ (d, 6 H, J = 7 Hz, δ 1.03), (CH_3)$_3$C– (s, 9 H, δ 1.48), $CH_3C_6H_4$– (s, 3 H, δ 2.43), $CH_3C_6H_4SO_2$– (4 H, δ 7.26–7.86). Anal. ($C_{20}H_{32}N_2O_6S$).

Glu-His-Trp-Ser-Tyr-Gly-Leu-δ-*N*-*i*-Pr-Orn-Pro-Gly-NH_2 (XIII). The protected decapeptide amide synthesized following the general procedure mentioned above was deblocked and purified following procedure A. The product was eluted at R_f 0.51 with 0.2 N HOAc on Sephadex G-15 and at R_f 0.21 on partition chromatography. The overall yield of the peptide was

39.8%: $[\alpha]^{23}D$ −53.0° (c 0.5, 0.2 N HOAc); TLC (silica gel) R_f^1 0.13, R_f^2 0.32, R_f^3 0.45; TLC (cellulose) R_f^1 0.69, R_f^2 0.90, R_f^3 0.91; TLE E(Glu), 0.26. Amino acid analysis gave Glu, 1.0; His, 0.98; Trp, 0.50; Ser, 0.87; Tyr, 0.99; Gly, 1.97; Leu, 1.0; δ-*N*-*i*-Pr-Orn[8], 1.0; Pro, 0.95; NH_3, 1.01. Anal. ($C_{57}H_{79}N_{15}O_{13}\cdot CH_3COOH\cdot 3H_2O$).

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Synthesis and Antiviral Activity of 5- and 5'-Substituted Thymidine Analogs

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The 5'-*O*-*p*-tolylsulfonyl derivatives of 5-chloro-, 5-bromo-, and 5-iodo-2'-deoxyuridine were synthesized and converted into the corresponding 5-halo-5'-azido-2',5'-dideoxyuridines (**5**–**7**). Reduction of 5-chloro-5'-azido-2',5'-dideoxyuridine (**5**) afforded 5-chloro-5'-amino-2',5'-dideoxyuridine (**10**, ACIU); however, similar efforts to prepare 5-bromo-5'-amino-2',5'-dideoxyuridine (**11**) and 5-iodo-5'-amino-2',5'-dideoxyuridine (**12**) by reduction of the corresponding 5'-azido precursor resulted in the formation of 5'-amino-2',5'-dideoxyuridine (**9**). 5-Bromo-5'-amino-2',5'-dideoxyuridine (**11**, ABrU) and 5-iodo-5'-amino-2',5'-dideoxyuridine (**12**, AIU) were prepared by halogenation of the 5-mercuriacetate of 5'-amino-2',5'-dideoxyuridine. The 5'-amino-2',5'-dideoxy analogs of 5-methyl-, 5-chloro-, 5-bromo-, and 5-iodo-2'-deoxyuridine possess antiviral activity against herpes simplex virus but exhibit no inhibitory activity against sarcoma 180 (murine) or Vero (monkey) cells in culture.

Baker et al.[1] first indicated that amino sugar nucleosides can possess biological activity. They reported that the activity of puromycin against a mammary adenocarcinoma and *Trypanosoma equipserdum* in mice is due to the in vivo enzymatic formation of N^6-dimethyl-3'-amino-adenosine. This nucleoside was found also to be an inhibitor of RNA synthesis as well as of cell division.[2] Other nucleosides that possess an amino substitutent in place of a sugar hydroxyl have been synthesized subsequently[3d,5] and some have been reported to exhibit antiviral or antineoplastic activity. Since thymidine analogs, such as 5-iodo-2'-deoxyuridine, 5-bromo-2'-deoxyuridine, 5-trifluoromethyl-2'-deoxyuridine, 5-ethyl-2'-deoxyuridine, 6-azathymidine, etc.,[3] have been shown to inhibit neoplastic cell and/or virus replication, the synthesis and biological activity of 5'-amino- (and 5'-azido-) thymidine