ethylacetic anhydride by general procedure C: NMR (methanol-d_4) δ 0.76–1.0 (m, 24 H), 1.22–1.70 [m, 13 H includes δ 1.30 (d), $J \simeq 7$ Hz], 2.0–2.2 (m, 2 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.89 (br m, 1 H), 3.98 (m, 1 H), 4.14 (d, 1 H, $J \simeq 7$ Hz), 4.24 (q, 1 H, $J \simeq 7$ Hz).

***N*-(*tert*-Butoxycarbonyl)-L-valyl-L-valyl-4(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (9).** The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and the symmetrical anhydride of Boc-L-Val-OH by general procedure C: NMR (methanol-d_4) δ 0.78–1.10 (m, 24 H), 1.24–1.78 [m, 18 H, includes δ 1.44], 1.87–2.20 (m, 2 H), 2.33 (d, 2 H, $J \simeq 7$ Hz), 3.78–4.38 (m, 5 H).

***N*-(*tert*-Butoxycarbonyl)-D-valyl-L-valyl-4(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (10).** The title compound was prepared as described for **9** starting with Boc-D-Val-OH: NMR (methanol-d_4) δ 0.80–1.02 (m, 24 H), 1.25–1.47 [m, 16 H, includes δ 1.43], 1.50–1.66 (m, 2 H), 1.95–2.22 (m, 2 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.85–4.02 (m, 3 H), 4.33 (d, 1 H, $J \simeq 7.5$ Hz), 4.23 (q, 1 H, $J \simeq 7$ Hz).

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (11).** The title compound was prepared from HCl·Val-Val-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride by general procedure C: NMR (methanol-d_4) δ 0.75–1.0 (m, 30 H), 1.23–1.43 [m, 7 H, includes δ 1.30 (d), $J \simeq 7$ Hz], 1.49–1.68 (m, 3 H), 1.95–2.15 (m, 4 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.89 (broad, 1 H), 3.99 (m, 1 H), 4.14 (m, 2 H), 4.25 (q, 1 H, $J \simeq 7$ Hz).

Acknowledgment. This work was supported in part by grants from the National Institutes of Health (AM 20100) and Merck Sharp & Dohme. We thank Dr. Paul G. Schmidt for 270-MHz NMR spectra of the inhibitors. We thank Dr. R. Bott for the preliminary X-ray data of the *R. chinensis* pepsin–pepstatin complex.

Synthesis and Biological Activity of Some Very Hydrophobic Superagonist Analogues of Luteinizing Hormone-Releasing Hormone[1]

John J. Nestor, Jr.,*[†] Teresa L. Ho,[†] Richard A. Simpson,[†] Bonnie L. Horner,[†] Gordon H. Jones,[†] Georgia I. McRae,[‡] and Brian H. Vickery[‡]

Institute of Organic Chemistry and Institute of Biological Sciences, Syntex Research, Palo Alto, California 94304.
Received September 14, 1981

The effect of increased hydrophobicity at position 6 of luteinizing hormone-releasing hormone (LH-RH) has been investigated by the incorporation of a series of 15 very hydrophobic, unnatural D-amino acids at this position. The unnatural amino acids studied can be considered analogues of phenylalanine with carbocyclic aromatic side chains consisting of substituted phenyl (e.g., 2,4,6-trimethylphenyl, *p*-biphenyl) or polycyclic aromatic (e.g., naphthalene, anthracene) units. When enzymatic resolution (subtilisin Carlsberg) of the most hydrophobic amino acids failed, the racemic amino acids were incorporated, and the diastereomeric LH-RH analogues were resolved by preparative high-performance liquid chromatography. The analogues were synthesized by the solid-phase technique. All of the synthetic compounds were very potent LH-RH superagonists, but [6-(3-(2-naphthyl)-D-alanine)]LH-RH, [6-(3-(2-naphthyl)-D-alanine),7-(N^{α}-methylleucine)]LH-RH and [6-(3-(2,4,6-trimethylphenyl)-D-alanine)]LH-RH appear to be among the most potent LH-RH agonist analogues yet reported when tested in a rat estrus cyclicity suppression assay designed to show the paradoxical antifertility effects of these compounds [$ED_{50} \approx 7 \times 10^{-8}$ g; twice daily in saline]. These analogues are twice as potent as [D-Trp^6,ProNHEt^9]LH-RH in this assay system (i.e., ~200 times the potency of LH-RH).

The isolation and determination of the structure of luteinizing hormone-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, LH-RH) from porcine[2] and ovine[3] hypothalami have led to the synthesis of a large series of analogues,[4,5] some of which have exhibited dramatically enhanced agonist ("superagonist") or antagonist potency. With further pharmacological study it has become clear that such compounds offer promising routes to the control of reproduction in both males and females. Of particular interest to us has been the demonstration that chronic treatment with pharmacological doses of long lived superagonist LH-RH analogues leads to "paradoxical" antifertility[6] and antisteroidal[7] effects which appear to be due to the desensitization of target cells in the anterior pituitary[8] and the gonads.[9] These results and their implications for fertility control led us to undertake a synthetic program designed to yield more potent superagonist LH-RH analogues with a better pharmacodynamic profile.

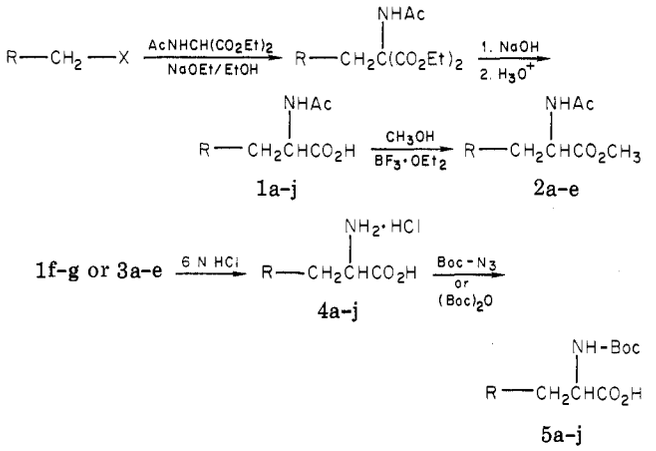
Previous analogue programs have demonstrated that the primary sites for successful modification of the LH-RH structure to yield superagonist analogues are at positions 6, 7, and 10. At position 6, D-amino acids led to major increases (3–40 times) in potency,[10] with an apparent preference for lipophilic amino acids. D-Trp gave the most potent analogues[11,12] at the start of these studies, and [D-Trp^6,ProNHEt^9]LH-RH was therefore chosen as the

[†] Institute of Organic Chemistry.
[‡] Institute of Biological Sciences.

(1) Contribution no. 610 from the Institute of Organic Chemistry, Syntex Research. Some of these data were presented in a preliminary form at the 7th American Peptide Symposium, Madison, WI, June 1981. The unnatural amino acids used in this study have been given the following abbreviations: Tmo, 3-(3,4,5-trimethoxyphenyl)alanine; Cha, 3-cyclohexylalanine; Pfp, 3-(pentafluorophenyl)alanine; Nal(1), 3-(1-naphthyl)alanine; Nal(2), 3-(2-naphthyl)alanine; Mtf, 3-[*m*-(trifluoromethyl)phenyl]alanine; Ptf, 3-[*p*-(trifluoromethyl)phenyl]alanine; Tmp, 3-(2,4,6-trimethylphenyl)alanine; Bna, 3-(1-bromo-2-naphthyl)alanine; Daa, 3-(9,10-dihydro-9-anthryl)-alanine; Bpa, 3-(*p*-biphenylyl)alanine; Fla, 3-(2-fluorenyl)alanine; Ana, 3-(9-anthryl)alanine; Dca, 3-(dicyclohexylmethyl)-alanine; Bha, 3-(benzhydryl)alanine. The abbreviations for natural amino acids and protecting groups follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1971)].
(2) H. Matsuo, Y. Baba, R. M. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971).
(3) R. Burgus, M. Butcher, M. Amoss, N. Ling, M. W. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale, and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 278 (1972).
(4) J. Rivier, M. Brown, C. Rivier, M. Ling, and W. Vale in "Peptides 1976", A. Loffett, Ed., Editions de l'Universite de Bruxelles, Brussels, 1976, pp 427–451, and references cited therein.
(5) A. V. Schally, D. H. Coy, and C. A. Meyers, *Annu. Rev. Biochem.*, **47**, 89 (1978), and references cited therein.

0022-2623/82/1825-0795$01.25/0

Scheme I[a]



[a] For compounds 1-5: a, R = benzhydryl; b, R = *m*-(trifluoromethyl)phenyl; c, R = *p*-(trifluoromethyl)phenyl; d, R = 2-fluorenyl; e, R = 3,4,5-trimethoxyphenyl; f, R = 9-anthryl; g, R = 2,4,6-trimethylphenyl; h, R = 1-bromo-2-naphthyl; i, R = 2-naphthyl; j, R = 1-naphthyl.

standard in our assay system (potency 144 times LH-RH, in vitro[11]). At position 7, *N*-methylleucine (NMeLeu) substitution[13] led to a small increase in activity, with some enhancement of resistance to enzymatic degradation.[11] At position 10, replacement of the Gly residue by NHEt,[14] $NHCH_2CF_3$,[15] or azaglycinamide[16] led to a moderate increase in activity.

We have investigated the interaction of these sites of modification while keeping the primary focus on position 6. Our approach has been to synthesize analogues with unnatural, very hydrophobic D-amino acid residues at position 6 with the hope of achieving prolonged biological half-life and increased potency.

Chemistry.[17] All of the peptides were synthesized by the Merrifield solid-phase method.[18,19] Decapeptide analogues were synthesized on benzhydrylaminopolystyrene–1% divinylbenzene resin,[20] and nonapeptide ethylamide analogues were synthesized on chloromethyl-

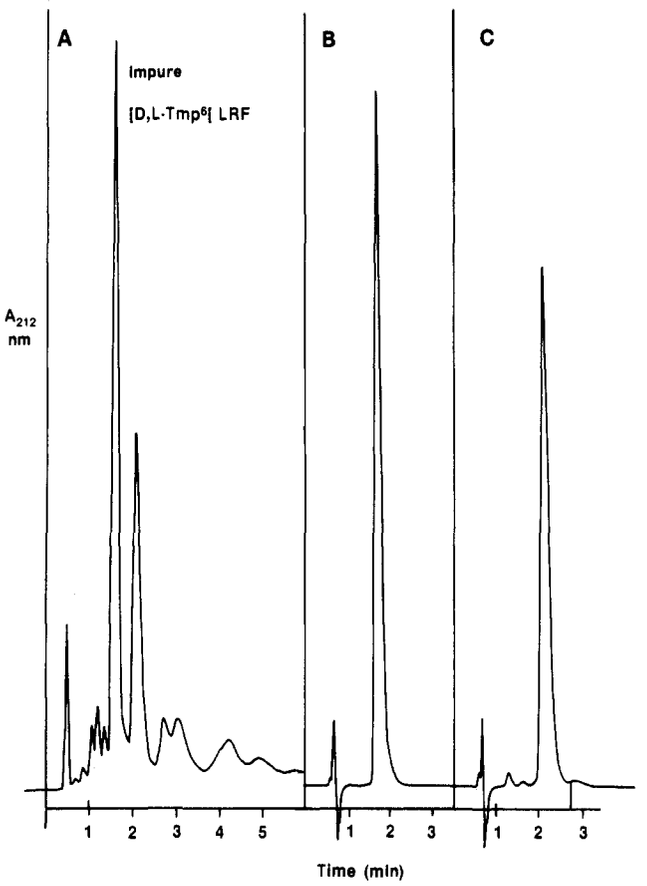
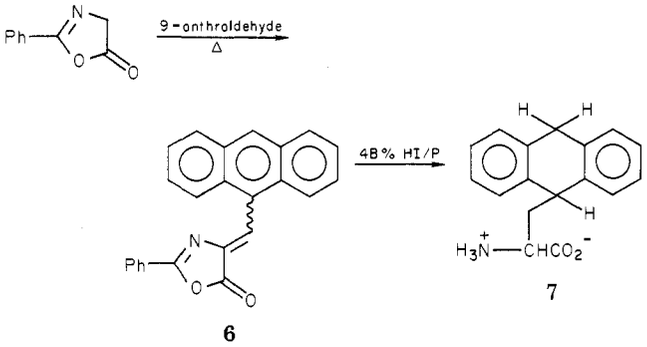


Figure 1. Analytical HPLC traces showing diastereomer resolution by prep-HPLC: (A) Impure [DL-Tmp^6]LH-RH from HF cleavage; (B) [L-Tmp^6]LH-RH and (C) [D-Tmp^6]LH-RH from prep-HPLC of diastereomeric mixture. Prep-HPLC conditions: 34% CH_3CN/H_2O (0.06 N NH_4OAc, pH 4.5). Analytical HPLC conditions: 50% CH_3CN/H_2O (0.03 N NH_4OAc, pH 4.5). See Experimental Section for further details.

Scheme II



polystyrene–1% divinylbenzene resin.[21] N^{α}-*tert*-butoxycarbonyl (Boc) protection[22] was used on all amino acids except <Glu, which was unprotected. The side-chain protection of amino acids was as follows: Arg(Tos),[23] Tyr(2,6-Cl_2Bzl),[24] Ser(Bzl), His(Tos).[25] Anhydrous liquid HF was used for the final deprotection.[26] The crude peptides were purified by preparative high-performance

(6) A. Corbin and C. W. Beattie, *Endocr. Res. Commun.*, **2**, 445 (1975).
(7) B. H. Vickery in "Antihormones", M. K. Agarwal, Ed., Elsevier/North Holland, New York, 1981, in press.
(8) J. Sandow, W. V. Rechenberg, G. Gerzabek, and W. Stoll, *Fertil. Steril.*, **30**, 205 (1978).
(9) A. J. W. Hsueh and G. F. Erickson, *Science*, **204**, 854 (1979).
(10) M. W., Monahan, M. S. Amoss, H. A. Anderson, and W. Vale, *Biochemistry*, **12**, 4616 (1973).
(11) W. Vale, C. Rivier, M. Brown, N. Ling, M. Monahan, and J. Rivier, in "Clinical Endocrinology", 5th Suppl, I. McIntyre, Ed., Blackwell Scientific Publications, Oxford, 1976, p 2615.
(12) D. H. Coy, J. A. Vilchez-Martinez, E. J. Coy, and A. V. Schally, *J. Med. Chem.*, **19**, 423 (1976).
(13) N. Ling and W. Vale, *Biochem. Biophys. Res. Commun.*, **63**, 801 (1975).
(14) M. Fujino, S. Kobayashi, M. Obayashi, S. Shinagawa, T. Fukuda, C. Kitada, R. Nakayama, I. Yamazaki, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Commun.*, **49**, 863 (1972).
(15) D. H. Coy, J. A. Vilchez-Martinez, E. Coy, M. Nishi, A. Arimura, and A. V. Schally, *Biochemistry*, **14**, 1848 (1975).
(16) A. S. Dutta, B. J. A. Furr, M. B. Giles, B. Valcaccia, and A. L. Walpole, *Biochem. Biophys. Res. Commun.*, **81**, 382 (1978).
(17) These compounds and procedures are protected by U.S. Patent 4 234 571 (1980) to J. J. Nestor, G. H. Jones, and B. H. Vickery.
(18) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(19) J. M. Stewart and J. D. Young, in "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, 1969.
(20) P. G. Pietta and G. R. Marshall, *Chem. Commun.*, 650 (1970).
(21) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).
(22) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 4427 (1957).
(23) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).
(24) D. Yamashiro and C. H. Li, *Int. J. Pept. Protein Res.*, **4**, 181 (1972).
(25) T. Fujii and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **43**, 3954 (1970).
(26) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **38**, 1412 (1965).

Table I. Amino Acid Intermediates

no.	formula	M_r	yield, %	mp, °C	$[\alpha]^{25}_D$ (*c*, MeOH), deg	anal.
1a	$C_{18}H_{19}NO_3$	297.36	84	174-175		C, H, N
1b	$C_{12}H_{12}NO_3F_3$	275.24	61	120-122		C, H, N
1c	$C_{12}H_{12}NO_3F_3$	275.24	67	198-200		C, H, N
1d	$C_{18}H_{17}NO_3$	295.34	85	225-227		C, H, N
1e	$C_{14}H_{19}NO_6$	297.31	61	189-190		C, H, N
1f	$C_{19}H_{17}NO_3$	307.35	67	270-271		C, H, N
1g	$C_{14}H_{19}NO_3$	249.31	83	210-212		C, H, N
1h	$C_{15}H_{14}NO_3Br$	336.20	86	204-206		C, H, N
2a	$C_{19}H_{21}NO_3$	311.39	90	113-114		C, H, N
2b	$C_{13}H_{14}NO_3F_3$	289.26	86	63-65		C, H, N
2c	$C_{13}H_{14}NO_3F_3$	289.26	89	110-112		C, H, N
2d	$C_{19}H_{19}NO_3$	309.37	95	170-171		C, H, N
2e	$C_{15}H_{20}NO_6$	310.33	88	129-131		C, H, N
3a	$C_{19}H_{21}NO_3$	311.39	84	113-114	−3.6 (0.7)	C, H, N
3b	$C_{13}H_{14}NO_3F_3$	289.26	80	68-70	−10.0 (1)	C, H, N
3c	$C_{13}H_{14}NO_3F_3$	289.26	76	93-95	−11.1 (1)	C, H, N
3d	$C_{19}H_{19}NO_3$	309.37	95	172-172.5	−46.9 (0.6)	C, H, N
3e	$C_{15}H_{20}NO_6$	310.33	71	130-132	−16.6 (0.7)	C, H, N
4a	$C_{16}H_{17}NO_2 \cdot 0.5HCl$	273.55	91	235-237	−23.3 (0.6)	C, H, N
4b	$C_{10}H_{10}NO_2F_3 \cdot HCl$	269.66	95	208-210	−19.7 (0.5)	C, H, N
4c	$C_{10}H_{10}NO_2F_3 \cdot {}^1/_3HCl$	245.35	91	190-192	−1.7 (0.5)	C, H, N
4d	$C_{16}H_{15}NO_2 \cdot {}^2/_3HCl$	277.15	81	264-269	−4.4 (0.6)	C, H, N
4e	$C_{12}H_{17}NO_5 \cdot 0.6HCl$	277.61	94	188-190	2.5 (0.2)	C, H, N
4f	$C_{17}H_{15}NO_2 \cdot HCl$	301.78	86	<290 dec	DL	C, H, N
4g	$C_{12}H_{17}NO_2 \cdot 0.1H_2O$	209.08	89	235-237	DL	C, H, N
4h	$C_{13}H_{12}NO_2Br \cdot 1.5HCl$	348.86	98	258-260	DL	C, N; H[a]
5a	$C_{21}H_{25}NO_4$	355.44	87	153-154	9.1 (1)	C, H, N
5b	$C_{15}H_{18}NO_4F_3$	333.32	91	100-102	−7.1 (1)	C, H, N
5c	$C_{15}H_{18}NO_4F_3$	333.32	84	117-118	−4.8 (1)	C, H, N
5d	$C_{21}H_{23}NO_4$	353.42	97	161-163 dec	−29.9 (0.5)	C, H, N
5e	$C_{17}H_{25}NO_7$	355.40	72	74-76	−7.4 (1)	C, H, N
5f	$C_{22}H_{23}NO_4$	377.44	77	140-142	DL	C, H, N
5g	$C_{17}H_{25}NO_4$	307.40	92	153-154	DL	C, H, N
5h	$C_{18}H_{20}NO_4Br$	394.28	91	167-169	DL	C, H, N

[a] H: calcd, 3.90; found, 4.31.

liquid chromatography (prep-HPLC).

Boc-D-Cha-OH,[27] Boc-D-Bpa-OH,[28] Boc-D-Nal(2)-OH,[28] Boc-D-Nal(1)-OH,[28,29] Boc-D-Pfp-OH,[30] and racemic Ac-Bha-OH[31] (**1a**) were prepared as described (for abbreviations see ref 1). Amino acids **1b–h** were prepared from diethyl acetamidomalonate and the corresponding aralkyl halide by a modification[32] of the Sorensen procedure[33] as shown in Scheme I. Resolution of the racemic *N*-acetyl methyl esters **3a–d** was performed by enzyme-catalyzed hydrolysis of the L-esters by subtilisin Carlsberg as described by Bosshard and Berger.[34] This enzyme gave much faster resolution of the more hydrophobic amino acids [e.g., Nal(2)] than did acylase or α-chymotrypsin.[28,29] Enzymatic resolution failed for some of the most bulky amino acids. In those cases where this resolution method failed (**4f–h**, **7**), the Boc-protected racemic amino acids were incorporated into the peptide, and the deprotected, diastereomeric products were easily separated by prep-HPLC[35] (Figure 1). In each case studied, the later peak[36,37] contained the D 6-position (biologically active) analogue. Some of the earlier running, L 6-position analogues were assayed and were found to be inactive at the doses tested (e.g., [L-Ana[6]]LH-RH was inactive at 0.4 μg/injection).

Amino acid **8** was obtained by the Erlenmeyer[38] azlactone route after reductive hydrolysis[39] with HI/P (Scheme II). Reduction of resolved H-Bha-OH[31] yielded 3-(dicyclohexylmethyl)-D-alanine (**9**).

Bioassay. The analogues were tested in an estrus suppression assay designed to show the paradoxical antifertility effects of these compounds. Adult female rats were injected (sc) twice daily for 14 days with a solution of test compound in 0.1% BSA–0.9% saline vehicle. The percent of rats showing complete estrus suppression (persistent diestrus, determined by daily vaginal lavage) from day 4 onward was plotted against log dose, and the ED_{50} for complete suppression of estrus was calculated (Tables II and III).

Biological Results and Discussion

The analogues listed in Tables II and III were designed to test the hypothesis that more potent LH-RH agonists could be obtained by substantially increasing the hydrophobicity in position 6. The capacity factor[40] (k') from analytical reversed phase HPLC (Altex Ultrasphere C-18, 40% CH_3CN/H_2O, eluent 0.03 M in NH_4OAc, pH 7) has

(27) E. Waser and E. Brauchli, *Helv. Chim. Acta*, 7, 740 (1924) and M. C. Khosla, R. A. Leese, W. L. Maloy, A. T. Ferreira, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, 15, 792 (1972).
(28) Y. Yabe, C. Miura, H. Horikoshi, and Y. Baba, *Chem. Pharm. Bull.*, 24, 3149 (1976).
(29) K. U. Prasad, R. W. Roeske, F. L. Weitl, J. A. Vilchez-Martinez, and A. V. Schally, *J. Med. Chem.*, 19, 492 (1976).
(30) J. L. Fauchere and R. Schwyzer, *Helv. Chim. Acta*, 54, 2078 (1971).
(31) J. Altman, R. Mosberg, and D. Ben-Ishai, *Tetrahedron Lett.*, 3737 (1975).
(32) H. R. Snyder, J. F. Shekleton, and C. D. Lewis, *J. Am. Chem. Soc.*, 67, 310 (1945).
(33) S. P. L. Sorensen, *Z. Physiol. Chem.*, 44, 448 (1905).
(34) H. R. Bosshard and A. Berger, *Helv. Chim. Acta*, 56, 1838 (1973).
(35) R. Burgus and J. Rivier, in ref 4, p 85.
(36) E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50, 1353 (1978).
(37) B. Larsen, B. L. Fox, M. F. Burke, and V. J. Hruby, *Int. J. Pept. Protein Res.*, 13, 12 (1979).
(38) E. Erlenmeyer, *Ann. Chem.*, 275, 1 (1893).
(39) C. R. Harington and W. McCartney, *Biochem. J.*, 21, 852 (1927).
(40) k' = (retention volume − void volume)/void volume.

Table II. LH-RH Analogues: Position 6 Substitutions

no.	LH-RH analogue[a]	mp, °C	$[\alpha]^{25}_D$ (c, HOAc),[b] deg	formula[c]	M_r	HPLC: k'[d]	TLC[e] BAW	TLC[e] BEAW	estrus suppression ED_{50}, μg	estrus suppression potency[f]
11	[D-Tmo^6]-	168–170	−30.5 (0.1)	$C_{67}H_{91}N_{17}O_{18}$	1422.58	0.59	0.26	0.51	0.09	1.4[g]
12	[D-Cha^6]-	162–164	−15.2 (0.2)	$C_{64}H_{91}N_{17}O_{15}$	1388.55	1.45	0.30	0.61	0.3	0.5
13	[D-Pfp^6]-	168–170	−31.1 (0.2)	$C_{64}H_{80}N_{17}O_{15}F_5$	1422.46	1.73	0.32	0.62	0.12	1.2
14	[D-Nal(1)^6]-	173–175	−28.1 (0.8)	$C_{68}H_{87}N_{17}O_{15}$	1382.56	1.91	0.28	0.64	0.3	0.5
15	[D-Nal(2)^6]-	188–190	−27.4 (0.9)	$C_{68}H_{87}N_{17}O_{15}$	1382.56	1.95	0.32	0.62	0.07	1.9
16	[D-Mtf^6]-	163–165	−29.6 (0.4)	$C_{65}H_{84}N_{17}O_{15}F_3$	1343.51	2.05	0.28	0.61	0.13	1.1
17	[D-Ptf^6]-	168–170	−30.1 (0.2)	$C_{65}H_{84}N_{17}O_{15}F_3$	1343.51	2.14	0.31	0.62	0.11	1.4
18	[D-Tmp^6]-	178–185	−42.1 (1.0)	$C_{67}H_{91}N_{17}O_{15}$	1374.58	2.18	0.31	0.61	0.07	2.0
19	[D-Bna^6]-	176–185	−43.8 (0.1)	$C_{68}H_{86}N_{17}O_{15}Br$	1461.46	2.33	0.33	0.60	0.15	1.0
20	[D-Daa^6]-	178–180	−20.3 (0.7)	$C_{72}H_{91}N_{17}O_{15}$	1434.64	3.18	0.32	0.59	0.2	0.7
21	[D-Bpa^6]-	177–185	−37.5 (1)	$C_{70}H_{89}N_{17}O_{15}$	1408.60	3.23	0.30	0.64		1.4[h]
22	[D-Fla^6]-	183–192	−25.8 (1)	$C_{71}H_{89}N_{17}O_{15}$	1420.61	3.50	0.31	0.63		1.0[h]
23	[D-Ana^6]-	187–195	−16.9 (1)	$C_{72}H_{89}N_{17}O_{15}$	1432.62	4.09	0.28	0.61	0.27	0.5
24	[D-Dca^6]-	172–174	−30.5 (0.1)	$C_{70}H_{101}N_{17}O_{15}$	1420.70	26.27	0.34	0.63	0.14	0.9[g]

[a] Unnatural amino acid abbreviations are given in ref 1. Acceptable amino acid analyses were obtained for all LH-RH analogues (see Experimental Section). [b] Optical rotations were measured in glacial HOAc at the concentration indicated (w/v %). [c] Formulas are given for an assumed composition of the molecule as the monoacetate, but the actual composition will contain various amounts of water and acetic acid. [d] k' = (retention volume − void volume)/void volume; conditions in Experimental Section. [e] BAW = 1-BuOH/HOAc/H_2O, 4:1:5 (upper phase); BEAW = 1-BuOH/EtOAc/HOAc/H_2O, 1:1:1:1. [f] Potency is calculated relative to the ED_{50} of **25** as standard (ED_{50} = 0.12–0.14 μg/injection; potency = 1). [g] Average ED_{50} for **25** used. [h] One injection/day assay used.

been used as a measure of the hydrophobicity of the analogues, and they have been arranged in order of increasing hydrophobicity in Tables II and III. It is evident from these data that analogues with very dramatically increased hydrophobicity have been prepared, when compared with the standard, [D-Trp^6,ProNHEt^9]LH-RH (Table III).

It has been suggested by Rivier[41] that there is a direct correlation between biological activity and retention time on reversed phase HPLC when performed at pH 7. Very recently[42] it has been suggested that a quantitative structure–activity relationship (QSAR) exists between potency of 6-position substituted LH-RH analogues and the calculated hydrophobicity of the amino acid side chain in position 6.

Inspection of the potencies of our monosubstituted 6-position analogues reveals that there is not a simple, direct relationship between k' and biological activity. Although [D-Nal(2)^6]LH-RH and [D-Nal(1)^6]LH-RH are essentially identical in hydrophobicity, as measured by k', there is a 4-fold difference in potency. The relatively high potency of [D-Tmo^6]LH-RH is also suprising in this regard. In the case of both the [D-Nal(2)^6]LH-RH/[D-Nal(1)^6]LH-RH and [D-Ptf^6]LH-RH/[D-Mtf^6]LH-RH pairs, the less potent analogue is the one which brings a side-chain branch point closer to the peptide backbone. On the other hand, [D-Tmp^6]LH-RH is an analogue with severe potential steric interaction between the side chain and the polypeptide backbone, yet it is one of our most potent analogues. In a recent QSAR study[42] there was a suggestion that side-chain steric bulk has a slight negative effect on potency, but this appears to be a significant factor only for [D-Ala^6]LH-RH and [6-D-phenylglycine]LH-RH in their data.

In a qualitative sense, it appears that the relatively compact, hydrophobic side chains give the most potent analogues, while the larger, more hydrophobic side chains yield analogues with somewhat reduced potencies. These studies have not, however, shown that any D-amino acid substitutions, even quite bulky ones (e.g., [D-Ana^6]LH-RH), introduce prohibitive steric requirements. This is, perhaps, to be expected in view of the observation that [D-Lys^6]-LH-RH can be conjugated to poly(Glu) with retention of the superagonist activity of the monomer.[43]

The analogues in Table III were studied in an attempt to demonstrate the multiplicative effects[4,5] of modifications at positions 7 and 10 on biological potency seen in earlier LH-RH analogue studies. A comparison between [D-Nal(2)^6]LH-RH and [D-Nal(2)^6,ProNHEt^9]LH-RH shows that the ProNHEt modification, which had been found to give a 3- to 5-fold increase in activity for less hydrophobic analogues,[4,5] leads to a substantial decrease in potency. The substantial increase in apparent hydrophobicity caused by the ProNHEt modification, coupled with a slight decrease in potency, again contradicts a simple relationship between hydrophobicity and biological activity.

The NMeLeu substitution[13] was coupled with two of our best 6-position substitutions. It can be seen that the addition of this single 7-position modification to the successful Nal(2) modification to give [D-Nal(2)^6,NMeLeu^7]-LH-RH has only a small effect on biological potency, while its incorporation into the triply modified analogue, [D-Nal(2)^6,NMeLeu^7,ProNHEt^9]LH-RH, has led to a substantial increase in activity relative to [D-Nal(2)^6,ProNHEt^9]LH-RH. When incorporated into an analogue with the sterically hindered Tmp to give [D-Tmp^6,NMeLeu^7]-LH-RH, there is a substantial decrease in activity. There is, therefore, no clear trend with this substitution for this series of analogues.

Previous studies have shown that the replacement of the Trp at position 3 by Nal(1),[28] but not by Nal(2),[28,29] gives an approximate 2-fold increase in biological activity. In [L-Nal(1)^3,D-Nal(2)^6,ProNHEt^9]LH-RH, this substitution has not shown a significant positive effect on potency when compared with [D-Nal(2)^6,ProNHEt^9]LH-RH, but Nal(1) again has been shown to effectively replace Trp in position 3.

Our results show that, although the dominant factor in obtaining LH-RH analogues of the highest potency is the

(41) J. Rivier, C. Rivier, M. Perrin, J. Porter, and W. Vale, in "Peptides 1980", Proceedings of the 16th European Peptide Symposium, K. Brunfeldt, Ed., Scriptor Publisher ApS, Copenhagen, 1981, p 566.
(42) L. Nadasdi and K. Medzihradszky, *Biochem. Biophys. Res. Commun.*, **99**, 451 (1981).
(43) M. S. Amoss, M. W. Monahan, and M. S. Verlander, *J. Clin. Endocrinol. Metab.*, **39**, 203 (1974).

Table III. LH-RH Analogues: Multisubstitutions

no.	LH-RH analogue[a]	mp, °C	$[\alpha]^{25}_D$, (c, HOAc),[b] deg	formula[c]	M_r	HPLC:[d] k′	TLC[e] BAW	TLC[e] BEAW	estrus suppression ED$_{50}$, μg	estrus suppression potency[f]
25	[D-Trp^6,ProNHEt^9]-			$C_{66}H_{87}N_{17}O_{14}$	1342.55	1.29	0.34	0.64	standard[j]	1.0
26	[D-Nal(2)^6,ProNHEt^9]-	180–190	−57.2 (1)[i]	$C_{68}H_{88}N_{16}O_{14}$	1353.56	3.55	0.37	0.64	0.22	0.9
27	[D-Nal(2)^6, NMeLeu^7]-	183–190	−81.3 (1)	$C_{69}H_{89}N_{16}O_{15}$	1382.58	3.73	0.32	0.61	0.07	1.9
28	[D-Tmp^6,NMeLeu^7]-	178–186	−77.0 (0.5)	$C_{68}H_{93}N_{17}O_{15}$	1388.61	5.13	0.33	0.63		1.1[h]
29	[D-Bha^6,ProNHEt^9]-	176–206	−33.7 (1)[i]	$C_{71}H_{92}N_{16}O_{14}$	1393.63	5.27	0.33	0.65	0.11	0.9
30	[Nal(1)^5,D-Nal(2)^6,ProNHEt^9]-	160–170	−45.3 (1)	$C_{70}H_{89}N_{15}O_{14}$	1364.59	6.09	0.37	0.66	0.16	0.9
31	[D-Nal(2)^6,NMeLeu^7,ProNHEt^9]-	170–185	−81.1 (0.7)[i]	$C_{69}H_{90}N_{16}O_{14}$	1367.59	6.73	0.38	0.63	0.12	1.8

[a–h] See corresponding footnotes in Table II. [i] Optical rotation performed in 10% HOAc. [j] References 11 and 12.

hydrophobicity[41,42] of the amino acid in position 6, the relationship between hydrophobicity and biological activity is not a simple one. The previously studied modifications at positions 3, 7, and 10 which gave improved activities were shown to be ineffective when combined with our most potent 6-position modifications. As of this date, the most potent LH-RH superagonists reported by other groups are [imBzl-D-His^6]LH-RH[44] and [6-D-pentamethylphenylalanine]LH-RH.[42] While we have not tested these analogues in our assay system, their previously reported potencies vs. [D-Trp^6,ProNHEt^9]LH-RH and [D-Pfp^6]LH-RH (i.e., potencies of approximately 2.0 relative to **25** and 1.8 relative to **13**, respectively) indicate that our analogues **15**, **18**, and **27** are among the most potent LH-RH superagonist compounds yet reported (~200 times LH-RH in this assay).

The application of these substitutions to the LH-RH antagonist series is under active investigation. The coupling of these modifications with Aza-Gly^{10} substitution[16] is also being investigated.

Experimental Section

General Methods. Melting points were obtained on a Thomas-Hoover apparatus and are corrected. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in a 1-dm microcell at 25 °C. Thin-layer chromatography (TLC) was performed in a solvent vapor saturated chamber on 5 × 20 cm glass plates coated with a 250-μm layer of silica gel GF (Analtech). The plates were visualized by UV absorption, followed by chlorination (Cl_2) and 1% KI/starch spray. Silica gel column chromatography was performed on E. Merck silica gel 60 (70–230 mesh) with gravity flow.

All solvents used for synthetic work were of AR quality (Mallinckrodt) and were used as received. Boc-protected L-amino acids were purchased from various sources. Commercially available precursors were purchased for the synthesis of 9-(bromomethyl)anthracene,[46] 2-(bromomethyl)fluorene,[47] and 4-(trifluoromethyl)-α-bromotoluene.[48] The other aralkyl halides used were available from Aldrich or Tridom/Fluka.

Analytical HPLC was performed under isocratic conditions on a Chromatronix Model 3500 equipped with a 20-μL loop injector (Rheodyne) and an Altex Ultrasphere 5-μm C-18 reversed phase column (4.6 × 250 mm). The eluent was filtered (0.5-μm Teflon filter, Millipore) and vacuum degassed after preparation from CH_3CN (Burdick and Jackson, UV) and quartz distilled water. The eluent contained 40% CH_3CN and was 0.03 M in NH_4OAc (Tridom/Fluka, puriss) at pH 7. The column effluent was monitored at 214 nm with a Schoeffel SF770 spectroflow detector.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman 119CL analyzer in the single column mode after 18–24 h hydrolysis in 4 N $MeSO_3H$–0.2% 3-(2-aminoethyl)indole reagent[45] (Pierce Chemical Co.). The buffer sequence pH 3.25 (50 min), pH 4.12 (67 min), pH 6.25 was used, but the elution time was lengthened to a total of 360 min for the more hydrophobic amino acids. Bna, Bpa, Fla, Ana, Daa, Dca, and Bha were not eluted from the amino acid analyzer under the conditions described here. The presence of an equimolar amount of Bna (λ_{max} 286 nm, ε 6397), Bpa (λ_{max} 253 nm, ε 17 581), Fla (λ_{max} 303 nm, ε 10 549), and Ana (λ_{max} 387 nm, ε 8464) in respective LH-RH analogues was determined by the UV absorption of solutions of the analogues in 10% HOAc/CH_3OH. The high-wavelength UV absorption of unsubstituted LH-RH is λ_{max} 280 nm (ε ~6400; Trp, Tyr). For the LH-RH analogues the results are: **19**, λ_{max} 284 nm (ε 11 347); **21**, λ_{max} 255 nm (ε 21 833); **22**, λ_{max} 303 nm (ε 10 264); **23**, λ_{max} 388 nm (ε 8606). Daa, Dca, and Bha do not have useful UV spectra for quantitation. Their presence in the LH-RH analogues can be inferred from the very hydrophobic nature of the molecules (k′ values).

(44) W. W. Vale, C. Rivier, M. Perrin, and J. Rivier, in "Peptides—Structure and Biological Function", E. Gross and J. Meienhofer, Eds., Pierce Chemical Co., Rockford, IL, 1979, p 781.

(45) R. J. Simpson, M. R. Neuberger, and T. Y. Lin, *J. Biol. Chem.*, **251**, 1936 (1976).

Amino Acid Synthesis. The *N*-Ac DL-amino acids (**1a–3**) were prepared by the Sorensen[33] route (Figure 1), essentially as described by Snyder et al.[32] The reaction was followed by TLC ($CH_2Cl_2/CH_3OH/HOAc$, 9:1:1) until the starting materials were consumed. The resultant mixture of mono- and dicarboethoxy intermediates was saponified and decarboxylated as described.[32] Crystallization from EtOAc/hexane yielded the desired intermediates (Table I).

Resolution Procedure.[34] The *N*-Ac DL-amino acid (**1a–d**; 60 mmol) was refluxed in 250 mL of dry MeOH containing $BF_3{\cdot}OEt_2$ (126 mmol, 2.1 equiv) for 1 h.[49] The solvent was evaporated, and the residue was partitioned between H_2O and EtOAc. The EtOAc extracts were washed with 5% $NaHCO_3$, H_2O, and 4% $NaHSO_4$. The organic layer was dried ($MgSO_4$), filtered, and concentrated. The *N*-Ac DL-amino acid methyl esters (**2a–3**) were crystallized from EtOAc/hexane (Table I).

The ester (**2a–e**; 30 mmol) was dissolved in a mixture of 120 mL of dimethyl sulfoxide, 100 mL of H_2O, and 20 mL of 0.01 M KCl which was adjusted to pH 7 with 1 N NaOH. The enzyme, Protease, type VIII (subtilisin Carlsberg, Sigma Chemical Co.), was added (45 mg), and the reaction mixture was stirred magnetically (~24 h) while the pH was maintained at 7 with 1 N NaOH on a pH stat (Radiometer). When the theoretical quantity of NaOH had been consumed, the solution was diluted with 120 mL of H_2O. The aqueous layer was extracted with 3 × 100 mL of EtOAc to obtain the *N*-Ac D-amino acid ester (**3a–e**); see Table III. Resolution with this enzyme has given results comparable to those obtained by resolution with α-chymotrypsin [e.g., Ac-D-Nal(2)-OCH_3: $[\alpha]^{25}_D$ −42.7° (*c* 0.8, EtOH) [lit.[50] $[\alpha]^{25}_D$ −43.5° (*c* 0.8, EtOH)].

The resolved ester was hydrolyzed in 6 N HCl at reflux for 2 h, and the solution was concentrated to dryness to yield the D-amino acid hydrochloride (**4a-e**; Table I).

In those cases where no base was consumed and the enzymatic hydrolysis did not proceed, the *N*-Ac DL-esters (**3f–g**) or *N*-Ac DL-acids (**1e–h**) were hydrolyzed with 6 N HCl to the racemic amino acids (**4e–h**; Table I).

3-(9,10-Dihydro-9-anthryl)-DL-alanine (7). A sample of 2-phenyloxazol-5-one (3.22 g, 20 mmol), prepared[51] from hippuric acid, was treated with 9-anthraldehyde (1.03 g, 5 mmol) at 100–110 °C for 20 min to form a yellow solid. The solid was dissolved in EtOAc and washed with 5% $NaHCO_3$, H_2O, and brine. The EtOAc layer was dried (Na_2SO_4), concentrated, and passed through a silica gel column in CH_2Cl_2. Concentration of the earliest eluting major band yielded 1.03 g (59%) of the somewhat unstable azlactone (**6**) as a bright orange solid (*m/e* 349), which was carried directly to the hydrolysis step.

To a solution of the azlactone (7.0 g, 20 mmol) in 25 mL of Ac_2O was added red P (3.97 g, 0.13 g atom) and (dropwise, exothermic) 47% HI (25 mL, 0.15 mol).[39] The mixture was heated for 4 h at 140–150 °C, cooled, and filtered on a glass frit to remove excess P. The solid was rinsed with HOAc and the combined filtrate was stripped to dryness. The resultant solid was dissolved in H_2O and stripped to dryness 3 times. The yellow solid was dissolved in 300 mL of H_2O, washed 2 times with Et_2O, treated with charcoal on the steam bath, filtered, and brought to pH 6 with NH_4OH. The solution was cooled, and the resultant white solid was filtered to yield 3.8 g (71%) of 7. Recrystallization from H_2O gave white needles: mp 234–235 °C; NMR (CF_3CO_2H) δ 2.5 (m, 2 H, $CHCH_2CH$), 4.1 (m, 2 H, Ar–CH_2–Ar), 4.4 (m, 2 H, Ar–CH–Ar and H_2NCHCO_2H), 7.0 (m, 2 H, exchangeable with D_2O, NH_2), 7.4 (s, 8 H, Ar). Anal. ($C_{17}H_{17}NO_2{\cdot}1.5H_2O$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-3-(9,10-dihydro-9-anthryl)-DL-alanine (8).** A solution of 7 (2.9 g, 11 mmol) in 40 mL of dioxane and 11 mL of 1 N NaOH was treated with di-*tert*-butyl dicarbonate[52] [$(Boc)_2O$; 2.59 g, 11.9 mmol] and MgO (0.43 g, 11 mmol) at 25 °C overnight. The mixture was filtered, and the filtrate was concentrated to remove dioxane. The solution was diluted with H_2O, washed with Et_2O, and acidified (pH 2) with $NaHSO_4$. The product was extracted with EtOAc and washed with H_2O, 4% $NaHSO_4$, H_2O, and brine. The EtOAc layer as dried ($MgSO_4$), filtered, and concentrated. The crude solid was recrystallized from EtOAc/hexane to yield 3.2 g (86%) of racemic **8** as a white solid: mp 205–206 °C; NMR (acetone-d_6) δ 1.5 (s, 9 H, *t*-Bu), 4.1 (m, 2 H, Ar–CH_2–Ar), 4.3 (m, 2 H, Ar–CH–Ar and H_2NCHCO_2H), 6.5 (m, 1 H, exchangeable with D_2O, NH), 7.3 (m, 8 H, Ar). Anal. ($C_{22}H_{25}NO_4$) C, H, N.

3-(Dicyclohexylmethyl)-D-alanine (9). A solution of **5a** (0.9 g, 2.5 mmol) in a mixture of 25 mL of 12 N HCl, 40 mL of H_2O, and 115 mL of HOAc was hydrogenated at 50 psi for 72 h at room temperature in the presence of 0.8 g of PtO_2. The reaction mixture was filtered on Celite and concentrated to dryness to yield 0.82 g (100%) of **9** as a white solid: mp 246–248 °C; $[\alpha]^{25}_D$ −10.6° (*c* 0.2, HOAc); NMR (Me_2SO-d_6), no aromatic resonances. Anal. ($C_{16}H_{29}NO_2{\cdot}HCl{\cdot}1.5H_2O$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-3-(dicyclohexylmethyl)-D-alanine (10).** A sample of **9** (0.7 g, 2.3 mmol) was treated with $(Boc)_2O$ by the general method described above to yield 0.58 g (70%) of **10** as a white powder: mp 138–140 °C; $[\alpha]^{25}_D$ 40° (*c* 1, MeOH). Anal. ($C_{21}H_{37}NO_4{\cdot}1.3H_2O$) C, H, N.

Peptide Synthesis. Peptide synthesis was carried out in a Beckman 990 peptide synthesizer which had been modified to allow the programming of an audio signal at the completion of each coupling cycle. Completeness of coupling was monitored by the Kaiser test.[53] The coupling program used was essentially as described,[54] with 50% CF_3CO_2H/CH_2Cl_2 used for deprotection and *N,N'*-dicyclohexylcarbodiimide or *N,N'*-diisopropylcarbodiimide used as the coupling reagent.

For nonapeptide analogues, Boc-Pro-OH was attached to the chloromethyl resin (Lab Systems) by the Cs salt procedure.[55] For decapeptide analogues, Boc-Gly-OH was attached to the benzhydrylamino resin (Beckman) by an overnight DCC coupling in the presence of 1.5 equiv of 1-hydroxybenzotriazole.[56]

Ethylamide analogues were removed from the resin by aminolysis in liquid ethylamine for 24 h, followed by filtration and extraction of the resin beads with MeOH. Final deprotection of the nonapeptide ethylamides was performed with at least 100 equiv of redistilled (CoF_3) anhydrous liquid HF[25] in the presence of 10% anisole as scavenger in a Kel-F cleavage apparatus (Peninsula Labs). A cleavage time of 1 h at 0 °C was found to give the best results. The HF was removed through a CaO trap under vacuum while the cleavage vessel was maintained at 0 °C. The oily residue was washed 3 times with Et_2O and extracted 3 times with HOAc. The extract was filtered, and the filtrate was concentrated. The crude peptide HF salt was converted to the acetate form by passage through an AG3X (Bio-Rad) column in the OAc form. The crude peptide acetate was lyophilized and carried directly into purification by preparative HPLC. Decapeptide analogues were deprotected and removed from the resin in one step by HF treatment as described above.

Purification. Portions of the crude peptide (~225 mg) were dissolved in eluent (<5 mL) and loaded on a 2.5 × 100 cm glass column (Altex) packed with Licroprep RP-18 HPLC packing (25–40 μm) from E. Merck. It was sometimes necessary to add a few drops of HOAc to the loading solvent to obtain a clear solution. The eluent was filtered (0.5 μm, Millipore) and vacuum degassed after preparation from CH_3CN (Burdick and Jackson, UV) and glass distilled water. The amount of CH_3CN, the pH, and the buffer salt concentration were varied to optimize resolution for each specific case, but a typical composition is 32% CH_3CN (0.06 M in NH_4OAc at pH 7.0). The column was eluted at ~20 mL/min (~150 psi) using an Altex Model 110 pump or

(46) M. Bullpitt, O. Kitching, D. Doddrell, and W. Adcock, *J. Org. Chem.*, **41**, 760 (1976).
(47) D. C. Morrison, *J. Org. Chem.*, **24**, 463 (1959).
(48) C. Vanderstelt, A. B. H. Funcke, H. M. Tersteege, and W. Th. Nauta, *Arzneim.-Forsch.*, **15**, 1251 (1965).
(49) T. Yamada, N. Isono, A. Inui, T. Miyazawa, S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Jpn.*, **51**, 1897 (1978).
(50) T. N. Pattabiraman and W. B. Lawson, *J. Biochem.*, **126**, 659 (1972).
(51) M. Crawford and W. T. Little, *J. Chem. Soc.*, 729 (1959).
(52) D. S. Tarbell, Y. Yamamoto, and B. M. Pope, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 730 (1972).
(53) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(54) H. Takashima, V. du Vigneaud, and R. B. Merrifield, *J. Am. Chem. Soc.*, **90**, 1323 (1968).
(55) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(56) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

an FMI Model RP-D-SSY pump. The eluent was monitored at 218 nm with a Schoeffel 770 detector which had been modified to bypass the heat exchanger.

The major peak, or in the case of the diastereomeric products the second major peak,[36,37] was cut and pooled for maximum purity. The solvent was stripped, and the bulk of the NH_4OAc in the residue was sublimed under high vacuum (~40 °C). The residue was lyophilized 3 times from H_2O to yield the pure analogue. Peptides were analyzed for purity (>95%) primarily by analytical HPLC as described above. TLC was performed on silica gel plates in the solvent systems 1-BuOH/HOAc/H_2O (4:1:5, upper phase, BAW) and 1-BuOH/EtOAc/HOAc/H_2O (1:1:1:1, BEAW). Samples of ~50 μg were applied, developed, and visualized as described.

Estrus Suppression Assay. Adult female Sprague–Dawley rats, each weighing approximately 160 g, were distributed equally be weight into groups of 10 and housed 5 per cage. They were maintained in 14:10 light/dark cycle in air-conditioned quarters and given unlimited access to food and water. The rats were injected subcutaneously, twice daily (0800 and 1630 h), with 0.1 or 0.2 mL/injection of 0.1% BSA–saline vehicle containing the test compound in solution. At least four doses at 2-fold dilutions were tested per compound. All assays included negative (vehicle) and positive (25) controls. Injections were administered for 14 consecutive days, during which time daily vaginal lavages were taken from each rat to determine the stage of the estrous cycle. The percent of rats showing complete estrus suppression (i.e., only diestrous cytology) from the 4th day on was plotted against the log dose, and the ED_{50} for complete estrus suppression was calculated in units of micrograms per injection.

Acknowledgment. We gratefully acknowledge the technical assistance of Ms. C. Tsai, Ms. P. Kirchgatter, Ms. K. Bergstrom, Ms. A. Worden, and Mr. R. Seidenberg in conducting the bioassays. We are indebted to Ms. J. Fazzari and Ms. S. Williams for HPLC and amino acid analyses. Dr. S. Unger provided helpful discussion of QSAR considerations. The encouragement and advice of Dr. J. G. Moffatt is gratefully acknowledged.

Species- or Isozyme-Specific Enzyme Inhibitors. 6.[1] Synthesis and Evaluation of Two-Substrate Condensation Products as Inhibitors of Hexokinases and Thymidine Kinases

Alexander Hampton,* Ton T. Hai, Francis Kappler, and Ram R. Chawla

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received January 4, 1982*

Syntheses are described of P^1-(adenosine-5′)-P^3-(glucose-6) triphosphate (Ap_3 glucose), Ap_4 glucose, and P^1-(adenosine-5′)-P^3-(thymidine-5′) triphosphate (Ap_3T). The compounds were not substrates of any of the enzymes used in the present studies. Ap_3 glucose and Ap_4 glucose were inhibitors of yeast hexokinase (HK) and the rat isozymes HK I–III; in general, they had less affinity for the enzymes than the substrates ATP and glucose. Inhibition constants (K_i values) of Ap_3T with rat mitochondrial thymidine kinase (M-TK) and rat cytoplasmic TK (C-TK) were determined for variable thymidine (TdR) with a constant saturating level of ATP and for variable ATP with constant saturating TdR. Ap_3T was a potent and selective inhibitor of M-TK [K_M (TdR)/K_i = 1.6, K_M (ATP)/K_i = 38 with variable ATP; K_M (TdR)/K_i = 0.06, K_M (ATP)/K_i = 1.4 with variable TdR] relative to C-TK [K_M (TdR)/K_i = 0.006, K_M (ATP)/K_i = 0.7 with variable ATP; K_M (TdR)/K_i = 0.001, K_M (ATP)/K_i = 0.12 with variable TdR]. Inhibition of M-TK and C-TK by Ap_3T differed qualitatively and quantitatively from inhibition under the same conditions by the metabolic feedback inhibitor TdR 5′-triphosphate.

Evidence, summarized previously,[2] indicates that fetal isozyme-selective inhibitors of key enzymes in cell metabolism are of interest as potential starting points in the design of antineoplastic drugs. In systems so far studied, it was found that isozyme-selective inhibitors could be generated fairly frequently by attaching a single small substituent at various atoms in turn of a substrate of the target enzyme.[1,3,4] This procedure usually led to inhibitors of weak or moderate potency. In the case of one target enzyme (adenylate kinase), it has proved possible to produce a potent isozyme-selective inhibitor by incorporating the substituent into a two-substrate condensation product which itself is a potent but nonselective inhibitor of isozymes of that enzyme.[5] This report describes the synthesis of several two-substrate condensation products and their evaluation as inhibitors of thymidine kinase (TK) or hexokinase (HK), certain forms of which are of interest as targets in cancer chemotherapy. The potential value of specific inhibitors of either the cytoplasmic form (C-TK) or the mitochondrial form (M-TK) of TK has been discussed previously.[1] That HK may be an appropriate target in cancer chemotherapy is suggested by evidence that the level of this enzyme in experimental hepatomas tends to increase in parallel with increasing growth rate and degree of malignancy.[6] Of the four main isozymic variants of HK, HK II and III are minor components of most rat normal tissues[7,8] and major components of most rat tumor tissues[8,9] so far examined. Potent and selective inhibitors of HK II and III, hence, might exhibit antineoplastic activity or potentiate the activity of other agents.

The synthesis is described of P^1-(adenosine-5′)-P^3-(glucose-6) triphosphate (Ap_3 glucose), which is a condensation product of the HK substrates ATP and glucose. The tetraphosphate homologue, Ap_4 glucose, has also been synthesized, as well as P^1-(adenosine-5′)-P^3-(thymidine-5′)

(1) For paper 5 in this series, see Hampton, A.; Chawla, R. R.; Kappler, F. *J. Med. Chem.* **1982**, *25*, 644.
(2) Hampton, A.; Kappler, F.; Maeda, M.; Patel, A. D. *J. Med. Chem.* **1978**, *21*, 1137.
(3) Hampton, A.; Kappler, F.; Chawla, R. R. *J. Med. Chem.* **1979**, *22*, 1524.
(4) Hampton, A.; Picker, D. *J. Med. Chem.* **1979**, *22*, 1529.
(5) Hampton, A.; Kappler, F.; Picker, D. *J. Med. Chem.* **1982**, *25*, 638.
(6) Weber, G. *Cancer Res.* **1976**, *36*, 3189.
(7) Katzen, H. M. *Adv. Enzyme Regul.* **1967**, *5*, 335.
(8) Farron, M. *Enzyme* **1972**, *13*, 233.
(9) Shatton, J. B.; Morris, H. P.; Weinhouse, S. *Cancer Res.* **1969**, *29*, 1161.

0022-2623/82/1825-0801$01.25/0