from the resin support, with simultaneous side-chain deprotection, by acidolysis with anhydrous hydrogen fluoride containing anisole (~15% v/v) and dithiothreitol (~0.3% w/v) as scavengers.

Purification. The crude peptides were subjected initially to gel permeation chromatography on Sephadex G25 (2.5 × 100 cm) with 50% acetic acid eluent. Final purification was effected by preparative, reversed-phase, high-performance liquid chromatography on C_{18} bonded silica gel (LRP-1, Whatman, 2.5 × 45 cm) eluted with a linear acetonitrile gradient with a constant concentration of trifluoroacetic acid (0.1% v/v). The linear gradient was generated with a Chromat-A-Trol Model II (Eldex Laboratories Inc) gradient maker. The separations were monitored at 280 nm and by thin-layer chromatography (TLC) on silica gel plates (Merck F60). The purity of the final peptides was assessed by HPLC and TLC in five solvent systems, and the results are given in Table IV. Reversed-phase HPLCs were recorded with a 5-μm Vydac phenyl support (4.6 × 250 mm, 5 μm, 30-nm pore size, Liquid Separations Group). Buffer A, 0.1 M triethylammonium phosphate, pH 2; buffer B, 20% buffer A in acetonitrile. A linear gradient of 10% B to 70% B over 30 min was employed for all the analyses at a flow rate of 1.5 mL min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide were assessed by an LKB 2220 recording integrator. Each peptide produced only one spot in each of the following solvent systems when visualized by both Ehrlich and chlorine/starch–iodide reagents:[14] 1, ethyl acetate–pyridine–acetic acid–water, 10:5:1:3; 2, ethyl acetate–pyridine–acetic acid–water, 5:5:1:3; 3, butan-1-ol–acetic acid–water–ethyl acetate, 1:1:1:1; 4, butan-1-ol–acetic acid–water, 4:1:1; and 5, propan-2-ol–1 M acetic acid, 2:1. Amino acid analyses were performed with an LKB 4150 analyzer, equipped with an LKB 2220 recording integrator, after the peptides were hydrolyzed in vacuo (110 °C, 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[15] (Pierce). The results of the amino acid analyses are given in Table V.

Biological Assays. The antiovulatory activity of each analogue was determined in Sprague–Dawley rats in a standard assay[16] using a 40% propane-1,2-diol–0.9% saline vehicle, in which the peptides were freely soluble. The compounds were injected subcutaneously at noon on the day of proestrus and the oviducts examined for the presence of ova on the following day. The results are expressed as the percentage of (n) rats that did not ovulate at a dose of x μg of analogue.

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(14) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; p 120.

(15) Simpson, R. J.; Neuberger, M. R.; Liu, T. Y. *J. Biol. Chem.* **1976**, *251*, 1936.

(16) Vilchez-Martinez, J. A.; Coy, D. H.; Coy, E. J.; Arimura, A.; Schally, A. V. *Endocrine Res. Commun.* **1976**, *3*, 231.

Effect of Reductive Alkylation of D-Lysine in Position 6 on the Histamine-Releasing Activity of Luteinizing Hormone-Releasing Hormone Antagonists[1]

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The reductive alkylation of the D-Lys side chain in position 6 of the LH-RH antagonist [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH was investigated in an attempt to reduce the histamine-releasing activity inherent to most potent antagonists while retaining high antiovulatory activity. The protected parent analogue was prepared by conventional solid-phase peptide synthesis. After selective removal of the Lys Fmoc side-chain protection, the resin-bound peptide was readily and conveniently alkylated at the ε amino group with various aldehydes and ketones in the presence of $NaCNBH_3$. The analogues were then cleaved from the resin with simultaneous deprotection by anhydrous hydrogen fluoride and purified to homogeneity in two stages: gel permeation followed by preparative reversed-phase liquid chromatography. The analogues were assayed in standard rat antiovulatory and in vitro histamine-release assays. Simple alkyl groups such as ethyl, isopropyl, neopentyl, and cyclohexyl caused little reduction in histamine-releasing activity while exhibiting antiovulatory activity similar to that of the parent peptide. The presence of benzyl and substituted benzyl groups resulted in substantial losses of both histamine-releasing and antiovulatory activities. Thus, results showed that alterations in the hydrophobicity and size of the position-6 side chain have little effect on histamine-releasing activity or antiovulatory activity as long as a high degree of basicity is retained.

Since the elucidation of the structure of the luteinizing hormone-releasing hormone (LH-RH) Glp-His-Trp-Ser-Tyr-Gly-Arg-Leu-Pro-Gly-NH_2 by Matsuo et al.[2] in 1971, many hundreds of analogues have been synthesized in the search for ever more potent agonists and antagonists. The antagonists are of particular interest for the control of fertility by the blockade of ovulation and also in the control of hormone-dependent tumors. Recently, however, the antagonists, typified by the compound [*N*-Ac-D-Nal[1],D-*p*ClPhe[2],D-Trp[3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH[3] have been shown to cause transient edema of the face and extremities when injected subcutaneously into rats at 50–100 times the effective antiovulatory dose.[4] Additionally, many compounds are mast cell secretagogues, release histamine, and

(1) Abbreviations used in this paper for amino acids, protecting groups, and peptides follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature and Symbols as described in the following: *Eur. J. Biochem.* **1972**, *27*, 201; *J. Biol. Chem.* **1975**, *250*, 3215. Glp, pyroglutamic acid; D-Nal, 3-(2-naphthyl)-D-alanine; D-pClPhe, 4-chlorophenyl-D-alanine.

(2) Matsuo, H.; Baba, Y.; Nair, R. M. H.; Arimura, A.; Schally, A. V. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1334.

(3) Hocart, S. J.; Nekola, M. V.; Coy, D. H. *J. Med. Chem.* **1985**, *28*, 967.

(4) Schmidt, F.; Sundaram, K.; Thau, R. B.; Bardin, C. W. *Contraception* **1984**, *29*, 283.

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are able to induce a cutaneous anaphylactoid-like response in rats, causing a dose-related wheal reaction.[5] Other peptides, most notably those containing several closely spaced basic residues, are also known to cause the release of histamine. These include substance P, somatostatin, and neurotensin, and the phenomenon is clearly linked to the presence of highly basic Lys and Arg residues.[6-8] In a preliminary study of the structure–activity relationship of the histamine-release potential of the LH-RH antagonists, we decided to investigate the effects of changing the hydrophobicity and basicity of the basic side chain at position 6.

Results and Discussion

Chemistry. Since only a limited number of naturally occurring basic amino acids are available commercially, we developed a rapid in situ solid-phase procedure in which the exposed side-chain amino group of Lys is reductively alkylated by a carbonyl compound in the presence of sodium cyanoborohydride. This technique was used previously[9] in this laboratory for the solid-phase synthesis of $\psi[CH_2NH]$ peptides using reductive alkylation of α-amino groups with an amino acid aldehyde. By suitable choice of aldehyde or ketone, a range of analogues containing amino acids of varying hydrophobicities and basicities can be produced. We chose to use D-Lys for the LH-RH analogues since it has a side chain of similar size to D-Arg and previous studies have shown that shorter basic side-chains in position 6 are poorly tolerated.[10]

D-Lys was incorporated as Boc-D-Lys(Fmoc) during peptide synthesis, and the masking group was removed from the fully assembled protected resin-bound peptide by treatment with 50% piperidine in dimethylformamide (DMF). A considerable reaction time (16 h) was required for complete removal, which is in stark contrast to the cleavage of the $N\alpha$Fmoc group, the latter being much more labile to base.[11] The deprotected resin was dispersed in DMF containing 1% acetic acid, and the exposed ϵ-amino group was then subjected to reductive alkylation with a variety of ketones and aldehydes, listed in Table I. An excess of the carbonyl compound was added to form an intermediate Schiff base, which was reduced to the desired N^{ϵ}-alkylated Lys derivative by the addition of $NaCNBH_3$.

The alkyl aldehydes and most of the ketones reacted readily and gave very weak rose colored Kaiser ninhydrin tests[12] within 1 h. The larger ketones were somewhat less reactive and required repeated reductions to achieve the same Kaiser color (see Table I). The aryl aldehydes were much less reactive presumably because the Schiff bases derived from aryl aldehydes are acid-sensitive. This lability is enhanced by the presence of electron-withdrawing substituents so that the Schiff base derived from 4-chlorobenzaldehyde was even unstable to 0.01% acetic acid. Consequently, all the aryl alkylations were performed in neat DMF for extended periods and generally gave more positive Kaiser tests. Indole-3-carboxaldehyde was sufficiently unreactive to require heating to 60 °C to promote alkylation.

Table I. Synthesis of N-Alkylated Lys Derivatives

peptide	amino acid	carbonyl compound	alkylation conditions[a]
III	D-N^{ϵ}methylLys	formaldehyde[b]	1 h
IV	D-N^{ϵ}ethylLys	acetaldehyde	1 h
V	D-N^{ϵ}neopentylLys	trimethylacetaldehyde	1 h
VI	D-N^{ϵ}isopropylLys	acetone	2 × 1 h
VII	D-N^{ϵ}(1-ethylpropyl)Lys	pentan-3-one	3 × 1 h
VIII	D-N^{ϵ}(1-propylbutyl)Lys	heptan-4-one	4 × 1 h
IX	D-N^{ϵ}cyclopentylLys	cyclopentanone	1 h, 2 h
X	D-N^{ϵ}cyclohexylLys	cyclohexanone	1 h
XI	D-N^{ϵ}benzylLys	benzaldehyde	4 × 1 h
XII	D-N^{ϵ}(4-methylbenzyl)Lys	4-tolualdehyde	16 h
XIII	D-N^{ϵ}(4-hydroxybenzyl)Lys	4-hydroxybenzaldehyde	16 h
XIV	D-N^{ϵ}(4-chlorobenzyl)Lys	4-chlorobenzaldehyde	4 × 1 h, 16 h
XV	D-N^{ϵ}(4-trifluoromethyl)benzylLys	4-(trifluoromethyl)benzaldehyde	16 h
XVI	D-N^{ϵ}(2-naphthylmethyl)Lys	2-naphthaldehyde	1 h, 16 h
XVII	D-N^{ϵ}(3-indolylmethyl)Lys	indole-3-carboxaldehyde	1 h, 3 h @ 60 °C

[a] Reaction time required to give a negative, or almost negative, Kaiser test. [b] 37% aqueous formaldehyde solution. [c] Reaction produced multiple products that could not be separated by preparative HPLC.

After cleavage and deprotection with anhydrous hydrogen fluoride, all crude peptides gave a major peak by TLC and most were readily purified to homogeneity as judged by TLC and analytical HPLC (see Table II). The only exception was the peptide containing D-N^{ϵ}propylLys[6], which consisted of several closely eluting peaks, perhaps the result of an aldol condensation of propionaldehyde in the dilute acid prior to alkylation. As this peptide could not be purified to homogeneity, it was not considered further. Since N^{ϵ}-methylated amino acids have negligible color values in standard ninhydrin-based amino acid analysis, the alkylated Lys derivatives were calculated with the Leu color value. All peptides gave rise to only one nonstandard peak and generally gave acceptable analyses, within ±10% of the theoretical values for all amino acids. The exceptions were N^{ϵ}(4-chlorobenzyl)Lys and N^{ϵ}(2-naphthylmethyl)Lys, which did not elute before the column regeneration step despite prolonged elution with the pH 10 buffer, N^{ϵ}(4-trifluoromethyl)benzylLys, which was only partially resolved from NH_3, and N^{ϵ}neopentylLys, which coeluted with NH_3 (see Table III).

Biology. The analogue [N-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH was chosen for this initial investigation of parameters governing the antiovulatory activity (AOA) and the histamine-release potential of position 6 since it has high antiovulatory activity (56% blockade at 0.5 μg) and could be produced in good yield.[13]

Presently, the majority of LH-RH antagonists of high antiovulatory activity have histamine-releasing ED_{50} values in the range 0.1–3 $\mu g\ mL^{-1}$.[14] This is in contrast to LH-

(5) Morgan, J. E.; O'Neil, C. E.; Coy, D. H.; Hocart, S. J.; Nekola, M. V. *Int. Arch. Allergy Appl. Immunol.* **1986**, *80*, 70.
(6) Lagunoff, D.; Martin, T. W. *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 331.
(7) Theorides, T. C.; Douglas, W. W. *Eur. J. Pharmacol.* **1981**, *73*, 131.
(8) Kerouac, R.; St-Pierre, S.; Rioux, F. *Peptides* **1984**, *5*, 695.
(9) Sasaki, Y.; Coy, D. H. *Peptides*, in press.
(10) Hocart, S. J.; Coy, D. H.; Coy, E. J.; Horvath, A.; Nekola, M. V. In *Peptides, Structure and Function, Proceedings of the Eighth American Peptide Symposium*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; p 337.
(11) Atherton, E.; Gait, M. J.; Sheppard, R. C.; Williams, B. J. *Bioorg. Chem.* **1979**, *8*, 351.
(12) Kaiser, E.; Colescott, R. Ll.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(13) Hocart, S. J.; Nekola, M. V.; Coy, D. H. *J. Med. Chem.* **1987**, *30*, 735.

Table II. Peptide Chromatograph and Purity Data

peptide	HPLC		TLC				
	t_R, min	purity, %	R_f 1	R_f 2	R_f 3	R_f 4	R_f 5
I	15.0	98.7	0.19	0.70	0.58	0.27	0.49
II	17.6	99.1	0.33	0.79	0.66	0.43	0.58
III	15.2	99.7	0.13	0.63	0.51	0.18	0.39
IV	15.8	99.0	0.16	0.64	0.53	0.19	0.41
V	16.4	97.3	0.31	0.77	0.65	0.35	0.52
VI	15.5	98.3	0.21	0.73	0.56	0.24	0.49
VII	16.4	96.4	0.26	0.75	0.62	0.33	0.51
VIII	18.6	99.2	0.35	0.81	0.56	0.45	0.53
IX	16.5	98.4	0.26	0.75	0.61	0.32	0.50
X	16.4	97.3	0.28	0.77	0.65	0.35	0.52
XI	18.3	97.7	0.49	0.87	0.71	0.45	0.57
XII	17.4	98.7	0.32	0.79	0.69	0.43	0.56
XIII	16.6	93.5	0.33	0.80	0.72	0.47	0.58
XIV	20.9	98.3	0.56	0.93	0.76	0.55	0.65
XV	19.5	97.9	0.36	0.83	0.72	0.46	0.58
XVI	19.1	94.3	0.32	0.80	0.72	0.46	0.58
XVII	17.5	95.1	0.28	0.76	0.66	0.41	0.58

Table III. Amino Acid Analyses

peptide	Ser	Pro	Ala	Tyr	Phe	Nal	Lys	Arg	X[a]	t_R[b]
I	0.89	0.95	1.00	1.05	3.01	0.98	0.95	0.95		60.5
II	0.98	1.07	1.00	0.96	2.75	0.97	0.88	0.93		59.4
III	0.93	0.95	1.00	0.96	2.89	2.16[c]		0.93	*c*	59.6[c]
IV	0.91	0.93	1.00	1.09	3.01	1.01		0.95	0.91	62.4
V	0.92	0.94	1.00	1.03	2.91	0.98		0.95	2.22	63.7[d]
VI	0.90	0.98	1.00	1.08	2.91	1.03		0.94	1.10[e]	63.9
VII	0.90	0.98	1.00	1.08	2.91	1.03		0.94	0.97	73.4
VIII	0.93	1.06	1.00	0.98	2.77	0.92		0.96	0.94	64.9
IX	0.94	1.02	1.00	0.99	2.74	1.02		0.93	0.89	66.9
X	0.89	0.95	1.00	0.95	2.90	0.93		0.94	0.92	73.5
XI	0.93	0.92	1.00	1.05	2.95	0.96		0.98	0.85	70.3
XII	0.89	0.89	1.00	0.95	3.05	0.97		0.95	1.09[f]	68.7[f]
XIII	0.95	1.08	1.00	0.97	3.06	1.01		0.97	0.98	61.3
XIV	0.95	0.98	1.00	1.03	2.94	0.98		0.98	*g*	*g*
XV	0.88	0.89	1.00	1.03	2.78	0.93		1.10	1.27[h]	64.0
XVI	0.95	1.09	1.00	0.97	2.74	1.09		1.00	*g*	*g*
XVII	0.89	0.96	1.00	0.98	2.80	0.95		1.05	0.83	60.6

[a] All alkylated Lys derivatives calculated as Leu. [b] Retention time in minutes on analyzer. [c] N^{ϵ}MethylLys and Nal coeluted. [d] N^{ϵ}NeopentylLys and ammonia coeluted. [e] N^{ϵ}IsopropylLys and Nal partially resolved. [f] N^{ϵ}(4-Methylbenzyl)Lys and arginine partially resolved. [g] Not eluted under conditions of analysis. [h] N^{ϵ}(4-Trifluoromethyl)benzylLys and ammonia partially resolved.

RH, with an ED_{50} of 328 ± 62, and the superagonist [D-Trp^6]LH-RH, with an ED_{50} of 46 ± 7. Thus the ED_{50} of an antagonist would have to be increased approximately 100-fold for it to be considered as a potential contraceptive agent, without major side effects. It was hoped that modifications to the basic side chain in position 6 might permit the retention of the desired antiovulatory activity while reducing the histamine-release potential via subtle changes in the side-chain basicity and hydrophobicity.

The parent analogue (I), containing D-Lys^6, had an ED_{50} of 0.37 ± 0.1 and blocked ovulation in 88% of rats at a dose of 1 μg (see Table IV). The ED_{50} was hardly improved by the incorporation of D-Lys(Fmoc)^6 (II, ED_{50} = 1.0 ± 0.1, 22% AOA @ 3 μg), but the antiovulatory activity was diminished markedly by this neutral hydrophobic residue. Neither the incorporation of a primary alkyl group on D-Lys^6 (peptides III–V) nor the incorporation of symmetrical secondary alkyl groups (peptides VI–X) caused a major change in the histamine-release activity of the antagonists. However, it was noticed that the ED_{50} increased as the size of the branched alkyl increased, despite a concomitant increase in the expected basicity of the residue. This slight increase in the ED_{50} was also associated with a decrease in antiovulatory activity, perhaps the result of charge shielding by the increasingly flexible, larger alkyl groups. Indeed, the rigid cycloalkylated D-Lys^6 peptides IX and X had marginally lower ED_{50} values than similar acyclic analogues (VII and VIII) and their antiovulatory activities were greater.

Arylation of the side chain was found to be much more effective in increasing the ED_{50} values. The incorporation of a benzyl moiety in the side chain increased the ED_{50} approximately 10-fold (XI, ED_{50} = 5.65 ± 0.11; 20% AOA @ 3 μg) but also reduced the antiovulatory activity. Para substitution with electron-donating groups (peptides XII and XIII) decreased the ED_{50} values 10-fold relative to that of the parent aryl analogue XI, apparently due to the increased basicity of the side chain. The inclusion of D-N^{ϵ}(4-chlorobenzyl)Lys^6 (XIV, ED_{50} = 53.18 ± 4.41, 0% AOA @ 12 μg) gave a poorly soluble peptide that exhibited a greater than 100-fold increase in the ED_{50} relative to the D-Lys^6 analogue (I). However, the antiovulatory activity was also abolished at the 12-μg dose level. The substitution of other large hydrophobic aryl groups (peptides XV–XVII) caused little change in the ED_{50} values relative to the control and also resulted in diminished antiovulatory activities.

In summary, the D-N^{ϵ}benzylLys^6 (XI) and D-N^{ϵ}(4-chlorobenzyl)Lys^6 (XIV), analogues of lower apparent basicities than the D-Lys^6 analogue (I), had much higher

(14) Karten, M. J.; Hook, W. A.; Siraganian, R. P.; Coy, D. H.; Folkers, L.; Rivier, J. E.; Roeske, R. W. In *LH-RH and its Analogs: Contraceptive and Therapeutic Applications Part II*; Vickery, B. H., Nestor, J. J., Hafez, E. S. E., Eds.; MTP: Hingham, MA, in press.

Table IV. Antiovulatory and Histamine-Release Activities of Analogues with the General Formula [*N*-Ac-D-Nal[1],D-Phe[2,3],X[6],Phe[7],D-Ala[10]]LH–RH

peptide	X	antiovulatory activity[a]	in vitro histamine release ED_{50}[b]
I	D-Lys	100 @ 3 (10) 88 @ 1 (8)	0.37 ± 0.1
II	D-Lys(Fmoc)	22 @ 3 (9)	1.00 ± 0.1
III	D-N^{ϵ}methylLys	72 @ 3 (7)	0.16 ± 0.01
IV	D-N^{ϵ}ethylLys	90 @ 3 (10)	0.15 ± 0.01
V	D-N^{ϵ}neopentylLys	91 @ 3 (11) 25 @ 1 (8)	0.19 ± 0.01
VI	D-N^{ϵ}isopropylLys	30 @ 1 (10)	0.15 ± 0.01
VII	D-N^{ϵ}(1-ethylpropyl)Lys	86 @ 3 (7)	0.27 ± 0.01
VIII	D-N^{ϵ}(1-propylbutyl)Lys	50 @ 3 (8)	0.56 ± 0.01
IX	D-N^{ϵ}cyclopentylLys	100 @ 3 (10) 60 @ 1.5 (10)	0.24 ± 0.01
X	D-N^{ϵ}cyclohexylLys	100 @ 3 (10)	0.32 ± 0.02
XI	D-N^{ϵ}benzylLys	0 @ 6 (8) 20 @ 3 (10)	5.65 ± 0.11
XII	D-N^{ϵ}(4-methylbenzyl)Lys	80 @ 6 (10)	0.53 ± 0.02
XIII	D-N^{ϵ}(4-hydroxybenzyl)-Lys	20 @ 3 (10)	0.43 ± 0.03
XIV	D-N^{ϵ}(4-chlorobenzyl)Lys	0 @ 12 (11)	53.18 ± 4.41
XV	D-N^{ϵ}(4-trifluoromethyl)-benzylLys	13 @ 3 (8)	1.63 ± 0.11
XVI	D-N^{ϵ}(2-naphthylmethyl)-Lys	0 @ 3 (10)	0.68 ± 0.05
XVII	D-N^{ϵ}(3-indolylmethyl)Lys	30 @ 3 (10)	0.56 ± 0.03

[a] Expressed as the percentage of (n) rats blocked at a dose of x μg. [b] Expressed as the mean ED_{50} ± standard error in units of micrograms/milliliter.

ED_{50} values but greatly reduced antiovulatory activities. The neutral D-Lys(Fmoc)[6] analogue (II) had a marginally improved ED_{50}, but again, the antiovulatory activity was severely reduced. We conclude that alterations in the hydrophobicity of the position-6 side chain have little effect on histamine-releasing activity or antiovulatory activity as long as a high degree of basicity is retained. It will, however, be interesting to extend this approach to alkylated Lys[8] analogues and to analogues alkylated in both positions 6 and 8.

Experimental Section

Materials. 4-Methylbenzhydrylamine hydrochloride resin[15] (ca. 0.7 meq g^{-1}) was obtained from Vega Biotechnologies Inc. Most *tert*-butoxycarbonyl (Boc) protected amino acids were purchased from Bachem Inc. The reactive side chains of the amino acids were masked as follows: Arg, N^{g}-tosyl; Lys, N^{ϵ}-fluorenylmethoxycarbonyl; Ser, *O*-benzyl; Tyr, *O*-2-bromobenzyloxycarbonyl. Boc-3-(2-naphthyl)-D-alanine was provided by the Southwest Foundation for Research and Education, San Antonio, TX, through the courtesy of Dr. Marvin Karten, Center for Population Research, Contraceptive Development Branch, National Institutes of Health, Bethesda, MD. All reagents and solvents were ACS grade or better and used without further purification.

Peptide Synthesis. The parent protected peptide [*N*-Ac-D-Nal[1],D-Phe[2,3],Ser(OBz)[4],Tyr(2-BrZ)[5],D-Lys(Fmoc)[6],Phe[7],Arg(Tos)[8],Pro[9],D-Ala[10]]LH-RH was synthesized on 4-methylbenzhydrylamine functionalized (0.7 meq g^{-1}), 1% cross-linked polystyrene resin[15] on a 3-mmol scale utilizing a Vega Model 50 synthesizer, using a modified solid-phase procedure.[16] All protected amino acids were coupled by using *N*,*N*′-diisopropylcarbodiimide[17] until completion, as judged by the Kaiser ninhydrin test.[12] After the amino acids were coupled, Boc deprotection was effected by using 20% boron trifluoride etherate in glacial acetic acid.[3] Following neutralization with 10% triethylamine, the synthetic cycle was repeated to assemble the resin-bound, protected, acetylated peptide.

Side-Chain Modification. The Fmoc side-chain protection was removed from Lys by treatment with 50% piperidine in DMF for 16 h followed by thorough washing and drying under nitrogen. The deprotected resin was split into aliquots (0.2 mmol), and the exposed Lys amino group was reductively alkylated with an aldehyde or ketone (10 mmol) in the presence of $NaCNBH_3$ (2 mmol) in DMF (25 mL) containing 1% acetic acid at ambient temperature (DMF alone was employed for the aryl aldehydes).

Peptide Cleavage. The decapeptides were cleaved from the resin support, with simultaneous side-chain deprotection, by acidolysis using anhydrous hydrogen fluoride containing anisole (~15% v/v) and dithiothreitol (~0.3% w/v) as scavengers for 1 h at 0 °C. The hydrogen fluoride was removed under a stream of nitrogen, and the crude peptide was precipitated with ether and collected by filtration.

Purification. The crude peptides were subjected initially to gel permeation chromatography on Sephadex G25 (2.5 × 100 cm) with 50% acetic acid eluent. Final purification was effected by preparative, reversed-phase, high-performance liquid chromatography on C_{18} bonded silica gel (Vydac C_{18}, 10–15 μm, 1.0 × 45 cm) eluted with a linear acetonitrile gradient with a constant concentration of trifluoroacetic acid (0.1% v/v). The linear gradient was generated by using a Chromat-A-Trol Model II (Eldex Laboratories Inc.) gradient maker. The separations were monitored at 280 nm, by thin-layer chromatography (TLC) on silica gel plates (Merck F60), and by analytical HPLC. The fractions containing the product were pooled, concentrated in vacuo, and subjected to filtration. Each peptide was obtained as a fluffy white powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed by HPLC and TLC in five solvent systems, and the results are given in Table II. Analytical reversed-phase HPLCs were recorded by using a Vydac C_{18} support (4.6 × 250 mm, 5 μg, 30-nm pore size, Liquid Separations Group). Buffer A was 0.1 M triethylammonium phosphate, pH 3, containing 5% acetonitrile; buffer B was 20% buffer A in acetonitrile. A linear gradient of 20% B to 80% B over 30 min was employed for all the analyses at a flow rate of 1.5 mL min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide were assessed by an LKB 2220 recording integrator. Each peptide produced only one spot in each of the following solvent systems at a loading of ~10 μg when visualized by UV or chlorine/starch–iodide:[18] 1, ethyl acetate–pyridine–acetic acid–water, 10:5:1:3, 2, ethyl acetate–pyridine–acetic acid–water, 5:5:1:3, 3, butan-1-ol–acetic acid–water–ethyl acetate, 1:1:1:1, 4, butan-1-ol–acetic acid–water, 4:1:1; and 5, propan-2-ol–1 M acetic acid, 2:1.

Amino Acid Analysis. The peptides were hydrolyzed in vacuo (110 °C, 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[19] (Pierce). Amino acid analyses were performed on the hydrolysates by using an LKB 4150 analyzer, equipped with an Ultropac 11 column (6 × 215 mm) and a Shimadzu C-R3A recording integrator with in-house software. The buffer sequence pH 3.20 (13.5 min), pH 4.25 (27 min), pH 10.00 (borate; 33 min) and temperature sequence 50 °C (5 min), 55 °C (5 min), 58 °C (30.5 min), 65 °C (7 min), 80 °C (26 min) were used. Standard retention times were as follows: His, 55.0; Nal, 59.3; Lys, 60.3; NH_3, 63.7; and Arg, 66.3 min, respectively. The unknown amino acids were calculated as Leu, and acceptable values were obtained for all residues. The results are given in Table III.

Biological Assays. The antiovulatory activity of each analogue was determined in Sprague–Dawley rats in a standard assay[20] using a 40% propane-1,2-diol–0.9% saline vehicle. The results (given in Table IV) are expressed as the percentage of (n) rats

(15) Matsueda, G. R.; Stewart, J. M. In *Peptides: Chemistry, Structure and Biology*; Walter, R., Meinenhofer, J., Eds.; Ann Arbor Science: Ann Arbor, 1975; p 333.
(16) Coy, D. H.; Coy, E. J.; Schally, A. V.; Vilchez-Martinez, J. A.; Debeljuk, L.; Carter, W. A.; Arimura, A. *Biochemsitry* **1974**, *13*, 323.
(17) Sarantakis, D. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 336.
(18) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; p 120.
(19) Simpson, R. J.; Neuberger, M. R.; Lin, T. Y. *J. Biol. Chem.* **1976**, *251*, 1936.
(20) Vilchez-Martinez, J. A.; Coy, D. H.; Coy, E. J.; Arimura, A.; Schally, A. V. *Endocr. Res. Commun.* **1976**, *3*, 231.

that did not ovulate at a dose of x μg of analogue. The in vitro histamine-release activity of each analogue was determined by using peritoneal cells from male Sprague-Dawley rats in a standard assay,[14] and the results are given as the ED_{50} values expressed in micrograms/milliliter (standard compound 48/80 has an ED_{50} of 0.58 in this assay system). The results are given in Table IV.

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Synthesis and Pharmacological Evaluation of γ-Aminobutyric Acid Analogues. New Ligand for $GABA_B$ Sites[1]

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Baclofen (β-*p*-chlorophenyl-GABA) is the only selective agonist for the bicuculline-insensitive $GABA_B$ receptor. We report the synthesis of new GABA analogues and baclofen analogues. In vitro, two compounds, 4-amino-3-benzo[*b*]furan-2-ylbutanoic acid (**9g**) and 4-amino-3-(5-methoxybenzo[*b*]furan-2-yl)butanoic acid (**9h**), showed an affinity for the $GABA_B$ receptor. The results obtained with racemic compounds of benzofuran structure, new for this series, and the surprising inactivity of compound **3a** (4-amino-3-(4-hydroxyphenyl)butanoic acid) permit the proposal of an hypothesis for the structure-activity relationships with regard to $GABA_B$ receptor.

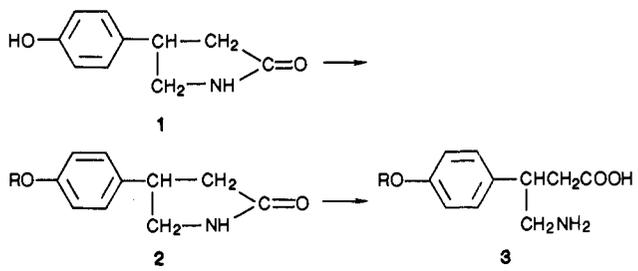
γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system.[2,3] GABA is involved in the regulation of a variety of physiological mechanisms[4,5] and implicated in the pathophysiology of several central nervous system diseases.[6] Therefore, a variety of compounds with properties of GABA have been investigated,[7-9] essentially GABA agonists, GABA antagonists, and GABA uptake inhibitors. Two subclasses of receptors for GABA have been defined and designated $GABA_A$ and $GABA_B$ receptors.[10,11] $GABA_A$ receptors are selectively activated by the GABA analogue muscimol and blocked by the convulsants such as bicuculline or picrotoxin. A selective agonist for the $GABA_B$ receptor is β-*p*-chlorophenyl-GABA (baclofen).[11] Until now, recent papers have investigated essentially agonists and antagonists of $GABA_A$ receptor. In contrast for $GABA_B$ receptor, few compounds were studied and activities and consequently structure-activity relationships were practically unknown.[12] The present paper describes the synthesis of new baclofen racemic analogues and the binding studies at $GABA_A$ and $GABA_B$ receptors.

Chemistry

Scheme I illustrates the procedure used for the synthesis of compounds **3a-e**. Lactam 1 (prepared according to a procedure described elsewhere[13]) was treated with alkyl chloride or alkylaryl chloride in absolute alcohol with sodium to give ethers **2**. The hydrolysis of **2a-e** in alkaline condition furnished the GABA analogues **3a-e**. The compounds are characterized as free base or hydrochloride.

The analogues of GABA with benzofuran or benzoxazol structure were synthesized according to Scheme II. A Reformatsky reaction of compounds **4** gave the α,β-unsaturated esters **5**. Esters **5** were treated with NBS in dry CCl_4 to furnish the bromo esters **6**, which were treated with a large excess of liquid ammonia in THF to give the unsaturated lactams **7**. The hydrogenation of **7g-h** at atmospheric pressure lead to compounds **8g,h**. Compounds **8f,i,j** were prepared by hydrogenation in an autoclave of

Scheme I



a, R=H; **b**, R=*i*-Pr; **c**, R=$CH_2C_6H_5$; **d**, R=CH_2-4-FC_6H_4; **e**, R=CH_2-5-Cl-2-thienyl

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(1) Preliminary communication, Berthelot, P.; Vaccher, C.; Musadad, A.; Debaert, M.; Luyckx, M. 16th Meeting of the Groupe d'Etudes Structure-Activité (GESA), Dax, France, May 27-30, 1986.
(2) Enna, S. J., Ed. *The GABA Receptors*; Humana: New Jersey, 1983.
(3) Krogsgaard-Larsen, P.; Scheel-Kruger, J.; Kofod, H., Eds. *GABA-Neurotransmitters, Pharmacochemical, Biochemical and Pharmacological Aspects*; Munksgaard: Copenhagen, 1979.
(4) De Feudis, F. V. *Neurochem. Int.* **1981**, *3*, 113.
(5) Grognet, A.; Hertz, F.; De Feudis, F. V. *Gen. Pharmacol.* **1983**, *14*, 585.
(6) Mandel, P.; De Feudis, F. V. Eds. *Advances in Biochemical Psychopharmacology*; Raven: New York, 1983; Vol. 37.
(7) Krogsgaard-Larsen, P. *J. Med. Chem.* **1981**, *24*, 1377.
(8) Muller-Uri, C.; Singer, E. A.; Fleischnacker, W. *J. Med. Chem.* **1986**, *29*, 125.
(9) Mann, A.; Humblet, C.; Chambon, J. P.; Schlichter, R.; Desarmenien, M.; Feltz, P.; Wermuth, C. G. *J. Med. Chem.* **1985**, *28*, 1440.
(10) Bowery, N. G.; Hill, D. R.; Hudson, A. L.; Doble, A.; Middlemiss, D. H.; Shaw, J.; Turnbull, M. *Nature (London)* **1980**, *283*, 92.
(11) Hill, D. R.; Bowery, N. G. *Nature (London)* **1981**, *290*, 149.
(12) Schlewer, G.; Wermuth, C. G.; Chambon, J. P. *Eur. J. Med. Chem.-Chim. Ther.* **1984**, *19*(2), 181.

0022-2623/87/1830-0743$01.50/0