Bisamidines of 2,6-Diaminoanthraquinone as Antiamebic Agents

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A series of bisamidines of 2,6-diaminoanthraquinone was synthesized and tested against cecal and hepatic forms of *Entamoeba histolytica* infections in rats and hamsters, respectively. A number of compounds were found to have good activity against infections in both species.

Metronidazole, the current primary drug[1] used in the treatment of human amebiasis, has demonstrated carcinogenicity in test animals.[2,3] We have found a new series of bisamidines of 2,6-diaminoanthraquinone, **2a–y**, which do not contain a nitro functionality and which are orally active against both hepatic and cecal amebic infections. A monoformamidine of 2-aminoanthraquinone, **3a**, with reported antiparasitic activity[4] was synthesized and was inactive when tested in our antiamebic screens. A number of positional isomeric bisamidines of diaminoanthraquinone were also synthesized and were likewise inactive (Table I).

Chemistry. The transformation of 2,6-diaminoanthraquinone into the amidines was accomplished by one or two of the following methods (Schemes I and II).

Method A. 2,6-Diaminoanthraquinone reacted with a complex which was formed from phosphorus oxychloride and an *N,N*-dialkylamide or *N*-alkylamide in acetonitrile.[5]

Method B. 2,6-Diaminoanthraquinone reacted with a 1-aza-2-methoxy-1-cycloalkene in a mixture of acetic acid and *N,N*-dimethylacetamide.[6]

Method C. 2,6-Diaminoanthraquinone reacted with an ortho ester in the presence of sulfuric acid or acetic anhydride[7] to give a diimidate which then reacted with various amines.[8] A series of unsubstituted amidines was prepared by reaction of the diimidate with ammonium bromide in *N,N*-dimethylformamide (Scheme II).

Method D. 2,6-Diaminoanthraquinone reacted with an *N,N*-dialkylamide dialkyl acetal with or without a solvent.[9]

Method E. 2,6-Diaminoanthraquinone reacted with an amide, which also served as a solvent, in the presence of at least 2 equiv of an arylsulfonyl halide.[10]

Acetylation of monosubstituted amidines was accomplished by heating in acetic anhydride for several minutes (Scheme III).

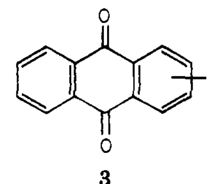
Pharmacology. The amidines were first evaluated against experimental *Entamoeba histolytica* infections in rats at a dosage of 100 mg/kg. Those compounds found active at that dose level in rats were titrated to the lowest active dose and were also evaluated against experimental *E. histolytica* infections in hamsters at a dosage of 100 mg/kg. Active compounds in the hamster were titrated to the lowest active dosage level.

Results and Discussion

The high activity against both cecal and hepatic infections was centered in the lower bis(alkanamidines) and bis(cycloalkanamidines) containing hydrogen or a lower alkyl on the nitrogens; in other words, the most active compounds are bis(formamidines), bis(acetamidines), bis(propionamidines), and bis(cyclopropionamidines) which have an *N*-methyl, *N*-ethyl, *N,N*-dimethyl, or *N,*-*N*-diethyl in the amidine chain, exemplified by compounds **4a,b,f,l,g,v**, although a number of other compounds had activity in both tests (cf. Table II). Only 2,6-disubstituted anthraquinones were found to be active (cf. Table I).

Side effects as evidenced by a general listlessness, accompanied by a reduction in food and water intake, were most evident at higher doses in the formamidine series.

Table I. Isomeric Amidines Prepared



3

No.	R	Mp, °C
3a	2-N=CHN$(CH_3)_2$	167-169[a,b]
3b	1,5-[N=CHN$(CH_3)_2$]$_2$	223-225[b]
3c	1,4-[N=CHN$(CH_3)_2$]$_2$	206-209[b]
3d	1,5-[N=C(CH_3)N$(CH_3)_2$]$_2$	205-206[c]
3e	1,4-[N=C(CH_3)N$(C_2H_5)_2$]$_2$	120-122[c]

[a] Lit.[2] mp 163-165 °C. [b] Prepared by method D. [c] Prepared by method A.

These side effects were not evident in the acetamidine, propionamidine, or cycloamidine series at doses up to 150 mg/kg. Activity diminished or was not evident when large alkyl or aryl residues were present in the amidine chain. There was one exception, *N′,N‴*-(2,6-anthraquinonylene)bis[4-chloro-*N,N*-dimethylbenzamidine], which was the only arylamidine tested which displayed activity against both cecal and hepatic infestations. The *N,N*-diethyl-*p*-chlorobenzamidine and the corresponding *N,*-*N*-diethyl- and *N,N*-dimethylbenzamidines were inactive against cecal infestations in rats. Similar side effects appeared in the series of monosubstituted amidines. The side effects were evident and activity diminished even further in the series of unsubstituted amidines.

A number of bisamidines of 2,6-diaminoanthraquinone have shown activity against experimental *E. histolytica* cecal infections in rats and hepatic infections in hamsters comparable to the activity of metronidazole. Compounds **2b,f,p,s,v** were found to be nonmutagenic when tested in the Ames test. Compound **2b** was nonmutagenic when tested in the Dominant lethal test. In light of the carcinogenicity of metronidazole[2,3] and the lack of mutagenicity of the bisamidines in the Ames and Dominant lethal tests, further testing was warranted. Preclinical toxicology and additional efficacy evaluations of specific compounds are in progress.

Experimental Section

All chemicals were used as received except for drying over molecular sieves. Melting points were determined on a Mel-Temp apparatus. NMR spectra were determined with a Varian Model HA-100 spectrometer; chemical shifts (δ) are reported in parts per million relative to internal tetramethylsilane.

Diethyl *N,N′*-(2,6-Anthraquinonylene)diformimidate. 2,6-Diaminoanthraquinone (35.79, 0.15 mol) was mixed with 100 mL of triethyl orthoformate containing 5 drops of concentrated H_2SO_4. The mixture was heated to reflux and the ethanol was removed as it formed over a 24-h period. The reaction mixture was cooled to -10 °C. The solid which formed was collected by filtration, washed with 2B alcohol, and air-dried. Recrystallization from dimethylformamide produced 7.1 g of brown crystals, mp 235-250 °C.

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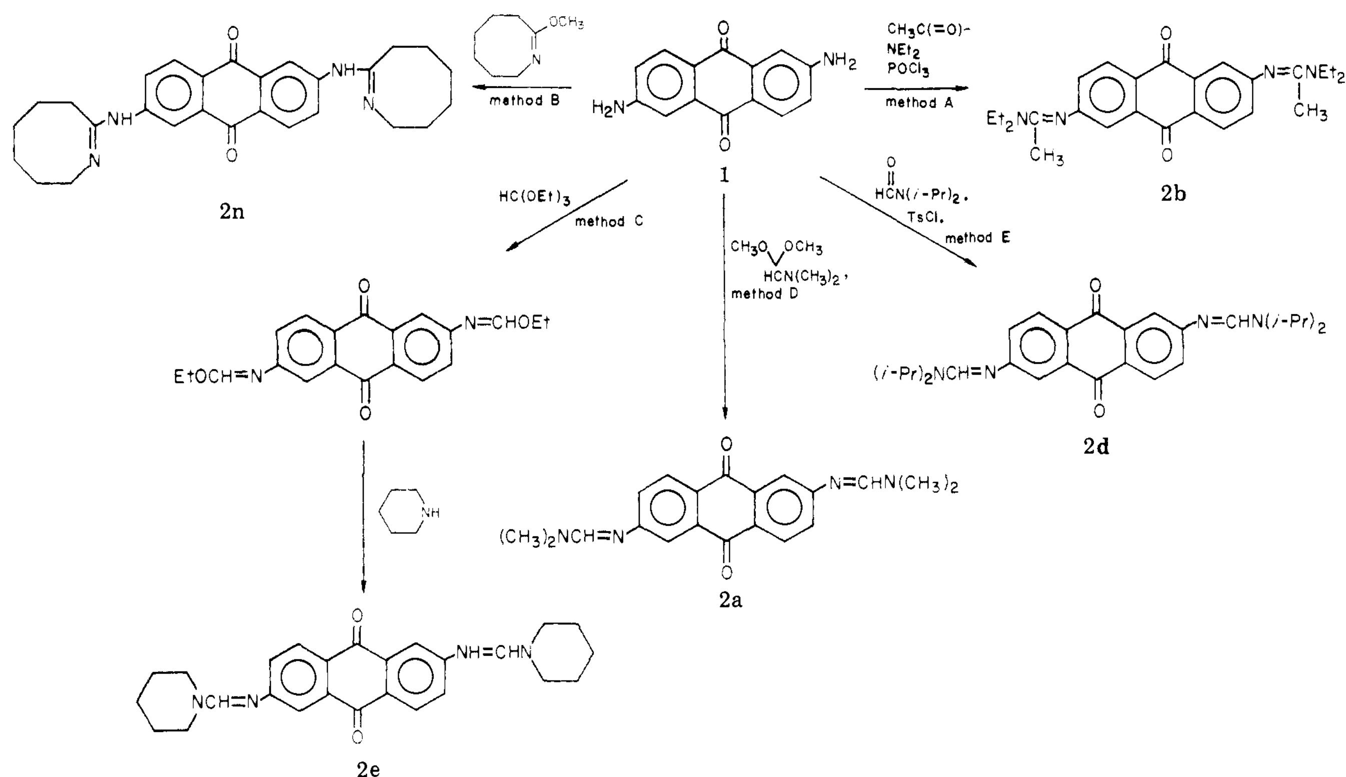
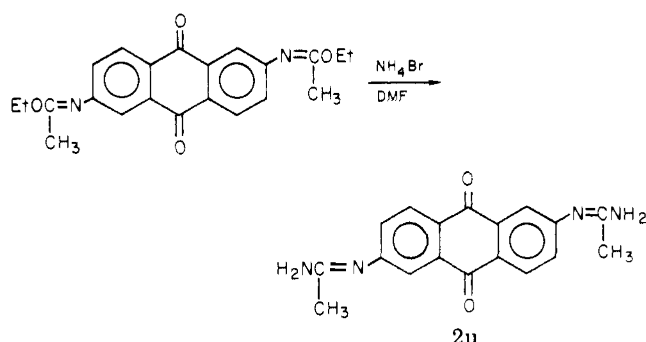
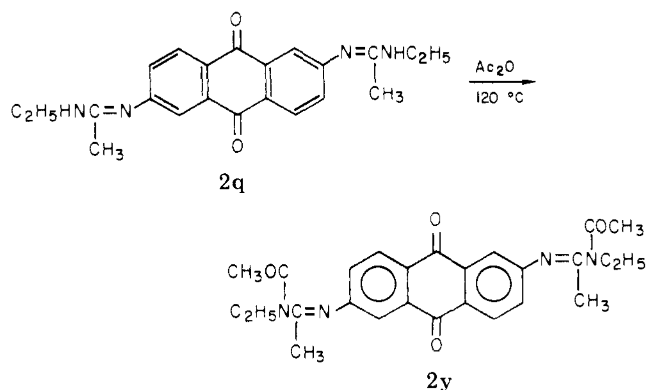
Scheme I



Scheme II



Scheme III



2,6-Bis[(piperidinomethylene)amino]anthraquinone (2e) (Method C). Diethyl *N,N'*-(2,6-anthraquinonylene)diformimidate (5.25 g), 4.0 mL of piperidine, and 100 mL of dimethylformamide were heated together on a steam bath overnight. The mixture was cooled and filtered, and the solid was washed with dimethylformamide and 2B alcohol and dried in vacuo at 78 °C over P_2O_5, producing an orange solid, mp 229–233 °C.

***N',N'''*-(2,6-Anthraquinonylene)bis[*N,N*-dimethylformamidine] (2a) (Method D).** A suspension of 7.15 g of 2,6-diaminoanthraquinone in 17.68 g of *N,N*-dimethylformamide diethyl acetal was stirred and heated in an oil bath at 150 °C for 17 h. The by-product, ethanol, distilled out. Dimethylformamide (30 mL) was added and the hot mixture was filtered. The solid was washed with acetone to give red-brown rods. These rods were recrystallized from 30 mL of dimethylformamide and dried 4 h at 80 °C to give a pale yellow solid: mp 242–245 °C (yield 7.8 g); NMR (TFA) δ 3.50 (s, 6, CH_3), 3.68 (s, 6, CH_3), 7.75 (d of d, 2, J = 8.8 and 1.3 Hz), 8.5 (m, 4), 8.8, 8.9 (CH=N's, 2).

***N',N'''*-(2,6-Anthraquinonylene)bis[*N,N*-diethylacetamidine] (2b) (Method A).** To a solution of 276.0 g of dried *N,N*-diethylacetamide in 800 mL of acetonitrile, which was cooled in an ice-water bath to 5–10 °C, was added dropwise 87.4 mL of phosphorus oxychloride over a period of 10–15 min. The ice-water bath was removed and the resulting mixture was stirred at room temperature for 1 h. 2,6-Diaminoanthraquinone (95.2 g, 0.4 mol) was added and the resulting mixture was stirred without heating for 1 h and then at 50 °C for 7.5 h. The mixture was cooled to room temperature and was poured into 1 L of ice-water. The aqueous solution was diluted to 3 L and was made basic with 5 N sodium hydroxide. The orange-yellow crystals formed were collected by filtration; then the material was washed with water and air-dried. The product was dissolved in 450 mL of chloroform, filtered, and then precipitated with about 400 mL of hexane to give 133 g of orange crystals: mp 173–177 °C; NMR (Me_2SO-d_6) δ 1.4 (t, 12), 2.1 (s, 6), 4.2 (q, 8), 7.3 (aryl, 6).

2,6-Bis(3,4,5,6,7,8-hexahydro-2-azocinylamino)anthraquinone (2n) (Method B). A mixture of 11.9 g of 2,6-diaminoanthraquinone, 28.0 g of 1-aza-2-methoxy-1-cyclooctene, and 6.0 g of glacial acetic acid in 50 mL of *N,N*-dimethylacetamide was refluxed in an oil bath for 2 h. The mixture was cooled to room temperature and 100 mL of diethyl ether was added. The product formed was filtered and washed copiously with dimethyl sulfoxide, then alcohol, and finally ether to give greenish yellow crystals. This material was dissolved in chloroform and filtered. The filtrate was evaporated to about 30 mL and the final product was precipitated out with ether. Filtration and washing with ether gave 8.39 g of orange-yellow crystals, mp 290–292 °C.

***N',N'''*-(2,6-Anthraquinonylene)bis[4-chloro-*N,N*-dimethylbenzamidine] (2p) (Method A).** To a stirred solution of 31.75 g of *N,N*-dimethyl-*p*-chlorobenzamide in 100 mL of acetonitrile cooled at 5–15 °C in an ice-water bath was added 10.8 mL of phosphorus oxychloride over a 30-min period. The ice-water bath was removed and stirring continued at room

Table II. Antiamebic Activity[a]

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	R_1	R_2	Lowest active dose, mg/kg: Cecal infection	Hepatic infection	Mp, °C	Method of prepn
2a	H	$-N(CH_3)_2$	20	10	242-245	D
2b	CH_3	$-N(C_2H_5)_2$	10	2.5		A
2c	H	$-N(CH_3)C_8H_{17}$	50	I[b]	81-88	A
2d	H	$-N(i\text{-}C_3H_7)_2$	20	100	206-208	E
2e	H	$c\text{-}NC_5H_{10}$	20	100	229-233	C
2f	CH_3	$-N(CH_3)_2$	5	2.5	300-303	A, D
2g	C_2H_5	$c\text{-}N(CH_2CH_2)_2N\text{-}4\text{-}CH_3$	20	I	208-210	C
2h	C_2H_5	$c\text{-}NC_5H_{10}$	50	I	183-185	C
2i	CH_3	$c\text{-}NC_5H_{10}$	50	100	114-116	C
2j	C_2H_5	$-N(CH_3)C_4H_9$	20	50	80-82	E
2k	C_3H_7	$-N(CH_3)_2$	20	50	159-162	A
2l	C_2H_5	$-N(CH_3)_2$	10	2.5	206-209	A
2m	C_6H_5	$c\text{-}NC_5H_{10}$	10	I	264-266	C
2n	CR_1R_2	(cyclic ring structure with N)	20	I	290-292	B
2o	$C(CH_3)_3$	$-N(C_2H_5)_2$	20	50	144-146	A
2p	$p\text{-}ClC_6H_4$	$-N(CH_3)_2$	20	50	296-298	A
2q	CH_3	$-NHC_2H_5$	10	I	271-273	A
2r	$CH_2CH(CH_3)_2$	$-N(CH_3)_2$	20	50	180-182	A
2s	$c\text{-}C_4H_7$	$-N(CH_3)_2$	20	50	234-236	A
2t	CH_3	$-NHCH_3$	10	I	266-269	A
2u	CH_3	$-NH_2$	10	I	298-300	C
2v	$c\text{-}C_3H_5$	$-N(CH_3)_2$	10	50	241-243	A
2w	$c\text{-}C_3H_5$	$-N(C_2H_5)_2$	20	I	166-168	A
2x	C_2H_5	$-NH_2$	I	I	250-252	C
2y	CH_3	$-N(C_2H_5)OAc$	I	I	187-190	
Metronidazole			10	10		
Nitrimidazine			20	100		
Tinidazole			5	25		

[a] All new compounds have correct analyses and proper supporting spectra. [b] Inactive.

temperature for 30 min; then 11.9 g of 2,6-diaminoanthraquinone was added and stirring continued at room temperature for 1 h and then at 60 °C for 20 h. The reaction mixture was then cautiously poured into a mixture of 500 mL of ice-water and stirring continued for 1 h, gradually adding 75 mL of 10 N sodium hydroxide. The red solid was collected by filtration, washed with water, and dried in vacuo at 80 °C. The dried material was slurried in 300 mL of chloroform and filtered. The filtrate was washed twice with water, dried over magnesium sulfate, filtered, and concentrated in vacuo to a syrup. This material was slurried with 25 mL of methyl alcohol and the orange-red crystals that formed were collected by filtration. The product was washed with diethyl ether, giving 19.2 g, mp 296-298 °C.

***N′,N‴*-(2,6-Anthraquinonylene)bis(acetamidine) (2u) (Method C).** Ammonium bromide (25.48 g, 0.26 mol) was dissolved in 150 mL of DMF, 19.6 g (0.052 mol) of diethyl *N*,-*N′*-(2,6-anthraquinonylene)diformimidate was added, and the resultant mixture was heated at 100 °C for 20 h. The suspension was cooled in an ice bath and the solid collected, washed with ether, and dried in vacuo. The solid was slurried in 400 mL of H_2O and basified with 10 N sodium hydroxide. The solid was collected, washed with water, dried, and recrystallized from DMF to give 4.0 g of yellow crystals, mp 298-300 °C.

***N′,N‴*-(2,6-Anthraquinonylene)bis[*N,N*-diisopropylformamidine] (2d) (Method E).** A mixture of 2,6-diaminoanthraquinone (5.95 g), *p*-toluenesulfonyl chloride (16.9 g), and diisopropyl formamide (50 mL) was heated on a steam bath for 2.5 h. A 150-mL portion of 2B alcohol was added; the mixture heated to reflux and filtered while hot. The filtrate was cooled to -10 °C and a brown solid recovered and dried at 78 °C in vacuo over P_2O_5. This solid was dissolved in 300 mL of hot water and then 20 mL of 1 N sodium hydroxide was added. The solid which formed was recrystallized from 150 mL of methanol and dried at 78 °C in vacuo over P_2O_5, giving an orange solid, mp 206-208 °C.

Biology. The amidines were active in treating cecal and hepatic amebic infections in warm-blooded animals. Two tests which establish this activity are as follows.

Organism. The organism used in both tests was the National Institutes of Health 200-μ strain of *E. histolytica*. This strain and an unidentified microorganism were cultured in Cleveland-Collier medium at 37 °C. This medium consisted of a liver infusion agar base overlaid with a horse serum-saline mixture (1:6) to which was added a few milligrams of sterile rice powder. The amebas were transferred to fresh medium twice weekly.

Cecal Infections in Female Albino Wistar Rats. Pooled overlay (0.25 mL) containing large numbers of amebas was injected into the cecum of anesthetized weanling rats during laparotomy. Treatment was begun on the day after inoculation. The compounds were dissolved or suspended in 0.2% aqueous agar and administered once daily, by gavage, for 5 consecutive days. Six days after inoculation of the amebas, the rats were sacrificed and a scraping from the cecal wall of each rat was mixed with a drop of 0.85% saline and examined microscopically for amebas. A rat was considered cured if no amebas were seen. The cure or clearance rate (number cured/number treated) for each regimen was calculated and corrected for nonspecific cures observed in the untreated infected controls. An active dose was the lowest dose, in terms of mg/kg/day, which cleared or cured 50% or more of the rats so treated. The results of the compounds of the series appear in Table II, together with results obtained using known effective drugs for comparison.

Hepatic Infections in Female Golden Hamsters. A piece of ameba-laden absorbable sponge (about 5 × 5 × 1 mm) was inserted between the middle lobes of the livers of anesthetized hamsters during laparotomy. Untreated hamsters usually die from the resulting infection about 7 days after inoculation. Treatment was started on the day of inoculation as soon as the hamsters recovered from the surgical anesthetic. The test compounds were dissolved or suspended in 0.2% aqueous agar and administered once daily, by gavage, for 5 consecutive days. Effective regimens prevented mortality. Survival rates were corrected for nonspecific survival observed in untreated groups. An active dose was the lowest dose, expressed in mg/kg/day, which protected 50% or more of the hamsters so treated as evidenced by survival 14 days after inoculation. The results of the compounds appear in Table II, together with the effective dose of known effective drugs for comparison.

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Branched-Chain Analogues of Luteinizing Hormone-Releasing Hormone

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Benzoyl-, acetylsalicylyl-, indomethacinyl-, pyroglutamylhistidyl-, and pyroglutamyl-D-phenylalanyl-D-tryptophanylseryltyrosyl groups were attached to a moderately active inhibitory analogue of LH-RH, [D-Phe[2],D-Trp[3],D-Lys[6]]-LH-RH, via the ε-amino group of the lysine residue. The resulting compounds were assayed for anti-LH-RH activity and for their ability to block ovulation in the rat. The decrease in polarity and increase in size of the lysine side chain resulting from addition of the aromatic acyl groups gave almost no increase in inhibitory activity. Addition of the dipeptide, <Glu-His, also had little effect on potency. However, incorporation of the pentapeptide sequence to give a branched pentadecapeptide with essentially two N termini resulted in antiovulatory activity greater than the parent peptide or any other analogue thus far tested by us. The corresponding agonist peptide, [N^ϵ-(<Glu-His-Trp-Ser-Tyr)-D-Lys[6]]-LH-RH, was also synthesized and tested for LH- and FSH-releasing activity. Surprisingly, it was no more active than [D-Lys[6]]-LH-RH itself, suggesting that an intact C terminus as well as an N terminus is necessary for the full expression of gonadotropin release.

The molecular factors responsible for the agonist or antagonist activities of LH-RH analogues are presently under intensive investigation, and the field has been the subject of a number of review articles.[2] Antagonist peptides have been made by the deletion of histidine[3] in position 2 of the decapeptide (Figure 1) or, better still, the substitution of D-phenylalanine in the same position.[4] The tryptophan residue in position 3 is essential for agonist, but not antagonist, activity. For instance, analogues containing L-phenylalanine or D-tryptophan in position 3 have decreased intrinsic LH-RH activities but still retain virtually full ability to compete for LH-RH receptors.[5,6]

Among the most interesting agonist peptides to have been discovered are those containing D-amino acids in place of glycine in position 6 of LH-RH.[7] The most potent peptides in this series are those containing aromatic D-amino acids[8] with in vivo gonadotropin-releasing activities 10–15 times higher than the natural hormone. Use has been made of this observation in the design of some very potent inhibitory analogues, such as [D-Phe[2],Phe[3],D-Phe[6]]-LH-RH[5] and [D-Phe[2],D-Trp[3],D-Phe[6]]-LH-RH,[6] which contain the position 2, 3, and 6 modifications within the same molecule. These compounds represent some of the most effective LH-RH antagonists presently known and are capable of blocking ovulation in the rat at doses of 4–6 mg/kg of body weight.

The reason for the increased biological activities of the D-amino acid[6] analogues is not wholly clear at present. It was originally proposed[7] that an improved receptor-binding conformation was responsible, a view which is supported by minimum free-energy calculations on LH-RH analogues.[9,10] Using this argument, one would assume that the D-aromatic amino acid[6] peptides are the most active due to improved conformations resulting from steric effects on the large side chains. It has also been found[11,12] that this type of analogue is more resistant than LH-RH to cleavage by tissue peptidases; however, this does not appear to offer an explanation for increased biological activity since several superactive LH-RH agonists with position 6 modifications have been found[13] not to exhibit prolonged plasma half-lives.

One approach for investigating structural requirements of the side chain of the position 6 D-amino acid, other than by the substitution of a wide array of naturally occurring and exotic amino acids, is to introduce various moieties by reaction with a suitable functional side group in this

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