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Nonapeptide Ethylamide Inhibitors of the Luteinizing Hormone-Releasing Hormone (LH-RH) Having a D-Alanyl Residue in Position 6 and Variations at Positions 2 and 3[1]

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A series of ten analogues, of structure des-Gly[10]-[amino acid[2],amino acid[3],D-Ala[6]]-LH-RH ethylamide, was synthesized by solid-phase methods. L-Aromatic and alkylamino acids were substituted into position 2 and alkylamino acids into position 3. Highest in vitro inhibition of LH-RH action was obtained with analogues having aromatic residues in position 2. Des-Gly[10]-[Trp[2],Leu[3],D-Ala[6]]-LH-RH ethylamide inhibited the action of 0.6 ng/mL of LH-RH, in an isolated pituitary assay, at a dosage as low as 1 μg/mL, and the corresponding Phe[2] analogue inhibited the effect of 0.3 ng of LH-RH at 0.1 μg/mL. The Trp[2] analogue inhibited ovulation in rats at the dosage of 1.5 mg per rat.

As may be predicted, a priori, an LH-RH inhibitor, which is a good candidate for antiovulation studies in which spontaneous ovulation is suppressed, should have high in vitro potency of inhibition, minimal in vitro agonist activity, no ovulation-inducing activity, and relatively prolonged bioactivity. The first reported inhibitory analogues of LH-RH, des-His[2]-LH-RH and [Gly[2]]-LH-RH, reported by Vale et al.,[2a] several 2- or 3-monosubstituted LH-RH analogues, reported by Monahan et al.,[2b] and [Leu[2],Leu[3]]-LH-RH, reported by Humphries et al.,[3] did not satisfy all of these requirements. Of first concern was the very low observed in vitro inhibitory potencies of all of these analogues. The high agonist activity associated with [Gly[2]]-LH-RH[2a] also eliminated this inhibitor from further consideration.

Analogue programs were initiated in several laboratories to achieve ways of altering the LH-RH sequence in order to increase inhibition potency. Monahan et al.[2b] reported that the incorporation of a D-Ala residue into position 6 of LH-RH led to an increase in agonist potency and that des-His[2]-[D-Ala[6]]-LH-RH was threefold more potent as an inhibitor than des-His[2]-LH-RH in a monolayer, in vitro assay.

Fujino et al.[4] reported that the replacement of the C-terminal Pro-Gly-NH_2 moiety by a Pro-NHEt group, as in des-Gly[10]-LH-RH ethylamide, increased the agonist potency by three- to fivefold, and later studies[5] showed that the activity of this analogue was prolonged. Coy et al.[6,7] found that the incorporation of this modification into the des-His[2]-LH-RH sequence, which gave des-His[2],des-Gly[10]-LH-RH ethylamide, produced an analogue which could inhibit the release of LH and FSH by LH-RH, in vivo, in rats; des-His[2]-LH-RH appeared to be inactive in this assay.

Incorporation of both the 6 position[2b] and C-terminal position[4] modifications into the des-His[2]-LH-RH sequence has given active inhibitors.[8-10]

We decided to synthesize some LH-RH analogues based upon our [Leu[2],Leu[3]]-LH-RH sequence,[3] having a D-Ala residue in position 6[2b] and also the C-terminal modification,[4] and to assay these analogues in the isolated pituitary assay. Wan et al.[11] have shown that the inhibitory potency of [Leu[2],Leu[3],D-Ala[6]]-LH-RH was up to tenfold higher than that of [Leu[2],Leu[3]]-LH-RH, in vitro. In particular, we were interested in varying the amino acid residues in positions 2 and 3 to further enhance inhibitory potency.

After this work was completed, Rees et al.[12] reported upon the advantage for inhibition of incorporating a D-Phe residue into position 2 of the LH-RH sequence, and Corbin and Beattie[13] showed that [D-Phe[2],D-Ala[6]]-LH-RH was an effective inhibitor of ovulation in rats and rabbits. However, Yardley et al.[14] later showed that although [D-Phe[2],D-Ala[6]]-LH-RH and des-Gly[10]-[D-Phe[2],D-Ala[6]]-LH-RH ethylamide both strongly inhibited the action of LH-RH, in vitro, in monolayer cultures, with the ethylamide analogue being the more potent inhibitor, only [D-Phe[2],D-Ala[6]]-LH-RH was effective in preventing ovulation in 4-day cycling rats. Studies have shown that this apparent lack of interassay correspondence could be rationalized when the ability of the inhibitors to suppress the proestrous preovulatory surge of LH was considered, for then only [D-Phe[2],D-Ala[6]]-LH-RH was effective.[14]

Experimental Section

Synthetic procedures with the Beckman Model 990 peptide synthesizer have been described.[15] Product yields (percent) were estimated from the starting amino acid-resin. On chromatography, the product of the major peaks was examined by TLC. Usually only those fractions corresponding to the upper parts of the peaks were taken, and consequently the percentage yields will be low. TLC on silica gel was used to evaluate product purity, with baths from the systems R_f[1], 1-BuOH–AcOH–EtOAc–H_2O (1:1:1:1 v/v);

R_f^2, EtOAc–Py–AcOH–H_2O (5:5:1:3 v/v); R_f^3, 2-propanol–1 N AcOH (2:1 v/v); R_f^4, $CHCl_3$–MeOH–concentrated NH_4OH (60:45:20 v/v); R_f^5, 1-BuOH–AcOH–H_2O (4:1:5 v/v upper phase); R_f^6, 1-BuOH–Py–AcOH–H_2O (30:20:6:24 v/v); R_f^7, EtOH–H_2O (7:3 v/v). Peptide spots were negative to ninhydrin and positive to chlorine–*o*-tolidine reagent. Amino acid analyses, on ca. 0.5-mg samples hydrolyzed in 6 N HCl in evacuated and sealed ampules for 18 h at 110 °C, were performed on a Beckman Model 119 amino acid analyzer. Optical rotations for these peptide acetate salts were measured on a Perkin-Elmer 141 digital read-out polarimeter. The peptides were purified as described for the Leu^2,Gly^3 analogue, except where indicated.

Des-Gly^{10}-[Leu^2,Gly^3,D-Ala^6]-LH-RH Ethylamide. Boc-Pro-resin ester (11.2 g, 0.49 mequiv/g of Pro) was submitted to five coupling cycles to give the hexapeptide resin ester, Boc-Ser(Bzl)-Tyr(*o*-BrZ)-D-Ala-Leu-Arg(Tos)-Pro-resin ester (16.86 g). Two grams of the hexapeptide resin ester was consecutively coupled with Boc-Gly, Boc-Leu, and <Glu-OH. The resultant nonapeptide resin ester was stirred with anhydrous $EtNH_2$ for ca. 6 h at 0 °C. The $EtNH_2$ was removed and the protected nonapeptide ethylamide was extracted into MeOH. Concentration of the methanolic extract, followed by addition of EtOAc, precipitated the protected nonapeptide ethylamide (300 mg). Treatment for 1 h at 0 °C with anhydrous HF containing 10–20% anisole[15,16] gave the free peptide ethylamide, which was purified by chromatography over Sephadex LH-20 (125 × 1.5 cm) using H_2O–1-BuOH (100:6 v/v) and ion-exchange chromatography on Bio-Gel CM-2 (25 × 1.5 cm) with an NH_4OAc gradient of 1 mM, pH 4.5, to 80 mM to give 147 mg (22.3%). Amino acid analysis gave Glu 1.0, Leu 2 × 1.1, Gly 1.0, Ser 0.92, Tyr 0.94, Ala 0.85, Arg 1.0, Pro 1.0; R_f^1 0.66, R_f^2 0.75, R_f^3 0.73; $[\alpha]^{24}_D$ −31.88° (*c* 11.67, MeOH).

The following five LH-RH analogues were prepared from 2 g of the same hexapeptide resin ester; the Phe^2,Nva^3-LH-RH analogue was synthesized from 2.4 g of this resin.

Des-Gly^{10}-[Leu^2,Abu^3,D-Ala^6]-LH-RH ethylamide: yield 176.6 mg (26%) (protected ethylamide 335 mg); amino acid analysis gave Glu 0.99, Leu 2 × 1.04, Ser 0.96, Tyr 1.05, Ala 0.99, Arg 0.85, Pro 1.09; R_f^1 0.69, R_f^2 0.85, R_f^3 0.78; $[\alpha]^{24}_D$ −34.91° (*c* 8.995, MeOH).

Des-Gly^{10}-[Leu^2,Nva^3,D-Ala^6]-LH-RH ethylamide: yield 160 mg (23.3%) (protected ethylamide 295.9 mg); amino acid analysis gave Glu 0.98, Leu 2 × 0.92, Ser 0.93, Tyr 1.09, Ala 1.07, Arg 1.00, Pro 1.07; R_f^1 0.63, R_f^2 0.88, R_f^3 0.66, R_f^4 0.69, R_f^5 0.39; $[\alpha]^{24}_D$ −38.85° (*c* 10.09, MeOH).

Des-Gly^{10}-[Leu^2,Nle^3,D-Ala^6]-LH-RH ethylamide: yield 137.7 mg (19.7%) (protected ethylamide 235 mg); amino acid analysis gave Glu 1.02, Leu 2 × 1.03, Ser 0.98, Tyr 1.01, Ala 1.01, Arg 0.89, Pro 1.03; R_f^1 0.74, R_f^2 0.85, R_f^3 0.80; $[\alpha]^{24}_D$ −42.12° (*c* 8.239, MeOH).

Des-Gly^{10}-[Trp^2,Leu^3,D-Ala^6]-LH-RH Ethylamide. This analogue was purified by partition chromatography over Sephadex G-25 (125 × 1.5 cm) with the system 1-BuOH–AcOH–H_2O (4:1:5 v/v), followed by preparative silica gel TLC of a portion with the same solvent system (upper phase): yield 48 mg; amino acid analysis gave Glu 1.15, Leu 2 × 0.99, Ser 0.87, Tyr 1.07, Ala 1.02, Arg 0.96, Pro 0.93; R_f^1 0.08, single spot on electrophoresis (500 V, 1 h, pH 6.5) on silica gel; $[\alpha]^{24}_D$ −32.72° (*c* 10.056, MeOH).

Des-Gly^{10}-[Phe^2,Nva^3,D-Ala^6]-LH-RH Ethylamide. Purification was as described for the Trp^2,Leu^3-LH-RH analogue: yield 72 mg; amino acid analysis gave Glu 1.03, Phe 1.05, Ser 0.91, Tyr 1.01, Ala 0.97, Leu 2 × 0.96, Arg 1.03, Pro 1.03; R_f^1 0.77, single spot on electrophoresis; $[\alpha]^{24}_D$ −37.97° (*c* 8.585, MeOH).

Des-Gly^{10}-[Phe^2,Leu^3,D-Ala^6]-LH-RH Ethylamide. Purification was by partition chromatography on Sephadex G-25 with the system 1-BuOH–AcOH–H_2O (4:1:5 v/v): yield 123 mg (11.5%); amino acid analysis gave Glu 1.04, Phe 0.92, Leu 2 × 1.1, Ser 0.99, Tyr 1.0, Ala 1.01, Arg 0.92, Pro 0.92; R_f^1 0.64, R_f^2 0.87, R_f^3 0.77, R_f^4 0.87, R_f^6 0.62, R_f^7 0.64; $[\alpha]^{24}_D$ −50.23° (*c* 8.322, MeOH).

Des-Gly^{10}-[Val^2,Leu^3,D-Ala^6]-LH-RH Ethylamide. Boc-Pro-resin ester (4 g, 0.55 mequiv/g of Pro) was taken through six consecutive coupling cycles to give Boc-Leu-Ser(Bzl)-Tyr(*o*-BrZ)-D-Ala-Leu-Arg(Tos)-Pro-resin ester (4.93 g). A portion (2.4 g) was submitted to two further coupling cycles to give the nonapeptide resin ester which produced on work-up: 218 mg (18.8%); amino acid analysis gave Glu 1.1, Val 0.98, Leu 2 × 1.08, Ser 1.05, Tyr 1.05, Ala 0.98, Arg 1.02, Pro 0.99; R_f^1 0.69, R_f^2 0.77, R_f^3 0.76; $[\alpha]^{24}_D$ −67.09° (*c* 8.928, MeOH).

The following analogues were similarly prepared from 1.2 g of the heptapeptide resin ester.

Des-Gly^{10}-[Ile^2,Leu^3,D-Ala^6]-LH-RH ethylamide: yield 147 mg (25.7%); amino acid analysis gave Glu 1.08, Ile 0.95, Leu 2 × 1.1, Ser 0.85, Tyr 0.97, Ala 0.99, Arg 0.92, Pro 1.01; R_f^1 0.76, R_f^2 0.96, R_f^3 0.79, R_f^4 0.67, R_f^6 0.83, R_f^7 0.71.

Des-Gly^{10}-[Leu^2,Leu^3,D-Ala^6]-LH-RH ethylamide: yield 153 mg (26.7%); amino acid analysis gave Glu 0.90, Leu 3 × 1.1, Ser 0.82, Tyr 0.95, Ala 0.90, Arg 1.0, Pro 1.04, R_f^1 0.75, R_f^2 0.94, R_f^3 0.78, R_f^4 0.67, R_f^6 0.88, R_f^7 0.68; $[\alpha]^{24}_D$ −45.23° (*c* 12.38, MeOH).

Biological Assays. In vitro studies were performed using pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). The LH and FSH agonist and antagonist activities were determined by incubating, for a total of 6 h, two pituitaries at 37 °C in 1 mL of lactated Ringer's solution (Travenol Laboratories) in 10-mL teflon beakers in a Dubnoff shaker. Medium was removed each hour for RIA[15] for LH and FSH, and fresh medium was added. The LH-RH analogue was added to the incubation medium at I_3, I_4, I_5, and I_6, and LH-RH was added (5 min after the peptide) at I_5 and I_6. Values were calculated in terms of nanograms of the standards LH-LER-1240-2 (0.60 NIH-LH-S1 unit/mg) and 2.1 × NIH-FSH-S1 units/mg.

Antiovulation assays were performed by counting, on estrus, the number of ova shed by 4-day cycling rats after a single sc injection of the LH-RH analogue in corn oil between 12 and 12:30 p.m. on proestrus. The control rats received 0.3 mL of the vehicle.

Results and Discussion

The results of the in vitro studies, in the isolated rat pituitary assay, are shown in Table I.

In the sequence, des-Gly^{10}-[Leu^2,amino acid^3,D-Ala^6]-LH-RH ethylamide, increasing the length of the side chain in position 3 has produced an increase in inhibitory potency, in vitro, in going from Gly to Abu and then to Nva and Nle. The Nva and Nle analogues partially inhibited LH release at the 1 μg/mL dosage, and inhibition was complete at 10 μg/mL. The Abu analogue required a 100 μg/mL dosage for complete inhibition of LH release and the Gly analogue appeared inactive at this dosage. Substitution with Leu in position 3 gave an analogue which completely inhibited LH release at 1 μg/mL. The incorporation of the C-terminal modification of Fujino et al.[4] has, therefore, brought about an increase in the in vitro inhibition potency of the [Leu^2,Leu^3,D-Ala^6]-LH-RH sequence by up to tenfold. The analogues, [Val^2,Leu^3,D-Ala^6]-LH-RH[11] and des-Gly^{10}-[Val^2,Leu^3,D-Ala^6]-LH-RH ethylamide, however, have comparable potencies.

Analogues with the highest in vitro potencies, described here, have L-aromatic amino acid residues in position 2. In the sequence, des-Gly^{10}-[amino acid^2,Leu^3,D-Ala^6]-LH-RH ethylamide, potent in vitro inhibition of the effect of 0.3 ng of LH-RH was found at 1 μg/mL when Phe was in position 2, and partial inhibition of LH release occurred at 0.1 μg/mL. Partial inhibition at 1 μg/mL and complete inhibition at 10 μg/mL were determined for the Trp^2 analogue when assayed against 0.6 ng/mL of LH-RH. The corresponding Leu^2 analogue completely inhibited LH release at 1 μg/mL, but the Val^2 and Ile^2 analogues, which have branching at the β carbon in position 2, were only about one-tenth as active.

In an attempt to increase in vitro inhibitory potency, des-Gly^{10}-[Phe^2,Nva^3,D-Ala^6]-LH-RH ethylamide was synthesized, which embodied one of the more active 3-substitutions (Nva) with one of the more active 2-substitutions (Phe). The analogue completely inhibited the LH release as induced in isolated pituitaries from 0.6 ng/mL of LH-RH, at a dosage of 1 μg/mL.

Some of the more active analogues, in vitro, have been assayed for inhibition of ovulation in rats. The most active

Table I. In Vitro Agonist and Antagonist Activity of the Analogues[a]

Peptide analogue	Dose: Peptide, μg/mL of medium	Dose: LH-RH, ng/mL of medium	LH: Δ ng/mL of medium	LH: SEM (±)	LH: p	FSH: Δ ng/mL of medium	FSH: SEM (±)	FSH: p
des-Gly^{10}-[Leu^2,Gly^3,D-Ala^6]-		0.3	257	56		3107	390	
LH-RH ethylamide	100	0.3	169	14	ns	3307	641	ns
			-42	14		-704	189	
	100		-6	14	ns	-782	580	ns
des-Gly^{10}-[Leu^2,Abu^3,D-Ala^6]-		0.3	111	14		4494	703	
LH-RH ethylamide	10	0.3	150	13	ns	3484	625	ns
	100	0.3	45	20	<0.05	1785	408	<0.01
			26	12		656	221	
	100		1	10	ns	866	293	ns
des-Gly^{10}-[Leu^2,Nva^3,D-Ala^6]-		0.3	227	42		6652	430	
LH-RH ethylamide	0.1	0.3	213	45	ns	5124	1348	ns
	1	0.3	93	34	0.02	3902	595	~0.001
	10	0.3	45	14	<0.001	1996	1353	<0.01
des-Gly^{10}-[Leu^2,Nle^3,D-Ala^6]-		0.3	111	14		4494	703	
LH-RH ethylamide	1	0.3	70	14	0.05	2245	345	<0.02
	10	0.3	28	13	0.001	1632	220	<0.01
	100	0.3	12	9	<0.001	-516	306	<0.001
			26	12		656	211	
	100		13	9	ns	-282	287	ns
des-Gly^{10}-[Val^2,Leu^3,D-Ala^6]-		0.3	257	56		3107	390	
LH-RH ethylamide	1	0.3	272	75	ns	4321	449	ns
	10	0.3	63	21	<0.01	1640	502	<0.05
			-42	14		-704	189	
	10		8	9	0.01	490	334	0.01
des-Gly^{10}-[Leu^2,Leu^3,D-Ala^6]-		0.3	219	43		5418	1264	
LH-RH ethylamide	0.1	0.3	166	98	ns	5111	986	ns
	1	0.3	60	14	<0.01	3981	373	ns
	10	0.3	45	20	<0.01	1460	340	0.01
			28	15		289	26	
	100		73	27	ns	712	368	ns
des-Gly^{10}-[Ile^2,Leu^3,D-Ala^6]-		0.3	219	43		5418	1264	
LH-RH ethylamide	1	0.3	186	37	ns	3326	663	ns
	10	0.3	-53	40	<0.001	1755	516	<0.01
			28	15		289	26	
	100		61	35	ns	-227	236	ns
des-Gly^{10}-[Trp^2,Leu^3,D-Ala^6]-		0.6	235	33		7448	1283	
LH-RH ethylamide	0.1	0.6	193	47	ns	7371	674	ns
	1	0.6	73	17	<0.01	3092	975	<0.05
	10	0.6	36	10	<0.01	-1	320	<0.001
			45	15		-614	613	
	100		18	14	ns	564	418	ns
des-Gly^{10}-[Phe^2,Leu^3,D-Ala^6]-		0.3	207	16		5795	715	
LH-RH ethylamide	0.1	0.3	130	19	<0.01	3119	496	0.01
	1	0.3	77	20	<0.001	1564	704	~0.001
	10	0.3	47	22	<0.001	549	280	<0.001
			15	9		510	277	
	100		59	15	ns	1380	243	<0.05
des-Gly^{10}-[Phe^2,Nva^3,D-Ala^6]-		0.6	275	57		6712	658	
LH-RH ethylamide	0.1	0.6	221	78	ns	3984	696	<0.02
	1	0.6	32	27	<0.01	2695	532	0.001
	10	0.6	-7	18	<0.001	-883	294	<0.001
			-48	38		-1079	331	
	100		-66	5	ns	-204	154	<0.05

[a] For brevity, not all dosages have been reported.

inhibitor of ovulation was des-Gly^{10}-[Trp^2,Leu^3,D-Ala^6]-LH-RH ethylamide, which inhibited the ovulation of five of six rats after a single sc injection of 1.5 mg per rat of the peptide in corn oil. The ovulation of two of five rats was also inhibited by a 750 μg per rat injection. The antiovulatory activities of these analogues were lower than that of [D-Phe^2,Pro^3,D-Trp^6]-LH-RH, which inhibited the ovulation of 4-day cycling rats in the same assay by 100% at 750 μg[17] and also effectively suppressed ovulation when infused into rats from a sc implanted minipump at a rate of 375 μg/day for 4 days.[18]

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Potential Carcinostatics. Synthesis and Biological Properties of *d*- and *l*-β,β-Difluoroaspartic Acid and β,β-Difluoroasparagine[1]

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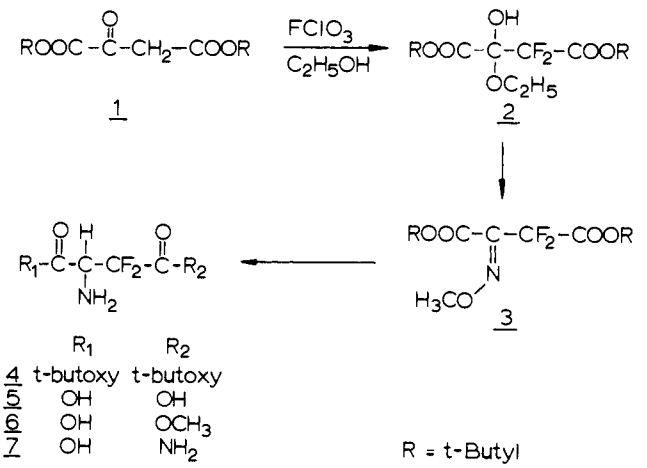
Di-*tert*-butyl β,β-difluorooxaloacetate, prepared by fluorination of di-*tert*-butyl oxaloacetate with perchloryl fluoride, was converted to di-*tert*-butyl β,β-difluoroaspartate via its *O*-methyl oxime, followed by reduction. The *tert*-butyl ester was hydrolyzed to give a mixture of *dl*-β,β-difluoroaspartic acid, which was resolved via its brucine salts. *dl*-Difluoroaspartic acid was converted into β,β-difluoroasparagine by monoesterification and subsequent ammonolysis. Racemic β,β-difluoroaspartic acid inhibits aspartate aminotransferase. Cell growth of 3T3-F cells was slightly inhibited by *l*-β,β-difluoroaspartic acid while the *d* enantiomer was without effect in this test system.

Interest in β,β-difluoroaspartic acid arises in view of its role as a potential inhibitor of the various aspartate-utilizing enzymes. Moreover, conversion of difluoroaspartic acid to a *reactive metabolite*, by interaction with the active site of an enzyme (e.g., via loss of HF), would allow it to act as a suicide enzyme inactivator.[2] Our special interest in β,β-difluoroaspartic acid and its suitable purine derivatives stems from the rationale that such compounds could be anticipated to behave as a reversible/irreversible inhibitor(s) of adenylosuccinate synthetase and for the adenylosuccinate lyase system.[3] The investigation forms a part of our current program on modified nucleoside analogues directed to the development of potential cytostatic agents. Interest in difluoroaspartic acid was also derived from the fact that the compound should serve as a precursor of the corresponding difluoroasparagine, which might be tested for activity in asparagine-dependent leukemias.[4] This communication describes the synthesis of optically active β,β-difluoroaspartic acids (*d*- and *l*-**5**) and racemic β,β-difluoroasparagine (**7**) and the results of preliminary biological studies of these compounds.

Chemistry. Preparation of di-*tert*-butyl β,β-difluorooxaloacetate (**2**) was accomplished via a direct fluorination of the corresponding di-*tert*-butyl oxaloacetate (**1**). Under carefully controlled conditions, using perchloryl fluoride[5] ($FClO_3$) as fluorinating reagent, **2** could be obtained. Condensation of **2** with *O*-methylhydroxylamine gave the oxime **3**.[6] Reduction of the oxime to the corresponding di-*tert*-butyl ester of difluoroaspartic acid (**4**) was carried out under a variety of conditions. The best results were obtained using aluminum amalgam in a mixture of water and diethyl ether.[7] To prepare the free racemic difluoroaspartic acid (**5**), its *tert*-butyl ester **4** was refluxed in trifluoroacetic acid. Structure **5** was confirmed by its spectroanalytical data. To obtain the enantiomers



	R_1	R_2
4	t-butoxy	t-butoxy
5	OH	OH
6	OH	OCH_3
7	OH	NH_2

of **5**, the racemic mixture was converted into the diastereomeric brucine salts. Fractional crystallization from acetone–water yielded, upon treatment of the crystalline fraction with ammonia, the ammonium salt of the enantiomer with a negative rotation. The (+) enantiomer was obtained as a crystalline product from the mother liquor. Selective esterification of *dl*-**5**, using thionyl chloride and methanol,[8] provided the monomethyl ester **6** as its hydrochloride. The IR spectrum of **6** exhibited a high carbonyl absorption at 1790 cm^{-1}, indicating that the desired carboxyl group had been esterified. Treatment of **6** with methanolic ammonia resulted in the formation of β,β-difluoroasparagine (**7**).