Isolation of **Helenalin**. Fraction F (2.07 g) was chromatographed on Sephadex LH-20 (90 g, 2.5 × 70 cm) in chloroform. Elution between volumes 250–385 ml gave colorless needles of helenalin (1.25 g, R_f 0.50, 1.4% dry weight of flowers): mp 170.5–174.5°; mass spectrum (70 eV) 262 (M^+), 244, 234, 151, 137, 124 (base peak), 109, and 96; ir (KBr) 1765, 1710, 1465, 1301, 1275, 1060, and 825 cm^{-1}; pmr ($CDCl_3$) δ 0.97 (3 H, s), 1.25 (3 H, d, J = 6 Hz), 1.62–2.53 (3 H, m), 3.04 (1 H, br s), 3.27 (1 H, br s), 3.58 (1 H, m), 446 (1 H, br s), 4.98 (1 H, d t, J = 7, 2 Hz), 5.81 (1 H, d, J = 3 Hz), 6.03 (1 H, ddd, J = 6, 5, 3 Hz), 6.37 (1 H, d, J = 3 Hz), and 7.69 (2 H, ddd, J = 6, 5, 2 Hz). Further elution of the column gave between volumes 385–560 ml, 0.40 g of a compound which after recrystallization from acetone–ligroine was shown (as above) to be autumnolide.[6]

The methanol-soluble fraction D (4.23 g) was chromatographed on Sephadex LH-20 (430 g, 5 × 85 cm) in methanol. Elution between volumes 1184–1304 ml gave a yellow gum (1.05 g) which, on trituration with methanol, gave a solid (0.070 g), mp 169–170°, identical (as above) with an authentic specimen of mannitol. Further elution gave a compound in volumes 1312–1512 ml which after crystallization from 95% ethanol melted at 240–243° (0.060 g) and was not further identified.

Large-Scale Isolation of Helenalin from *Helenium autumnale*. The flowers, leaves, and stems of air-dried *H. autumnale* L. var. *montanum* (Nutt.) Fern. (10.96 kg) were broken into 2-in. sections, placed in muslin bags, and submerged in chloroform (50 l.) for 24 hr at room temperature. The solution was filtered and concentrated (*in vacuo*) to 4 l. at room temperature. The chloroform extract was washed with H_2O (4 × 1 l.). After filtering the $CHCl_3$ solution through a cotton plug (premoistened with $CHCl_3$) the solvent was removed (*in vacuo*) at room temperature to give a greenish brown gum (470 g) which was extracted with methanol (5 × 500 ml). The methanol extract was filtered, diluted with 300 ml of water, and extracted with ligroine (4 × 900 ml). Evaporation of solvent from the ligroine extract gave 95 g of black oily residue. The aqueous methanol solution was reduced (*in vacuo*) to 0.5 l. and diluted with water to 3 l. The resulting aqueous solution was extracted with $CHCl_3$ (4 × 500 ml). The combined chloroform extract was filtered through a cotton plug (premoistened with $CHCl_3$) and concentrated at room temperature to dryness. The residue (338 g) was dissolved in a minimum amount of hot benzene (100 ml) and the solution was poured slowly into a rapidly stirred solution of diethyl ether (1200 ml). The ether solution was decanted, filtered, decolorized with charcoal, and allowed to evaporate slowly at room temperature. Crystalline helenalin separated and was recrystallized from acetone–ligroine to yield 17 g of pure material. Further slow evaporation of the ether solution afforded (after recrystallization) another 22 g of pure helenalin. The mother liquors were added to the remaining ether-soluble material to give (after evaporation of solvent) 74 g of a brown semisolid which contained (by tlc analysis) considerable quantities of both helenalin and autumnolide. In addition, substantial quantities of both compounds remained (by tlc analysis) in the ether-insoluble material (225 g).

Preparation of Helenalin Esters. The acetate and propionate esters of helenalin were prepared (typically) by mixing 1.0 g of helenalin with 15 ml (large excess) of the anhydride in 25 ml of anhydrous pyridine. After stirring the mixture for 48 hr solvent was removed (*in vacuo*). The residue was triturated with methanol and hexane and both were evaporated (*in vacuo*). A solution of the residue in acetone was decolorized with charcoal and recrystallized from acetone–hexane to give the solid derivatives.

A modification of the method of Parish and Stock[12] was used for preparation of the remaining helenalin esters as follows. In a typical example, helenalin (1.0 g) was added to a solution prepared from benzene (25 ml), trifluoracetic anhydride (10 ml), and the respective acid (5.0 g). After 24 hr at room temperature (reaction followed by tlc) 10% aqueous NaOH (50 ml) was added. The mixture was stirred for 1 hr and the benzene solution was decanted, washed with saturated $NaHCO_3$ solution and saturated sodium chloride solution, and dried over $MgSO_4$. Solvent was removed *in vacuo* and the residue was recrystallized from acetone–hexane. All of the esters gave satisfactory ir, nmr, and elemental analysis and a single spot on a thin-layer chromatogram.

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Synthesis and Biological Activities of Analogs of the Luteinizing Hormone-Releasing Hormone (LH-RH) Modified in Position 2

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After the primary structure of luteinizing hormone-releasing hormone (LH-RH) from porcine[1] and ovine[2] hypothalami was shown to be <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 and had been synthesized, reports on various structural modifications rapidly began to appear. One of these investigations[3] indicated that changes at the 2 position of the LH-RH molecule diminished or eliminated the agonist activity and elicited LH-RH antagonism. In the interim further reports of modification of LH-RH in this position have appeared in the literature,[4-11] but with the exception of [D-Ala^2]-LH-RH (Monahan, *et al.*[9]) no other derivative of LH-RH wherein histidine was replaced by a D-amino acid has come to our attention.

Table I. Chemical and Physical Properties of Synthetic LH-RH Analogs

No.	Peptide	Amino acid analyses[a]									[α]D[b]	R_f on tlc[c]
		Glu		Trp[d]	Ser	Tyr	Gly	Leu	Arg	Pro		
1	LH-RH	1.06	0.97[e]	0.89	0.84	1.02	1.96	1.00	1.02	1.11	−52.9[f]	0.05[g]
2	Des-His²-LH-RH[q]	1.02		0.96	0.82	1.01	2.01	1.00	0.98	1.02	−47.3[h]	0.13[g]
3	[Trp²]-LH-RH[r]	1.05		1.67	0.71	1.02	2.02	1.00	1.02	1.13	−47.5	0.22[g]
4	[D-Trp²]-LH-RH	1.11		2.00	1.00	1.00	2.13	1.00	1.03	1.00	−43.4	0.71[i]
5	[D-His²]-LH-RH[s]	1.03	0.90[e]	0.95	0.98	1.02	2.00	0.88	0.93	1.17	−43.8	0.52[j]
6	[Tyr²]-LH-RH	1.07		0.88	0.78	2.11	1.97	1.00	1.03	0.97	−46.9	0.22,[g] 0.63[j]
7	[D-Phe²]-LH-RH	1.00	1.00[k]	0.84	0.70	0.98	2.00	1.00	1.03	1.04	−42.2	0.34[g]
8	[Arg²]-LH-RH[t]	1.01		1.05	0.69	1.06	1.96	1.00	2.05	0.95	−48.2[l]	0.04[g]
9	[D-Arg²]-LH-RH	1.03		0.82	0.73	1.03	2.05	1.00	2.05	0.98	−43.3	0.04,[g] 0.65[j]
10	[Leu²]-LH-RH[u]	1.06		0.85	0.73	1.08	2.09	2.00	1.05	1.04	−54.0[m]	0.28[g]
11	[D-Leu²]-LH-RH	1.06		0.83	0.86	0.88	1.94	2.00	0.93	1.00	−41.5	0.67[i]
12	[Thr²]-LH-RH	1.12	0.96[n]	0.83	0.93	1.14	2.07	1.00	1.06	1.00	−58.9	0.16[g]
13	[D-Val²]-LH-RH	1.04	0.95[o]	0.92	0.85	0.96	2.11	1.00	1.03	1.08	−33.0	0.73[p]

[a]All peptides were hydrolyzed in 6 *N* HCl containing 0.5% thioglycolic acid for 4 hr at 145° in a closed system under nitrogen. All analyses were performed on a Durrum D-500 high-speed amino acid analyzer. [b]All optical rotations were performed on a Carl Zeiss LEP-A2 photoelectric precision polarimeter in 1% acetic acid solution with the exception of the rotation for compound **5**, which was performed in distilled water at a concentration of about 1% at ambient temperature. [c]Thin-layer chromatography was carried out on fluorescent silica gel plates (Brinkmann silica gel F). The plates were scanned under uv light (*ca.* 260–300 mμ) and spots were visualized by iodine vapor and Pauly and Ehrlich spray. [d]All Trp values are adjusted upward, by a factor previously determined, to account for the loss of Trp during the above described hydrolysis. [e]His. [f][α]^{25}D −50.0° (*c* 1, 1% AcOH).[14] [g]1-Butanol–acetic acid–water (4:1:1). [h][α]^{25}D −47° (*c* 0.9, 1% AcOH).[4] [i]1-Butanol–acetic acid–ethyl acetate–water (1:1:1:1). [j]1-Butanol–acetic acid–pyridine–water (4:1:1:2). [k]Phe. [l][α]^{23}D −51.2° (5% AcOH);[6] [α]^{25}D −47° (*c* 1, 1% AcOH).[6a] [m][α]^{31}D −43.4° (*c* 1.4, 1 *M* AcOH).[8] [n]Thr. [o]Val. [p]Chloroform–methanol–acetic acid (85:10:5). [q]See ref 4. [r]See ref 9. [s]See †. [t]See ref 6. [u]See ref 8.

Table II. *In Vitro* LH Agonist and Antagonist Activity of LH-RH Analogs

Peptide	Min releasing concn, μg/ml	Releasing act. rel to LH-RH, %	Min concn antagonizing LH-RH, μg/ml (antagonist:agonist)[a]
LH-RH	0.0005	100.0	
Des-His²-LH-RH	50.0	0.001	5.0 (1000:1)
[Trp²]-LH-RH	<0.01 > 0.001	>5.0 < 50	Inactive[b]
[D-Trp²]-LH-RH	>50.0	<0.001	5.0 (1000:1)
[D-His²]-LH-RH	0.005	10.0	Inactive[c]
[Tyr²]-LH-RH	0.01	5.0	Inactive[d]
[D-Phe²]-LH-RH	>50.0	<0.001	1.0 (200:1)
[Arg²]-LH-RH	10	0.005	Inactive[b]
[D-Arg²]-LH-RH	<1.0 > 0.1	>0.05 < 0.5	Inactive[e]
[Leu²]-LH-RH	0.1	0.5	Inactive[d]
[D-Leu²]-LH-RH	<5 > 1	>0.01 < 0.05	Inactive[e]
[Thr²]-LH-RH	0.5	0.1	Inactive[f]
[D-Val²]-LH-RH	0.025	2.0	Inactive[d]

[a]LH-RH studied at 0.005 μg/ml. [b]Highest level tested, 50 μg/ml. [c]Highest level tested, 100 μg/ml. [d]Highest level tested, 5 μg/ml. [e]Highest level tested, 10 μg/ml. [f]Highest level tested, 1000 μg/ml.

This report focuses predominantly on the synthesis and biological activity of such D-amino acid derivatives, but L-amino acid derivatives were also synthesized for comparison of biological activity with their 2-antipodic congeners. Synthesis and biological activities of the following LH-RH derivatives are described: Des-His²-LH-RH, [Trp²]-LH-RH, [D-Trp²]-LH-RH, [D-His²]-LH-RH, [Tyr²]-LH-RH, [D-Phe²]-LH-RH, [Arg²]-LH-RH, [D-Arg²]-LH-RH, [Leu²]-LH-RH, [D-Leu²]-LH-RH, [Thr²]-LH-RH, and [D-Val²]-LH-RH.

Synthesis. All peptides were synthesized by the solid-phase method[12] on benzhydrylamine resin[13] by a procedure essentially as described by Rivier, *et al.*,[5] and by Monahan, *et al.*,[14] with the exception of [D-His²]-LH-RH, which was synthesized by classical methods.† A notable exception to the procedures described by Monahan, *et al.*, and Rivier, *et al.*, was the introduction of the tyrosine and glycine moiety as a dipeptide [Boc-Tyr(Bzl)-Gly]. The *tert*-butyloxycarbonyl (Boc) group was used for the protection of all amino functions, and the hydroxyl functions of threonine, serine, and tyrosine were appropriately protected by their benzyl ethers, whereas the guanidino group of arginine was protected as its nitro derivative. The imidazole of the histidine moiety was protected with a tosyl group. Cleavage of the peptide from the resin was accomplished by the use of liquid hydrogen fluoride with the addition of 25–30% anisole to diminish destruction and derivatization of sensitive amino acids. Purification of the crude peptides was carried out on ion-exchange columns (CMC-52, Whatman) eluted with linear aqueous ammonium acetate gradient and/or by partition chromatography on Sephadex in the system 1-butanol–acetic acid–water (4:1:5) (BAW 4:1:5) and/or by gel filtration on Sephadex G-25 or G-15 with aqueous acetic acid as eluent. Columns were monitored by the absorption of their effluent at 280 mμ and also by thin-layer chromatography. The purity of the final products was determined by the shape of the elution curve, thin-layer chromatography, and amino acid analysis. The physical constants of the peptides investigated can be seen in Table I.

Biological Activities. All peptides were assayed for LH secretion by monolayer cultures of dispersed rat pituitary cells[15,16] and compared with LH-RH. The amount of hormone released was determined by double-antibody ra-

† D. Sarantakis and W. McKinley, unpublished results.

dioimmunoassay using reagents kindly provided by NIAMDD Rat Pituitary Hormone Distribution Program. All the compounds were tested for their ability to inhibit the secretion of LH as stimulated by LH-RH.[10]

It was found that peptides containing L-aromatic amino acid moieties in the 2 position of LH-RH, such as [Trp^2]-LH-RH and [Tyr^2]-LH-RH, are strong LH-releasing agents, showing at least 5% of the standard activity (Table II). This confirms findings by others that L-aromatic amino acids inserted in the 2 position of LH-RH do not decrease the releasing activity of the parent molecule to a substantial degree. In agreement with us, Monahan, *et al.*,[9] found 40% activity for [Trp^2]-LH-RH. [Phe^2]-LH-RH has been reported to have strong releasing activity (4–7%[6a] and 4%[9] *in vitro* and 1.4% *in vivo*[8]). Methyl substitution of the imidazole moiety of histidine in LH-RH has been found to result in 2% activity for the 1 position and 6% for the 3 position.[7] Replacement of the histidine moiety with L-β-(pyrazolyl-3)alanine was reported to result in a derivative with 19% of the activity of LH-RH in rats.[11]

In contrast to the L analogs, the D-aromatic amino acid analogs [D-Trp^2]-LH-RH and [D-Phe^2]-LH-RH exhibited a minimum of releasing activity (less than 0.001%), yet could be shown to antagonize the LH release induced by LH-RH *in vitro* at a ratio of 1000:1 and 200:1, respectively (see Table II). [D-His^2]-LH-RH, however, showed exceptional releasing activity (10%), although some racemization during the synthesis might account for this.‡

L-Aliphatic amino acid analogs were generally of low releasing activity, and none of those reported in Table II showed antagonist activity at the doses tested. Fujino, *et al.*,[6a] reported [Arg^2]-LH-RH to have 0.1% and Arnold, *et al.*,[6b] reported 0.03% of the activity of LH-RH; Yanaihara, *et al.*,[8] reported a similar level of *in vivo* activity (0.18%) for [Leu^2]-LH-RH; these data are in agreement with our findings. Monahan, *et al.*,[9] claimed some releasing activity, with moderate antagonist activity, for [Asp^2]-LH-RH and [Cys^2]-LH-RH.

Substitution of the corresponding D-aliphatic amino acids in the 2 position ([D-Arg^2]-LH-RH and [D-Leu^2]-LH-RH) gave weakly releasing compounds. [D-Ala^2]-LH-RH[9] probably falls in this same category; yet some antagonist activity was claimed for this compound. [D-Val^2]-LH-RH was an exception, being a rather strong releasing compound.

Experimental Section

All protected amino acids were purchased from Bachem Laboratories, Marina Del Rey, Calif., or from Fox Chemical Co., Los Angeles, Calif.

The following specific experimental details will serve to illustrate the conditions employed in the synthesis of all of the peptides described in this paper.

Boc-Gly-benzhydrylamine Resin. Benzhydrylamine resin (25 g) was washed thoroughly with *ca.* 200 ml of CH_2Cl_2, TFA–CH_2Cl_2 (1:1) (twice), CH_2Cl_2, MeOH, DMF [12.5% in $(Et)_3N$ (twice)], MeOH (twice), and CH_2Cl_2 (twice). The resin was then suspended in 75 ml of CH_2Cl_2–DMF (1:1) and 3.5 g (20 mequiv) of Boc-Gly was added, followed by 24 ml of 1 *M* DCC (24 mequiv) in two portions, 30 min apart, with gentle rocking. Rocking was continued overnight, the solvents were removed, and the resin was washed with MeOH and CH_2Cl_2. A sample was withdrawn and found to be negative in the ninhydrin test. Acid hydrolysis of a sample in 6 *N* HCl for 4 hr at reflux and subsequent quantitative amino acid analysis showed it to be substituted by glycine to the extent of 0.30 mequiv/g of resin.

Boc-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-benzhydrylamine Resin. The above resin was deblocked with TFA–CH_2Cl_2 (1:1) three times, each time for 10 min, and was then washed with CH_2Cl_2, MeOH, DMF [12.5% in $(Et)_3N$ (twice, each time for 10 min)], MeOH (twice), and CH_2Cl_2 (twice). A sample of the resin showed a very strong ninhydrin response. The resin was suspended as previously, and 6.45 g (30 mequiv) of Boc-Pro was added (a fourfold excess), followed by 36 ml of 1 *M* DCC in CH_2Cl_2 (36 mequiv) in three portions over a period of 1 hr. Rocking was then continued overnight. In essentially the same fashion, Boc-Arg(NO_2) (9.57 g, 30 mequiv), Boc-Leu (6.93 g, 30 mequiv), and Boc-Tyr(Bzl)-Gly (12.84 g, 30 mequiv) were added. After the appropriate washings the resin was removed from the reaction vessel and dried *in vacuo* for 48 hr: yield 32.5 g.

Boc-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro Gly-benzhydrylamine Resin. The synthesis was continued on half of the above resin (16.25 g) by the addition of Boc-Ser(Bzl) (4.4 g, 15 mequiv), followed by Boc-Trp (4.56 g, 15 mequiv). After washing and drying, the resin weighed 17.73 g.

<Glu-Trp-Trp-Ser(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-benzhydrylamine Resin. After the incorporation of Boc-Trp, the deblocking step was carried out under the addition of 5% ethanedithiol in TFA–CH_2Cl_2 (1:1 mixture). A sample of 3.93 g of the above peptide was deblocked and then coupled with 0.912 g (3 mequiv) of Boc-Trp (4 ml, 1 *M* DCC) and, after the appropriate washings and deprotection steps, with 0.38 g (3 mequiv) of <Glu (4 ml, 1 *M* DCC). The resin was again removed from the vessel and dried *in vacuo*: yield 3.95 g.

<Glu-Trp-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. The above peptide resin (3.95 g) was suspended in 35 ml of liquid HF and 10 ml of anisole and stirred for 45 min at 0°. The HF was then removed by aspirator and the anisole by washing with ether. The resin was subsequently extracted with 10% HOAc and filtered. The aqueous phase was lyophilized overnight and 1.1 g of crude peptide was recovered as a fluffy powder.

The peptide was put on a column (2.8 cm o.d. and 28 cm height) of 60 g of CMC52 (Whatman) prepared in 0.01 *M* NH_4OAc at pH 4.5. The elution was carried out with a linear gradient of NH_4OAc, pH 4.5 (0.01–0.4 *M* in NH_4OAc, 750 ml each). Fractions of 12 ml each were taken and the effluent was monitored on a Beckmann DU instrument at 280 mμ. A major fraction was eluted as a broad peak in fractions 48–75 (455.7 mg).

The above material was then dissolved in 10 ml of the upper phase of BAW (4:1:5) and applied to a partition column prepared on a bed of Sephadex G-25 fine (2.6 cm o.d. and 90 cm height) previously equilibrated with lower phase and eluted with upper phase, and 9-ml fractions were collected. In fractions 20–24 a single sharp peak was eluted (254 mg) which had the physical constants reported in Table I.

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Synthesis and Antiprotozoal Activity of 1-(3-Chloro-2-hydroxypropyl)-Substituted Nitroimidazoles

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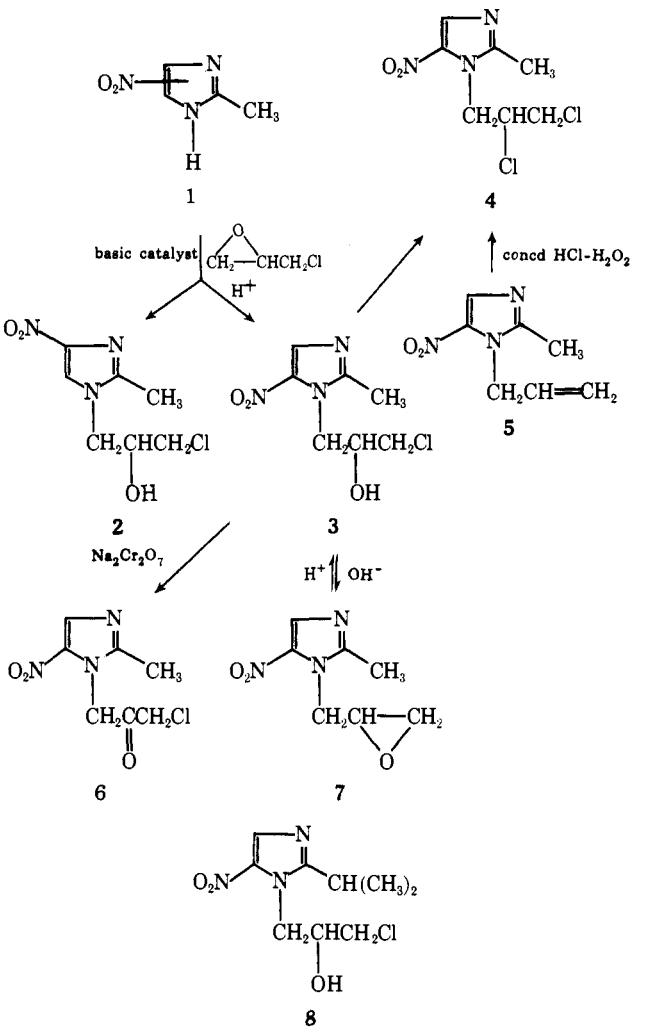
Nitroimidazoles are chemotherapeutically important as antiprotozoal and antibacterial agents.[1] For example, metronidazole† [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is both an amebicide[2] and trichomonacide[3] while azomycin (2-nitroimidazole) exhibits antibiotic properties.[4] Other compounds of this type are effective against a variety of protozoan infections.[1,5] A partial list includes dimetridazole (1,2-dimethyl-5-nitroimidazole) which is used against trichomonas in cows and *H. meleagridis* infections in fowl, ronidazole (1-methyl-2-carbamylmethyl-5-nitroimidazole) and ipronidazole (1-methyl-2-isopropyl-5-nitroimidazole) which are potent histomonastats, some 2-styryl-5-nitroimidazoleswhich are effective against trichomonas, and flunidazole [1-(2-hydroxyethyl)-2-(*p*-fluorophenyl)-5-nitroimidazole], a trichomonacide which is also an active local and oral amebicide in test animals. In view of this wide continued interest in this class of compounds,[5] we now wish to report the synthesis and antiprotozoal activity of a number of 1-(3-chloro-2-hydroxypropyl)-substituted nitroimidazoles and related compounds.

Chemistry. Treatment of 2-methyl-4- (or 5-) nitroimizole (**1**) with epichlorohydrin in the presence of a base furnished 1-(3-chloro-2-hydroxypropyl)-2-methyl-4-nitroimidazole (**2**) while the reactants under acidic conditions formed the 5-nitro-substituted isomer **3.** The structure of **3,** readily assigned by uv spectroscopy and pK measurements,[6] was confirmed by conversion with $POCl_3$ and PCl_5 into the dichloro derivative **4** which was also obtained by chlorination of the known[7] 1-allyl-2-methyl-5-nitroimidazole (**5**). The 5-nitro isomer **3** was also transformed by oxidation into the chloro ketone **6** and by aqueous alkali into the epoxide **7** which regenerated **3** upon treatment with HCl. Using a procedure similar to that for **3,** the 2-isopropyl homolog **8** was also prepared (Scheme I).

Alternatively, treatment of 2-methyl-4- (or 5-) iodo-5- (or 4-) nitroimidazole[6] with epichlorohydrin in refluxing ethanol afforded a separable mixture of the iodinated isomers **9** and **10** while azomycin[8] was converted under these conditions to the chlorohydroxy derivative **11.**

†Flagyl.

Scheme I



Biological Results. Compounds **2–4** and **7–11** were tested *in vivo* and compared with metronidazole for their activity against the *Trichomonas vaginalis* and *T. foetus* infections in mice and the intracecal *Endamoeba histolytica* infection in rats according to previously cited test methodologies.[9] The desired concentrations of the compound were administered in 1.0-ml volumes in water or 1% methylcellulose as follows: *T. vaginalis*, sc by infiltration at the site of infection 2 and 24 hr after infection or po 1, 24, 48, and 72 hr after infection; *T. foetus*, po 1, 24, and 72 hr after infection; *E. histolytica*, po once daily for 3 days. The results of treatment were evaluated as follows: *T. vaginalis*, the presence or absence of subcutaneous lesions 24 hr after the last treatment; *T. foetus*, survival of the mice for 14 days after infection; *E. histolytica*, the presence or absence of organisms in the cecum 6 days after infection. The CD_{50} and LD_{50} values were calculated by the method of Reed and Muench.[10]

From the results shown in Table I, it can be seen that the compounds exhibited varying degrees of activity against