delicato, Dr. L. Hatfield, and Dr. R. Schirmer of these laboratories for helpful conversations.

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## 3-Hydroxyisoxazole-5-hydroxamic Acid

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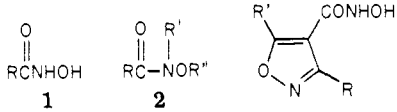
The synthesis of the title compound, 3-hydroxyisoxazole-5-hydroxamic acid (**4b**), by two procedures is described. The first, involving the treatment of dimethyl acetylenedicarboxylate with hydroxylamine, had previously been reported to give the 3-hydroxyisoxazole-5-carboxylic acid (**4a**). In the second, treatment of chlorofumaroyl dichloride with hydroxylamine also gave the intermediate chlorofumarodihydroxamic acid (**6**). Compound **6** was found to have some activity against P388 lymphocytic leukemia.

Since hydroxamic acids **1** have many different kinds of biological activities[1] we were interested in combining this functionality with that of the 3-hydroxyisoxazole moiety[2] in order to investigate the antibacterial and anticancer activity of this structure.

Hydroxamic acids are well known as strong chelating agents of Cu(II) and Fe(III) ions. Various aromatic and heterocyclic hydroxamic acids show powerful activity against mycobacteria and fungi.[3,4] Alkylated hydroxamic acids **2** also show antibacterial and antifungal activities.[5] Hydroxamic acid derivatives of isoxazole **3** are also known[6]

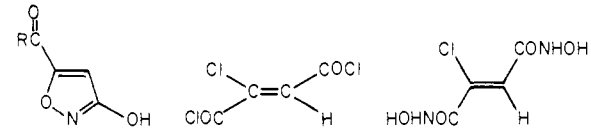



1 2 3

to show in vitro bacteriostatic activity. Of great interest also is the antileukemic activity of *N*-hydroxyurea[7] and the antimicrobial, fungicidal, and herbicidal activities of its derivatives.[8] Finally, simple aliphatic and aromatic hydroxamic acids are known to be potent inhibitors of ureas.[9]

In 1968, Nakamura[10] reported the synthesis of the acid **4a** and its methyl ester **4c** by the low-yield conversion of dimethyl acetylenedicarboxylate into **4a** with hydroxylamine in strongly basic solution. In our hands, this reaction yielded a very crude hydroxamic acid **4b** which could be converted into the crystalline methyl ester **4c** in acceptable yield. Saponification of **4c** gave the same acid (**4a**) reported by Nakamura in about 20% overall yield.

Several methods, including the treatment of $\beta$-keto esters with hydroxylamine[11] and the hydrolysis of 3-haloisoxazoles,[12] are available for the synthesis of 3-



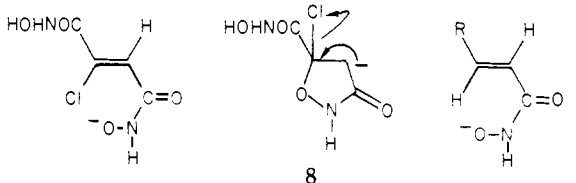

4a, R = OH
b, R = NHOH
c, R = $OCH_3$

5 6

hydroxyisoxazoles, but none of these was appropriate to the synthesis of the desired hydroxamic acid, **4b**. An alternative procedure which yielded not only **4b** but also the intermediate dihydroxamic acid **6** proceeded through the diacid chloride **5**.[13] This compound was readily prepared from monopotassium acetylenedicarboxylate by treatment with HCl followed by thionyl chloride. When **5** was treated with 2 equiv of hydroxylamine at room temperature under basic conditions, only a red oil giving a positive $FeCl_3$ test for the hydroxamic acid function was obtained. However, when the reaction was carried out carefully at 0 °C under a nitrogen atmosphere, the solid dihydroxamic acid **6** was obtained in 67% yield. The ring closure of **6** to the isoxazole **4b** was carried out at room temperature in sodium hydroxide solution under a nitrogen atmosphere in 45% yield. This product (**4b**) had an infrared spectrum consistent[14] with the 3-hydroxyisoxazole structure. Methanolysis of the pure **4b** followed by saponification of the resulting ester gave the acid **4a** in approximately 60% yield. The infrared and NMR spectra of **4a** were consistent with the structure, while its melting point, 238 °C sublimes, differed appreciably from those given in the literature.[1b,11]

To our knowledge the mechanism of this kind of ring closure (**6** → **4b**) has not been investigated. It seems

unlikely that the ring was formed by direct intramolecular hydroxamate ion (7) displacement of the vinylic chlorine atom, but rather by addition of the hydroxamate ion to the double bond giving the anion 8 which eliminated



7 8 9

chloride ion giving, after tautomerization, the hydroxyisoxazole **4b**. According to Baldwin's rules,[17] however, the 5-endo-trigonal closure of the anion **7** is disfavored. Indeed, our attempts to prepare a 3-isoxazolidone by closure of the anion **9** some years ago failed completely, as predicted now by Baldwin. If **8** is, indeed, a disfavored intermediate, then we might postulate dehydrochlorination of **7**, under the strongly basic conditions used, followed by the allowed[17] 5-endo-digonal closure to the observed product. It is possible, however, that the enhanced stability of the somewhat aromatic isoxazole ring in **4b** makes the ring closure of **7** sufficiently more favorable than that of **9** to allow formation of **4b** through intermediate **8**.

The hydroxamic acids **4b** and **6** were essentially inactive[18] against a broad spectrum of some 33 microorganisms including gram-positive and gram-negative bacteria, fungi, and mycobacteria. They also showed no activity against L1210 leukemia in mice (100 mg/kg) while **6** showed some activity (T/C = 154) against P388 lymphocytic leukemia in mice (100 mg/kg).

## Experimental Section

**Materials and Instrumentation.** Acetylenedicarboxylic acid monopotassium salt was purchased from Aldrich Chemical Co. and used without further purification. Dimethyl acetylenedicarboxylate was prepared according to that described in ref 15 and distilled at 76–85 °C (10 mm) [lit. 95–98 °C (19 mm)]. Chlorofumaric acid, mp 191–193 °C (lit.[16] 192–193 °C), was prepared according to the literature[16] from monopotassium acetylenedicarboxylate. All solvents were reagent grade and used without further purification unless otherwise stated. Melting points were taken on a Nalge-Kofler micro hot stage and are uncorrected. Infrared spectra (Nujol and KBr pellet) were taken on a Perkin-Elmer 257 recording spectrophotometer, and NMR spectra were obtained on Perkin-Elmer R-20 and Varian HA-100 spectrometers.

**Chlorofumarodihydroxamic Acid (6).** Chlorofumaroyl dichloride[13] (46.7 g, 0.251 mol, prepared from chlorofumaric acid by refluxing in thionyl chloride) in 100 mL of dimethoxyethane was added slowly with stirring to a solution of hydroxylamine hydrochloride (38.8 g, 0.55 mol) and sodium hydroxide (66.4 g, 1.66 mol) in 500 mL of water in a 1000-mL two-necked flask. The addition was carried out in an ice bath under a nitrogen atmosphere. After the addition was complete, the mixture was allowed to stand for 30 min at room temperature and acidified to pH 1 with concentrated hydrochloric acid, and the solvent was evaporated in vacuo. The resulting brown solid was dried in vacuo overnight, extracted with 3000 mL of hot 2-propanol, and filtered. The filtrate was evaporated in vacuo to yield 49.3 g of crude **6**. The crude material was recrystallized from 2000 mL of 2-propanol to yield 23.1 g (51%) of **6** in several crops. Washing with acetone removed residual color to give pure **6** (30%): mp 156–157 °C dec; IR (KBr) 1650–1610 (–CONHOH), 1520 $cm^{-1}$ (C=C); NMR ($D_2O$) $\delta$ 7.15 (1 H, s, vinyl); dark purple color with ferric chloride. Anal. ($C_4H_5N_2O_4Cl$) C, H, N, Cl.

**3-Hydroxyisoxazole-5-hydroxamic Acid. (4b). A. From Dimethyl Acetylenedicarboxylate.** Hydroxylamine hydrochloride (24.3 g, 0.35 mol) was dissolved in 4.0 N NaOH (175 mL, 0.7 mol) and the solution cooled in an ice bath. Dimethyl acetylenedicarboxylate (20 g, 0.14 mol) in 200 mL of absolute methanol was then added to the above solution slowly with stirring. The mixture was stirred overnight at room temperature and acidified to pH 3 with concentrated HCl, and the solvent was evaporated in vacuo to yield a dark solid residue. The solid was dried in vacuo, extracted with 1500 mL of hot acetone, and filtered, and the filtrate was evaporated to near dryness in vacuo. The remaining acetone solution was diluted with 650 mL of anhydrous ether and the mixture was stirred to yield a powdery brown precipitate. The precipitate was filtered and dried in vacuo to yield 10 g (55%) of crude **4b**: mp ~170 °C; IR identical with that of an authentic sample from method B.

**B. From 6.** A suspension of chlorofumarodihydroxamic acid (**6**) (35.2 g, 0.196 mol) in 448 mL of water was stirred under a nitrogen atmosphere for 15 min. Sodium hydroxide (210 mL of 4.0 N solution, 0.84 mol) was then added and the mixture allowed to stand overnight at room temperature under a nitrogen atmosphere. The mixture was acidified to pH 1 with concentrated HCl and evaporated in vacuo, and the brown residue was dried in vacuo. The solid was extracted with 500 mL of hot *p*-dioxane and filtered, and the filtrate was evaporated in vacuo to near dryness. The resulting oil was stirred with 250 mL of acetone and filtered, and the precipitate was dried in vacuo to yield 13.5 g of **4b**. A second crop of **4b** was obtained by concentrating the acetone filtrate to 100 mL, allowing the solution to stand overnight, and filtering to yield an additional 2.4 g of **4b** (total yield 56%). An analytical sample was recrystallized from water: mp 202–203.5 °C dec; IR (Nujol) 3400–2400 (–OH, –NH–), 1655 (–CONHOH), 1625, 1530, 1465, 935 $cm^{-1}$ (isoxazole ring); dark purple color with ferric chloride; fluffy white precipitate with silver nitrate (isoxazole ring) which redissolves in dilute $HNO_3$; NMR ($Me_2SO$-$d_6$) $\delta$ 6.96 (1 H, s, vinyl). Anal. ($C_4H_4N_2O_4$) C, H, N.

**Methyl 3-Hydroxyisoxazole-5-carboxylate (4c).** 3-Hydroxyisoxazole-5-hydroxamic acid (**4b**) (3.0 g, 0.021 mol) was suspended in 50 mL of absolute methanol in a three-neck flask. Dry HCl was bubbled through the refluxing solution for 1.5 h and the refluxing was continued for another 24 h. The solution was then filtered and the filtrate evaporated in vacuo to yield a gummy brown residue which was dried in vacuo. The residue was sublimed at 115–130 °C (0.5 mm) to yield 1.8 g (61%) of crude product, mp 145–165 °C. An analytical sample was recrystallized from benzene–hexane: mp 167–168 °C (lit.[10] 166–168 °C); IR (Nujol) 3300–2400 (–OH), 1730 (ester C=O), 1620, 1520, 935 $cm^{-1}$ (isoxazole ring); NMR (acetone-$d_6$) $\delta$ 6.62 (1 H, s, vinyl), 3.89 (3 H, s, $CH_3O$).

**3-Hydroxyisoxazole-5-carboxylic Acid (4a).** Methyl 3-hydroxyisoxazole-5-carboxylate (8.7 g, 0.061 mol) was dissolved in 1.0 N NaOH (130 mL, 0.13 mol) and allowed to stand for 1 h. The solution was acidified to pH 1 with concentrated HCl, stirred overnight, and then evaporated in vacuo to yield a white solid. The solid was extracted with 350 mL of acetone and filtered, and the filtrate was evaporated in vacuo to yield 7.1 g (90%) of **4a**. An analytical sample was recrystallized from glacial acetic acid: mp 238–241 °C sublimes; IR (Nujol) 3400–2300 (–OH), 1690 (acid C=O), 1615, 1510, 1440, 940 $cm^{-1}$ (isoxazole ring); NMR (acetone-$d_6$) $\delta$ 6.61 (1 H, s, vinyl).

**Testing Procedure.** Antibacterial testing was carried out by placing 6.35-mm filter paper disks impregnated with 1% methanolic solutions of the test compounds onto seeded agar plates. Zones of inhibition were measured from the periphery of each disk to the edge of inhibited growth.

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# Inhibition of the Activity of the Luteinizing Hormone-Releasing Hormone (LH-RH) by Analogues with Variations at Positions 2, 3, and 6 and the Carboxyl Terminus[1]

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In the isolated rat pituitary assay, [Thr$^2$,Leu$^3$]-LH-RH, [Leu$^2$,Ala$^3$,D-Ala$^6$]-LH-RH, and des-Gly$^{10}$-[Abu$^2$,Ala$^3$,D-Ala$^6$]-LH-RH ethylamide inhibited the LH release due to 0.3 ng/mL of added LH-RH at a 10 μg/mL dosage. Under these same assay conditions, des-Gly$^{10}$-[Ile$^2$,Ala$^3$,D-Ala$^6$]-LH-RH ethylamide was about one-tenth as active, and no inhibition was observed by [Leu$^2$,Ser$^3$]-LH-RH or [Leu$^2$,Asn$^3$]-LH-RH at a 100 μg/mL dosage. The corresponding results from FSH inhibition assays, in vitro, are also reported.

An inhibitory LH-RH sequence formed by dual structural variation at the biologically important 2 and 3 positions[2a] appears to be one of considerable promise for analogue design in the search for more potent and effective LH-RH inhibitors and antiovulation agents. The first lead to inhibitors based on this sequence was [Leu$^2$,Leu$^3$]-LH-RH. Humphries et al.[2b] reported that this analogue inhibited the release of LH and FSH, in vitro, as induced by 0.3 ng/mL of LH-RH from isolated rat pituitaries, at a dosage of 100 μg/mL. No agonist activity was observed up to a 100 μg/mL dosage with this analogue. Vale et al.[3] had earlier reported that des-His$^2$-LH-RH showed weak inhibition with a monolayer culture in vitro technique.

Later studies by Wan et al.[4] showed that [Leu$^2$,Leu$^3$,-D-Ala$^6$]-LH-RH and [Val$^2$,Leu$^3$,D-Ala$^6$]-LH-RH inhibited, in vitro, at a dosage of 10 μg/mL and had a ratio of inhibitor–LH-RH of 30 000:1.

Bowers et al.[5] found that [chlorambucil$^1$,Leu$^2$,Leu$^3$,D-Ala$^6$]-LH-RH irreversibly inhibited the action of LH-RH, in vitro, on isolated pituitaries.

We now report six new LH-RH analogues, having sequences related to the dually substituted [Leu$^2$,Leu$^3$]-LH-RH.[2b] Some of these new analogues contain a D-Ala residue in the 6 position[6] and a C-terminal Pro$^9$-NHEt unit[7] in an attempt to increase potency or duration of inhibition.

After this work was completed, Rees et al.[8] reported that [D-Phe$^2$]-LH-RH inhibited, in vitro, in a monolayer assay system at a higher potency than des-His$^2$-LH-RH.

## Experimental Section

Amino acid derivatives were purchased from Beckman Instruments, Palo Alto, Calif., or from Bachem Inc., Marina del Rey, Calif. Amino acid analyses, on samples hydrolyzed in 6 N HCl at 110 °C for 18 h, were performed with a Beckman Model 119 amino acid analyzer equipped with an Infotronics Model CRS-210 automatic digital integrator. Optical rotations were measured on a Perkin-Elmer 141 digital read-out polarimeter.

During column chromatography, the peptide peaks were located at 254 nm using an ISCO UV monitor. The product of the major peak was examined by TLC and fraction cuts were made, prior to lyophilization, for purity at the expense of product yield. Product yields, based on starting amino acid-resin, therefore, will tend to be low.

Homogeneity of the peptides was demonstrated on silica gel plates, using baths from the following TLC systems: $R_f^1$ EtOAc–$H_2O$–AcOH–1-BuOH (1:1:1:1); $R_f^2$ EtOH–$H_2O$ (7:3); $R_f^3$ $CHCl_3$–MeOH–concentrated $NH_4OH$ (60:45:20); $R_f^4$ EtOAc–pyridine–AcOH–$H_2O$ (5:5:1:3); $R_f^5$ 1-BuOH–pyridine–AcOH–$H_2O$ (30:20:6:24); $R_f^6$ 2-propanol–1 N AcOH (2:1). The solvent fronts were allowed to travel for 10–15 cm and spots were negative to ninhydrin and positive to chlorine–*o*-tolidine reagent and to iodine. The peptides were homogeneous on thin-layer electrophoresis on silica gel plates at 500 V with the systems pyridine–AcOH–$H_2O$ (30:1:270) (pH 6.5) and 1 M AcOH (pH 2.8).

**Coupling Program.** Reactions were performed with a Beckman Model 990 peptide synthesizer. The swollen resin, on to which was covalently attached the C-terminal amino acid as its $N^{\alpha}$-Boc derivative, was washed three times with $CH_2Cl_2$. After a 2-min prewash with 30% trifluoroacetic acid (TFA) in $CH_2Cl_2$ (v/v), the $N^{\alpha}$-protecting group was removed by stirring the resin with 30% TFA in $CH_2Cl_2$ (v/v) for 30 min. The amino acid-resin-trifluoroacetate salt remaining was given six washes (2 min each) with $CH_2Cl_2$ and two prewashes (2 min each) with 10% $NEt_3$ (redistilled from NaOH pellets) in $CH_2Cl_2$ (v/v). The resin salt was neutralized by stirring the resin with 10% $NEt_3$ in $CH_2Cl_2$ (v/v) for 10 min. The resulting amino acid-resin was washed five times (2 min each) with $CH_2Cl_2$. A solution of the next amino acid derivative to be incorporated, as its $N^{\alpha}$-Boc derivative and