

Mechanism of Action of Retinyl Compounds on Wound Healing IV: Effect of Desmethylretinoic Acid and Its Vinylogs on Granuloma Formation

K. H. LEE^{*}, CHERNG-CHYI FU, and MARVIN JUNG

Abstract □ The effect of side-chain length of desmethylretinoic acid on granuloma formation induced by implantation of cotton pellets was studied. As in the homologous series of retinoic acid studies, compounds with side chains shorter than retinoic acid were not active while compounds with side chains as long or longer than retinoic acid were active. The compound with a chain two carbons longer than retinoic acid was the most active. The hydroxyproline and hexosamine contents of the granuloma were analyzed. The mechanisms of action of these active compounds on wound healing are discussed.

Keyphrases □ Retinyl compounds—desmethylretinoic acid and vinylogs, wound-healing mechanism of action, effect on granuloma formation □ Desmethylretinoic acid and vinylogs—wound-healing mechanism of action, effect on granuloma formation □ Vitamin A-related compounds—desmethylretinoic acid and vinylogs, wound-healing mechanism of action, effect on granuloma formation □ Granuloma formation—effect of desmethylretinoic acid and vinylogs

It was reported previously that a few vitamin A (retinol) related compounds promote skin wound healing (1–3) and tissue regeneration (4) in rats. The structural relationship of a few naturally occurring compounds related to retinol was also studied (4). Recently, the effect of the length of the side chain of retinoic acid on granuloma formation induced by implanted cotton pellets was reported (5); compounds with side chains shorter than that of retinoic acid possessed no activity while compounds with longer side chains showed activity. The compound with a side chain carbons longer than retinoic acid showed higher activity than retinoic acid (5).

The present work concerned the effects of chain length of desmethylretinoic acid on granuloma formation induced by implanted cotton pellets. Compounds with side chains shorter than desmethylretinoic acid were not active. Compounds with side chains two or more carbons longer than desmethylretinoic acid were more active than desmethylretinoic acid and retinoic acid.

Both hydroxyproline and hexosamine contents of the granulomas induced by implanted cotton pellets in the presence of active compounds were greatly increased as compared with those present in the control granulomas. These active compounds are effective wound-healing agents. The mechanisms of action of these compounds on wound healing are discussed.

EXPERIMENTAL

Materials—The following were used: crystalline hydroxy-L-proline¹; D-glucosamine hydrochloride², grade A; sodium hydride, 57% oil dispersion; lithium aluminum hydride powder³, 97%;

triethyl phosphite⁴, 97%; β -ionone⁴, n_D^{20} 1.584; trimethyl phosphoacetate⁴; ethyl bromoacetate⁴, 75%; tetrahydrofuran⁴, 99.5%; *p*-dimethylaminobenzaldehyde⁵, reagent grade; ether⁶, anhydrous; phosphoric acid NF, 85%; manganese sulfate⁶, monohydrate, analytical grade; potassium permanganate⁶ USP, crystals; and dental cotton roll⁷, size I.

Implantation of Cotton Pellet—The methods used in the preparation and implantation of cotton pellets were essentially the same as described previously (5).

On the 7th day after implantation, the animals were killed with ether. The granulomas were carefully removed and immediately weighed on a precision torsion balance⁸. After drying in an oven at 68° for 48 hr, the dry weight of the granuloma was taken.

Chemical Analysis—The granulomas were digested in the Pyrex digestion tube (5), and 1 ml of 6 *N* HCl was added to each granuloma. Each tube was sealed *in vacuo*, and the content was hydrolyzed at 140° in a constant-temperature heating block for 3 hr. After cooling to room temperature, the seal of the tube was broken and the content was carefully neutralized with 6 *N* NaOH.

The content was then filtered. The original digesting tube and the filter were rinsed several times with small portions of distilled water, and the filtrate with the washings was brought to volume. Aliquots were taken for hydroxyproline and hexosamine analysis according to the methods described by Woessner (6) and Cessi and Piliego (7), respectively.

Chemical Synthesis—The methods used for the synthesis of desmethylretinoic acid vinylogs basically followed published schemes (8). All reactions were carried out under nitrogen. Direct light was also avoided. The synthesis was carried out as shown in Scheme I.

2,6,6-Trimethyl-1-(2'-carboxy-1'-vinyl)cyclohex-1-ene (I) (Desmethyl C₁₂ Acid)—The synthesis of this acid was described in detail previously (5).

2,6,6-Trimethyl-1-(4'-carboxybuta-1',3'-dienyl)cyclohex-1-ene (V) (Desmethyl C₁₄ Acid)—1. Synthesis of the C₁₂ alcohol (II)—Fifteen grams of I (0.0773 mole) was dissolved in 80 ml of anhydrous ether. Lithium aluminum hydride (4.4 g, 0.116 mole) was placed in a round-bottom, three-necked flask with enough anhydrous ether to make a slurry. The mixture was brought to -15° with the aid of dry ice in acetone. The C₁₂ acid solution was added dropwise into the mixture with stirring. The temperature was kept below -5°.

After all of the acid solution had been added, the reaction mixture was kept at room temperature with stirring for 1 hr. The mixture was then brought to 0°, and dilute sulfuric acid (1 *M*) was added dropwise to destroy any remaining lithium aluminum hydride. The mixture was then extracted with ether. The combined ether extract was washed once with water and then dehydrated with anhydrous magnesium sulfate overnight. The ether extract was filtered, and evaporation of ether gave 12.1 g (87% yield).

2. Synthesis of the C₁₂ aldehyde (III)—A solution of 12.1 g (0.066 mole) of the C₁₂ alcohol in anhydrous carbon tetrachloride was added dropwise into a flask containing 121.1 g of active manganese dioxide (9) and 500 ml of anhydrous carbon tetrachloride with vigorous stirring. The mixture was kept at room temperature with stirring for 4 hr. The manganese dioxide then was filtered by suction and washed several times with carbon tetrachloride. The carbon tetrachloride of the combined filtrates was evaporated, and the yield of the C₁₂ aldehyde was 12 g (100%). The C₁₂ aldehyde

¹ Sigma Chemical Co., St. Louis, Mo.

² Calbiochem, Los Angeles, Calif.

³ Alpha Inorganics, Beverly, Mass.

⁴ Aldrich Chemical Co., Milwaukee, Wis.

⁵ Eastman Kodak Co., Rochester, N.Y.

⁶ Mallinckrodt Chemical Works, St. Louis, Mo.

⁷ Johnson and Johnson, New Brunswick, N.J.

⁸ Roller-Smith.



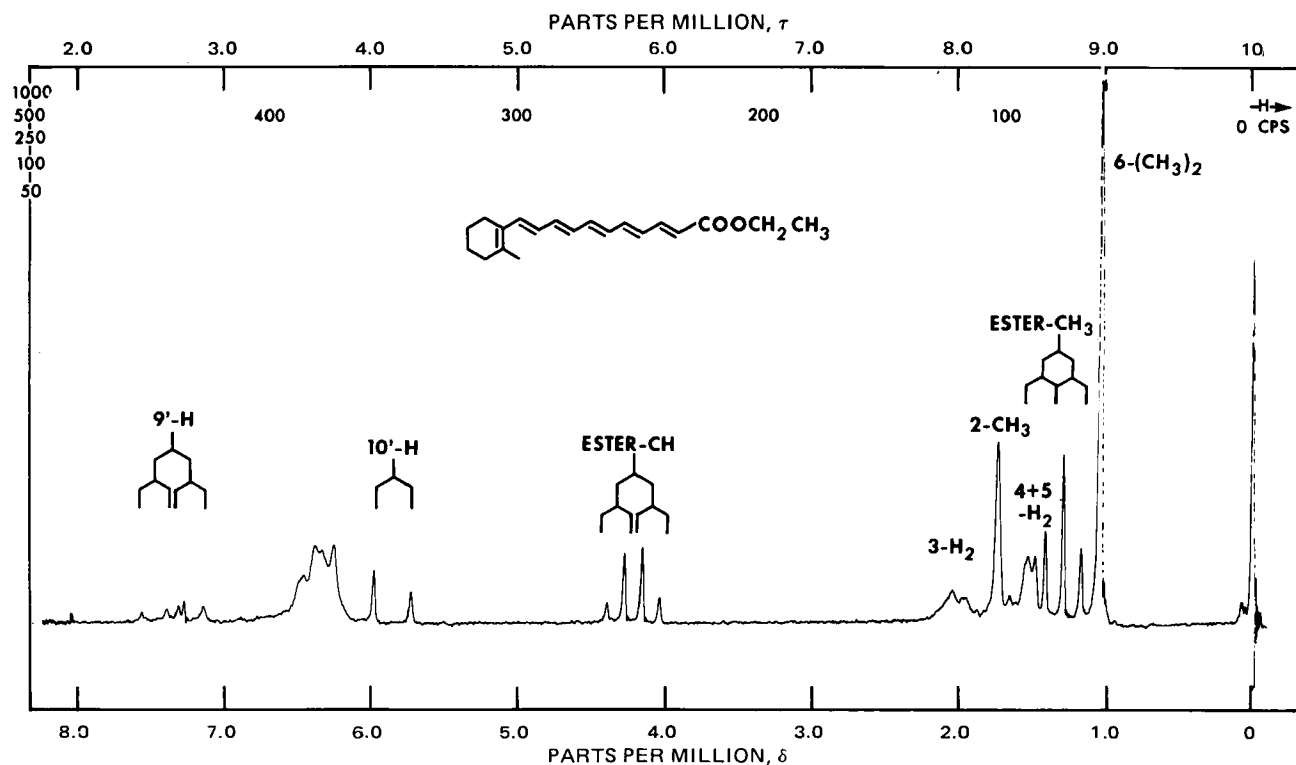


Figure 1—NMR spectrum of XIII.

(95% ethanol): $\lambda_{\max}$ 375 nm (ϵ 49,000).

Anal.—Calc. for $C_{20}H_{26}O_2$: C, 80.33; H, 8.72. Found: C, 80.55; H, 8.67.

2,6,6-Trimethyl-(12'-carboxyundeca-1',3',5',7',9',11'-hexaenyl)-cyclohex-1-ene (XVI) (Desmethyl C_{22} Acid)—The desmethyl C_{22} acid and ethyl ester (XV) were synthesized from IX and triethyl phosphonosorbate (XIV), and a 9.1% yield was obtained, mp 128–129°; NMR ($CDCl_3$) (Fig. 2): δ 1.04 (s, gem-dimethyl), 1.3 (t, ester methyl), 1.72 (s, C-2 methyl), 4.22 (q, ester methylene), 5.85 (d,

C-12 proton, $J = 15$ Hz), and 7.38 (q, C-11 proton).

Anal.—Calc. for $C_{24}H_{32}O_2$: C, 81.81; H, 9.09. Found: C, 81.61; H, 8.98.

The desmethyl C_{22} acid (XVI) was obtained from hydrolysis of the desmethyl C_{22} ethyl ester in alcoholic potassium hydroxide solution, mp 193.5–195°; UV (95% ethanol): $\lambda_{\max}$ 396 nm (ϵ 74,200).

Anal.—Calc. for $C_{12}H_{28}O_2$: C, 81.48; H, 8.64. Found: C, 81.44; H, 8.67.

2,6,6-Trimethyl-1-(14'-carboxydodeca-1',3',5',7',9',11',13'-hept-

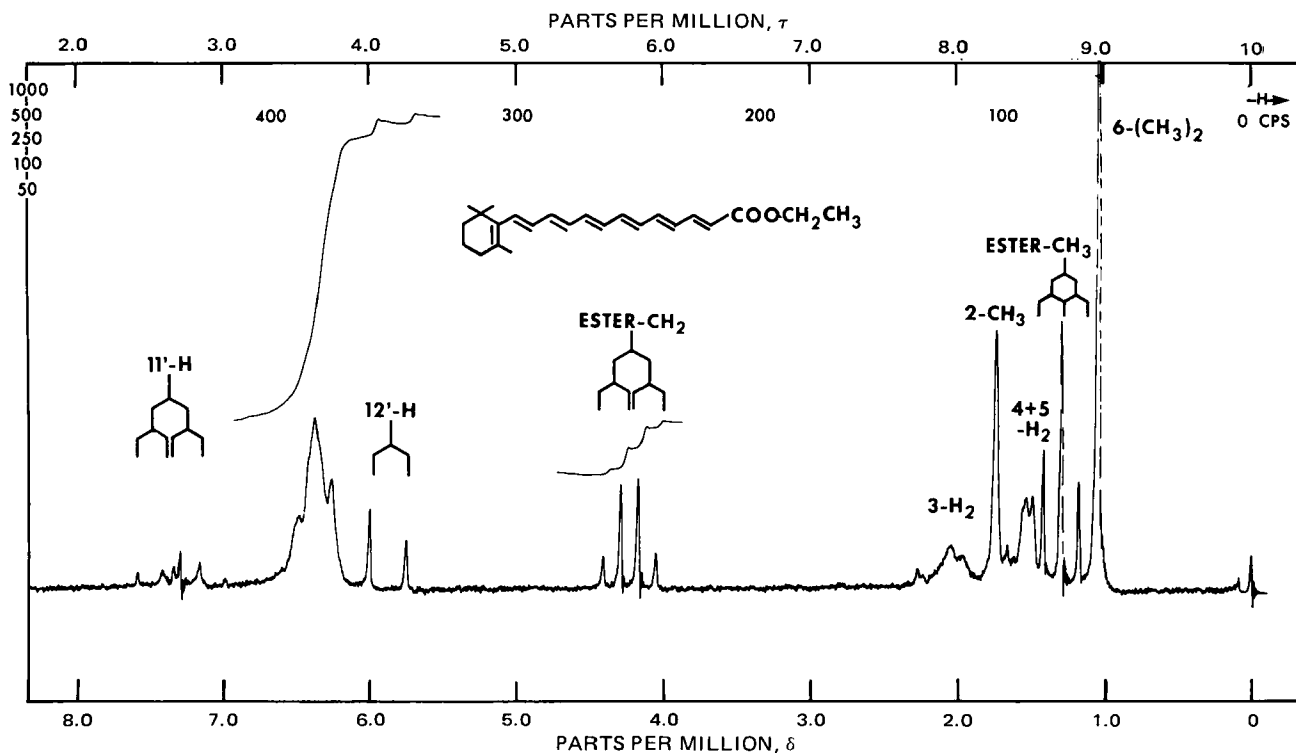


Figure 2—NMR spectrum of XVI.

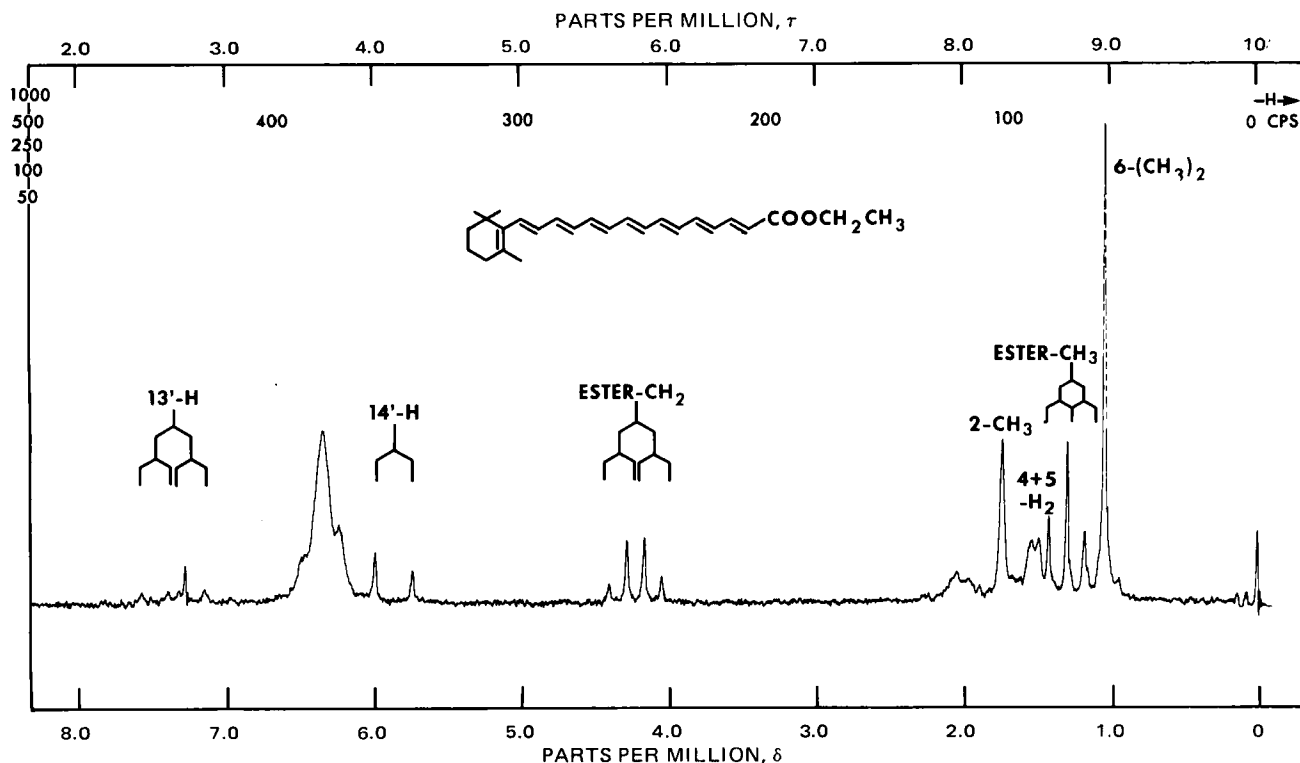


Figure 3—NMR spectrum of XX.

aenyl)cyclohex-1-ene (XX) (Desmethyl C_{24} Acid)—The desmethyl C_{24} acid and ethyl ester (XIX) were synthesized from the desmethyl C_{18} aldehyde (XVIII) and XIV, and a 2.2% yield was obtained, mp 158–159°; NMR ($CDCl_3$) (Fig. 3): δ 1.04 (s, gem-dimethyl), 1.3 (t, ester methyl), 1.72 (s, C-2 methyl), 4.22 (q, ester methylene), 5.85 (d, C-14 proton, $J = 15$ Hz), and 7.38 (q, C-13 proton).

Anal.—Calc. for $C_{26}H_{34}O_2$: C, 82.54; H, 8.99. Found: C, 82.36; H, 8.95.

Desmethyl C_{24} (XX) was obtained from hydrolysis of the desmethyl C_{24} acid ethyl ester in alcoholic potassium hydroxide solution, mp 202.5–203°; UV (95% ethanol): λ_{max} 415 nm (ϵ 85,200).

Anal.—Calc. for $C_{24}H_{30}O_2$: C, 82.38; H, 8.57. Found: C, 81.98; H, 8.30.

Triethyl Phosphonocrotonate (XIV)—Twenty-five grams of ethyl 4-bromocrotonate was placed in a three-necked flask and heated to 125° in an oil bath. Triethyl phosphite (21.5 g) was

added dropwise, and the reaction was continued for 4 hr. Distillation *in vacuo* yielded 25 g of triethyl phosphonocrotonate (81.3%), bp 104–108°, 0.22 mm Hg.

RESULTS AND DISCUSSION

The structural formulas for the synthetic compounds are shown in Table I. The effects of these compounds on granuloma formation induced by implantation of cotton pellets are shown in Table II. Compounds with a side chain shorter than that of desmethylretinoic acid were not active while compounds with a side chain as long as or longer than that of desmethylretinoic acid were active. These findings are in agreement with the previous findings (4, 5) that, besides the 2,6,6-trimethylcyclohex-1-ene ring, the length of the side chain is also important for activity.

Desmethylretinoic acid was more active than retinoic acid. However, XIII, the compound with a chain two carbons longer than

Table I—Structural Formulas of Desmethylretinoic Acid Vinylologs

Compound Number	Structure	Trivial Names
V		Desmethyl β - C_{14} acid
VII		Desmethyl β - C_{16} acid
XI		{ Desmethyl β - C_{18} acid or desmethylretinoic acid
XIII		
XVI		Desmethyl β - C_{22} acid
XX		Desmethyl β - C_{24} acid

Table II—Effect of Desmethylretinoic Acid Vinyls on Cotton Pellet-Induced Granuloma in Rats

Compound Number	Number of Animals	Granuloma Wet Weight, mg		Granuloma Weight, Experimental/Control	Granuloma Dry Weight, mg		Experimental/Control (p)
		Experimental	Control		Experimental	Control	
VII	6	238.6 ± 12.1	220.5 ± 12.2	1.1	29.0 ± 2.1	27.2 ± 2.1	1.1
XI	14	331.1 ± 14.8	208.8 ± 5.9	1.5	43.1 ± 2.6	25.5 ± 1.5	1.7
XIII	43	430.2 ± 8.1	205.0 ± 2.7	2.2	68.0 ± 1.5	23.9 ± 0.0	2.9
XVI	20	373.9 ± 8.6	202.9 ± 4.6	1.8	60.5 ± 1.6	24.9 ± 1.2	2.4
XX	6	340.3 ± 22.8	209.2 ± 4.4	1.6	51.6 ± 3.1	31.1 ± 2.0	1.7

desmethylretinoic acid, was considerably more active than its counterpart with two methyl groups on the side chain [β -C₂₂ acid, 2,6,6-trimethyl-(10'-carboxy-3',7'-dimethyldeca-1',3',5',8',9'-pentaenyl)cyclohex-1-ene] (5).

Compound XIII was the most active compound synthesized. Compound XIV was less active than XIII but was still more active than desmethylretinoic acid (XI or retinoic acid). It was also more active than its counterpart with two methyl groups on the side chain (β -C₂₄ acid) (5). Even XX was a reasonably active compound.

Compounds XIII, XVI, and XX are new compounds. The NMR spectra of the esters of these acids are shown in Figs. 1-3.

Grindlay and Waugh (10) introduced a simple method for studying tissue regeneration. These workers used a polyvinyl sponge disk as a foreign body to induce granuloma formation or tissue regeneration. Jackson *et al.* (11) believed that the repair of connective tissue is the most basic feature in wound healing, and they used granulomas induced by the implanting polyvinyl sponge technique to study wound healing. It was shown previously that only retinyl derivatives that are active in increasing tensile strength of the healing wound also increase the size and weight of granuloma induced by either the cotton pellet or polyvinyl sponge (4, 5). Granuloma formation is a useful quantitative method for studying wound healing. All of the active synthetic compounds studied promote healing.

Sandberg and Zederfeldt (12) reported that the rate of gain in

tensile strength and hydroxyproline in granuloma was directly related in rats or rabbits. For the present study, the effect of the active compounds on granuloma formation and the increase of hydroxyproline are shown in Tables III-VI. Meier *et al.* (13) used a cotton pellet as a foreign body to induce granuloma. Application of cortisone resulted in a diminution of granuloma size, which can be expressed quantitatively by determining its fresh or dry weight. Since then the method has been adapted as a standard assay for anti-inflammatory agents. Active retinyl compounds enhance granuloma formation, and it seems justified to call these compounds "inflammatory agents" (5, 14).

The effects of active retinyl derivatives on mucopolysaccharide synthesis were reported (4, 5). In this study, all compounds that stimulated granuloma formation also promoted mucopolysaccharide synthesis (Tables III-VI).

Inflammation and mucopolysaccharide synthesis are the two known important features in wound healing. Active retinyl derivatives reported previously (2, 14) and active compounds in the present experiment stimulated granuloma formation and promoted hydroxyproline and mucopolysaccharide synthesis. These results suggest that active retinyl compounds promote wound healing by inducing inflammation and increase the mucopolysaccharide synthesis mechanism of action.

Vitamin A or retinol has three distinct but unrelated major physiological functions. It is involved in the vision mechanism, it promotes growth, and it helps maintain a healthy epithelium. It is

Table III—Effect of XI on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control		Experimental
Number of animals	7		7
Granuloma dry weight, mg	25.1		48.8
Experimental/control	1.9		
Hydroxyproline weight, μ g	291		425
Experimental/control	1.5		
Hydroxyproline, μ g/granuloma, mg	11.5		8.7
Hexosamine weight, μ g	24.3		5.3
Experimental/control	2.1		
Hexosamine, μ g/granuloma, mg	9.7		10.5

Table IV—Effect of XIII on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control		Experimental
Number of animals	11		11
Granuloma dry weight, mg	21.3		67.3
Experimental/control	3.15		
Hydroxyproline weight, μ g	277		736
Experimental/control	2.66		
Hydroxyproline, μ g/granuloma, mg	12.9		10.9
Hexosamine weight, μ g	201		480
Experimental/control	2.36		
Hexosamine, μ g/granuloma, mg	9.4		7.0

Table V—Effect of XVI on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control		Experimental
Number of animals	8		8
Granuloma dry weight, mg	23.1		71.2
Experimental/control	3.06		
Hydroxyproline weight, μ g	331		875
Experimental/control	2.64		
Hydroxyproline, μ g/granuloma, mg	14.3		12.3
Hexosamine weight, μ g	233		505
Experimental/control	2.13		
Hexosamine, μ g/granuloma, mg	7.1		10.5

Table VI—Effect of XX on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control		Experimental
Number of animals	6		6
Granuloma dry weight, mg	30.0		47.9
Experimental/control	1.6		
Hydroxyproline weight, μ g	350		600
Experimental/control	1.7		
Hydroxyproline, μ g/granuloma, mg	11.7		12.6
Hexosamine weight, μ g	284		460
Experimental/control	1.6		
Hexosamine, μ g/granuloma, mg	9.5		9.6

well known that retinol can change reversibly to its aldehyde form (retinal) and irreversibly to its acid form (retinoic acid) in animal tissues. Retinal is responsible for the vision mechanism, but retinoic acid is not involved in this mechanism. Retinol, retinal, and retinoic acid promote growth, and any other change in structure of these molecules abolishes the growth-promoting activity.

Desmethylretinoic acid does not promote growth since it does not have methyl groups on the side chain. Being an acid, it cannot be involved in the vision mechanism. Desmethylretinoic acid promotes wound healing, and its action on epithelium is still not known. The relationships between healing and supporting a healthy epithelium are being studied.

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ACKNOWLEDGMENTS AND ADDRESSES

Received December 18, 1974, from the School of Pharmacy, University of California, San Francisco, CA 94143

Accepted for publication July 17, 1975.

Supported in part by Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, Calif.

* To whom inquiries should be directed.

Solvent-Dependent Conformational System in Hydroxyureas Detected by NMR Spectroscopy

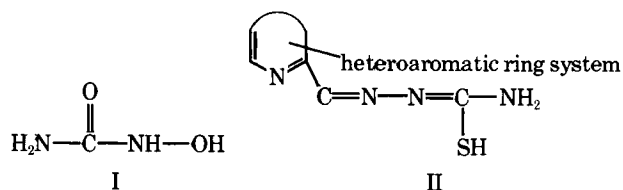
GEORGE R. PARKER *, NANCY K. HILGENDORF, and JAMES G. LINDBERG *

Abstract □ The proton magnetic resonance spectra of the antileukemia agent hydroxyurea and substituted hydroxyureas in several solvents were recorded and correlated with structural features. Solvent-dependent differences in conformational preferences due to effects on internal hydrogen bonding, a temperature-dependent conformational feature, and the exchangeability of protons with deuterium oxide and acetone- d_6 were observed. The conformational features consistent with the spectral data are discussed.

Keyphrases □ Hydroxyurea and substituted hydroxyureas—NMR spectra correlated with structural features, solvent-dependent differences observed □ NMR spectroscopy—hydroxyurea and substituted hydroxyureas, spectra correlated with structural features, solvent-dependent differences observed □ Solvent-dependent conformational system—hydroxyurea and substituted hydroxyureas, detected by NMR spectroscopy □ Antileukemic agents—hydroxyurea, NMR spectra correlated with structural features

NMR spectroscopy has become a powerful tool for the determination of organic molecular structure, configurational stereochemistry, and conformational properties of molecules (1). Knowledge concerning the conformational properties of drugs at their site of action is essential to the understanding of the complex molecular mechanism of action (2).

An NMR analysis was conducted on a series of substituted analogs of the antileukemia agent hydroxyurea (I) to gain some insight into the conformational properties that might explain the relative biological inactivity of the 3-*N*-substituted analogs. Hydroxyurea has been observed to inhibit DNA synthesis in various biological systems (3–5) and exhibits



activity against L-1210 lymphoid leukemia (6). The action of this compound has been attributed to the inhibition of the iron-dependent, allosterically controlled enzyme ribonucleoside diphosphate reductase (7–9) via a mechanism similar to that observed in a series of α -*N*-formylheteroaromatic thiosemicarbazones (II) (10).

In contrast to the rather large number of active analogs of II (72 of 97 compounds rated active *in vitro* and 51 of 97 active *in vivo*), only the ethyl and *n*-propyl analogs substituted at the 3-*N*-position and the methyl and ethyl 1-*N*-analogs of hydroxyurea were active *in vivo* against L-1210 lymphoid leukemia¹. These biological data could be rationalized in terms of *in vivo* instability, poor transportability to the site of action, steric effects at the site of action, or conformational factors relevant to drug action, which this paper reports as a possibility in substituted hydroxyureas.

The molecular mechanism of action of I and II has

¹ Personal communication from the Cancer Chemotherapy National Screening Center of the National Cancer Institute.