

Mechanism of Action of Retinyl Compounds on Wound Healing III: Effect of Retinoic Acid Homologs on Granuloma Formation

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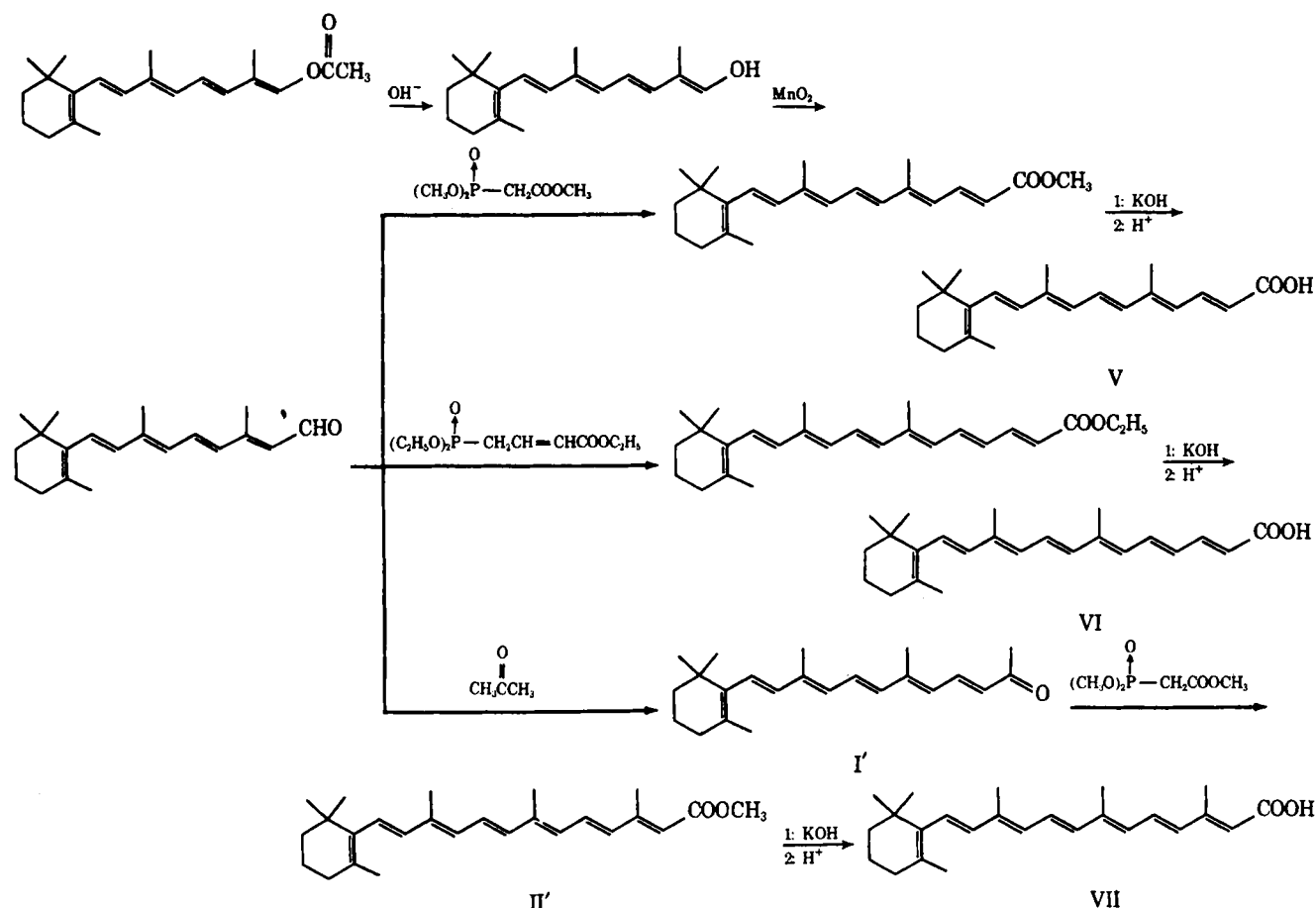
Abstract □ A series of homologs of retinoic acid was synthesized. The activities of these compounds on granuloma formation induced by implanted cotton pellets were studied. The compound (β -C₂₂ acid) with two more carbons on the side chain of retinoic acid is the most active. The hexosamine and hydroxyproline contents in the granuloma affected by these compounds are also increased. The mechanisms of action of these compounds on wound healing are discussed.

Keyphrases □ Retinoic acid analogs—synthesis, effect and mechanism on granuloma formation □ Wound healing—synthesis of retinoic acid analogs, effect and mechanism on granuloma formation □ Granuloma formation—synthesis, effect and mechanism of retinoic acid analogs □ Structure-activity relationships—effect of retinoic acid analogs containing 2,6,6-trimethylcyclohex-1-ene ring on granuloma formation

Previous reports showed that a few retinol-related compounds promote skin wound healing in rats (1-4). The structure-activity relationships of a few naturally

occurring compounds related to retinol were studied (3). The fact that retinol, retinyl acetate, and retinoic acid are all active in promoting wound healing indicates that the primary alcohol group is not essential. β -Carotene, the natural precursor of retinol, is active. Lycopene has essentially the same structure as β -carotene except that it does not have a closed ring at either end of the molecule. Lycopene is inactive. β -Ionone has essentially the same trimethylcyclohexene ring as retinol or β -carotene, but it has a shorter side chain. β -Ionone is active, but it is less active than retinol. α -Ionone, with only the position of a double bond in the ring different from β -ionone, is not active. It is clear that the 2,6,6-trimethylcyclohex-1-ene ring is essential for activity.

The present study concerned the structure-activity relationship of side-chain length of retinoic acid and tissue regeneration. The growth of granuloma induced by the implanted cotton pellet technique, as



Scheme I

described previously (4), was used. Jackson *et al.* (5) pointed out that the repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by polyvinyl sponges to study healing. Sandberg and Zederfeldt (6) found that the rate of gain in tensile strength and hydroxyproline in granuloma was directly related in both rats and rabbits. We have found that only those compounds that are active in increasing tensile strength also increase the size and weight of granuloma induced by either cotton pellet or polyvinyl sponge. Granuloma formation is a useful quantitative method to study wound healing.

EXPERIMENTAL¹

Materials and Special Chemicals—Materials and chemicals used in this study included the following: crystalline hydroxy-L-proline²; retinoic acid³, all-*trans*, Sigma grade, Type XX; D-glucosamine hydrochloride⁴, A grade; sodium hydride⁵, 57% oil dispersion; lithium aluminum hydride powder⁶, 97.5%; triethyl phosphite⁶, 97%; β -ionone⁴, n_D^{20} 1.584°; trimethylphosphonoacetate⁶; ethyl 4-bromocrotonate⁶, 75%; tetrahydrofuran⁶, 99.5%; 1,2-dimethoxyethane⁶; *p*-dimethylaminobenzaldehyde⁶, reagent grade; ether⁷, anhydrous; phosphoric acid NF⁷, 85%; and dental cotton roll⁸, size I.

Implantation of Cotton Pellet—The effect of retinoic acid and its homologs (I–VII, Table I) on granuloma formation in rats was evaluated by the cotton pellet method (7). Cotton pellets are disks sliced from dental rolls with a sharp razor blade. Disks weighing 20 ± 0.5 mg. were placed in petri dishes for sterilization in a steam autoclave for 40 min. at 115°. The sterilized cotton disks were handled with sterilized instruments and an aseptic technique.

Growth of granulation tissue in cotton pellets was induced by subcutaneous implantation at two symmetrical dorsolateral sites on Sprague–Dawley male rats, weighing 120 ± 2 g., under ether anesthesia.

The cotton pellet implanted on the right side contained 2 mg. of the test compound, and the cotton pellet implanted on the left side served as the control. The compound under study was dissolved in tetrahydrofuran, and 0.05 ml. of the solution was introduced into the pellet from a 0.25-ml. syringe with a size 20 needle. The tetrahydrofuran was completely evaporated in a vacuum desiccator connected to a life vacuum line under total darkness. Two hours was sufficient to evaporate all of the tetrahydrofuran. On the 7th day after implantation, the granulomas formed were carefully removed and the wet weight was measured right after removal. The granulomas formed were dried at 65° for 3 days, and the dry weights of the granulomas were measured.

Chemical Analysis—All of the dried granulomas resulting from a test compound of one experiment were combined in a digestion tube. One milliliter of 6 *N* HCl was added for each granuloma. The control dried granulomas were digested in a separate tube.

The lower part of the digestion tube is 25 mm. in diameter and 100 mm. in length; the upper part of the tube is 10 mm. in diameter and 100 mm. in length. It can be easily made from a 25 × 200-mm. Pyrex test tube. The digestion tube was sealed *in vacuo*, and the contents were hydrolyzed at 115° in a constant-temperature heating block or in an oil bath for 4 hr. After cooling to room temperature, the seal of the tube was broken and the contents were carefully neutralized with 6 *N* NaOH. The contents were then filtered with

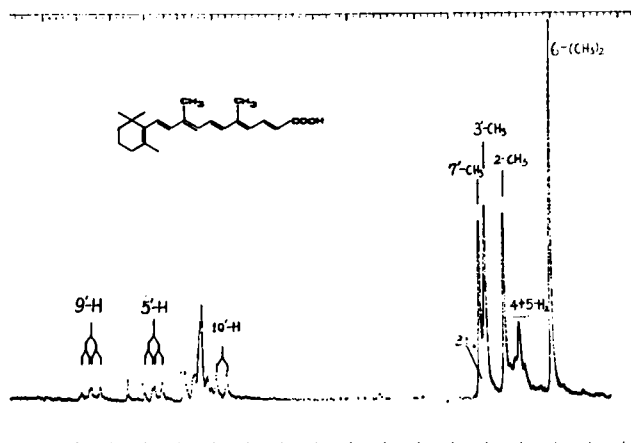


Figure 1—NMR spectrum of β -C₂₂ acid.

the aid of suction. The original digestion tube and the filter were rinsed several times with small portions of distilled water, and the filtrate was brought to volume in a suitable volumetric flask. Aliquots were used for hydroxyproline and hexosamine analysis according to the methods described by Woessner (8) and Cessi and Piliego (9), respectively.

Synthesis—Small quantities of analytically and spectroscopically pure Compounds II and III were used as received⁹. More of these compounds were synthesized by following essentially the published methods (10) with good success. The method for synthesizing Compound I was modified. Compounds V and VI are new. Compounds V, VI, and VII were synthesized from retinol acetate according to Scheme I.

2,6,6-Trimethyl-1-(2'-carboxy-1'-vinyl)cyclohex-1-ene (I) (β -C₁₂ Acid)—Sodium hydroxide, 240 g. (6 moles), was dissolved in water to make 600 ml. solution in a 2-l. conical flask with a magnetic stirrer. The weight of the flask and its contents was recorded. The sodium hydroxide was then cooled to -5° in an acetone–dry ice bath. Chlorine gas was bubbled into the sodium hydroxide solution until 126 g. had been introduced. The temperature was kept at 0° . This takes about 1 hr. The solution was then poured into a 4-l. beaker, and 100 g. (0.52 mole) of β -ionone was added into the beaker in a slow stream with stirring. The stirring was continued at room temperature after the addition of β -ionone for 3 hr.

Methanol (120 ml.) was slowly added to the contents in the beaker with stirring. Vigorous reaction was started about 3 min. after the addition of methanol, and chloroform bubbled out from the solution. The mixture was kept just below 80° by adding crushed ice. After the reaction subsided, the mixture was cooled to 2 – 3° . The mixture was neutralized slowly with concentrated phosphoric acid (approximately 200 ml.). The final pH was adjusted to about 3.5. The β -C₁₂ acid solidified and separated out of the solution and was filtered in a Büchner funnel. The acid was dissolved in ether. The ether solution was extracted with 10% sodium hydroxide solution four times, and the ether layer was discarded. The alkaline extract was acidified with 6 *N* HCl slowly at cold temperature to pH 4. The β -C₁₂ acid was extracted with ether, and the extract was dried overnight with anhydrous magnesium sulfate. The dried ether was filtered by gravity and washed with anhydrous ether, and the ether of the combined extracts was evaporated. The yield of crude C₁₂ acid was 92 g. (92%). The crude acid was recrystallized from 60% ethanol, and the yield was about 74 g. (74%), m.p. 107.5 – 108.0° (uncorrected) [lit. (10) m.p. 105.5 – 108°]; UV absorption spectrum (ethanol): $\lambda_{\max} = 278$ nm., $\epsilon = 10,000$; IR spectrum (KBr): C=O 1690 cm.⁻¹, C=C 1620 cm.⁻¹; NMR spectrum (CDCl₃): gem-dimethyl $\delta = 1.09$ (singlet), C₍₂₎—CH₃ $\delta = 1.80$ (singlet), C_(2')—H $\delta = 5.90$ (doublet), and C_(1')—H $\delta = 7.60$ (doublet).

Anal.—Calc. for C₁₂H₁₈O₂: C, 74.19; H, 9.34. Found: C, 73.88; H, 9.16.

2,6,6-Trimethyl-1-(10'-carboxy-3',7'-dimethyldeca-1',3',5',7',9'-pentaenyl)cyclohex-1-ene (V) (β -C₂₂ Acid)—Sodium hydride (3.1 g. 57% in mineral oil, 0.07 mole) was washed with ether and then sus-

¹ The NMR spectra of the synthesized compounds were measured with either a Varian A-60 analytical spectrometer or Jeolco J N M-4H-100 spectrometer. A Cary 15 double-beam UV spectrophotometer and Perkin-Elmer IR spectrometer were used to measure UV and IR spectra. Elemental analysis was performed by the Chemistry Department's Microchemical Analysis Laboratory, University of California, Berkeley, Calif.

² Sigma Chemical Co., St. Louis, Mo.

³ Calbiochem, Los Angeles, Calif.

⁴ Alpha Inorganics, Beverly, Mass.

⁵ Aldrich Chemical Co., Milwaukee, Wis.

⁶ Eastman Kodak Co., Rochester, N. Y.

⁷ Mallinckrodt Chemical Works, St. Louis, Mo.

⁸ Johnson & Johnson, New Brunswick, N. J.

⁹ Supplied by Professor H. O. Huisman, University of Amsterdam, The Netherlands.

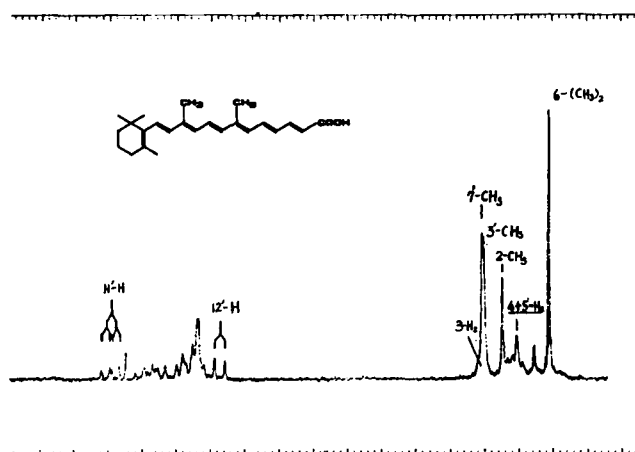


Figure 2—NMR spectrum of β - C_{24} acid.

pendent in dry tetrahydrofuran at 0° . Trimethylphosphonoacetate (15.7 g., 0.07 mole) was added slowly to this suspension so that the temperature remained below 5° . The reaction mixture was kept at 0° with stirring for 30 min. Retinal (10 g., 0.035 M) was added as a solution in tetrahydrofuran over 15 min. The reaction mixture was kept at 0° for 30 min. and then at 35° for an additional 30 min. Saturated sodium chloride solution was cautiously added to the mixture and cooled to 0° . Extraction (petroleum ether, b.p. 30 – 60°), drying over anhydrous magnesium sulfate, and removal of solvent gave a dark-red oil containing the ethyl ester of β - C_{22} acid. The ester was immediately hydrolyzed in 100 ml. of ethanol and 100 ml. of a 20% potassium hydroxide solution at room temperature overnight. The reaction mixture was diluted with water, and the non-acidic impurities were extracted with petroleum ether. The aqueous layer was extracted with ether after acidifying with hydrochloric acid to pH 4, and the ether extract was dried over anhydrous magnesium sulfate. After removing the solvent, β - C_{22} acid (9 g., 79%) was isolated. Recrystallization from 80% ethanol three times gave pure *trans*- β - C_{22} acid, m.p. 180.5 – 181.0° (uncorrected); UV absorption spectrum (ethanol): $\lambda_{\max} = 378$ nm., $\epsilon = 46,400$; IR spectrum (KBr): $C=O$ 1680 cm^{-1} , $C=C$ 1600 cm^{-1} ; NMR spectrum ($CDCl_3$) (Fig. 1): gem-dimethyl $\delta = 1.04$ (singlet), $C_{(3)}-CH_3$ $\delta = 1.72$ (singlet), $C_{(3')} - CH_3$ $\delta = 2.00$ (singlet), $C_{(10')} - H$ $\delta = 5.88$ (doublet) ($J = 13$ Hz., $C_{(5')} - H$ $\delta = 6.88$ (quartet), and $C_{(9')} - H$ $\delta = 7.80$ (quartet) ($J = 13$ Hz.).

Anal.—Calc. for $C_{22}H_{30}O_2$: C, 80.87; H, 9.20. Found: C, 80.77; H, 9.25.

2,6,6-Trimethyl-1-(12'-carboxy-3',7'-dimethyldodeca-1',3',5',7',9',11'-hexaenyl)cyclohex-1-ene (VI) (β - C_{24} Acid)— β - C_{24} acid was synthesized essentially as β - C_{22} acid except that 17.5 g. (0.07 mole) of triethylphosphonocrotonate was used to replace trimethylphosphonoacetate for 10 g. (0.035 mole) of retinal. The β - C_{24} ester was refluxed in alcohol (50%) containing 10% potassium hydroxide under nitrogen for 3 hr. The yield was 10 g. (81%). After recrystallization from 80% ethanol three times, pure β - C_{24} acid was obtained, m.p. 188.0 – 188.5° (uncorrected); UV absorption spectrum (cyclohexane): $\lambda_{\max} = 410$, $\epsilon = 69,800$; IR spectrum (KBr): $C=O$ 1680 cm^{-1} , $C=C$ 1600 cm^{-1} ; NMR spectrum ($CDCl_3$) (Fig. 2): gem-

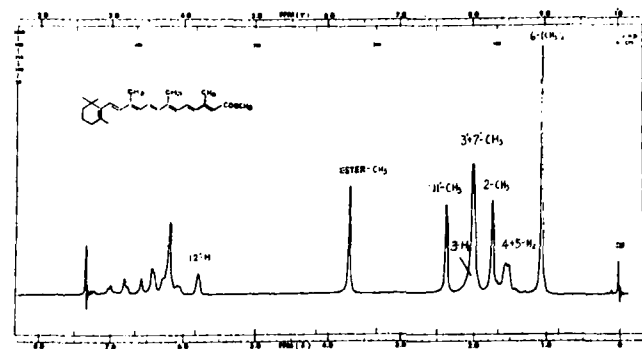


Figure 3—NMR spectrum of β - C_{25} acid methyl ester.

Table I—Structural Formulas of Retinoic Acid Homologs Studied

Compound Number	Structure	Trivial Names
I		All- <i>trans</i> - β - C_{12} acid
II		All- <i>trans</i> - β - C_{14} acid
III		All- <i>trans</i> - β - C_{17} acid
IV		All- <i>trans</i> - β - C_{20} acid
V		All- <i>trans</i> - β - C_{22} acid
VI		All- <i>trans</i> - β - C_{24} acid
VII		All- <i>trans</i> - β - C_{25} acid

dimethyl $\delta = 1.04$ (singlet), $C_{(3)}-CH_3$ $\delta = 1.72$ (singlet), $C_{(3')} - CH_3$ $\delta = 2.00$ (singlet), $C_{(7')} - CH_3$ $\delta = 2.02$ (singlet), $C_{(12')} - H$ $\delta = 5.87$ (doublet) ($J = 13$ Hz.), and $C_{(11')} - H$ $\delta = 7.49$ (quartet) ($J = 13$ Hz.).

Anal.—Calc. for $C_{24}H_{32}O_2$: C, 81.77; H, 9.15. Found: C, 81.82; H, 9.09.

3,5,7,11-Dodecapentaen-2-one-6,10-dimethyl-12-(2',6',6'-trimethyl-1'-cyclohexene-1'-yl) (I') (β - C_{22} Ketone)—A mixture of 28 g. (0.098 mole) of retinal, 75 ml. of petroleum ether (b.p. 30 – 60°), 300 ml. of acetone, and 100 ml. of 0.5 N NaOH solution was refluxed for 2.5 hr. After cooling, the reaction mixture was poured into 500 ml. of ice water and 35 ml. of 2 N H_2SO_4 . The organic layer was separated and the aqueous layer was extracted with petroleum ether. The combined organic phases were washed with water and dried over anhydrous magnesium sulfate. Evaporation of solvent gave 15 g. of solid residue (46.3%). It was crystallized from hexane, yielding 11.3 g. (34.8%), m.p. 104 – 105° [lit. (11) m.p. 104 – 105°]; UV absorption spectrum (cyclohexane): $\lambda_{\max} = 392$, $\epsilon = 52,700$; IR spectrum (KBr): $C=O$ 1650 cm^{-1} , $C=C$ 1600 cm^{-1} ; NMR spectrum ($CDCl_3$): gem-dimethyl $\delta = 1.03$ (singlet), $C_{(3')} - CH_3$ $\delta = 1.73$ (singlet), $C_{(10)} - CH_3$ $\delta = 2.00$ (singlet), $C_{(6)} - CH_3$ $\delta = 2.10$ (singlet), and $C_{(1)} - CH_3$ $\delta = 2.30$ (singlet).

2,6,6-Trimethyl-1-(12'-carbomethoxy-3',7',11'-trimethyldodeca-1',3',5',7',9',11'-hexaenyl)cyclohex-1-ene (II')—Trimethylphosphonoacetate, 5.46 g. (0.03 mole), in 20 ml. tetrahydrofuran was added dropwise to a well-stirred suspension of 0.72 g. (0.03 mole) sodium hydride in 200 ml. tetrahydrofuran. The temperature was kept at -5 – 0° for 1 hr. and at room temperature for 1 hr. A solution of β - C_{22} ketone, 4.86 g. (0.015 mole), in 20 ml. tetrahydrofuran was then added dropwise. The mixture was refluxed for 5 hr. under nitrogen. It was then poured into 500 ml. of water and extracted with petroleum ether. The organic phase was washed with water. After removal of the solvent, 8 g. of crystalline residue was obtained. It was recrystallized from 95% ethanol to give 4 g. of β - C_{25} acid methyl ester (70.1%), m.p. 108.5 – 109° (uncorrected); UV absorption spectrum (cyclohexane): $\lambda_{\max} = 402$, $\epsilon = 68,200$; IR spectrum (KBr): $C=O$ 1700 cm^{-1} , $C=C$ 1600 cm^{-1} ; NMR spectrum ($CDCl_3$) (Fig. 3): gem-dimethyl $\delta = 1.01$ (singlet), $C_{(3)} - CH_3$ $\delta = 1.70$ (singlet), $C_{(3')} + C_{(7')} - CH_3$ $\delta = 2.00$, $C_{(11')} - CH_3$ $\delta = 2.35$, ester- CH_3 $\delta = 3.7$ (singlet), and $C_{(12')} - H$ $\delta = 5.8$ (singlet).

2,6,6-Trimethyl-1-(12'-carboxy-3',7',11'-trimethyldodeca-1',3',5',7',9',11'-hexaenyl)cyclohex-1-ene (VII) (β - C_{25} Acid)—A mixture of 4 g. (0.011 mole) β - C_{25} acid methyl ester, 5 g. potassium hydroxide,

Table II—Effect of Retinoic Acid Homologs on Cotton Pellet-Induced Granuloma

Compound Number	Number of Animals	Compound Applied	Body Weight Change, Average	Granuloma Wet Weight, mg.—Experimental	Granuloma Wet Weight, mg.—Control	Granuloma Weight—Experimental/Control (<i>p</i>)	Granuloma Dry Weight, mg.—Experimental	Granuloma Dry Weight, mg.—Control	Experimental/Control (<i>p</i>)
I	6	C ₁₈	+48	213.8 ± 7.5	215.3 ± 5.1	1.0	28.3 ± 3.1	26.8 ± 2.9	1.0
II	6	C ₁₈	+38	214.5 ± 2.3	215.4 ± 5.0	1.0	27.6 ± 1.0	27.8 ± 0.8	1.0
III	12	C ₁₇	+50	220.5 ± 8.2	218.0 ± 6.0	1.0	30.2 ± 2.7	29.9 ± 3.0	1.0
IV	12	C ₂₀	+50	332.2 ± 4.9	218.7 ± 2.5	1.5 (<0.001)	59.9 ± 1.7	35.1 ± 1.9	1.5 (<0.001)
V	12	C ₂₂	+47	438.0 ± 5.4	225.9 ± 7.4	1.9 (<0.001)	69.8 ± 2.6	36.1 ± 2.5	1.9 (<0.001)
VI	10	C ₂₄	+46	302.0 ± 12.7	216.5 ± 6.9	1.4 (<0.001)	47.4 ± 2.1	31.3 ± 2.2	1.5 (<0.001)
VII	7	C ₂₅	+61	278.4 ± 26.4	198.7 ± 4.3	1.4 (<0.005)	35.0 ± 2.7	20.8 ± 1.3	1.6 (<0.001)

25 ml. water, and 150 ml. ethanol was stirred under nitrogen in a 50° water bath for 4 hr. After diluting with water, the nonacidic impurities were extracted with ether and the water layer was acidified with sulfuric acid. Extraction with ether and evaporation of ether gave 3.5 g. of orange-red crystalline residue. It was crystallized from isopropyl alcohol to give 1.5 g. (90.0%) of pure β -C₂₅ acid, m.p. 197–198°; UV absorption spectrum (cyclohexane): $\lambda_{\max}$ = 405 nm., ϵ = 70,300; IR spectrum (KBr): C=O 1700 cm.⁻¹, C=C 1600 cm.⁻¹.

Anal.—Calc. for C₂₅H₃₄O₂: C, 81.96; H, 9.29. Found: C, 81.95; H, 9.47.

The acid was converted into methyl ester by treatment with diazomethane and its NMR spectrum is identical with that shown in Fig. 3.

RESULTS AND DISCUSSION

The structural formulas for the compounds studied 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 retinoic acid did not stimulate granuloma formation at the amount of 2 mg./pellet, while compounds with a side chain longer than that of retinoic acid stimulated granuloma formation. These findings are in agreement with the previous finding (4) that, besides the 2,6,6-trimethylcyclo-

hex-1-ene ring, the length of the side chain is also important for activity. Compound V is the most active.

Meier *et al.* (7) introduced a simple quantitative method for measuring the degree of inflammation. They used a cotton pellet as a foreign body to induce granuloma formation. Application of cortisone caused a diminution of granuloma size, which can be expressed quantitatively by determining the weight of the granuloma formed. Since then, this method has been extensively used for assaying steroid and nonsteroid anti-inflammatory agents. Retinoic acid and its active homologs, like retinol (4), cause an increased granuloma mass induced by cotton pellets. These active compounds are "inflammatory agents."

Grindlay and Waugh (12), at about the same time, applied essentially the same implantation technique to study tissue regeneration. Jackson *et al.* (5) pointed out that the repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by implanted polyvinyl sponges to study wound healing. Sandberg and Zederfeldt (6) found that the rate of gain in tensile strength and the rate of hydroxyproline synthesis in granuloma were directly related in both rats and rabbits. This was confirmed previously in this laboratory (4). Granuloma formation is a technique used for measuring inflammation as well as wound healing.

Inflammation and mucopolysaccharide synthesis are the two known important features in wound healing. Anti-inflammatory

Table III—Effect of β -C₂₀ Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	6	
Granuloma dry weight, mg.	36	61.2
Experimental/control	1.7	
Hydroxyproline weight, mcg.	395.6	662.5
Experimental/control	1.67	
Hydroxyproline, mcg./granuloma, mg.	11	10.8
Hexosamine weight, mcg.	330	493
Experimental/control	1.50	
Hexosamine, mcg./granuloma, mg.	9.17	8.1

Table IV—Effect of β -C₂₂ Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	8	
Granuloma dry weight, mg.	31.8	70.4
Experimental/control	2.2	
Hydroxyproline weight, mcg.	343.7	637.5
Experimental/control	1.85	
Hydroxyproline, mcg./granuloma, mg.	10.8	9.1
Hexosamine weight, mcg.	306	640
Experimental/control	2.1	
Hexosamine, mcg./granuloma, mg.	9.6	9.1

Table V—Effect of β -C₂₄ Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	6	
Granuloma dry weight, mg.	30.2	47.9
Experimental/control	1.6	
Hydroxyproline weight, mcg.	350	600
Experimental/control	1.7	
Hydroxyproline, mcg./granuloma, mg.	11.6	12.5
Hexosamine weight, mcg.	284	460
Experimental/control	1.62	
Hexosamine, mcg./granuloma, mg.	9.4	9.6

Table VI—Effect of β -C₂₅ Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	7	
Granuloma dry weight, mg.	20.8	35
Experimental/control	1.6	
Hydroxyproline weight, mcg.	287.5	412.5
Experimental/control	1.44	
Hydroxyproline, mcg./granuloma, mg.	13.8	11.8
Hexosamine weight, mcg.	223	333
Experimental/control	1.5	
Hexosamine, mcg./granuloma, mg.	10.7	9.5

agents retard healing by their anti-inflammatory and inhibitory actions on mucopolysaccharide synthesis (2). Retinoic acid reverses the healing retardation action of anti-inflammatory agents (13). Active retinoic acid homologs studied in the present paper promote wound healing by increasing granuloma mass.

The healing-promoting action of these active retinoic acid homologs is further evidenced by the increase of hydroxyproline and hexosamine contents of the granuloma (Tables III-VI). Hydroxyproline is an important constituent of collagen, which is an essential component of connective tissue. Hexosamine is a component of mucopolysaccharide, and the role of mucopolysaccharide in healing was extensively discussed previously (2). As shown in Tables III-VI, both the hydroxyproline and hexosamine contents were increased in the granuloma with either one of the active compounds. This fact indicated that these compounds increased connective tissue regeneration. However, the amount of hydroxyproline or hexosamine per unit of weight of granuloma of the experimental was lower than that of the control. This fact indicated that these active compounds also stimulated inflammation.

It is suggested that active retinoic acid homologs, like retinol, promote healing by inducing inflammation and increase mucopolysaccharide and collagen synthesis mechanisms of action.

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▲ To whom inquiries should be directed.

Extraction and Separation of Anthraquinone Glycosides

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Abstract □ A method was developed for extracting anthraquinone aglycones and their corresponding glycosides from plant materials and for separating them in pure form. The method consists of rendering the glycosides and aglycones insoluble in chloroform and then removing, stepwise, the interfering extractable substances. The method makes use of the fact that the aglycones are chloroform soluble whereas the glycosides are not. The method was applied with good results to the isolation of the aglycones and glycosides of cascara and senna.

Keyphrases □ Anthraquinone aglycones and corresponding glycosides—extraction, separation from plant material □ Cascara bark—extraction, separation of anthraquinone aglycones and glycosides □ Senna leaves—extraction, separation of anthraquinone aglycones and glycosides

The methods that have been used to date for the isolation and purification of the anthraquinone derivative glycosides from cascara bark and senna leaflets fall into three categories. The first involves an aqueous or hydroalcoholic menstruum (1), and the second uses a pure solvent such as methanol (2, 3), ethanol (4), propyl alcohol, or isopropyl alcohol (1). The final method uses a solvent such as chloroform or ether (5) to remove certain interfering substances, and then extraction proceeds using an alcoholic menstruum. Purification of the various fractions is then accomplished by

recrystallization from one of these solvents. In some procedures, the fractions are acetylated in benzene (1, 3, 6) prior to being further purified by fractional crystallization.

In view of the fact that interfering plant constituents in cascara bark and senna leaflets made a clearcut separation of their constituents impossible by these methods, it was decided to try to develop a new procedure which would produce purer fractions of the anthracene derivatives, particularly derivatives of hydroxyanthraquinones, both free and in the form of glycosides.

EXPERIMENTAL

Extraction Procedure—The extraction procedure chosen (Scheme I) is based on the fact that the hydroxyanthraquinone derivatives and their corresponding glycosides are present both free and as magnesium, potassium, and sodium salts combined either through a hydroxy or a carboxylic acid group. In addition, the separation method capitalizes on the differential solubility in chloroform of the glycosides and their aglycones as well as other anthracene derivatives. The glycosides are insoluble in chloroform whereas the free aglycones, as well as the other anthracene derivatives, are chloroform soluble.

The extraction procedure consisted of macerating the ground drug for 24 hr. with 5% acetic acid. The purpose of this step was to liberate the free anthraquinones and their corresponding glycosides from their magnesium, potassium, or sodium salts, in which form