TABLE IV

RADIOACTIVITY OF ISOLATED LIGNINS AND LIGNIN OXIDATION PRODUCTS AFTER ADMINISTRATION OF LABELED PHENYLALANINE, CINNAMIC ACID AND ACETATE TO WHEAT AND MAPLE

Compound administered[a]	Material recovered: Name	Sp. activity (mμc./mmole CO_2): Wheat	Maple	Sp. activity of lignin monomer (mμc./mmole): Wheat	Maple
L-Phenylalanine-R-C^{14}	Klason lignin	42.1	21.1	442[b]	222[b]
	Dioxane lignin	38.9	16.3	409[b]	171[b]
	Vanillin-NBH[d]	29.6	23.0	570[c]	444[c]
	Syringaldehyde-NBH[d]	28.8	10.7	800[c]	220[c]
Cinnamic acid-3-C^{14}	Klason lignin	30.9	..	324[b]	..
	Dioxane lignin	25.5	..	268[b]	..
	Vanillin-NBH[d]	20.9	..	314	..
	Syringaldehyde-NBH[d]	28.8	..	461	..
Acetic acid 2-C^{14}	Klason lignin	38.1	7.1	400[b]	75[b]
	Dioxane lignin	5.7	12.0	60[b]	126[b]
	Vanillin-NBH[d]	0.17	0.82	2.6	12
	Syringaldehyde-NBH[d]	0.33	1.22	5.3	19.5

[a] Wheat plants (equivalent to *ca.* 4.5 g. dry weight) were fed 4.0 μc. carbon-14 in 0.079 mmole of the compound. Maple twigs (dry wt. *ca.* 7.5 g.) received 6.5 μc. carbon-14 in 0.10 mmole. [b] Calculated on the basis of the average number of carbon atoms per lignin molecule equalling 10.5 (see text). [c] Results are multiplied by 9/7 to correct for loss of two skeletal carbons during oxidation. [d] NBH ≡ *m*-nitrobenzoylhydrazone.

Two possible explanations suggest themselves for this. It could have been due to a $C_6,C_1 + C_2$ condensation incorporating carbon-14 into carbons 1 and 2 of the side chain, which were then lost in the oxidation but recovered as isolated lignin. However, after feeding acetate to both species virtually inactive DHCA and DHSA were isolated (Table I), and as these contain all nine skeletal carbons, the above possibility was eliminated. The alternative explanation is that acetate has been incorporated into some artifact associated with the isolated lignins, which does not yield vanillin and syringaldehyde. These results underline the difficulties involved if isolated lignins are employed in lignification experiments.

Caution is needed when comparing the degree of utilization of such different compounds as acetate and substituted phenylpropanes. There may be considerable difference in pool sizes, of course, and feeding of equivalent amounts of the two types of compound has been shown in this Laboratory[21] to yield data which may not be closely comparable. But even allowing for this, the present results indicate that incorporation of acetate into the isolable aromatic ring or side chain of lignin is slight

Acknowledgments.—Microanalyses reported here were done by Mr. J. A. Baignée and analyses of carbon-14 by Mr. John Dyck. The technical assistance of Mr. J. P. Shyluk throughout the investigation is gratefully acknowledged.

(21) J. E. Watkin and A. C. Neish, unpublished data.

SASKATOON, SASK., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

Gibberellins from Flowering Plants. I. Isolation and Properties of a Gibberellin from *Phaseolus vulgaris* L.[1]

BY CHARLES A. WEST AND BERNARD O. PHINNEY

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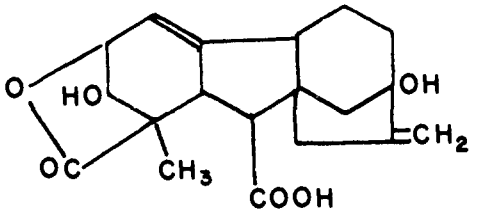
A procedure is described for the isolation of a gibberellin, bean factor II, from acetone–water extracts of immature bean seed (*Phaseolus vulgaris* L.). Bean factor II can be distinguished from the fungal gibberellins, gibberellic acid, gibberellin A_1 and gibberellin A_2, on the basis of its differential biological activity for dwarf maize mutants *dwarf-1* and *dwarf-5*, its chromatographic behavior and its infrared spectrum. Preliminary chemical studies suggest that bean factor II has a carboxylic acid and a lactone functional group, as well as sites of unsaturation not in conjugation with either of these.

The gibberellins, which are potent plant growth regulators,[2] were isolated as a crude crystalline mixture from culture filtrates of the vegetative stage of the fungus *Gibberella fujikuroi* (Saw.) Wr. by Yabuta and Sumiki in 1938.[3] Since that time four different gibberellins have been isolated in the pure state from that source, either as a free acid or methyl ester: gibberellic acid ($C_{19}H_{22}O_6$);[4] gibberellin A (gibberellin A_1) ($C_{19}H_{24}O_6$)[5]; gibberellin A_2 ($C_{19}H_{26}O_6$);[6] and gibberellin A_4 ($C_{18}H_{22}O_5$) or $C_{19}H_{24}O_5$).[7] A structural formula has been assigned to gibberellic acid by Cross, *et al.*[8] Gibberellin



(1) This project was supported in part by grants from Merck and Co., Inc., National Science Foundation (Grant 3526), and Research Corporation.

(2) B. B. Stowe and T. Yamaki, *Ann. Rev. Plant Physiol.*, **8**, 181 (1957).

(3) T. Yabuta and Y. Sumiki, *J. Agr. Chem. Soc. Japan*, **14**, 1526 (1938).

(4) P. J. Curtis and B. E. Cross, *Chem. Ind. (London)*, 1066 (1954).

(5) F. H. Stodola, K. B. Raper, D. I. Fennell, H. F. Conway, V. E. Sohns, C. T. Langford and R. W. Jackson, *Arch. Biochem. Biophys.*, **54**, 240 (1954); F. H. Stodola, G. E. N. Nelson and D. J. Spence, *ibid.*, **66**, 438 (1957).

(6) N. Takahashi, H. Kitamura, A. Kawaranda, Y. Seta, M. Takai, S. Tamura and Y. Sumiki, *Bull. Agr. Chem. Soc. Japan*, **19**, 267 (1955).

(7) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, *ibid.*, **21**, 396 (1957).

(8) B. E. Cross, J. F. Grove, J. MacMillan and T. P. C. Mulholland, *Proc. Chem. Soc.*, 221 (1958).

A_1 has been shown to be identical with one of the isomers resulting when ring A is saturated.[9]

A considerable body of evidence has been accumulated to support the idea that gibberellins or gibberellin-like substances act as natural growth regulators or hormones in flowering plants. Extracts of many seeds,[10-12] plant vegetative parts[13-17] and plant tissue cultures[18] are capable of inducing a growth response in plants indistinguishable from the response to gibberellin treatment. At least some of the active substances in these extracts seem to differ chemically from gibberellin A_1, gibberellin A_2 and gibberellic acid as evidenced by chromatographic studies.[10] Recently Macmillan and Suter have reported the isolation of gibberellin A_1 from extracts of runner bean seed (*Phaseolus multiflorus* L.).[19] Otherwise, none of the substances responsible for the biological activity of the plant extracts has been identified.

Extracts of immature bean seen (*Phaseolus vulgaris* L.) were found to be relatively active in the bioassay for gibberellin-like substances, and the active principle of these extracts was found to be similar in chromatographic behavior to the fungal gibberellins.[10] For these reasons this source was selected for investigation of the gibberellin-like substances of flowering plants. Two crystalline compounds, called for convenience bean factor I and bean factor II,[20] have been isolated in small quantities from this source. Bean factor I is identical with gibberellin A_1 in many of its properties and very similar to it in others. However, a positive identification of this factor must await the isolation of additional material and will be reported later. Bean factor II has biological, physical and chemical properties which distinguish it from the other reported gibberellins; its isolation and properties are the subject of this paper.

The procedure employed for the isolation of bean factor II involved a number of purification steps. The gibberellin-like substances were detected throughout by means of a bioassay based on the growth response of dwarf mutants of maize. Immature bean seed was extracted with a mixture of acetone and water (1:1). The active material was adsorbed on charcoal from aqueous solution and subsequently eluted with acetone in a step patterned after the isolation of gibberellic acid from fungal filtrates.[21] The eluate was chromatographed on a silicic acid: celite column developed with mixtures of ethyl acetate and chloroform. Assay of the fractions from this column on different maize mutants, *dwarf-1* (*d-1*) and *dwarf-5* (*d-5*) revealed that a group of the early fractions was relatively active in stimulating *d-5* growth but only slightly active for *d-1*, whereas a later group of fractions was relatively active for both mutants. The differential activity of the early fractions was unique since the fungal gibberellins and the gibberellin-like substances from flowering plants tested previously had been found to have approximately equal activity for both mutants. Thus, there were at least two different gibberellin-like substances present in the bean extract, and one of these differed from the fungal gibberellins in its biological properties. Subsequent purification steps, which were carried out separately for the two groups of fractions, included chromatography on a charcoal: celite column developed with mixtures of acetone and water and a countercurrent distribution between the two phases of a *n*-butyl alcohol and aqueous ammonia buffer (*p*H 8) mixture. Approximately two milligrams of bean factor I, which was equally active on *d-1* and *d-5* and approximately two mg. of bean factor II, which was at least ten times more active for *d-5* than *d-1*, were crystallized from the material recovered from the countercurrent distributions.

A comparison of the properties of bean factor II and those of the fungal gibberellins suggests that they possess many structural features in common. Figure 1 illustrates the many similarities in the infrared absorption spectra for bean factor II and gibberellic acid but also shows that distinct differences do exist. Table I summarizes the absorption maxima in the frequency range 1700 to 1800 cm.$^{-1}$ for gibberellic acid, gibberellin A_1, and bean factor II and the methyl esters of these compounds. The positions of the maxima are consistent with the presence of a carboxylic acid

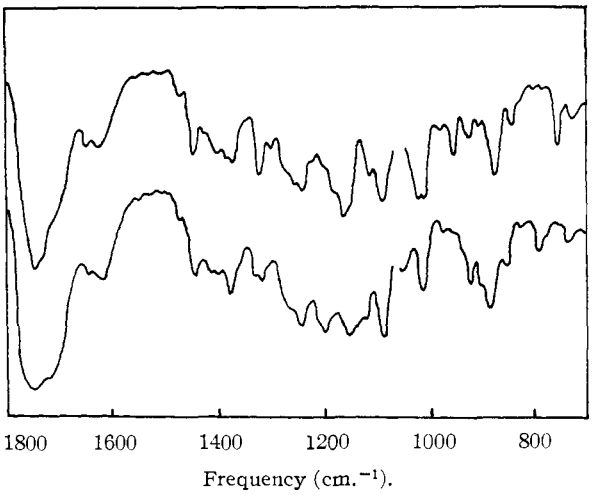


Fig. 1.—Infrared spectra of gibberellic acid (upper curve) and bean factor II (lower curve). Samples were prepared by pressing approximately 300 μgrams of the compound and 50 mg. of potassium bromide into a small pellet. Spectra were obtained on a Model 21, Perkin-Elmer recording infrared spectrophotometer.

(9) J. F. Grove, P. W. Jeffs and T. P. C. Mulholland, *J. Chem. Soc.*, 1236 (1958).

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(18) L. G. Nickell, *Science*, **128**, 88 (1958).

(19) J. MacMillan and R. J. Suter, *Naturwiss.*, **45**, 46 (1958).

(20) The problem of a suitable nomenclature for newly discovered members of this class of plant growth regulators is under discussion with other workers in the field.

(21) A. Borrow, P. W. Brian, V. E. Chester, P. J. Curtis, H. G. Hemming, C. Henehan, E. G. Jeffreys, P. B. Lloyd, I. S. Nixon, G. L. F. Norris and M. Radley, *J. Sci. Food Agr.*, **6**, 340 (1955).

TABLE I

INFRARED ABSORPTION MAXIMA, 1700–1800 CM.$^{-1}$

Compound	Supporting medium	Absorption maxima
Gibberellic acid	KBr	1745 (broad)
		1720 (shoulder)
Gibberellin A_1	KBr	1740
		1717
Bean factor II	KBr	1750 (broad)
		1717 (shoulder)
Gibberellic acid methyl ester	KBr	1765
		1730
Gibberellin A_1 methyl ester	$CHCl_3$	1765
		1725
Bean factor II methyl ester	$CHCl_3$	1760
		1725

and a γ-lactone in bean factor II of types which are assigned to the fungal gibberellins.

Other properties determined for bean factor II and the fungal gibberellins are collected in Table II. Hydrogenation studies carried out on 100 to 200 microgram samples in a Warburg–Bancroft respirometer with palladium on powdered charcoal as catalyst suggest that bean factor II and gibberellic acid possess the same degree of unsaturation; however, these results should be considered tentative because of the sample size and the lack of material for replication. The infrared spectra of both bean factor II and of gibberellic acid show two weak absorption maxima between 1600 and 1700 cm.$^{-1}$, which is again suggestive of two sites of unsaturation.

TABLE II

COMPARISON OF PROPERTIES OF FUNGAL GIBBERELLINS AND BEAN FACTOR II

	Gibb. acid	Gibb. A_1	Gibb. A_2	Bean fact. II
Paper chromatography[a] (R_{Ga}[b])				
n-Butyl alcohol–ammonia[c]	(1.0)	1.0	1.2	2.0
Pyridine–amyl alcohol–water[d]	(1.0)	1.0	1.1	1.2
Relative activity on maize mutants				
d-1	(100)	20	4	5
d-5	(100)	20	1	90
Fluorescence in H_2SO_4	Blue-green	None	None	None

[a] According to the procedure described.[10] [b] Ratio of the distance from the origin the compound had migrated to the distance a gibberellic acid control sample had migrated on the same chromatogram. Gibberellic acid has an average R_f of 0.25 in the butyl alcohol–ammonia system and 0.58 in the pyridine–amyl alcohol–water system. [c] Upper phase of a mixture of *n*-butyl alcohol and 1.5 *M* ammonium hydroxide (3:1). [d] Upper phase of a mixture of pyridine–*n*-amyl alcohol–water (35:35:30).

The failure of sulfuric acid solutions of bean factor II to show the characteristic blue-green fluorescence seen with gibberellic acid indicates the absence of the unsaturated ring substituted with hydroxyl and lactone functional groups of the type attributed to ring A of gibberellic acid. Gibberellin A_1, which differs from gibberellic acid in that ring A is saturated, does not fluoresce appreciably when dissolved in concentrated sulfuric acid. A methanolic solution of bean factor II showed only a slight end absorption and no absorption maximum above 220 mμ. Thus, unsaturated sites in conjugation do not appear to be present.

The differential biological activity of bean factor II for the single-gene dwarf mutants of maize which respond to treatment with the fungal gibberellins may have implications concerning the biosynthetic pathway leading to the production of native gibberellins in maize. One interpretation of the stimulatory action of gibberellic acid and gibberellin A_1 on the growth of these mutants is that they replace a native gibberellin produced by normal plants but not by the mutants. In terms of this hypothesis bean factor II might be an intermediate which can be converted to an active gibberellin by *d-5* but not by *d-1*. Thus, the genetically controlled enzymatic block in *d-1* would be between the production of bean factor II and the active gibberellin. A more detailed presentation of the biological properties of bean factor II and their biochemical and genetical implications will be presented elsewhere.

Experimental

Bioassay for Gibberellin-like Substances.—Gibberellin activity was followed throughout the purifications by means of biological assays on two genetically different dwarf mutants of *Zea mays* L., *d*-1 and *d*-5, according to the procedure described.[10] The material to be assayed was dissolved in 0.1 ml. of water containing 0.05% Tween 80[22] and applied to the first unfolding seedling leaf. After four or five days the lengths of the first and second leaf sheaths were measured. An increase in the length of either of these sheaths of 25% or more over the largest untreated dwarf control was interpreted as a positive test for gibberellin-like substances. A quantitative assay was used to determine the relative activities on the different mutants.[23] Known weights of each compound were applied to at least ten plants at each of three concentration levels. After seven days the lengths of the leaf sheaths of treated plants were compared statistically with non-treated dwarf controls.

Isolation of Bean Factor II. A. Extraction of Bean Seed.—Approximately 25.2 kg. (wet weight) of immature bean seed (*Phaseolus vulgaris* L.) was soaked in 50 l. of acetone–water (1:1) for at least 12 hr. After the extract was removed by filtration, the seed was extracted twice more in a similar manner. The combined filtrates were evaporated to about 40 l. *in vacuo*.

B. Charcoal Adsorption and Elution.—The residual solution from extraction was stirred with 250 g of charcoal[24] and 250 g. of celite[25] for two hr. The mixture was filtered and the charcoal treatment was repeated on the filtrate. The combined charcoal-celite residues were washed on the funnel with 2.5 l. of water and were stirred with 5 l. of acetone-water (95:5) for two hr. After the mixture was filtered the elution of the charcoal–celite residue was repeated in a similar manner. One-millionth of the combined filtrates gave a strongly positive response in both the *d-5* and *d-1* bioassays. The volume of the combined acetone-water filtrates was reduced to 4.3 l. *in vacuo*.

C. Ethyl Acetate Extraction.—The residual charcoal–celite eluate, buffered with potassium acid phosphate (57.4 g.) and sufficient 3 *M* sodium hydroxide to adjust the *p*H to 7, was extracted with two portions of ethyl acetate (5 l.). This ethyl acetate extract, which showed only slight biological activity, was discarded. After the *p*H of the aqueous phase was adjusted to 2 with hydrochloric acid (concd.), the solution was extracted with four portions of ethyl acetate

(22) Sorbitan polyoxyethylene monolaurate. Obtained from Atlas Powder Co., Wilmington, Delaware.

(23) P. M. Neely and B. O. Phinney, *Plant Physiol.* (Suppl.), **32**, xxxi (1957).

(24) Darco G 60 Vegetable Charcoal obtained from the Matheson Co., Inc., East Rutherford, New Jersey.

(25) Hyflo Super-Cel obtained from Johns Manville Corp.

(16 l.). Only a small amount of the biologically active material remained in the aqueous phase. The ethyl acetate extract, which was very active in the bioassay, was evaporated to dryness *in vacuo*.

D. Silicic Acid Chromatography.—A mixture of 133 g. of silicic acid[26] and 67 g. of celite was tamped dry into a column 3 × 90 cm. which was washed with successive portions of ethyl ether (75 ml.), ethyl ether–acetone (75 ml.), ethyl ether (50 ml.) and chloroform (100 ml.). The residue from the ethyl acetate extract at *p*H 2 was adsorbed on 15 g. of silicic acid–celite (2:1), and the mixture transferred to the column. The column was developed with chloroform (1 l.), increasing concentrations of from 5 to 28% ethyl acetate in chloroform (2.2 l.), 30% ethyl acetate in chloroform (10.4 l.) and ethyl acetate (1 l.). Seventy-eight 200-ml. fractions were collected.

Evaporation of fractions 17–24 yielded 0.87 g. of a brown oil which was highly active in the bioassay for *d-5* but only slightly active for *d-1*. The active material in this fraction is referred to as bean factor II.

Evaporation of fractions 25–53 yielded 1.14 g. of a brown oil which was highly active in the bioassay for both mutants, *d-1* and *d-5*. The active material in this fraction is referred to as bean factor I. Fractions 54–73 also contained small quantities of bean factor I.

E. Charcoal Chromatography.—Sixty-seven g. of charcoal and 133 g. of celite were slurried in water, poured into a column 4.5 cm. in diameter, packed under a small pressure head of nitrogen to a height of 26 cm. and washed with water. The bean factor II fraction (0.87 g.) from silicic acid chromatography was added in 15 ml. of acetone–water (1:1) to the column which was then developed with 50% acetone in water (1 l.), increasing concentrations of from 60 to 90% acetone in water (0.8 l.) and acetone (1.4 l.). Thirty 100-ml. fractions were collected. Evaporation of fractions 16–23 yielded 0.07 g. of a pale yellow oil containing bean factor II.

F. Countercurrent Distribution.—The fraction from charcoal chromatography containing bean factor II (0.07 g.) was distributed between 10 ml. of the upper phase and 10 ml. of the lower phase of a two phase system resulting from the equilibration of equal volumes of *n*-butyl alcohol and 0.02 *M* ammonium hydroxide–ammonium chloride buffer (*p*H 8). These solutions were introduced into tube O of a countercurrent distribution machine,[27] and ninety-nine transfers were effected in this solvent system. The contents of tubes 16–40, which showed activity in the *d-5* bioassay, were acidified to *p*H 2 with hydrochloric acid (concd.), and the phases were separated. The lower phase was re-extracted with several portions of *n*-butyl alcohol. Evaporation of the combined *n*-butyl alcohol extracts yielded 33 mg. of an almost colorless oil.

G. Isolation of Crystalline Bean Factor II.—The bean factor II fraction (33 mg.) from countercurrent distribution was dissolved in acetone–water (95:5), and the solution was passed over a small charcoal column. The residue (15 mg.) obtained on evaporating the column effluent was extracted with warm ethyl acetate, and the solution filtered. The volume of the filtrate was reduced to 1 ml. in a stream of nitrogen and 1 ml. of petroleum ether was added.[28] A flocculent precipitate separated on standing. It was removed and petroleum ether was again added to incipient turbidity. On standing and cooling a crystalline precipitate separated. Two milligrams of bean factor II was recovered as platelets which melted with decomposition from 250–255°.

Methylation.—N-Nitrosomethylurea (25 mg.) dissolved in ethyl ether was treated with 1 ml. of 50% potassium hydroxide, and the diazomethane generated was distilled into ethyl ether according to a procedure described.[29] Approximately 0.5 mg. of bean factor II was triturated with the diazomethane solution. The solvent and excess diazomethane were removed by evaporation. The solid residues mixed with a small quantity of Florisil[30] were slurried in petroleum ether[28] and added to a Florisil column (1 × 5 cm.). The column was developed with 25% ethyl ether (10 ml.), 75% ethyl ether in petroleum ether (10 ml.), ethyl ether (60 ml.), and 5% ethanol in ethyl ether (60 ml.). The ethyl ether eluate and the first 20 ml. of the 5% ethanol in ethyl ether eluate were combined and evaporated to dryness. The oily residue was dissolved in 10 microliters of chloroform and transferred to a microcell for the determination of infrared absorption spectrum. The methylation of gibberellin A_1 was carried out in a similar manner.

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(26) Merck reagent grade obtained from Merck and Co., Rahway, New Jersey.

(27) Model 2-B, H. O. Post Scientific Instrument Co., Maspeth, N. Y.

(28) Skelly Solve B, boiling range 60–80°.

(29) "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1953, p. 165.

(30) A synthetic adsorbent obtained from Floridin Co., Tallahassee, Florida.

LOS ANGELES, CALIFORNIA

[JOINT CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WAYNE STATE UNIVERSITY AND THE INSTITUTO DE QUIMICA AGRICOLA, MINISTERIO DA AGRICULTURA, RIO DE JANEIRO]

Naturally Occurring Oxygen Heterocyclics. IV.[1] The Methylation of Pyronones[2]

BY DAVID HERBST, WALTER B. MORS, OTTO RICHARD GOTTLIEB AND CARL DJERASSI

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The contradictory reports in the literature on the diazomethane methylation of pyronones are reviewed and it is shown that such treatment of 6-methyl-2,4-pyrononе (triacetic lactone) and 6-phenyl-2,4-pyrononе leads in each case to a chromatographically separable mixture of the 2-methoxy-γ-pyrone and the 4-methoxy-α-pyrone. These results have a bearing on the structures of several naturally occurring pyrones and attention is again directed to the utility of infrared measurements in settling this point.

Recently, we have reported the isolation from different species of rosewood (*Aniba* species) of anibine (I),[3] 4-methoxyparacotoin (II)[3,4] and 5,6-dehydrokavain (III).[4] The skeletal structures were established rigorously by the course of the alkaline degradation, but the alternate isomeric

(1) Paper III, P. Crabbé, P. R. Leeming and C. Djerassi, THIS JOURNAL, **80**, 5258 (1958). The present paper should also be considered Part IV in the series "Chemistry of Rosewood." For part III see ref. 4.

(2) The work at Wayne State University was supported by grant number H-2574 from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service, while the investigations in Rio de Janeiro received financial aid from the Conselho Nacional de Pesquisas, Brazil.

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(4) O. R. Gottlieb and W. B. Mors, *J. Org. Chem.*, **24**, 17 (1959).