Antitumor Agents II: Tulipinolide, a New Germacranolide Sesquiterpene, and Costunolide. Two Cytotoxic Substances from *Liriodendron tulipifera* L.

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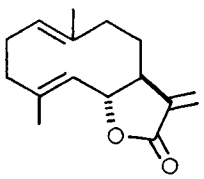
Abstract □ An alcoholic extract of the root bark of *L. tulipifera* which showed significant activity against the KB cell culture was systematically fractionated by solvent partitioning and column chromatography on silicic acid. Two active constituents, costunolide and a new germacranolide, tulipinolide, were isolated. Preliminary characterization of tulipinolide is described.

Keyphrases □ Antitumor agents—*Liriodendron tulipifera* □ Tulipinolide—isolation, identification □ Costunolide—isolation □ Column chromatography—separation □ TLC—separation □ Mass spectroscopy—identification □ NMR spectroscopy—identification □ UV spectrophotometry—identification

As a result of a random screening of botanical sources for antitumor activity, it was found that the alcoholic extract of *Liriodendron tulipifera* L. (family *Magnoliaceae*) showed a reproducible inhibitory activity against the cell culture (KB) of a human carcinoma of the nasopharynx.[1] A systematic fractionation of the extract resulted in the isolation of two cytotoxic germacranolide sesquiterpenes—the known compound costunolide (I), and a new substance which has been named tulipinolide.



I

The fractionation procedure is summarized in Fig. 1 and the cytotoxicity of the fractions is given in Table I. The powdered dried root bark of *L. tulipifera* was percolated with ethanol and the residue partitioned between chloroform and water. The active chloroform fraction was next partitioned between petroleum ether[2] and 10% aqueous methanol. Chromatography of the active 10% aqueous methanol residue on silicic acid gave, on the basis of dry weight analysis, five column fractions. The column was initially eluted with chloroform to give the first two fractions and the third and fourth fractions were obtained by eluting with 2% methanol in chloroform. The final fraction resulted when the column was washed with methanol.

The first active column fraction (F) yielded a crystalline cytotoxic substance, costunolide (I) (1) identified by the physical constants and the examination of the IR, UV, NMR, and mass spectra.[3] An authentic sample of

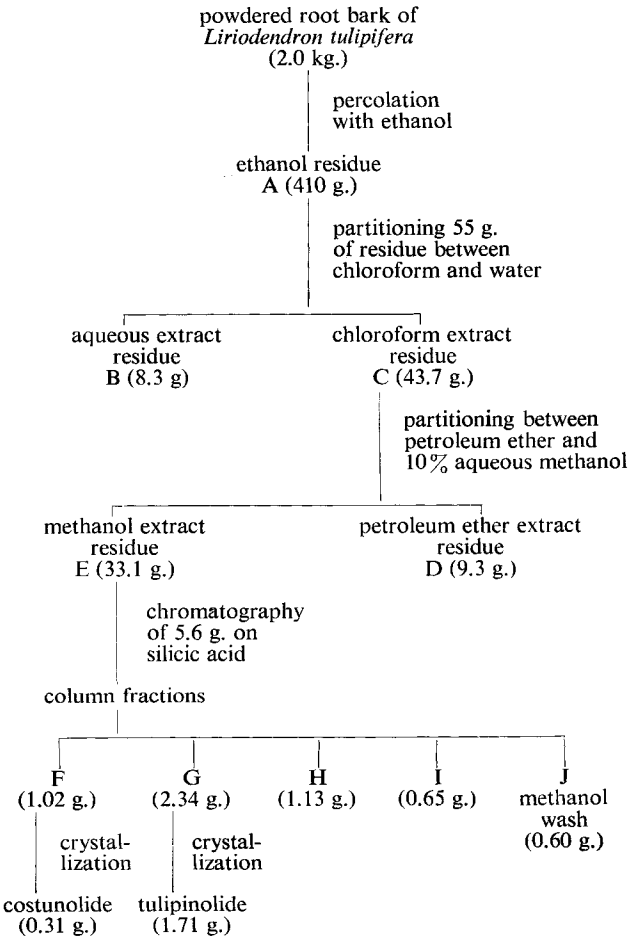


Figure 1—*Flow diagram for the fractionation of an alcoholic extract from* L. tulipifera.

[1] The KB cell culture assay was performed under the auspices of the Cancer Chemotherapy National Service Center (CCNSC) according to the procedure described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[2] Skellysolve B, Skelly Oil Co.

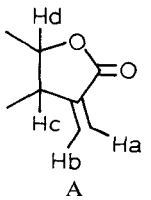
[3] The authors thank Dr. Rodger L. Foltz of Battelle Memorial Institute for the mass spectral data which was obtained on an AEI MS-9 double-focusing mass spectrometer.

costunolide was isolated from the root oil of the costus plant, *Saussurea lappa* Clarke (family *Compositae*)[4] based on the procedure given in this report for *L. tulipifera*. A direct comparison of the two compounds showed them to give the same IR spectrum, an undepressed mixture melting point, and the same mobility in the thin-layer chromatographic system. In addition, two derivatives, the dihydro- and the pyrazoline were prepared. The former gave physical constants in agreement with reported values and an IR spectrum identical to the published spectrum (1). The latter was prepared for the first time and was identical to the pyrazoline derivative obtained from authentic costunolide.

The column fraction G gave a second cytotoxic substance, tulipinolide which analyzed for $C_{17}H_{22}O_4$ (mol. wt. 290) and gave a saponification equivalent of 147 suggesting two hydrolyzable functional groups. The IR spectrum showed at least two intense but not well-resolved carbonyl peaks at $\nu_{max.}$ 1,760 and 1,742 cm.$^{-1}$, a medium strength but broad band at 1,660 cm.$^{-1}$, and a very intense and broad peak with a number of shoulders centered at 1,243 cm.$^{-1}$. When the spectrum was taken in acetonitrile the carbonyl region was clearly resolved into two peaks of almost equal intensity and eliminated the possibility of the presence of three bands. Since the IR spectrum of costunolide exhibited absorption at $\nu_{max.}$ 1,760, 1,660, 1,295, and 1,250 cm.$^{-1}$, it appeared that tulipinolide also contained an α,β'-unsaturated-γ-lactone function. The other two oxygens were ascribed to an acetate function and supported by the following evidence.

The mass spectrum of tulipinolide exhibited a base peak at *m/e* 43 (C_2H_3O), a very weak molecular ion peak at *m/e* 290 (0.014%), a somewhat stronger peak at *m/e* 248 (M-C_2H_2O, 0.32%), and the first sizeable high mass peak at *m/e* 230 (M-$C_2H_4O_2$, 5.2%)—a fragmentation pattern typical of aliphatic acetates (2). Confirmation for the acetate function was made by the isolation of acetic acid as the *p*-chlorophenacyl derivative from the hydrolysis products of tulipinolide.

The NMR spectrum of tulipinolide exhibited a pair of doublets at δ 6.34 and 5.84 (J about 3 and 1 cps.), typical for the two exocyclic methylene protons H_a and H_b of α,β'-unsaturated-γ-lactone group (3), and suggesting partial structure A. The broad multiplet cen-



A

tered at δ 3.08 (1H) was assigned to the H_c proton; a sharp singlet at δ 2.08 (3H) was designated for the acetate methyl group, and two barely split ($J \sim 1$ cps.) peaks at δ 1.71 (3H) and 1.58 (3H) were due to two olefinic methyl groups.

In the olefinic proton region and centered at δ 5.0 were present a collection of peaks that integrated for four protons. These were tentatively assigned to two vinyl protons, one proton on a carbon bearing an acetate and the H_d proton of the lactone. Costunolide showed a similar gathering of peaks at δ 4.8 but integrating for three protons.

Of the seven double-bond equivalents necessary to satisfy the molecular formula, three are accounted for by the α,β'-unsaturated-γ-lactone and one by the acetate. Two of the remaining three equivalents were considered as due to two double bonds and the last was assumed to result from a carbocyclic ring. The close similarity of the NMR spectrum of tulipinolide to costunolide served as a basis for these assignments and the data is best accommodated by a germacranolide structure.

The UV spectrum of tulipinolide consisted only of strong end absorption typical of other sesquiterpenes (3–5) possessing an unsaturated lactone and ruling out the possibility of a conjugated diene. The presence of the conjugated exocyclic methylene was confirmed by the preparation of a crystalline pyrazoline derivative (6) and by the formation of formaldehyde (isolated as the dimedone derivative) on ozonolysis. Further work on the structure of tulipinolide is in progress and will be reported at a later date.

A thin-layer chromatographic system was developed for detecting the cytotoxic substances and was of value in following the column separation. Silica Gel G was the adsorbent and isopropyl ether–chloroform (4:1) was the solvent system. Zone detection was made by exposure of the plates to iodine vapor. Costunolide and tulipinolide had R_f values of 0.53 and 0.37, respectively.

A sample of trunk bark of *L. tulipifera* L. gave an alcoholic extract that was inactive against the KB cells and when fractionated in the same manner as the root-bark extract yielded neither costunolide nor tulipinolide. It appears that the cytotoxic activity is not located throughout the entire plant but restricted to certain parts.

Table I—Cytotoxicity of Fractions from *L. tulipifera*

Fraction	ED_{50}, mcg./ml.	Fraction	ED_{50}, mcg./ml.
A	4.1	G	1.8
B	100	H	4.8
C	2.8	I	2.5
D	30	J	30
E	2.6	Costunolide	0.26
F	0.21	Tulipinolide	0.46

EXPERIMENTAL[5]

Plant Material—The root bark of *Liriodendron tulipifera* L. was collected in North Carolina in October of 1964 and 1965 and was provided through the courtesy of Dr. Robert E. Perdue, Jr., of the

[4] The authors acknowledge with thanks the sample of costus root oil from Mr. Robert J. Eiserle of Fritzsche Brothers Inc., New York, N. Y.

[5] Melting points were determined with a Thomas-Hoover Unimelt capillary melting point apparatus and were uncorrected. The IR spectra were obtained in chloroform on a Perkin-Elmer, model 237 spectrophotometer and the UV spectra were determined in ethanol on a Cary model 15 spectrophotometer. NMR spectra were recorded on a Varian A-60 instrument in deuterochloroform with tetramethylsilane as internal standard and chemical shifts reported in δ (p.p.m.) units. Optical rotations were obtained on a Zeiss-Winkel polarimeter. Silica Gel G (Merck) was obtained from Brinkmann Instruments, Westbury, N. Y. and silicic acid from Mallinckrodt Chemical Works, St. Louis, Mo. Skellysolve B is a petroleum ether fraction, b.p. 60–70°.

U. S. Dept of Agriculture, Beltsville, Md., under the agreement with the CCNSC. The trunk bark was a gift from Dr. Jonathan Hartwell of the CCNSC.

Extraction and Initial Fractionation—The powdered plant material (2.0 kg.) was extracted by percolation at room temperature with ethanol USP until the extract was almost colorless. Removal of the solvent by evaporation at reduced pressure and at 40° left a residue (A) of 410 g. A 55-g. sample of this residue was partitioned between 500 ml. of water and four 500-ml. portions of chloroform. The combined chloroform extract left a residue (C) of 43.7 g. when the solvent was removed at reduced pressure. The aqueous extract gave a residue (B) of 8.3 g. after freeze-drying. Partitioning of the active residue C between 500 ml. of 10% aqueous methanol and four 500-ml. portions of petroleum ether resulted in the activity concentrating in the aqueous methanol residue (E) which weighed 33.1 g. The petroleum ether residue (D) weighed 9.3 g.

Adsorption Chromatography of the 10% Aqueous Methanol Fraction (E)—A chromatographic column, 2.5 × 42 cm. from 100 g. of silicic acid-diatomaceous earth[6] (4:1) was poured as a slurry in chloroform. The adsorbant was activated at 120° for 1 hr. A 5.6-g. sample of the 10% aqueous methanol residue (E) dissolved in 25 ml. of chloroform was passed into the column and elution was started with chloroform. Effluent fractions of 15 ml. were taken mechanically and residue weights determined after the column holdup was discarded. The eluting solvent was changed to 2% methanol in chloroform after 43 effluent fractions were collected and an additional 47 fractions were taken. The remainder of the material on the column was removed by a wash with methanol. The column fractions were combined on the basis of the residue weight peaks giving a total of five pooled fractions, F (1.02 g.), G (2.34 g.), H (1.13 g.), I (0.65 g.), and J (0.60 g., methanol wash). The activity of the fractions is given in Table I.

Costunolide (I)—The fraction F (1.02 g.) was dissolved in about 25 ml. of hot isopropyl ether and after filtering and cooling yielded almost colorless needles, m.p. 104–105°. Recrystallization from hot absolute ethanol plus water to the point of slight turbidity gave colorless needles of costunolide (0.81 g.), m.p. 105–106°, $[\alpha]_D^{29}$ + 131° ± 1° (c, 0.69% in chloroform).

The IR spectrum possessed peaks at $\nu_{max.}$ 1,760 (α,β'-unsaturated-γ-lactone) and 1,670 cm.$^{-1}$ (olefinic double bond). The NMR spectrum showed a pair of doublets at δ 6.23 and 5.56 ($J \sim 3.5$ cps.) for the exocyclic methylene protons of an α,β'-unsaturated-γ-lactone and a collection of peaks centered at δ 4.7 for two vinyl protons and one proton on a carbon bearing the lactone ether oxygen. Two olefinic methyl groups were present at δ 1.71 and 1.42, both showing broadening and slight splitting ($J \sim 1$ cps.).

A direct comparison of the isolated substance with costunolide isolated from costus root oil showed them to give the same IR spectrum and an undepressed mixture melting point.

Anal.—Calcd. for $C_{15}H_{20}O_2$ (mol. wt. 232): C, 77.55; H, 8.68. Found: C, 77.02; H, 8.49. The molecular ion in the mass spectrum was found at *m/e* 232.

Dihydrocostunolide—Costunolide (80 mg.) isolated from *L. tulipifera* was dissolved in 14 ml. of absolute ethanol and added to a mixture of 50 mg. of 5% Pd on charcoal in 10 ml. of absolute ethanol and presaturated with hydrogen. After one equivalent of hydrogen was absorbed, the rate of hydrogen uptake decreased and the reaction was stopped. Removal of the catalyst by filtration and evaporation of the solvent left an oil (80 mg.) which was further purified by dissolving in ether and extracting with 1% aqueous sodium carbonate and water. The dried (Na_2SO_4) ether phase on evaporation of the solvent left an oil which on repeated crystallization from petroleum ether gave the crystalline dihydrocostunolide (45 mg.), m.p. 77–78°, $[\alpha]_D^{25}$ + 111° ± 1° (c, 1.87% in chloroform). The literature (1) values are m.p. 77–78°, $[\alpha]_D$ + 113.6° (c, 0.3% in chloroform). The IR spectrum was identical to the one reported for dihydrocostunolide (1).

Pyrazoline Derivative of Costunolide—Costunolide (40 mg.) from *L. tulipifera* was dissolved in 5 ml. of ether and added to a 2-ml. ethereal solution of diazomethane (20 mg.). The diazomethane was prepared from *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide[7] by the procedure of Vogel (7). The reaction solution was maintained at 5° for 3 days and the oil remaining after removal of the solvent crystallized from ether to give colorless needles, m.p. 154–155°, $[\alpha]_D^{25}$ + 516° (c, 0.6% in chloroform). The UV spectrum showed a peak at $\lambda_{max.}$ 350 mμ (ϵ 140) characteristic for the —N=N— group (8). The IR spectrum exhibited peaks at $\nu_{max.}$ 1,670 and 1,570 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{22}N_2O_2$ (mol. wt. 274): C, 70.04; H, 8.08; N, 10.21. Found: C, 69.61; H, 8.68; N, 10.49.

As expected, the same compound was obtained when costunolide from costus root oil was treated with diazomethane. Comparison was made by the IR spectrum and the mixture melting point.

Isolation of Costunolide from Costus Root Oil—The root oil (2.97 g.) of *Saussurea lappa* was partitioned between 100 ml. of 10% aqueous methanol and four 100-ml. portions of petroleum ether. The aqueous methanol fraction (1.0 g.) was chromatographed on a 50-g. silicic acid–diatomaceous earth (4:1) column (2.5 cm. diameter). Elution was with chloroform and 10-ml. effluent fractions were taken. The fractions containing R_f 0.53 material by thin-layer chromatographic analysis were combined to give 0.11 g. of a pale green oil which crystallized from alcohol and water to give costunolide (56 mg.), m.p. 105–106°, $[\alpha]_D^{26}$ + 127° ± 1° (c, 0.513% in chloroform). The literature (1) values are m.p. 106–107°, $[\alpha]_D$ + 128° (c, 0.45% in chloroform). The IR spectrum was identical to that reported for costunolide (1).

Tulipinolide—The silicic acid column fraction G was crystallized first from isopropyl ether and then from absolute ethanol to give rhomboidal plates of tulipinolide (1.71 g.), m.p. 181° (d), $[\alpha]_D^{28}$ + 260° (c, 1.0% in benzene). The IR spectrum showed major peaks at 3,015, 2,965, 2,935, 2,865, 1,760, 1,742, 1,660, 1,450, 1,380, 1,243 (very broad), 1,150, 1,050, 1,025, 998, 964, and 894 cm.$^{-1}$. The NMR spectrum exhibited a pair of doublets at δ 6.34 (1H) and 5.84 (1H) ($J \sim 3.0$ and 1.0 cps.), a collection of peaks centered at δ 5.0 (4H), a broad peak at δ 3.08 (1H), a sharp singlet at δ 2.08 (3H), two slightly split singlets at δ 1.71 (3H) and 1.58 (3H), with $J \sim 1$ cps., and an envelope of peaks centered at about δ 2.2 (6H).

Anal.—Calcd. for $C_{17}H_{22}O_4$ (M.W. 290): C, 70.34; H, 7.64. Found: C, 70.11; H, 7.59. The saponification equivalent was 147 and the mass spectrum exhibited peaks at *m/e* 290 (intensity 0.014%), 248 (0.32%), 230 (5.2%), and 43 (100%, base peak).

Alkaline Hydrolysis of Tulipinolide—Tulipinolide (0.45 g.) was added to 15 ml. of 0.54 *N* aqueous KOH and the mixture refluxed for 1 hr. The solution was filtered, cooled in ice, and neutralized with cold 1 *N* H_2SO_4 using litmus paper as the indicator. The neutral solution deposited crystalline material which was collected, washed with water, and recrystallized from 20% aqueous ethanol, m.p. 178.5–179°. It was optically inactive and the NMR spectrum indicated it was a mixture. Consequently, it was not further investigated at this time.

The filtrate from which the crystalline hydrolysis product was obtained was rendered distinctly acid with 1 *N* H_2SO_4 and steam-distilled. The distillate was made neutral with 0.1 *N* NaOH, concentrated to 1.5 ml. and treated with 0.2 g. of *p*-chlorophenacyl bromide and enough alcohol to dissolve it. After refluxing for 1 hr. on the steam bath and cooling, the *p*-chlorophenacyl derivative, 170 mg., crystallized. Recrystallization from aqueous ethanol gave a product, m.p. 70°, which showed no melting point depression when admixed with an authentic sample of *p*-chlorophenacyl acetate. The IR spectra of the two samples were superimposable.

Pyrazoline Derivative of Tulipinolide—Tulipinolide (100 mg.) was dissolved in 100 ml. of ether and treated with 5 ml. of an ethereal solution of diazomethane (400 mg.) prepared from *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide. After 1 day at 5° the solvent was evaporated and the product crystallized from ether (90 mg.), m.p. 157–158° (d), $[\alpha]_D^{29}$ + 605.7° (c, 1.04% in chloroform). The UV and the IR spectrum showed peaks at $\lambda_{max.}$ 351 mμ (ϵ 140) and $\nu_{max.}$ 1,670 and 1,570 cm.$^{-1}$, respectively.

Anal.—Calcd. for $C_{18}H_{24}N_2O_4$ (M.W. 332): C, 65.04; H, 7.28; N, 8.43. Found: C, 64.78; H, 7.23; N, 8.65.

Ozonolysis of Tulipinolide—Tulipinolide (67 mg.) was dissolved in 45 ml. of glacial acetic acid and treated with an excess of ozone. The reaction mixture was steam-distilled and the distillate collected in a flask containing 20 ml. of a cold saturated aqueous solution of dimedone. The following day the precipitated dimedone derivative (31 mg.) was collected and recrystallized from aqueous ethanol to give fine needles, m.p. 183–185°. A mixture melting point with an authentic sample of the dimedone derivative of formaldehyde was not depressed and the IR spectrum was the same for both.

Thin-layer Chromatographic Procedure—Silica Gel G plates, 250 μ thick were spotted with the samples to be analyzed and developed with the solvent system of isopropyl ether–chloroform (4:1). Detec-

[6] Celite 545, Johns-Manville Corp., New York, N. Y.
[7] Diazald, obtained from Aldrich Chemical Co., Milwaukee, Wis.

tion of the zones was made by placing the developed plates in a jar containing iodine crystals. Within 5 min. brown spots appeared. Costunolide and tulipinolide showed R_f values of 0.53 and 0.37, respectively.

REFERENCES

(1) A. S. Rao, G. R. Kelkar, and S. C. Bhattacharyya, *Tetrahedron*, **9**, 275(1960).

(2) J. H. Beynon, R. A. Saunders, and A. E. Williams, *Anal. Chem.*, **33**, 221(1961).

(3) W. Herz, P. S. Subramaniam, and T. A. Geissman, *J. Org. Chem.*, **33**, 3743(1968) and references therein.

(4) L. A. P. Anderson, W. T. deKock, K. G. R. Pachler, and C. v. d. M. Brink, *Tetrahedron*, **23**, 4153(1967).

(5) T. R. Govindachari, B. S. Joshi, and V. N. Kamat, *ibid.*, **21**, 1509(1965).

(6) P. G. Deuel and T. A. Geissman, *J. Am. Chem. Soc.*, **79**, 3778 (1957).

(7) A. I. Vogel, "A Text-book of Practical Organic Chemistry," 3rd ed., Longmans, Green & Co., New York, N. Y., 1956, p. 971.

(8) J. Hochmannova, V. Herout, and F. Sorm, *Collection Czech. Chem. Commun.*, **26**, 1826(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 18, 1968, from the *College of Pharmacy, The Ohio State University, Columbus, OH 43210*

Accepted for publication January 28, 1969.

This investigation was supported by Public Health Service Research grant CA-08133 from the National Cancer Institute.

Previous paper: R. W. Doskotch and C. D. Hufford, *J. Pharm. Sci.*, **58**, 186(1969).

Thin-Layer and Gas-Liquid Chromatography of Some Indanol Derivatives of Pharmaceutical Interest

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Abstract □ Various solvent systems and adsorbents used in the separation and identification of indanol derivatives in TLC have been reported. Quantitative estimation of the active principle in some pharmaceutical preparations by means of GLC are also included in this work.

Keyphrases □ Indanol derivatives—separation, identification □ TLC—separation, identification □ UV light—chromatographic spot visualization □ GLC—separation, identification

Some of the indanol derivatives especially 7-chlor-4-hydroxyl indan and 4-hydroxy-1,5,7-trimethyl indan have distinguished themselves because of showing excellent bactericidal, fungicidal, and amebicidal properties *in vitro* as well as *in vivo* (1–4). These compounds can be prepared by a ring cyclization (5) reaction or by substituting the corresponding indan (6). For a qualitative as well as a quantitative control of the purity of the starting materials and end products in the pharmaceutical specialties it was necessary to conduct some TLC and GLC experiments. Not much work (7, 8) has been done on indanols in comparison to the number of publications on the chromatography of phenols. It was found worthwhile to include some of the related indan derivatives in the present investigations.

EXPERIMENTAL

Thin-layer Chromatography

Adsorbents—Silica Gel G (Woelm TLC) and polyamide (Woelm TLC) were used. In either case the adsorbent was mixed with approximately 2% fluorescent indicator green before preparing the slurry. The plates can be used after drying them overnight at room temperature.

Solvent Systems—(I) Water-saturated chloroform; (II) benzene–chloroform–absolute alcohol, 4:1:1; (III) chloroform–absolute alcohol, 97:3; (IV) benzene; (V) and carbon tetrachloride.

Detection—Thin-layer plates should be dried after development and then viewed under UV light of 254 mμ. The substances show up as dark spots against a greenish fluorescent background. In case the fluorescent indicator or a UV lamp is not available, the plates should be sprayed with an aqueous potassium permanganate (1%) solution. Yellow spots against a violet-brown background indicate the position of various compounds.

Table I—Chromatographic Data for the Indanol Derivatives

Substances	R_f Values with Silica Gel G in Solvent Systems I	II	III	IV	V	R_f Values with Polyamide in Solvent System I
4-Hydroxy indan	0.31	0.84	0.78	0.25	Start	0.74
5-Hydroxy indan	0.22	0.82	0.72	0.18	Start	0.63
7-Chlor-4-hydroxy indan	0.28	0.78	0.72	0.23	Start	0.52
5,7-Dichlor-4-hydroxy indan	0.69	0.89	0.91	0.63	0.31	0.84
7-Chlor-4-hydroxy-indan-on(1)	0.60	0.91	0.94	0.44	0.08	0.90
5-Acetyl indan	0.60	0.92	0.94	0.34	0.05	Front
5-Amino indan	0.79	Front	0.94	0.83	0.38	Front
4-Hydroxy-1,5,7-trimethyl indan	0.59	0.89	0.85	0.44	0.07	0.87