since tocainide did not produce IV when treated with alkali or heat. The explanation is that a carbonyl group is present in the metabolite between the amino nitrogen and the glucuronide moiety.

A proposed reaction for metabolite formation *in vivo* is presented in Scheme II. Based on the proposed metabolite structure, the data can be explained easily. Acid or enzyme hydrolysis initially would yield the carbamic acid (V), which would then easily generate tocainide. A carbamic acid is in a constant and rapid equilibrium with an amine. A change in the pH or the carbon dioxide–bicarbonate concentration can easily shift the equilibrium. Conjugation of V with glucuronic acid produces the proposed metabolite (VI), tocainide carbamoyl *O*-β-D-glucuronide. This structure also provides a reasonable explanation for the facile conversion to IV by attack of the amide nitrogen on the carbonyl carbon with displacement of the glucuronide moiety as a leaving group. The uncharged species at pH 2.2 found in the electrophoretic studies also can be explained since the inclusion of the carbonyl group would result in a loss of basicity of the nitrogen atom.

The reaction of tocainide with carbon dioxide has ample precedent in the transport mechanism for carbon dioxide by hemoglobin; hemoglobin carbamic acid accounts for ~12% of the carbon dioxide transported by the blood of resting humans (12). Glycine and glycylglycine with pKa values of ~8.0 often have been used as models for the reaction of hemoglobin with carbon dioxide (12); tocainide has a pKa of 7.8. Among other factors, the degree to which any amine combines with carbon dioxide will depend on its pKa, the pH, and the carbon dioxide concentration (12).

In summary, a pathway for the conjugation of an amine has been proposed. It was fortunate that tocainide carbamoyl *O*-β-D-glucuronide yielded a characteristic degradation product (IV). Whether this pathway applies to amines other than tocainide can be resolved only by further research.

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Secondary Products of Itanoxone

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Received February 26, 1979, from the **P. Fabre S.A. Research Center, 17 avenue Jean Moulin, 81106 Castres, France*, and the ‡*U.E.R. of Pharmaceutical and Biological Sciences, 38240 Meylan, France.* Accepted for publication August 7, 1979.

Abstract □ Itanoxone synthesis by Friedel–Crafts reaction between itaconic anhydride and 2-chlorobiphenyl was studied. Five isomers corresponding to possible impurities were prepared and studied to perfect a reliable and practical method to detect these impurities in itanoxone.

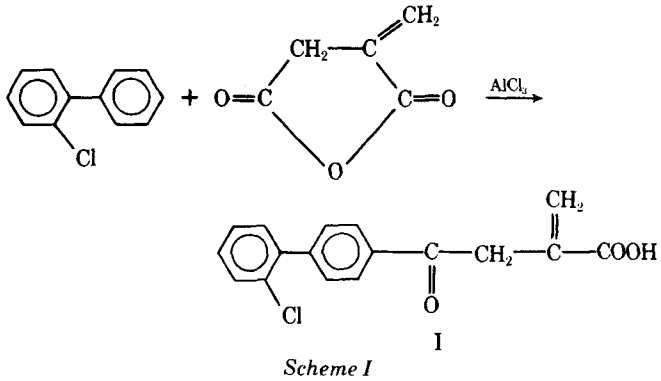
Keyphrases □ Itanoxone—synthesis, synthesis and detection of impurities □ Hypolipidemic agents—itanoxone, synthesis, synthesis and detection of impurities □ Hypouricemic agents—itanoxone, synthesis, synthesis and detection of impurities

The Friedel–Crafts reaction between itaconic anhydride and an aromatic derivative was reported (1–3). This reaction was adapted to many aromatic substrates and led to the synthesis of a chemical series with interesting pharmacological properties (4, 5). Pharmacological and toxicological studies yielded a new compound, 4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid (F 1379) (I), whose International General Designation is itanoxone. Compound I is a powerful hypolipidemic and hypouricemic agent (6–8).

The impurities that might be found in I were defined, isolated, or synthesized. With both chemical and physicochemical data of secondary products and samples, a detection method for these impurities in the end-product was investigated.

BACKGROUND

The Friedel–Crafts reaction between itaconic anhydride and 2-chlorobiphenyl gave I as the main product as reported previously (4, 5) (Scheme I).



I

Scheme I

0022-3549/80/0100-0049$01.00/0

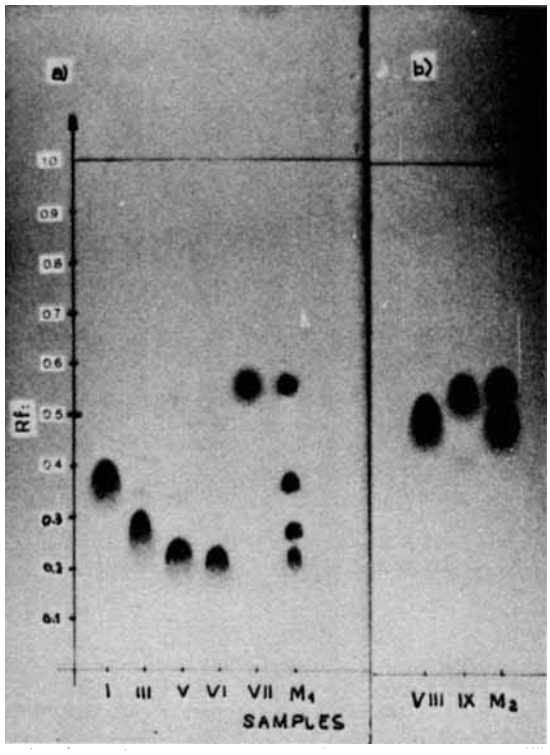
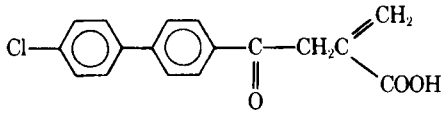


Figure 1—*Comparative thin-layer chromatograms of I, its isomers (III, V, VI, and VII), and M_1 (a mixture of I and its isomers) (a), and VIII and IX (hydrogenation products from III and I, respectively), and M_2 (a mixture of VIII and IX) (b).*



II

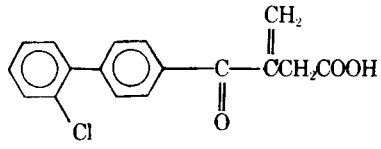
Impurities may be due to the raw material, unspecific reaction, or I degradation. The raw material, 2-chlorobiphenyl, may contain 4-chlorobiphenyl as an impurity. 2-Chlorobiphenyl, synthesized from 2-chloroaniline by a Gomberg–Bachmann–Hey reaction, must be analyzed for the 4-chlorobiphenyl impurity before itanoxone synthesis. After the Friedel–Crafts reaction with itaconic anhydride, 4-chlorobiphenyl gave another itanoxone isomer [4-[4′-(4-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid, II].

Secondary products are formed in the reaction since itaconic anhydride is asymmetric. Although the acylation is selective (1–3), it could give equally another derivative [4-[4′-(2-chlorophenyl)phenyl]-4-oxo-3-methylenebutanoic acid, III].

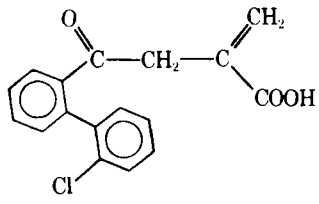
Furthermore, chlorobiphenyl acylation takes place preferentially in the *para*-position of the nonchlorinated ring, but the formation of IV is conceivable. This derivative has not been observed and was not prepared as a reference sample.

Degradation products also may be present as impurities. In I and III, the double bond can shift, as was found (1–3) with similar compounds. Shifting the double bond of I to an adjacent carbon gave a more highly conjugated isomer [4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methyl-2-butenoic acid, V].

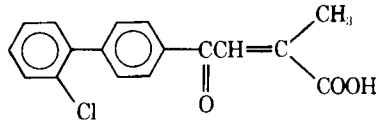
Compound V exists as two geometrical isomers, *Z* and *E*. Theoretically, the *Z* form is more stable in the cyclized form [5-[4′-(2-chlorophenyl)-phenyl]-5-hydroxy-3-methyl-2,5-dihydrofuran-2-one, VI] (1–3, 9, 10). Since VI is a lactone, V can be regarded as the *E*-isomer. Compound VI



III



IV



V

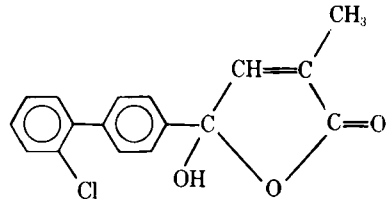
was prepared by sunlight isomerization of V (1–3, 10). Analogous reasoning applied to III led to the postulation of a derivative 5-[4′-(2-chlorophenyl)phenyl]-5-hydroxy-4-methyl-2,5-dihydrofuran-2-one (VII).

Compounds V and VII also may be obtained by Friedel–Crafts reaction of 2-chlorobiphenyl with citraconic anhydride, as reported for similar products (1–3, 10–12). This anhydride may be an impurity of itaconic anhydride, but not when the latter compound is crystallized. The isomerization of III or VII to the open *E* form is more difficult, as was shown (1, 11, 13) with similar compounds, and was not investigated. These derivatives are isomers with the formula $C_{17}H_{13}ClO_3$; their presence in I was not revealed by elemental analysis. These compounds, except IV, were synthesized.

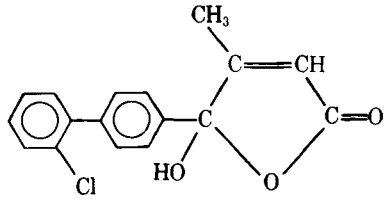
EXPERIMENTAL

General Methods—NMR spectra were recorded on a 60-MHz apparatus[1] with tetramethylsilane as an internal reference; the chemical shifts were expressed in δ relative to tetramethylsilane. IR[2] and UV[3] spectra were obtained in a double-beam spectrometer. Melting points were taken on a hot stage[4] and were not corrected. TLC utilized precoated silica gel plates[5] with chloroform–methanol (85:15); after development to 12–15 cm, the plate was exposed to 254-nm UV radiation or iodine vapor for visualization (Figs. 1*a* and 1*b*).

Synthesis of I—A solution of 47.3 g (0.25 mole) of 2-chlorobiphenyl and 28 g (0.25 mole) of itaconic anhydride (rhombic bipyramidal prisms, mp 68–69°) in 120 ml of methylene chloride was preheated to 30° and placed in a 500-ml round-bottom flask. The flask was fitted with a mechanical stirrer, a thermometer, a dropping funnel, and a reflux condenser connected to a hydrochloric acid absorber. Freshly ground anhydrous aluminum chloride (74 g, 0.55 mole) was added in portions. After boiling



VI



VII

[1] Model R-24B, Hitachi Perkin-Elmer, Hitachi Ltd., Tokyo, Japan.
[2] Model DK-2A, Beckman Instruments, GMBH, München 45, West Germany.
[3] Model 177, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England.
[4] Kofler stage, C. Reichert AG, Wein XVII, Austria.
[5] Silica gel 60 F 254 precoated plates, E. Merck, Darmstadt, West Germany.

under reflux for 3 hr, 350 ml of concentrated hydrochloric acid mixed with 200 g of crushed ice was added to the mixture.

The organic phase was separated and washed with water until the wash water was neutral. It then was poured into a mixture of 200 ml of acetone and 100 ml of 95% ethanol, which gave a first crop of crystals. These crystals were filtered off and rinsed with acetone (yield of 43.7 g). The mother liquor was evaporated to dryness, giving an oil (27 g) containing some crystals. This oil was taken up in 100 ml of 95% ethanol, and a second crop of crystals precipitated. These crystals were filtered off and washed with acetone (yield of 4.7 g). The solution (A) was retained.

The crystals were combined and purified by recrystallization from dioxane to give 46.5 g (62% yield) of I as small colorless needles, mp 212–214°; IR (potassium bromide): 1690 (COOH), 1675 (C=O), and 1610 (C=C aromatic) cm^{-1}; UV (95% ethanol): λ_{max} 267 (log ϵ 4.24) nm; NMR (dimethyl sulfoxide-d_6): δ 4.1 (s, 2H, $COCH_2$), 5.85 (d, 1H, J = 1.2 Hz, *trans*-HC=CCOOH), 6.3 (d, 1H, J = 1.2 Hz, *cis*-HC=CCOOH), 7.3–7.9 (m, 7H, COOH and aromatic protons), and 8.1 (d, 2H, J = 8 Hz, aromatic protons *ortho* to C=O) ppm; TLC: R_f 0.41.

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 67.52; H, 4.30; Cl, 11.76.

Isolation of III—Solution A was evaporated to dryness under reduced pressure, and the residue was taken up in 250 ml of ether. The ethereal solution was washed with water and then dried over anhydrous sodium sulfate. On evaporation, it gave a yellow-orange oil (22 g) containing some crystals. This oil was extracted continuously for 8 hr with petroleum ether in a soxhlet apparatus. The resulting solution contained only unreacted 2-chlorobiphenyl and a small quantity of I.

The insoluble matter was purified by recrystallization from boiling isopropyl ether and gave 1.58 g (2.1% yield) of III as colorless crystals, mp 120°; IR (potassium bromide): 1705 (COOH), 1650 (C=O), and 1630 and 1605 (C=C) cm^{-1}; UV (95% ethanol): λ_{max} 269 (log ϵ 4.15) nm; NMR (chloroform-d and dimethyl sulfoxide-d_6): δ 3.5 (s, 2H, CH_2COOH), 5.75 (d, 1H, J = 0.6 Hz, *trans*-HC=CC=O), 6 (d, 1H, J = 0.6 Hz, *cis*-HC=CC=O), 7.2–7.7 (m, 6H, aromatic protons), 7.9 (d, 2H, J = 10 Hz, aromatic protons *ortho* to carbonyl), and 9.6 (s, 1H, COOH) ppm; TLC: R_f 0.32.

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 68.02; H, 4.18; Cl, 11.74.

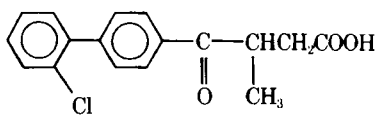
Determination of Structure of III—Compound III was hydrogenated to give a derivative [4-[4′-(2-chlorophenyl)phenyl]-4-oxo-3-methylbutanoic acid, VIII], which also could be obtained unequivocally by the action of citraconic anhydride on 2-chlorobiphenyl followed by reduction by a literature method (12).

Hydrogenation of III—A solution of 300.7 mg (10 mmoles) of III in 5 ml of ethanol was hydrogenated under normal pressure in the presence of 30 mg of 10% palladium-on-charcoal. After the catalyst was removed, the solvent was evaporated under reduced pressure, and the residue was recrystallized from boiling benzene–cyclohexane (2:1) to give 172 mg (58% yield) of colorless crystals, mp 139°; IR (potassium bromide): 1705 (COOH), 1680 (C=O), and 1605 (C=C aromatic) cm^{-1}; UV (95% ethanol): λ_{max} 266 (log ϵ 4.23) nm; NMR (dimethyl sulfoxide-d_6): δ 1.25 (d, 3H, J_{ad} = 7 Hz, $CHCH_{a3}$), 2.4 (q, 1H, J_{bd} = 6 Hz, J_{bc} = 16.5 Hz, CH_bCOOH), 2.9 (q, 1H, J_{cd} = 8 Hz, J_{cb} = 16.5 Hz, CH_cCOOH), 3.95 (m, 1H, CH_dCH_3), 7.2–7.7 (m, 6H, aromatic), 8 (d, 2H, J = 10 Hz, aromatic protons *ortho* to C=O), and 8.6 (s, 1H, COOH) ppm; TLC: R_f 0.46.

Anal.—Calc. for $C_{17}H_{15}ClO_3$: C, 67.44; H, 4.99; Cl, 11.71. Found: C, 67.64; H, 5.27; Cl, 11.61.

Preparation of VII—A suspension of 30 g (0.23 mole) of freshly ground anhydrous aluminum chloride in 80 ml of anhydrous carbon disulfide was treated dropwise with 11.2 g (0.1 mole) of citraconic anhydride. The solution was stirred for 30 min, and a solution of 18.8 g (0.1 mole) of 2-chlorobiphenyl in 30 ml of anhydrous carbon disulfide was added over 40 min. When the addition was complete, the mixture was refluxed for 2 hr.

The usual workup with hydrochloric acid and crushed ice gave 28 g of yellow-green crystals. When these crystals were recrystallized in boiling isopropyl ether, 15.5 g (52% yield) of VII was obtained as colorless crystals, mp 146°; IR (potassium bromide): 3280 (OH), 1740 (C=O lactone), and 1640 (C=C) cm^{-1}; UV (95% ethanol): λ_{max} 248 (log ϵ 4.16) nm: NMR (chloroform-d, dimethyl sulfoxide-d_6): δ 1.9 (s, 3H, CH_3), 5.7 (s, 1H, CH=CCH_3), and 7.8 (m, 9H, aromatic protons and OH) ppm; TLC: R_f 0.55.



VIII

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 67.41; H, 4.17; Cl, 11.81.

Hydrogenation of VII to VIII—An intimate mixture of 4.5 g (0.015 mole) of VII in 10 ml of acetic acid and 25 ml of water was refluxed in the presence of 3 g of zinc powder in a 100-ml round-bottom flask. After 1 hr, the mixture was filtered while still boiling. The insoluble matter was extracted several times with ethyl acetate, which also was used to extract the filtrate.

The combined organic extracts were washed with 50 ml of 5 N HCl and with water and then were dried over anhydrous sodium sulfate and evaporated. The residue was purified with boiling benzene–cyclohexane (2:1) and gave 2 g (45% yield) of VIII as colorless flakes, mp 139° (not depressed by mixing with the sample prepared by hydrogenation of III); IR (potassium bromide): 1705, 1680, and 1605 cm^{-1}; UV (95% ethanol): λ_{max} 266 (log ϵ 4.24) nm; NMR (dimethyl sulfoxide-d_6): δ 1.25, 2.4, 2.9, 3.95, 7.2–7.7, and 8 ppm; TLC: R_f 0.46.

Anal.—Calc. for $C_{17}H_{15}ClO_3$: C, 67.44; H, 4.99; Cl, 11.71. Found: C, 67.69; H, 5.09; Cl, 11.69.

Isomerization of I to V—Compound I was isomerized to V by the procedure of Lutz *et al.* (1) as adapted by Bailey and Dien (13) and Fateen *et al.* (3). Pure triethylamine (12.5 ml) was added to 5 g (16.6 mmoles) of I, suspended in 25 ml of water. The yellow solution that formed was stirred for 12 hr at room temperature. After a small quantity of insoluble matter was removed, the filtrate was diluted with 50 g of crushed ice and was acidified with 6 N HCl to pH 2–3. The yellow crystals were collected on a fritted filter, dried, and rinsed with distilled water until the wash water was neutral.

After drying, the crystals were recrystallized from 15 volumes of boiling isopropyl alcohol to give 3.5 g (70% yield) of V as yellow needles, mp 196°; IR (potassium bromide): 1690 (COOH), 1665 (C=O), 1630 and 1610 (C=C), and 975 (*trans*-CH_3C=C–H) cm^{-1}; UV (95% ethanol): λ_{max} 287 (log ϵ 4.25) nm; NMR (dimethyl sulfoxide-d_6): δ 2.15 (d, 3H, J = 1.4 Hz, CH_3) and 7.2–8.2 (m, 10H, other protons) ppm; TLC: R_f 0.28.

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 68.06; H, 4.52; Cl, 11.59.

Isomerization of V to VI—Compound VI was obtained by sunlight isomerization of V according to the procedure of Lutz *et al.* (1) as adapted by Bowden and Henry (10). Compound V (15 g, 50 mmoles) was dissolved in methanol–dioxane, and the solution was exposed to sunlight for 12 hr. After the solvents were evaporated, the residue was purified by extraction with anhydrous ether. Petroleum ether was added, and 12 g (80% yield) of VI as colorless crystals precipitated, mp 130°; IR (potassium bromide): 3400 (OH), 1730 (C=O lactone), and 1610 (C=C) cm^{-1}; UV (95% ethanol): λ_{max} 249 (log ϵ 4.12) nm; NMR (dimethyl sulfoxide-d_6): δ 2.0 (d, 3H, J = 1 Hz, CH_3), 7 (d, 1H, J = 1 Hz, =C–H), and 7.15–7.8 (m, 9H, aromatic protons and COOH) ppm; TLC: Rf 0.26.

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 67.64; H, 4.92; Cl, 11.62.

Synthesis of II—A solution of 20.6 g (0.1093 mole) of 4-chlorobiphenyl and 12.26 g (0.1093 mole) of itaconic anhydride in 70 ml of anhydrous methylene chloride was added with vigorous stirring to a suspension of 32.4 g (0.24 mole) of ground anhydrous aluminum chloride in 70 ml of anhydrous methylene chloride. After 2 hr at room temperature, the reaction mixture was refluxed for 3 hr and then left overnight at room temperature. The mixture was hydrolyzed in the usual manner. The organic phase was separated and rinsed several times by shaking with water. The solvent was removed under vacuum without heating.

The crystals obtained were rinsed with water until the wash water was neutral and were allowed to dry in the air. They finally were recrystallized from 11 volumes of boiling ethyl acetate to give 17 g (52% yield) of II as pale-beige powdery crystals, mp 192°; IR (potassium bromide): 1700 (COOH), 1675 (C=O), and 1630 and 1610 (C=C) cm^{-1}; UV (95% ethanol): λ_{max} 285 (log ϵ 4.47) nm; NMR (dimethyl sulfoxide-d_6): δ 4.05 (s, 2H, $COCH_2$), 5.75 (s, 1H, *trans*-HC=CCOOH), 6.3 (s, 1H, *cis*-HC=CCOOH), and 7.3–8.3 (m, 9H, other protons) ppm; TLC: R_f 0.38.

Anal.—Calc. for $C_{17}H_{13}ClO_3$: C, 67.89; H, 4.35; Cl, 11.78. Found: C, 67.12; H, 4.55; Cl, 11.49.

Hydrogenation of I to 4-[4′-(2-Chlorophenyl)phenyl]-4-oxo-2-methylbutanoic Acid (IX)—A semisolution of 54 g (0.179 mole) of I in 1080 ml of dioxane containing 36 ml of acetic acid was treated with 2.7 g of 10% palladium-on-charcoal in a 3-liter erlenmeyer flask connected to a hydrogen gas holder. After the apparatus was purged with nitrogen and hydrogen, the stirrer was started and hydrogenation began after induction for 40 min. The theoretical quantity of hydrogen was absorbed in 6 hr. The gas holder was disconnected, the catalyst was removed by

filtration through kieselguhr, and the filtrate was evaporated to dryness and gave beige crystals.

The solid was recrystallized from boiling alcohol, and 34.2 g (63% yield) of IX was produced as brilliant-beige needles on cooling, mp 172°. When IX was mixed with VIII, the melting point was depressed to 128–131°, and TLC gave a spot with two parts (Fig. 1*b*); IR (potassium bromide): 1700 (COOH), 1680 (C=O), and 1610 (aromatic C=C) cm^{-1}; UV (95% ethanol): λ_{max} 262.5 (log ϵ 4.21) nm; NMR (dimethyl sulfoxide-d_6): δ 1.25 (d, 3H, J = 7 Hz, $-CH_3$), 2.7–3.8 (m, 3H, $-COCH_2CHCOOH$), 7.2–7.8 (m, 7H, aromatic protons and COOH), and 8 (d, 2H, J = 8 Hz, aromatic protons *ortho* to the carbonyl) ppm; TLC: R_f 0.50.

Anal.—Calc. for $C_{17}H_{15}ClO_3$: C, 67.44; H, 4.99; Cl, 11.71. Found: C, 67.41; H, 5.10; Cl, 11.49.

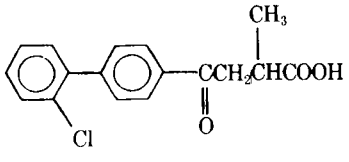
DISCUSSION

Structure of III—The results of the experimental analysis confirmed the structure of III. The IR spectrum of III compared to that of I showed a shift of the acidic carboxyl band toward the shortwave region, which indicates that the carboxyl group was attached to an sp^3 carbon. Moreover, the shift of the ketonic carbonyl band toward the longwave region suggested that this function was adjacent to a double bond. Furthermore, the UV spectrum showed the presence of an absorption maximum at 269 nm (which excluded the existence of conjugation between the carbonyl and the carboxyl groups as in V).

In comparison with I, there also was a slight bathochromic effect (λ_{max} 269 *versus* 267 nm), which indicated that IV was not present since in that case there would have been a conjugation breakdown leading to a hypsochromic effect. This observation was in agreement with the slight bathochromic effect observed previously (14) in passing from α-methylpropiophenone or α-methylbutyrophenone to α-methylenepropiophenone. Finally, the NMR spectrum gave an identical picture for the block representing the aromatic protons of I and of III, which proved that the two isomers had the *para* structure. Shielding was also noticeable for the two signals of the ethylenic protons, but this shielding was especially remarkable for the methylenic protons since the chemical shift passed from δ 4.1 for I to δ 3.5 for III. Such a shift could be explained only by chain isomerism. Its intensity made it possible to note the presence of I in III and to calculate the percentage of it by integration ($\simeq$4% in the sample obtained).

These results were confirmed by the conversion into the saturated derivative. The saturated derivative was identical to an authentic sample prepared unequivocally by chemical reduction of the hydroxylactone (VII) formed by the reaction between citraconic anhydride and 2-chlorobiphenyl. The hydrogenated derivative (VIII) differs from its isomer (IX) obtained by hydrogenating I (different IR and NMR spectra). The melting point of a mixture of VIII and IX showed a distinct depression. Upon TLC, the mixture of VIII and IX gave a stain consisting of two parts (Fig. 1*b*). Therefore, the formation of this isomer showed that, contrary to the opinion of several investigators (1–3, 13), opening of the itaconic anhydride ring by an aryl group does not give a single product but instead leads to the formation of the two isomers in unequal proportions. NMR carried out on a crude reaction mixture after hydrolysis, washing with water, and solvent evaporation showed that the ratio by weight of I to III was ~80:20.

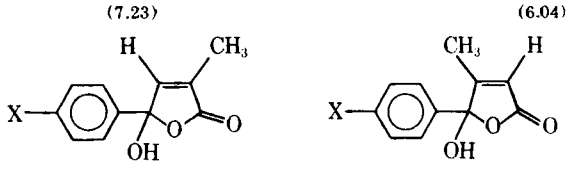
Structure of V—The method of formation of V showed that it is not easy to purify I by converting it into the water-soluble alkali metal salt followed by acidification after separating insoluble matter, unless the conditions are chosen carefully. The isomer structure was deduced from spectral analysis. The NMR spectrum agreed with a 4-aryl-4-oxo-2-methyl-2-butenoic acid. The presence of the doublet with δ 2.15, J = 1.4 Hz, was concordant with the values [δ 2.2, 2.14, and 2.18 (average)] reported (11, 12, 15) for similar aryl-substituted acids. With IR spectroscopy, two absorption bands were found at 1690 (COOH) and 1665 (C=O) cm^{-1}, which showed that the compound had an open structure. The stretching frequency observed at 975 cm^{-1} is characteristic of $=C-CH_3$ in the *trans*-$CH_3-C=C-H$ group (15, 16). As reported by various investigators (1, 3, 9–11), the *trans*-geometrical isomers of these acids are linked to the open form, the *cis*-isomer being stable only as the cyclized hydroxylactone.



IX

Structure of VII—The IR spectrum of VII showed the absence of a ketonic carbonyl group and the very strong absorption of the lactone group at 1740 cm^{-1}. In addition, the presence of a hydroxyl group was indicated by an absorption band at 3280 cm^{-1}. The UV spectrum confirmed this result by the absence of conjugation between the aromatic ring and the carboxyl group through the intermediary of the carbonyl group excited by the double bond (λ_{max} 248 nm). Finally, the NMR spectrum agreed with the values of the signals given (10, 12) by analogous compounds.

The NMR spectrum was close to that of the isomeric hydroxylactone, VI. However, the signal relating to the ethylenic proton was more depressed with VI (7.0 *versus* 5.8), which confirmed the position assigned to the methyl group in these two compounds. The values of the chemical shifts observed for the ethylenic protons in VI and VII were in good agreement with those given by Bowden and Henry (10) in a review of ring–chain tautomerism for the derivatives:



CONCLUSIONS

With the samples prepared as described and after comparative studies of R_f values, specific absorptions in the UV range, and specific NMR signals, a method was developed for detecting these impurities. The most suitable method for routine analysis is TLC on silica gel using methanol–chloroform (15:85) and developing to 15 cm. With these conditions, four isomers exhibited sufficient R_f differences to permit their detection in I (Fig. 1*a*). The respective R_f values for I, II, III, V, VI, and VII were 0.41, 0.38, 0.32, 0.28, 0.26, and 0.55. Nevertheless, considering the close R_f values for I and II, this method is not sufficiently accurate for the determination of II; in this case, GLC of 2-chlorobiphenyl provides a better control for the absence of II in I.

The most frequent impurity is V, which can be formed even when starting with pure I. Compound VI, formed with greater difficulty in the solid state, is present to a lesser extent in industrial samples. Compounds V and VI correspond to the evolution products of the I molecule, and their absence in the starting material does not exclude their detection in the finished pharmaceutical form. The other secondary products are manufacturing impurities, which must be tested for in the starting material. Their absence in the starting material is adequate to guarantee the quality of the pharmaceutical form since they cannot be formed spontaneously from I.

Systematic tests have shown that this method makes possible the detection of these main impurities in I concentrations as low as 0.2%. Levels as low as 1% of V can be detected in I by NMR using a routine 60-MHz apparatus. A standard range has been carried out in dimethyl sulfoxide-d_6 with I containing increasing quantities of V (0.5, 1, 2, 3, 5, and 7.5%); at 1%, the signal at δ 2.1 ppm clearly was distinct from the background noise. These UV or NMR physicochemical methods do not provide any advantage for detecting the impurities studied in comparison with TLC.

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Chemical Constituents of Gentianaceae XXVIII: Flavonoids of *Enicostemma hyssopifolium* (Willd.) Verd.

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Abstract □ The whole plant of *Enicostemma hyssopifolium* (Willd.) Verd. (Gentianaceae) was collected at different growth stages and was shown to contain seven flavonoids: apigenin (I), genkwanin (II), isovitexin (III), swertisin (IV), saponarin (V), 5-*O*-glucosylswertisin (VI), and 5-*O*-glucosylisoswertisin (VII). Compounds VI and VII previously were unreported in nature. The yields of the flavonoids varied with the growth stage. The biochemical and chemotaxonomic significance of these results is appraised.

Keyphrases □ Flavonoids—isolation from *Enicostemma hyssopifolium*, identification □ Medicinal plants—isolation of flavonoids from *Enicostemma hyssopifolium*, identification □ Gentianaceae—isolation of flavonoids from *Enicostemma hyssopifolium*, identification □ *Enicostemma hyssopifolium*—isolation and identification of flavonoids

The genus *Enicostemma* (Gentianaceae) is monotypic; the only recorded species is *E. hyssopifolium* (Willd.) Verd. (synonymous with *E. littorale* Blume) (1). The plant is found throughout India up to 450 m (1500 ft). Extracts of the plant are used in Indian medicine to treat cardiac dropsy, rheumatism, and certain mental disorders. Significant antipsychotic (3), anti-inflammatory (4), and anthelmintic (5) activities were reported for its major alkaloid, gentianine; the corresponding heteroside, swertiamarin, was reported to produce central nervous system depressant (6) and cardiostimulant (7) activity.

The chemicals previously reported in this species were monoterpene alkaloids (8–12), heterosides (6, 7, 10, 13, 14), and a triterpene (14). This paper describes the isolation and characterization of the flavonoids of this species. The biochemical significance of the chemical constituent changes during vegetation is appraised in light of its separation as a monotypic genus.

EXPERIMENTAL[1]

All solutions were dried with anhydrous sodium sulfate. Silica gel[2] (60–120 mesh) was used for column chromatography. TLC was performed on silica gel[3] with chloroform–acetic acid (98:2, Solvent 1) and *n*-butanol–acetic acid–water (4:1:2, Solvent 2). Iodine vapor and ferric chloride solution were used for staining. Glucose was detected by partition paper chromatography[4] using sodium metaperiodate–benzidine reagent for staining (15).

Dried and milled whole plants of *E. hyssopifolium*[5] at the flowering stage (5.2 kg) were extracted continuously with light petroleum ether (bp 60–80°) in a soxhlet apparatus for 30 hr. The defatted plant material was extracted with ethanol for 30 hr. The petroleum ether extract contained alkanes and alkanols and was not processed further. The alcoholic extract was concentrated under reduced pressure and processed as shown in Scheme I.

The relative percent yields of the individual compounds at various stages of plant development are given in Table I. Determination of the mixed minor entities was accomplished by preparative layer chromatography of mixture extracts and absorptiometry of the layers after the components in these layers were identified by the usual methods.

Treatment of Fraction A—Fraction A was dissolved in 250 ml of hot ethyl acetate, the solution was concentrated, and a yellow solid (14.2 g) was separated. The solid showed two major spots on TLC at R_f 0.68 and 0.74 (Solvent 2). The two components were separated by fractional crystallization from methanol.

Swertisin (IV)—The sparingly methanol-soluble solid crystallized from methanol–dioxane as cream-colored crystals (7.65 g), mp 240–242°; $[\alpha]_D^{24}$ −9° (c 0.33, pyridine); UV: λ_{max} (ethanol) 272 (log ϵ 4.28) and 334 (4.38) nm; IR: ν_{max} 3360, 1668, and 1610 cm^{-1}; NMR (deuterodimethyl sulfoxide): δ 7.88 (2H, d, J = 9 Hz, H-2′,6′), 6.80 (2H, d, J = 9 Hz, H-3′,5′), 6.54 (1H, s, H-8), 6.50 (1H, s, H-3), 4.78 (1H, broad, glucosyl H-1), and 3.98 (3H, methoxyl). The hexaacetate, formed with acetic anhydride and pyridine under reflux, crystallized from alcohol as colorless needles, mp 150–151°; mass spectrum: *m/e* 698 (M$^+$, relative intensity 8%), 656 (22), 639 (12), 597 (20), 537 (12), 463 (80), 421 (98), 313 (100), 297 (60), and 139 (20).

Methylation with ethereal diazomethane gave swertisin-4′,5-di-*O*-methyl ether, mp 298–300°. Acetylation of the di-*O*-methyl ether with acetic anhydride and pyridine under reflux afforded swertisin-di-*O*-methyl ether tetraacetate, mp 128–129°; mass spectrum: *m/e* 642 (M$^+$, 5%), 583 (100), 582 (22), 523 (8), 341 (55), 325 (62), 294 (5), and 139 (5). The physical and spectral properties of the parent compound and its derivatives were indistinguishable from those of swertisin (16, 17).

Isovitexin (III)—After separation of swertisin, the methanol mother liquor was concentrated and diluted with acetone. It was kept at ordinary temperature overnight, and a yellow solid precipitated. Repeated crystallization of the solid from acetone–methanol gave isovitexin (18, 19) as yellow needles (6.4 g), mp 229–230°; $[\alpha]_D^{28}$ +15.4° (c 0.37, ethanol); UV: λ_{max} (ethanol) 272 (log ϵ 4.45) and 338 (4.42) nm; IR: ν_{max} 3300 (broad), 1662, 1628, 1600, and 1588 cm^{-1}; NMR (deuterodimethyl sulfoxide): δ 7.95 (2H, d, J = 9 Hz, H-2′,6′), 6.95 (2H, d, J = 9 Hz, H-3′,5′), 6.72 (1H, s, H-3), 6.54 (1H, s, H-8), and 4.70 (1H, broad, glucosyl H-1); mass spectrum: *m/e* 414 (M − 18, 20%), 396 (8.5), 378 (14), 295 (18), 283 (100), and 165 (20). The 4′,5,7-tri-*O*-methyl ether tetraacetate, prepared as de-

[1] Melting points were taken on a Kofler block in open capillaries and are uncorrected. UV spectra were determined on a Cary model 14 recording spectrophotometer. IR spectra were taken in Nujol using a Perkin-Elmer model 337 spectrophotometer, and only the major bands are quoted. ^{1}H-NMR spectra were recorded on an XL 100 instrument with tetramethylsilane as the internal standard. Mass spectra were determined with an MS-9 or MS-50 spectrometer at 70 ev.
[2] British Drug Houses, Poole, England.
[3] Silica gel G, E. Merck, Darmstadt, West Germany.
[4] Whatman No. 50.
[5] The plants were collected from the Banaras Hindu University Campus area 12 times during June and August of 1973–1976. Voucher specimens have been preserved at the Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Banaras Hindu University.

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