[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, RUTGERS UNIVERSITY]

On the Toxin of Illicium Anisatum. I. The Isolation and Characterization of a Convulsant Principle: Anisatin[1]

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From the dried, powdered seeds and carpels of the Japanese star anise there has been isolated a pure, crystalline convulsant principle, *anisatin*, $C_{15}H_{20}O_8$. A study of the reactions of this compound and of its infrared absorption spectrum indicates that it is the anhydride of a pentahydroxydicarboxylic acid. Associated with anisatin are two physiologically inactive substances: *co-anisatin* and *ψ-anisatin* to which the tentative formulas $C_{13}H_{18}O_5$ and $C_{21}H_{32}O_8$, respectively, have been assigned.

The convulsant activity of the fruit of the Japanese star anise[2] has been known[3] for several centuries, but attempts to isolate the active principle have met with little success. In 1881, Eykman[4] reported isolating a few milligrams of impure, crystalline, toxic material ("sikimin" or "shikimin") which melted "at about 175°" but which he did not further characterize. More recently Siersch[5] reported a repetition of Eykman's work, which gave minute quantities of "shikimin," again not further characterized. Other workers[6,7] obtained only highly toxic concentrates.

The present paper describes the isolation of a pure, well-defined, crystalline substance of high toxicity for which the name *anisatin* is proposed.

By methods described in the Experimental part the convulsant principle was first isolated as the 2-propanolate, $C_{15}H_{20}O_8 \cdot {}^1/_2C_3H_8O$, $[\alpha]^{25}D$ −25° (*c* 2, ethyl acetate). The isolation was complicated by the presence of an impurity, co-anisatin, $C_{13}H_{18}O_5$, $[\alpha]^{23}D$ −24° (*c* 1, dioxane), which had approximately one-fourth the solubility of anisatin in isopropyl alcohol. In general, the progress of purification was followed in the last stages by determinations of purity according to the solubility method of Webb,[8] with isopropyl alcohol as the solvent.

Pure anisatin, $[\alpha]^{26}D$ −28° (*c* 2, dioxane), was obtained on recrystallization of the 2-propanolate from water. Recrystallization from the solvents tetrachloroethane and nitromethane gave the well-defined crystalline complexes $C_{15}H_{20}O_8 \cdot {}^1/_4C_2H_2Cl_4$ and $C_{15}H_{20}O_8 \cdot {}^1/_2CH_3NO_2$, respectively.

Anisatin is a neutral substance, sparingly soluble in water, from which solvent it may be recovered unchanged at *p*H's up to 6. It dissolves readily in dilute alkali, however, with the consumption of two moles of base. When the basic solution is acidified and extracted with ethyl acetate, there is obtained a physiologically inactive, monobasic acid, anisatinic acid, which is isomeric with anisatin. This acid, which has been characterized by preparation and analysis of its methyl ester, brucine salt and silver salt, is most probably a γ-lactonic acid. Thus, when the acid was dissolved in excess of alkali and the solution titrated rapidly with hydrochloric acid an inflection was reached *ca.* *p*H 8 which corresponded to consumption of nearly two moles of alkali. This was not a stable end-point, however, the *p*H tending to rise as the solution stood, a phenomenon consistent with the transition of the mono-sodium salt of a dibasic acid to the salt of a monobasic acid. If, on the other hand, the solution was acidified and back-titrated with base, a smooth titration curve resulted which indicated the presence of only one titratable carboxyl group. The acid further exhibits, *cf.* Fig. 1, absorption maxima in the infrared at 5.67 and 5.79 μ which are in harmony[9,10] with its description as a γ-lactonic acid.

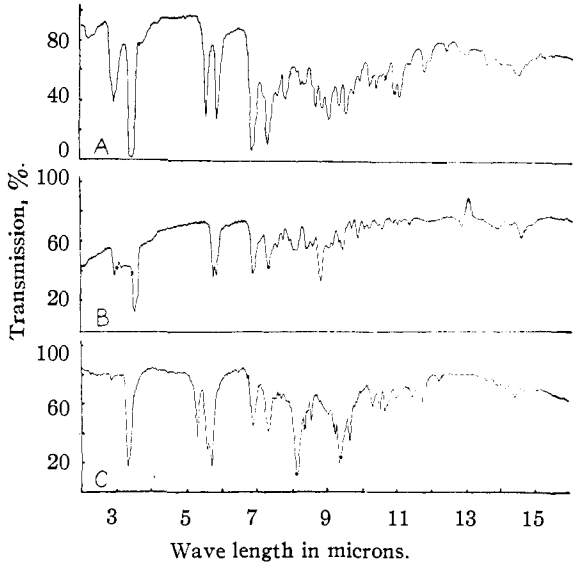


Fig. 1.—Infrared absorption curves (nujol mulls): A, anisatin (2-propanolate); B, anisatinic acid; C, anisatin triacetate.

Anisatin is quite stable in alcoholic solutions under ordinary conditions. However, prolonged treatment with anhydrous methanol in a sealed tube at 125° converted it to the methyl ester of anisatinic acid. At room temperature in a sealed tube with

(1) Presented in part at the Cleveland Meeting of the American Chemical Society, April 10, 1951.

(2) This plant, commonly known in Japan as *shikimi-no-ki* or *ashikimi* (evil fruit) and in China as *mang tsao* (mad herb), has often been termed *Illicium Religiosum* (Siebold or Zuccarini), but according to the recent taxonomical review of A. C. Smith (*Sargentia*, No. 7, 1 (1947)) should be designated *Illicium Anisatum*, Linnaeus. The material (dried seeds and carpels) used here was supplied by S. B. Penick and Co., New York, N. Y., and was collected by their agents in the Shikoku and Kyushu districts of Japan.

(3) *Cf.* S. Y. Chen, *Am. J. Pharm.*, **101**, 676 (1929), for a review of the early literature.

(4) J. F. Eykman, *Pharm. J.* and *Trans.*, **11**, 1046 (1881).

(5) E. Siersch, *Pharm. Zentralhalle*, **69**, 587, 601 (1928).

(6) K. K. Chen, *J. Am. Pharm. Assoc.*, **15**, 861 (1926).

(7) T. Q. Chou, *Chinese J. Physiol.*, **1**, 213 (1927).

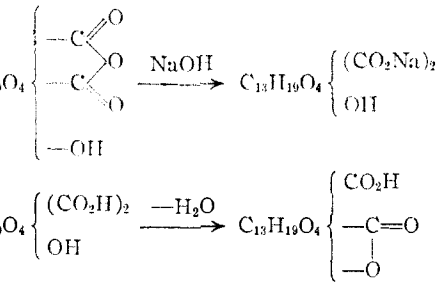
(8) T. J. Webb, *Anal. Chem.*, **20**, 100 (1948).

(9) J. F. Grove and H. A. Willis, *J. Chem. Soc.*, 877 (1951).

(10) R. S. Rasmussen and R. R. Brattain, THIS JOURNAL, **71**, 1073 (1949).

liquid ammonia it reacted slowly to give a salt from which anisatinic acid could be obtained.

This behavior suggests the presence in anisatin of a stable anhydride function and at least one hydroxyl group so placed that it can readily enter into lactone formation with one of the carboxyl groups produced by opening of the anhydride linkage: *e. g.*

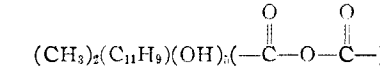


Confirmation for this hypothesis was obtained in two ways. First, the infrared absorption curves of anisatin (*cf.* Fig. 1) showed maxima in the carbonyl region at 5.51 and 5.87 μ which is characteristic of the anhydride linkage.[11] Second, on treatment with sodium methoxide in warm, anhydrous methanol, anisatin consumed one mole of sodium methoxide, behavior which is again characteristic of anhydrides,[12] but not of esters or lactones.

The relatively great stability of this linkage toward hydroxylic solvents (and even cold sodium methoxide) is somewhat surprising and suggests that it must be so situated as to present considerable steric hindrance to the approach of bases which normally cleave anhydrides. It is of interest to note that similar stability is exhibited by a number of the anhydrides of bicyclic dicarboxylic acids, *e.g.*, cantharidin,[13] camphoric anhydride[14] and apocamphoric anhydride.[15]

The remaining oxygens in anisatin appear to be hydroxylic. Thus, it forms a triacetate which gives a positive test for active hydrogen (2 moles) by the method of Zerewitinoff and which displays absorption maxima (*cf.* Fig. 1) at 2.81, 7.34 and 8.56 μ, characteristic of tertiary hydroxyl. The two non-acylable hydroxyl groups must be so located as to be singularly unreactive, for even under rigorous conditions anisatin triacetate is indifferent to the action of phosphorous oxychloride in pyridine and to mixtures of acetyl chloride and acetic anhydride.

In conclusion it is to be noted that the Kuhn–Roth C-methyl determination on (solvent-free) anisatin indicated two (1.5) such groups to be present. Accordingly, anisatin is most probably to be formulated as



This leads to the formulation of the "nucleus" as $C_{11}H_{18}$ which, since anisatin displays no unsaturation, must contain three rings. Studies on the nature of the nuclear structure are now in progress and will be reported at a later time.

(11) N. B. Colthup, *J. Optical Soc. Am.*, **40**, 397 (1950).

(12) W. M. D. Bryant and D. M. Smith, THIS JOURNAL, **58**, 2452 (1936).

(13) G. Marchiolo, *Bull. soc. chim. Farm.*, **62**, 65 (1923); *C. A.*, **17**, 2166 (1923).

(14) A. Laurent, *Ann.*, **22**, 141 (1837); O. Aschan, *Ber.*, **26**, 1639 (1893).

(15) J. E. Marsh and J. A. Gardner, *J. Chem. Soc.*, **59**, 650 (1891).

Experimental

Materials.—The trichloroethylene (b.p. 86–87°) used in the defatting experiments was a technical product obtained from the Donner and Smith Chemical Company, Newark, N.J. The ethanol and ethyl acetate for extraction were of C.P. grade and were used without further purification. The ethyl acetate used in chromatography was of reagent grade, dried over anhydrous magnesium sulfate and distilled. Chromatographic alumina (Fischer or Aluminum Co. of America, F-20), acid washed to *p*H 3 with dilute sulfuric acid and dried at 170° was used as an adsorbent. The plant material, obtained[2] from Japan, consisted of dried seeds and carpels which were ground to 30 mesh. The resulting meal was brown in color, oily to the touch and highly aromatic.

Bioassay.—The material to be assayed was dissolved (or suspended) in water, and each of a number of white mice (15–25 g.) was injected intraperitoneally with a volume of solution equal in one-hundredths of a milliliter to its weight in grams. Mice which had received dosages of the order of the MLD exhibited partial paralysis of the hind limbs shortly after injection. This was followed by generalized body trembling with convulsive seizures in from 15 to 30 minutes after injection. These seizures increased rapidly in severity and duration, death usually occurring within 90–120 minutes. Mice that survived this period, however, almost always recovered fully without seeming ill effects.

In general the order of toxicity was first roughly estimated with the aid of a few animals. Dosages over a suitable range were then administered (at intervals of about 10%) to larger groups (*e.g.*, 5 to 10) of animals, and the value of the LD 50 estimated by Behrens' method.[16] This was determined to be 1.1 ± 0.1 mg. for anisatin 2-propanolate (see below) and 1.1 ± 0.1 g. for the powdered crude drug (injected in a 2% aqueous starch suspension).

Defatting of the Crude Drug.—Seven hundred eighteen grams of crude, ground drug was stirred with 1440 ml. of trichloroethylene for three hours and filtered. The residue was washed thoroughly with 1150 ml. of fresh solvent. Evaporation of the combined solutions gave 73.6 g. (10.3%) of a green oil. This oil, suspended in 2% starch solution, caused no convulsions when injected in dosages up to 400 mg./kg. body weight.

Alcoholic Extraction of the Defatted Drug.—Twenty kg. of defatted ground drug was stirred with two successive 95-l. portions of 95% ethanol for two days and filtered. The volume of solution was reduced by distillation at atmospheric pressure to about 18 l. and allowed to stand. After several days 600 g. of precipitated, crude shikimic acid separated and was removed by filtration. On complete removal of the solvent the residue weighed 2.5 kg.; LD_{50} 250 mg.

Extraction with Ethyl Acetate.—Four hundred grams of dried alcoholic extract (LD_{50} 250 mg.), dissolved in 1200 ml. of water was neutralized to *p*H 6 with 110 g. of sodium bicarbonate. After filtration and washing the volume was 1,700 ml. This was extracted with four 1,700-ml. portions of ethyl acetate. Evaporation of the solvent gave 20 g. of a greenish gum, LD_{50} 20 mg.

Chromatographic Separation and Purification of Anisatin.—Two hundred grams of ethyl acetate extract, LD_{50} 28 mg., dissolved in 4 l. of ethyl acetate, was added to a column 100 cm. high which contained 3.5 kg. of alumina. Elution with 4,300 ml. of ethyl acetate removed 16.3 g. of material having negligible activity. An additional 2,300 ml. of solvent eluted the greater part of the activity in a fraction containing 24 g. (estimated by evaporation of an aliquot portion), LD_{50} 5.0 mg.

Evaporation of this fraction in steps yielded 8.21 g. of crystals which separated spontaneously. The rest of the solid material could be recovered by evaporating the solution almost to dryness and diluting with chloroform, but the precipitate then was amorphous. From the crystalline material there was obtained, after two recrystallizations from isopropyl alcohol, 2.9 g. of anisatin 2-propanolate which, by solubility analysis (see below), was shown to be about 80% pure.

(16) J. H. Burn, "Biological Standardization," Oxford University Press, Oxford, England, 1937, pp. 12–15.

From 2.6 g. of this material, on recrystallization from isopropyl alcohol (concn. 33 mg./g. solvent), was obtained 990 mg. of 93% pure material (Fig. 1, curve I). Recrystallization of this material (484 mg.) gave 214 mg. of 98+% pure anisatin 2-propanolate $[\alpha]^{25}D$ −25° (*c* 2, ethyl acetate), $[\alpha]^{25}D$ −27° (*c* 2, dioxane); m.p. (dec.)[17] 215–220°; solubilities in isopropyl alcohol (mg./g. of solvent): 9.9 (25°), 12.3 (30°).

Anal. Calcd. for $C_{15}H_{20}O_8 \cdot {}^1/_2C_3H_8O$: C, 55.33; H, 6.75; OC_3H_7, 8.25. Found[18]: C, 54.96; H, 6.97; OC_3H_7, 6.99[19] (average values of four analyses).

The molecular weight, determined by the micro-Rast method, was 251 by the cryoscopic method in dry acetophenone (K_f = 5.70 against benzoic acid and cholesterol), 332, by the micro-isopiestic method of Niederl,[20] 325. The calculated value for complete dissociation is 272.

Recrystallization of the 2-propanolate from water gave anisatin itself, $[\alpha]^{26}D$ −28° (*c* 2, dioxane); m.p. (dec.) 215–220°, LD_{50} *ca.* 1 mg.

Anal. Calcd. for $C_{15}H_{20}O_8$: C, 54.87; H, 6.14; mol. wt., 328. Found:[18] C, 54.96; H, 6.14; mol. wt. (Rast), 340 (average of two analyses).

To ascertain whether any decomposition could be occurring under the conditions of the Rast method, the camphor from one of the determinations was removed by sublimation at room temperature, the residue washed with ether and dried *in vacuo:* LD_{50} < 1.5 mg; m.p. 215–220° (no depression of m.p. on admixture with pure anisatin).

By recrystallization of the 2-propanolate from tetrachloroethane and from nitromethane were obtained stable crystalline solvates having m.p., toxicity and optical rotation closely resembling anisatin:

Anal. (a) Calcd. for $C_{15}H_{20}O_8 \cdot {}^1/_4C_2H_2Cl_4$: C, 50.28; H, 5.58; Cl, 9.58. Found[18]: C, 49.99; H, 5.68; Cl, 9.58 (averages of two analyses); mol. wt. (Rast), 325 (calcd. for complete dissociation 296). (b) Calcd. for $C_{15}H_{20}O_8 \cdot {}^1/_2CH_3NO_2$: C, 52.44; H, 6.04; N, 1.96. Found[18]: C, 52.14; H, 6.12; N, 2.01; mol. wt., (Rast), 261 (calcd. for complete dissociation, 239).

Anisatin showed itself to be indifferent to the routine classification tests[21] for carbonyl reagents. It also gave a negative iodoform test and could be recovered unchanged after 24 hours of treatment (in the dark) with excess of bromine in ethyl acetate.

Solubility Analysis.—Solubilities were determined by equilibrating weighed samples of material and isopropyl alcohol in sealed tubes at 25 ± 0.05°, withdrawing aliquot portions of the solution after three days and evaporating the aliquots to constant weight in tared micro-evaporating flasks purchased from the Research Glass Apparatus Co., Newark, N.J. Curve II, Fig. 2, shows a typical set of data. The solubility of the pure substance is estimated by extrapolating the sloping straight line segment to the zero point on the abscissa: 9.9 mg./g. solvent. The percentage of impurity is given by the slope of the line: 0.04, or 4% impurity. The second segment is horizontal (at a solution composition of 11.8 mg./g. of solvent) indicating that only one impurity is present, and that its solubility is 2 mg./g. solvent. Finally it may be inferred that a supersaturated solution of this sample must contain no more than 5.7 mg. of solid per g. of solvent in order for crystals of *pure* anisatin (2-propanolate) to separate from it.

Behavior of Anisatin toward Sodium Hydroxide.—Samples of anisatin (2-propanolate) of 3–10 mg. were allowed to stand in the cold for two hours (or heated for 15 minutes on the water-bath) in 0.1/ml. of water or aqueous acetone containing about 0.1 meq. of sodium hydroxide (accurately measured from a microburet). All operations were carried out under an atmosphere of nitrogen.

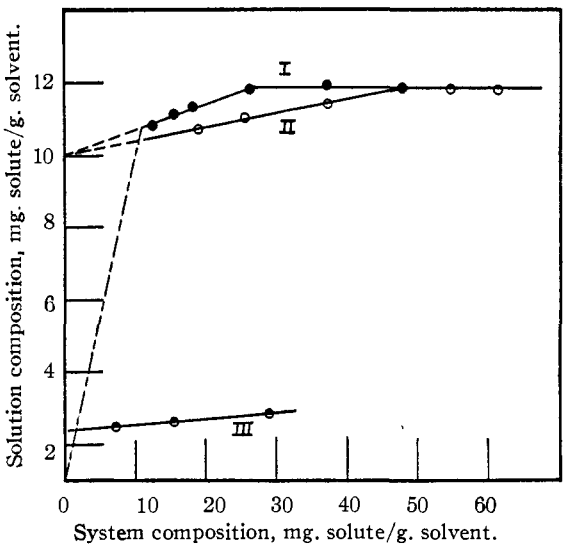


Fig. 2.—Solubility analyses in isopropyl alcohol at 25°: O, anisatin (2-propanolate), 96% pure; ●, anisatin (2-propanolate) 93% pure; ◓, co-anisatin.

Procedure A.—Phenolphthalein was added and the solution titrated with standard hydrochloric acid to the first end-point permanent for ten seconds. Great difficulty was encountered in achieving reproducibility, for the point of disappearance of the phenolphthalein color depended to a large extent on the rapidity of approach to the end-point. Moreover, this first end-point was unstable, the *p*H rising slowly as the solution was allowed to stand. The method of preparing the solution, however (*e.g.*, with or without acetone or heating), had no significant effect on the results. The average of eight determinations of the saponification equivalent by this method gave 205 ± 20 (calcd. for $(C_{15}H_{20}O_8 \cdot {}^1/_2C_3H_8O)_{1/2}$, 179).

Procedure B.—The solution was acidified with an accurately measured excess of *N* hydrochloric acid, phenolphthalein was added, and back-titration carried out with *N*/5 sodium hydroxide. Here no difficulty was encountered in determining reproducible values of the saponification equivalent: 349 ± 4 (average of five determinations); calcd. for $C_{15}H_{20}O_8 \cdot {}^1/_2C_3H_8O$, 358.

Anisatinic Acid.—A sample of anisatin 2-propanolate (459 mg., containing 10% of co-anisatin by solubility analysis) was dissolved in 12 ml. of water containing 6.10 meq. of barium hydroxide. After 14 hours 1.56 ml. of 4.10 *N* sulfuric acid was added and the barium sulfate removed by filtration. The aqueous solution was evaporated to dryness under diminished pressure below 50°. Extraction of the residue with chloroform–ethyl acetate yielded 425 mg. of solid, m.p. 210–215°. This was chromatographed on 12 g. of silicic acid (Eimer and Amend, C.P. grade) with 50% chloroform–ethyl acetate as the eluting agent. The main fraction, 384 mg., m.p. 215–219°, gave, on two recrystallizations from chloroform–ethyl acetate, 325 mg. of anisatinic acid, m.p. 218–221°, $[\alpha]^{25}D$ −46° (*c* 2, ethyl acetate). The acid was insoluble in benzene and petroleum ether, sparingly soluble in ethyl acetate, chloroform and ether and very soluble in ethanol and water.

Anal. Calcd. for $C_{15}H_{20}O_8$: C, 54.87; H, 6.14. Found[18]: C, 54.87; H, 6.32.

When the neutral equivalent was determined in the conventional fashion by titration with standard base to the phenolphthalein end-point a value of 322 ± 4 (four determinations) was obtained (calcd. for $C_{15}H_{20}O_8$ 328). A potentiometric titration gave the value 340 when the end-point was approached from the acid side. From the alkaline side, however, a value of about 210 was obtained. Estimation of the true equivalence point here was very difficult, for the *p*H of the solution was unstable and tended to rise rather rapidly as the solution stood. Therefore, the

(17) All melting points recorded in this paper were determined on the Kofler micro hot-stage.

(18) Microanalyses by J. Alicino, Squibb Institute for Medical Research, New Brunswick, N. J., and W. Manser, Zurich, Switzerland.

(19) The low (15%) value of isopropoxyl is typical of the behavior of this group in the Zeisel determination (*cf.* S. Siggia, "Quantitative Organic Analysis *via* Functional Groups," John Wiley and Sons, New York, N. Y., 1942, p. 30). In this Laboratory isopropyl benzoate was found to give a result 27% low when the boiling period was 60–90 min., 13% low when the boiling period was 120–150 min.

(20) J. B. Niederl, *et al.*, *Mikrochemie ver. Mikrochim. Acta*, **34**, 123 (1949).

(21) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1948, pp. 97, 98, 101, 106, 116.

value 210 represents an upper limit. Taken in conjunction with the titration data on alkaline solutions of anisatin the results indicate that the acid is a lactonic acid, formed almost immediately from the parent dibasic acid in neutral or acid solution.

The methyl ester was prepared by treating anisatinic acid (46 mg.) with an excess of ethereal diazomethane. Evaporation of the resulting solution gave a nearly quantitative yield of the ester which, after one recrystallization from ethanol, melted at 230–232°.

Anal. Calcd. for $C_{15}H_{19}O_7(OCH_3)$: C, 56.14; H, 6.48; OCH_3, 9.05. Found[18]: C, 56.20; H, 6.50; OCH_3, 8.91.

The brucine salt was prepared by adding brucine (41 mg.) to a warm solution of 31 mg. of anisatinic acid in 1.5 ml. of acetone. After 12 hours at room temperature 60 mg. of salt had precipitated, m.p. 230–234°. After two recrystallizations from absolute ethanol 29 mg. was obtained, m.p. 233–236° (sublimation at 220°).

Anal. Calcd. for $C_{15}H_{20}O_8 \cdot C_{23}H_{26}O_4N_2$: C, 63.07; H, 6.41; N, 3.88; OCH_3, 8.58. Found[18]: C, 62.69; H, 6.66; N, 3.87; OCH_3, 8.79.

The silver salt was prepared in nearly quantitative yield by treating a solution of the ammonium salt of anisatinic acid with the calculated quantity of silver nitrate. After one recrystallization from water it had a decomposition point of 250°.

Anal. Calcd. for $C_{15}H_{19}O_8Ag$: Ag, 24.80. Found: Ag, 24.92.

Action of Liquid Ammonia on Anisatin.—When anisatin (150 mg., 80% pure) was treated with liquid ammonia in a sealed tube for 40 hours at room temperature there was obtained, after evaporation of the ammonia, a water-soluble, neutral residue which liberated ammonia on warming with sodium carbonate. Acidification of the aqueous solution with hydrochloric acid, evaporation to dryness and extraction with chloroform gave 87 mg. of anisatinic acid, m.p. 215–220° (no depression of m.p. with an authentic specimen).

Action of Methanol on Anisatin.—A sample of anisatin 2-propanolate (105 mg.) was dissolved in 3 ml. of anhydrous methanol and heated in a sealed tube for 2 days at 125°. After removal of the solvent the resulting brown oil was taken up in chloroform and adsorbed on a column of silicic acid. Elution with chloroform gave 47 mg. of non-crystalline material. This was followed by elution with chloroform–ethyl acetate to give 19 g. of partially crystalline material which on recrystallization from ethanol gave 5 mg. of the methyl ester of anisatinic acid, m.p. 229–232° (no depression of m.p. on admixture with an authentic specimen of the methyl ester). Finally elution of the column with ethyl acetate gave 47 mg. of an acidic oil. This was treated with ethereal diazomethane and the resulting brown oil again chromatographed on silica as above. There was isolated an additional 8 mg. of the methyl ester, m.p. 229–232°.

Action of Sodium Methoxide on Anisatin.—The method used here was a modification of that of Bryant and Smith.[12] Samples weighing 3–8 mg. were dissolved in absolute methanol, and a measured excess of $N/4$ sodium methoxide in absolute methanol was added. The solution was heated at 50° for 30–60 minutes, then cooled and titrated with $N/10$ methanolic hydrogen chloride to the phenolphthalein endpoint. Results obtained on anisatin and some other substances were as follows:

Substances	Wt. of sample (mg.)	$NaOCH_3$ consumed (mmole)	Moles $NaOCH_3$ per mole sample
Anisatin 2-propanolate	7.00	0.0212	1.08
	4.30	.0121	1.01
Coanisatin	4.30	.0000	0.00
ψ-Anisatin	8.21	.0000	0.00
Camphoric anhydride	5.30	.0295	1.01
	4.94	.0284	1.05
Anisatinic acid	5.05	.0151	0.98

These results definitely indicate that anisatin is an anhydride. In conjunction with the studies reported below on ψ-anisatin, they suggest that this compound is a dilactone.

Action of Periodic Acid on Anisatin.—Samples of anisatin (5–12 mg.) were allowed to react with excess of periodic acid in acetic acid for varying lengths of time, after which potassium iodide was added and the liberated iodine titrated with thiosulfate.[22] The reaction was very slow. Thus, after one hour only 0.14 mole of HIO had been consumed, after 20 hours, 0.85 mole, and after 40 hours, 1.08 moles. These results indicate that anisatin contains a pair of vicinal hydroxyl groups of low reactivity (possibly *trans*).

Acetylation of Anisatin. Anisatin Diacetate.—Anisatin 2-propanolate (74.5 mg., 80% pure by solubility analysis) was treated with acetic anhydride and pyridine for six days at room temperature, followed by one hour on the steam-bath. When the solution was poured into water (2 ml.) 57 mg. of fine needles separated, which were recrystallized from chloroform–ether, m.p. 225–227°, $[\alpha]^{25}D$ −9.4° (*c* 2, ethyl acetate), $[\alpha]^{25}D$ 0.0 (*c* 1.5 chloroform).

Anal. Calcd. for $C_{15}H_{18}O_6(OAc)_2$: C, 55.33; H, 5.87; Ac, 20.77; mol. wt., 412. Found[18]: C, 55.02; H, 5.94; Ac (Kuhn), 20.91; mol. wt. (Rast), 440.

Anisatin Triacetate.—Anisatin 2-propanolate (110 mg., 85% pure by solubility analysis) was treated with acetic anhydride and pyridine for seven days at room temperature, followed by 2 hours on the steam-bath. The solution was then allowed to stand over sulfuric acid in a vacuum desiccator for two days. Ice-water was then added and the crystalline product removed by filtration, 104 mg., m.p. 230–234°. The melting point was not changed by recrystallization from chloroform–ether or by chromatography on silicic acid with 40% carbon tetrachloride–60% chloroform as the eluting agent; $[\alpha]^{25}D$ −4.4° (*c* 2, chloroform).

Anal. Calcd. for $C_{15}H_{17}O_5(OAc)_3$: C, 55.50; H, 5.77; Ac, 28.41; mol. wt., 454.4. Found[18]: C, 55.28; H, 5.84; Ac (Kuhn), 27.58; mol. wt. (Rast), 460.

The saponification equivalent, determined by adding excess of base, heating for 15 minutes and titrating with hydrochloric acid to the phenolphthalein end-point, was 87 (calcd. for $[C_{15}H_{17}O_5(OAc)_3]_{1/5}$ 91). The color of the indicator gradually returned. As this occurred more acid was added, until finally after 2 days a stable end-point was reached which corresponded to a saponification equivalent of 112 (calcd. for $[C_{15}H_{17}O_5(OAc)_3]_{1/4}$, 114). This behavior is in harmony with the formulations of anisatin as an anhydride and of anisatinic acid as a lactonic acid. The compound was completely indifferent to the action of excess periodic acid in acetic acid over a period of 52 hours at room temperature. Determination[18] of active hydrogen by the method of Zerewitinoff gave the value 0.45% (calcd. for $C_{21}H_{24}O_{11}(H)_2$, 0.44).

A sample of this triacetate was again subjected to the acetylation treatment outlined above, after which the acetyl value[18] was increased to 29.2%. The infrared absorption curve of the reacetylated material is given in Fig. 1.

In an attempt to remove the suspected tertiary hydroxyl group (or groups) samples of anisatin triacetate were subjected to the action of (i) acetyl chloride–acetic anhydride (1:1 volume ratio) for 2 hours at 50° (ii) phosphorus oxychloride–pyridine (1:2 volume ratio) for 6 hours on the steam-bath followed by two hours under reflux. The triacetate was recovered unchanged in 70% yield from the first experiment, in 85% yield from the second.

Co-anisatin.—In the course of repeated crystallizations of impure anisatin, appreciable amounts of rhombic-shaped crystals were sometimes left after all other material had dissolved. These were combined and recrystallized from isopropyl alcohol to give a pure (Fig. 2, curve III) sample having a solubility of 2.4 mg./g. of isopropyl alcohol. The substance melted with sublimation which began at 195°, the sublimate recrystallizing on the cover-glass to diamond-shaped crystals of m.p. 279–282°. Co-anisatin is a neutral substance, insoluble in water, cold isopropyl alcohol, methanol, acetone, nitromethane and acetonitrile, sparingly soluble in ethanol, ethyl acetate and dioxane, $[\alpha]^{23}D$ −24° (*c* 1, dioxane).

Anal. Calcd. for $C_{15}H_{18}O_5$: C, 61.42; H, 7.14; mol. wt., 254. Found[18]: C, 61.19; H, 7.01; mol. wt. (Rast), 249.

When the substance was treated with excess of standard alkali, by procedure A (see above) a saponification equiva-

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lent of 240 was obtained while by procedure B no detectable consumption of alkali was indicated. In another experiment co-anisatin (10 mg.) was dissolved in excess of 1 *N* sodium hydroxide, heated on the steam-bath for five minutes and allowed to stand at room temperature for 15 minutes. The solution was then acidified with hydrochloric acid and extracted with ethyl acetate. The material so obtained after recrystallization from methanol melted at 275–277° and gave no depression of the m.p. on admixture with an authentic specimen of co-anisatin.

ψ-Anisatin.—This substance was obtained from the ethyl acetate mother liquors after anisatin had crystallized from the early, more potent chromatographic fractions (see above) and was the chief constituent of the later fractions. It was dextrorotatory, $[\alpha]^{22}D$ + 59° (*c* 2, dioxane); m.p. (dec.) 200–210°. No physiological activity could be observed. Its solubility in ethyl acetate at 25° was determined to be 19.8 mg./g. of solvent. It was very soluble in isopropyl alcohol.

Anal. Calcd. for $C_{21}H_{32}O_8$: C, 61.6; H, 7.82; mol. wt., 412. Found[18]: C, 60.96; H, 7.61; mol. wt., 383 (cryoscopic, acetophenone).

When an alkaline solution of ψ-anisatin was heated for 15 minutes and the excess base titrated with alkali to the phenolphthalein end-point, a saponification equivalent of 258 was obtained. The end-point was not permanent, however, and after 2 days the quantity of base consumed corresponded to a saponification equivalent of 390. On the other hand, when excess hydrochloric acid was added to an alkaline solution which had been heated for 15 minutes and the excess acid titrated with base, a saponification equivalent of 431 was obtained. This behavior indicates that the product of hydrolysis may very well be a lactonic acid, stable in acid solution.

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N-Carboalkoxy Derivatives of Procaine

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The methods of synthesis of a series of dicarboalkoxy-bis-procaines, as well as a number of procaine derivatives of monourethans, are described. Preliminary pharmacological data indicate that the bis-compounds resemble procaine in anesthetic potency, but lack the central nervous stimulation characteristic of the latter. The mono-derivatives appear to be quite similar to procaine in their physiological behavior.

In spite of the relatively large number of dialkylaminoalkyl *p*-aminobenzoates which have been investigated for anesthetic activity, comparatively few are known in which the 4-amino group has been substituted. Certain 4-alkyl-,[3] 4-substituted alkyl[3] and 4-dialkylamino[3a,4] derivatives have been prepared. Various acyl and aroyl groups[5] have been introduced at the 4-amino nitrogen atom, and the latter has been diazotized and coupled to give azo compounds.[6] Recently Rao, Iyer and Guha[7] have condensed procaine with a number of diacid chlorides to obtain bis-derivatives. Krishnamacharlu, Iyer and Guha[8] have reported that they have substituted the amino group in procaine with urea, thiourea, cyanamide, guanidine and aminoguanidine.

Several investigators[9] have recognized the possi-

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(2) Abstracted in part from a thesis submitted by R. C. Nagler to the Graduate College of the University of Missouri, 1949, in partial fulfillment of the requirements for the degree of Master of Arts.

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