TABLE II

ALKALINE STABILITY OF GENTAMICIN, KANAMYCIN, NEOMYCIN, AND PAROMOMYCIN[a]

| Reflux time, hr. | Antibiotic activity, γ/ml. × 10³ Gentamicin | Kanamycin | Neomycin | Paromomycin |
|---|---|---|---|---|
| 0 | 5.8 | 9.5 | 6.0 | 7.8 |
| 19 | 5.7 | 11.2 | 6.5 | 8.0 |
| 25 | 5.8 | 9.6 | 6.3 | 8.5 |
| 48 | 5.7 | 10.0 | 6.3 | 7.8 |

[a] Conditions: boiling at reflux after addition of 10% by volume of 50% (w./w.) NaOH (19 *N*). Kanamycin, 10 mg./ml. as base; others, 10 mg./ml. as sulfates.

the effluent was dried to a residue and repeatedly triturated with methanol to remove salts. The regenerated antibiotic hydrochlorides were compared with the antibiotic starting materials (also as hydrochlorides) in a variety of chromatographic systems and were found to have identical $R_f$ values in each instance. Each regenerated compound and its parent were then reacetylated and chromatographed using the method of Pan and Dutcher[4] and in each case were found to be identical.

Hydrolysis experiments were carried out on each of the free antibiotics to determine if the bases were as stable as the N-acetyl derivatives used in these studies under the same reaction conditions. The high stability of the free bases under these rigorous conditions is confirmed by the maintenance of microbiological activity as summarized in Table II. These data demonstrate the stability of these antibiotics toward alkali even more dramatically than that reported by Leach, *et al.*,[5] who refluxed neomycin with excess barium hydroxide for 18 hr. with no decrease in activity.

The results obtained in the present study indicate that rigorous conditions of refluxing at high pH for long periods of time are required to cleave the acetyl groups from these antibiotic derivatives. Previous reports[6,7] on the inability to recover an active antibiotic by attempted deacetylation were probably due to inadequate hydrolytic conditions.

(4) S. C. Pan and J. O. Dutcher, *Anal. Chem.*, **28**, 836 (1956).
(5) B. E. Leach, W. H. DeVries, H. A. Nelson, W. G. Jackson, and J. S. Evans, *J. Am. Chem. Soc.*, **73**, 2797 (1951).
(6) R. L. Peck, C. E. Hoffhine, Jr., P. H. Gale, and K. Folkers, *ibid.*, **75**, 1018 (1953).
(7) J. D. Dutcher in "Neomycin: Its Nature and Practical Application," S. A. Waksman, Ed., Williams and Wilkins Co., Baltimore, Md., 1958, Chapter 7.

## 5,8-Isoquinolinediones. I. Synthesis of 5,8-Isoquinolinedione[1]

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*Received July 10, 1964*

The potential physiological activity of certain quinones, which are heterocyclic analogs of 1,4-naphthoquinone, has not been fully exploited. The biological activity of 6-(1-aziridinyl)-5,8-quinolinedione has been studied[2] and a number of substituted 5,8-quinolinediones have been reported to have physiological activity.[3-5] Although vitamin K antagonists have been the subject of several investigations,[6] no work has been done with the quinoline, quinoxaline, or isoquinoline analogs of 1,4-naphthoquinone. It was reported that 6-methyl-5,8-quinolinedione did not show any antihemorrhagic activity,[7] but no information could be found on the possible antivitamin K activity of this compound.

The recent interest in 5,8-quinolinediones led us to investigate the closely related 5,8-isoquinolinediones. The aim of our study was to explore the potential physiological activity of these compounds and also to compare the chemical reactivity of this system to that of the well-known 1,4-naphthoquinone system. Thus, we looked for a suitable route to synthesize first the unsubstituted compound, 5,8-isoquinolinedione.

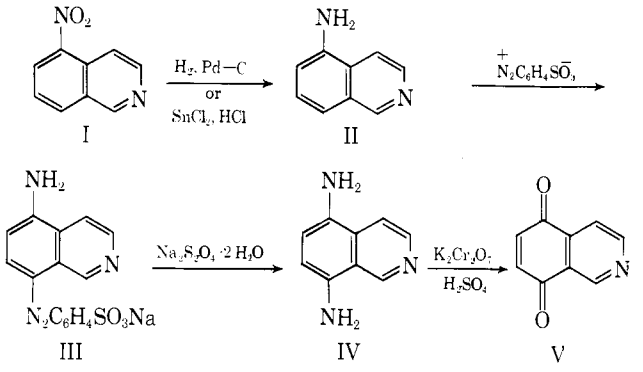
The synthesis of 5,8-isoquinolinedione by the oxidation of 5-amino-8-hydroxyisoquinoline was attempted by Fieser and Martin,[8] but the desired compound was not obtained. The only product isolated was reported to be the hydrochloride of isoquinoline-5,8-hydroquinone. The authors suggested that this compound might have resulted from a disproportionation of the quinone first formed.

More recently, the preparation of some substituted 5,8-isoquinolinediones has been reported[9] but only the picrates of these compounds were characterized.

We have attempted the preparation of 5,8-isoquinolinedione by the oxidation of the corresponding 5,8-diamine and have been able to isolate the pure quinone in good yields.

The synthesis of 5,8-isoquinolinedione was accomplished according to Scheme I. The first step of the

SCHEME I




synthesis was the nitration of isoquinoline according to known directions.[10] The pure product was reduced.[11,12]

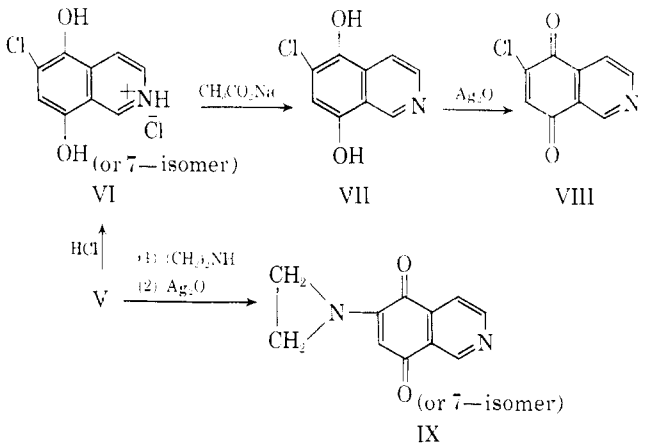
(1) This work was supported in part by a grant (AM-07684-01) from the National Institutes of Health, U. S. Public Health Service.

(2) S. Petersen, W. Gauss, and E. Urbschat, *Angew. Chem.*, **67**, 217 (1955).
(3) Farbenfab. Bayer Akt. Ges., British Patent 856,505 (1960); *Chem. Abstr.*, **55**, 12430*f* (1961).
(4) Farbenfab. Bayer Akt. Ges., British Patent 874,770 (1959); *ibid.*, **56**, 5968*g* (1962).
(5) A. Wagner, W. Beck, and A. Diskus, Austrian Patent 221,311 (1962); *ibid.*, **57**, 9823*h* (1962).
(6) A. Burger, "Medicinal Chemistry," 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1960, pp. 221–230, 650–654.
(7) A. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **46**, 421 (1941).
(8) L. F. Fieser and E. L. Martin, *J. Am. Chem. Soc.*, **57**, 1840 (1935).
(9) M. Lora-Tamayo, R. Madroñero, and M. Stud, *Chem. Ber.*, **95**, 2176 (1962).
(10) C. G. KeFévre and R. J. W. LeFévre, *J. Chem. Soc.*, 1470 (1935).
(11) J. J. Craig and W. E. Cass, *J. Am. Chem. Soc.*, **64**, 783 (1942).
(12) F. W. Bergstrom and R. E. Paterson, *J. Org. Chem.*, **10**, 481 (1945).

The other steps were similar to those used for the preparation of 6-methoxy-5,8-quinolinequinone.[13]

Isoquinoline-5,8-dione is a reasonably stable compound but its bright yellow color slowly fades and becomes brown on standing for several weeks. A solution of this quinone in water or alcohol slowly turns dark and finally deposits a black solid. This solid dissolves in acids forming a yellow solution and in alkalies forming a violet solution. This behavior suggests that this compound may be a quinhydrone.

To test the reactivity of this new quinone system, two addition reactions were tried. The addition of




anhydrous hydrogen chloride proceeded smoothly to yield a quantitative yield of the hydrochloride of a 6(or 7)-chloro-5,8-dihydroxyisoquinoline. This salt was converted to the free base which was oxidized with silver oxide to the corresponding chloroquinone. The addition of the chloride ion could occur either at the 6- or 7-positions, although the addition of hydrogen chloride to 5,8-quinolinedione has been shown to yield the 6-chloro isomer exclusively.[14]

Ethyleneimine was treated with 5,8-isoquinolinedione in the presence of silver oxide in order to oxidize directly the hydroquinone formed to the quinone. 6(or 7)-(1-Aziridinyl)-5,8-isoquinolinedione is an irritating compound.

Toxicity tests were run using 20-g. white Swiss mice (commercial source) and employing either single or multiple intraperitoneal doses of the compound dissolved in saline containing 5% v./v. ethanol. The observed single dose $LD_{50}$ was 25–35 mg./kg.; the multiple dose $LD_{50}$ for 5 daily injections was approximately 7 mg./kg. The compound showed no antitumor activity on the Gardner lymphosarcoma (C6-H3ED) and only a very slight antitumor activity on lymphocytic leukemia L1210. Its effect on Ehrlich carcinoma was a 50% extension of mean survival time at 5 mg./kg. intraperitoneally for 5 days.

Although the position of the substituents in compounds VIII and IX has not been proven with certainty, this matter will be the subject of future investigations.

## Experimental[15]

**5-Aminoisoquinoline** (II).—The reduction of 5-nitroisoquinoline[10] (I) may be carried out catalytically in a Parr hydrogenator using palladium on carbon as a catalyst, in ethanol.[11] Although this procedure may give good yields (86%), it requires a nitro compound of high purity. Reduction with stannous chloride and hydrochloric acid[12] gave more consistent results. The yield of pure product by this method was 78.5%, m.p. 128° (lit.[11] m.p. 128–129°).

**5,8-Diaminoisoquinoline** (IV).—5-Aminoisoquinoline (7.2 g., 0.05 mole) was dissolved in a mixture of 100 ml. of 1 *N* acetic acid and 100 ml. of a saturated sodium acetate solution. A slurry of diazotized sulfanilic acid[16] (12.2 g., 0.07 mole) was added to this solution at 5–10°, with stirring. After 30 min., the product was salted out with NaCl. The moist azo dye (III) was suspended in 300 ml. of water containing 8 g. of sodium hydroxide, heated to 50°, treated with solid sodium hydrosulfite (30 g.), and heated at 60° for 30 min. The solution was cooled, made alkaline, and extracted with ether. The ether solution yielded 51% of yellow, crystalline IV, m.p. 138–140°.

*Anal.* Calcd. for $C_9H_9N_3$: C, 67.91; H, 5.70; N, 26.40. Found: C, 67.68; H, 5.45; N, 26.52.

**5,8-Isoquinolinedione** (V).—5,8-Diaminoisoquinoline (5 g., 0.03 mole) was dissolved in 4 ml. of 12 *N* sulfuric acid and 200 ml. of water, then treated with a mixture of 30 ml. of 12 *N* sulfuric acid and a solution of potassium dichromate (10 g. of dichromate in 100 ml. of water). The resulting mixture was extracted continuously with chloroform, and the desired quinone precipitated by addition of petroleum ether (b.p. 30–60°) to the chloroform solution, after the chloroform solution had been reduced in volume. The yield of V was 50%. 5,8-Isoquinolinedione is a yellow, crystalline product which melts around 135–138° dec.; $\nu_{max}$ (KBr) 1659 cm.$^{-1}$ (C=O); $\lambda_{max}$ ($CH_3CN$) 325, 254, 244.5, and 237 mμ (log ε 3.52, 4.13, 4.27, and 4.28). It gives a positive Craven test.[17]

*Anal.* Calcd. for $C_9H_5NO_2$: C, 67.92; H, 3.15; N, 8.80. Found: C, 67.90; H, 3.09; N, 8.82.

**Reaction of V with Anhydrous HCl.**—5,8-Isoquinolinedione (2 g., 0.013 mole) was dissolved in 200 ml. of 1,2-dimethoxyethane and anhydrous HCl was passed into the solution for 2 hr. The reaction was exothermic and a yellow precipitate formed. This solid was collected by filtration and air-dried. A yield of 3 g. was obtained (100%). This compound melted at 190–195° dec. and was believed to be the hydrochloride of 6(or 7)-chloro-5,8-dihydroxyisoquinoline (VI). This salt was converted to the corresponding base by treatment with a saturated solution of sodium acetate. The free base separated as a violet solid and was collected by filtration and dried. It was insoluble in most solvents and did not have a definite melting point. It began to decompose at 195° and melted completely at 205° dec.

*Anal.* Calcd. for $C_9H_6ClNO_2$: C, 55.25; H, 3.07; Cl, 18.13; N, 7.16. Found: C, 55.43; H, 3.22; Cl, 18.06; N, 7.08.

6(or 7)-Chloro-5,8-dihydroxyisoquinoline (1 g., 0.005 mole) was suspended in 100 ml. of 1,2-dimethoxyethane, 1 g. of silver oxide was added, and the mixture was stirred for 2 hr. The solid was removed by filtration, the yellow filtrate was reduced to a small volume and poured into petroleum ether. The yellow crystalline solid (VIII) was collected by filtration and dried, 60% yield; m.p. 135–140° dec.; $\nu_{max}$ (KBr) 1662 cm.$^{-1}$ (C=O); $\lambda_{max}$ ($CH_3CN$) 300, 261.5, 254.5, and 242 mμ (log ε 3.64, 4.17 4.18, and 4.08). The yellow color of this compound changes slowly on standing. It gives a positive Craven test.[17]

*Anal.* Calcd. for $C_9H_4ClNO_2$: C, 55.81; H, 2.07; Cl, 18.32; N, 7.23. Found: C, 55.85; H, 2.04; Cl, 18.44; N, 7.15.

**Reaction of V with Ethyleneimine.**—5,8-Isoquinolinedione (1 g., 0.007 mole) was dissolved in 100 ml. of 1,2-dimethoxyethane. A solution of 0.8 ml. of ethyleneimine in 25 ml. of 1,2-dimethoxyethane was dropped into the stirred mixture over a period of 10 min. Stirring was continued for 30 min. more and 1 g. of silver oxide was added. Stirring was continued for 2 additional hr. at the end of which time the solid was removed by filtration, the solution was evaporated to a small volume and poured into an excess of petroleum ether. The brownish yellow solid which

(13) Y. T. Pratt and N. L. Drake, *J. Am. Chem. Soc.*, **77**, 37 (1955).
(14) Y. T. Pratt and N. L. Drake, *ibid.*, **82**, 1155 (1960).

(15) Microanalyses were performed by Dr. A. Bernhardt, Max Planck Institute, West Germany, and Galbraith Laboratories, Knoxville, Tenn. The melting points are uncorrected and were determined in a Thomas-Hoover capillary melting point apparatus. Infrared spectra were determined on a Perkin-Elmer 421 spectrophotometer. Ultraviolet spectra were measured in acetonitrile solution ($4–5 \times 10^{-5}$ *M*) on a Cary 14 spectrophotometer.

(16) L. F. Fieser, "Experiments in Organic Chemistry," 3rd Ed., D. C. Heath and Co., Boston, Mass., 1957, p. 192.

(17) R. Craven, *J. Chem. Soc.*, 1605 (1931).

formed was collected by filtration and dried to yield 0.8 g. (63.6%) of 6(or 7)-(1-aziridinyl)-5,8-isoquinolinedione (IX), m.p. 143–145°; $\nu_{max}$ (KBr) 1638 and 1670 cm.$^{-1}$ (C=O); $\lambda_{max}$ ($CH_3CN$) 318, 258, and 236 mμ (log ε 3.77, 4.16, and 4.12). This compound is irritating and should be handled with care. It gives a positive Craven test.[17]

*Anal.* Calcd. for $C_{11}H_8N_2O_2$: C, 66.00; H, 4.03; N, 13.99. Found: C, 65.94; H, 4.12; N, 13.98.

**Acknowledgment.**—The authors wish to thank Dr. Robert J. Rutman and his staff for the biological results.

## Tumor Inhibitors. IV.[1] Apocannoside and Cymarin, the Cytotoxic Principles of *Apocynum cannabinum* L.[2]

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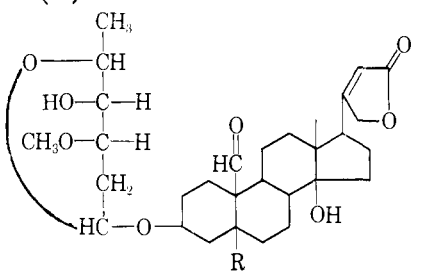
*Received July 14, 1964*

In the course of a continuing screening program for tumor inhibitors from plant sources, an alcoholic extract of the roots of *Apocynum cannabinum* L. was found to have significant inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB).[4-6] We report herein the fractionation of the active extract and the isolation and characterization of the active principles apocannoside (I) and cymarin (II).



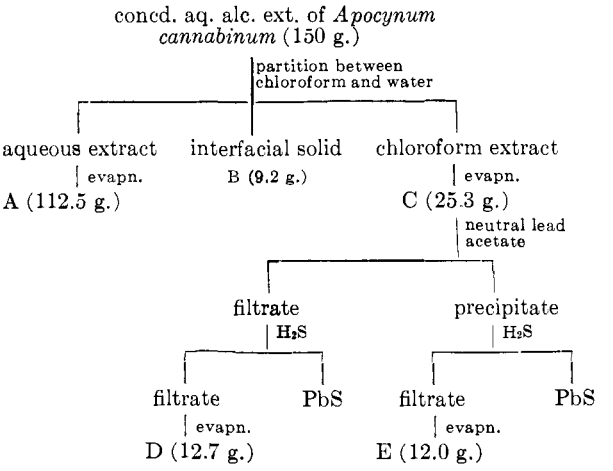
I, R = H
II, R = OH

Partition of the concentrated aqueous alcoholic extract of the roots of *A. cannabinum* between water and chloroform resulted in a concentration of the activity (Table I) in the chloroform phase (C, Scheme I). The material recovered from the chloroform layer was dissolved in methanol and treated with excess 10% methanolic neutral lead acetate solution. Removal of the precipitate by centrifugation and of the excess lead with hydrogen sulfide yielded a still more active extract (D).

TABLE I
CYTOTOXICITY OF FRACTIONS FROM *A. cannabinum*

| Fraction | $ED_{50}$, γ/ml. | Fraction | $ED_{50}$, γ/ml. |
|---|---|---|---|
| A | 3.0 | J | 45 |
| B | 1.5 | K | 26 |
| C | 0.55 | L | 0.062 |
| D | 0.6 | M | 0.021 |
| E | 30.4 | N | 0.45 |
| F | >100 | O | 5.0 |
| G | >100 | P | 0.037, 0.098 |
| H | >100 | Q | 30 |
| I | >100 | R | 0.0039, 0.016 |

SCHEME I
FRACTIONATION OF THE CYTOTOXIC PRINCIPLES OF *Apocynum cannabinum*




Further fractionation of fraction D was effected by adsorption chromatography on a silicic acid–Celite 545 column, whereby the activity was concentrated into two yellow oily fractions (L and M). Fraction L was crystallized from methanol–ether to yield apocannoside (I), characterized by comparison of the physical properties of the glycoside[7] and of its acetate[8] with reported values. Fraction M was crystallized from methanol–ether to yield cymarin (II), characterized by comparison of the physical properties of the glycoside[9] and of its aglycone, strophanthidin,[10] with reported values.

The biological testing data (Table I)[6] indicate that apocannoside and cymarin are chiefly responsible for the cytotoxicity of the extract of *A. cannabinum*. The cytotoxicity of the two glycosides is sufficiently high to warrant scheduling the compounds for testing in a variety of *in vivo* tumor systems. It is noteworthy that, although a survey of the literature has revealed

(1) Part III in the series: S. M. Kupchan, R. W. Doskotch, and P. W. Vanevenhoven, *J. Pharm. Sci.*, **53**, 343 (1964).

(2) This investigation was supported by grants from the National Cancer Institute (PHS Research Grant No. CA-04500-06) and the American Cancer Society (T-275). R. J. H. gratefully acknowledges receipt of a Wellcome Research Travel grant awarded by the Wellcome Trust.

(3) Author to whom inquiries should be addressed.

(4) Our original plant sample was gathered in Maryland in July, 1960. We acknowledge with thanks the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center.

(5) We thank Dr. Jonathan L. Hartwell of the National Cancer Institute for informing us of his independent observation of cytotoxicity toward KB cell culture of an aqueous alcoholic extract of *Apocynum cannabinum* L. ("Black Indian Hemp Root") available commercially from the Meer Corp., New York, N. Y., and for placing at our disposal a supply of this extract for fractionation studies.

(6) The evaluation of the KB assay results by the Cancer Chemotherapy National Service Center in sequential testing is such that a purified compound is considered active if the average $ED_{50}$ of two tests ≦ 4 γ/ml. and if this result is reproducible by a second screener. In the event that a compound has an $ED_{50}$ of < 1 γ/ml. in the first test, the second sequential test is omitted and it is submitted to a second screener for confirmation. The procedures were described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(7) T. Golab, C. H. Trabert, H. Jäeger, and T. Reichstein, *Helv. Chim. Acta*, **42**, 2418 (1959).

(8) C. H. Trabert, *Arzneimittel-Forsch.*, **10**, 197 (1960).

(9) W. A. Jacobs and A. Hofmann, *J. Biol. Chem.*, **67**, 609 (1926).

(10) N. K. Sen, J. K. Chakravarti, W. Kreis, C. Tamm, and T. Reichstein, *Helv. Chim. Acta*, **40**, 588 (1957).