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Phytochemical Investigation of *Adenium obesum* Forskal (Apocynaceae): Isolation and Identification of Cytotoxic Agents

JOSEPH J. HOFFMANN * and JACK R. COLE ˣ

Abstract □ An ethanol extract of *Adenium obesum* exhibited cytotoxic activity against the human epidermoid carcinoma of the nasopharynx test system. Constituents in the active extract were identified as the cardenolides somalin, honghelioside A, 16-acetylstrospeside, and honghelin and the flavonol 3,3′-bis(*O*-methyl)quercetin. Their identities were proven by IR, UV, proton magnetic resonance, and mass spectrometry; elemental analyses; preparation of derivatives; and melting-point determinations. An inactive triterpene, dihydroifflaionic acid, and an inactive flavonol, 3-*O*-methylkaempferol, also were isolated and identified.

Keyphrases □ *Adenium obesum*—ethanol extract of aboveground parts, various cardenolides and flavonol isolated and identified, evaluated for cytotoxic activity □ Cardenolides, various—isolated from ethanol extract of aboveground parts of *Adenium obesum*, evaluated for cytotoxic activity □ Flavonols—3,3′-bis(*O*-methyl)quercetin isolated from ethanol extract of aboveground parts of *Adenium obesum*, evaluated for cytotoxic activity □ Cytotoxic activity—evaluated in various cardenolides and flavonol isolated from ethanol extract of aboveground parts of *Adenium obesum*

In the continuing search for plants having antitumor properties, an ethanol extract of the stems, leaves, and flowers of *Adenium obesum* Forskal (Apocynaceae)[1] exhibited cytotoxic activity against the human epidermoid carcinoma of the nasopharynx (KB) test system[2].

DISCUSSION

The chloroform fraction, obtained from an ethanol extract of the ground stems, leaves, and flowers of *A. obesum* by partitioning between chloroform and water, was systematically fractionated by solvent extractions. The highly active ether-soluble fraction, consisting of seven major constituents, was subjected to multiple column chromatography, preparative TLC, and fractional crystallizations.

The seven compounds were identified as the triterpene dihydroifflaionic acid, the flavonols 3,3′-bis(*O*-methyl)quercetin and 3-*O*-methylkaempferol, and the cardenolides somalin, honghelioside A, 16-acetylstrospeside, and honghelin by means of IR, UV, proton magnetic resonance (PMR), and mass spectrometry; elemental analyses; preparation of derivatives; and melting-point determinations. In addition, the structures of the flavonols were substantiated by microanalytical UV spectroscopy. Somalin and honghelioside A, previously isolated from other *Adenium* species (1–5), were hydrolyzed to their respective genins and carbohydrate moieties.

Somalin, honghelin, honghelioside A, 16-acetylstrospeside, and 3,3′-bis(*O*-methyl)quercetin exhibited cytotoxic activities at levels < 0.01, 0.02, 0.035, 0.035, and 3.08 μg/ml, respectively. Dihydroifflaionic acid and 3-*O*-methylkaempferol were inactive. Activity in the KB test system is defined as $ED_{50} \leq 20$ μg/ml (6).

EXPERIMENTAL[3]

Isolation Procedure—The stems, leaves, and flowers (15 kg) of *A. obesum* were ground to a powder and extracted exhaustively in a Lloyd-type extractor with petroleum ether and then with 95% ethanol. The air-dried ethanol extract (1.2 kg) was partitioned between chloroform (10 liters) and water (10 liters). The air-dried chloroform fraction (468 g) was stirred mechanically with petroleum ether three times, the residue (324 g) was stirred mechanically with acetone three times, and the air-dried filtrate was stirred mechanically with ether three times.

The ether-soluble fraction (100 g) was subjected to a silica gel G (1.5 kg) column (7 × 81 cm) eluted with petroleum ether–ether–chloroform (1:3:1). Fractions were collected with an automatic fraction collector (25 ml/tube) and combined according to their TLC profiles. The first fractions (A) contained dihydroifflaionic acid. The next set of fractions (B) consisted of a mixture of two flavonols. The third set (C) contained somalin. The fourth set (D) consisted mainly of honghelioside A.

The fifth fraction (E, 50 g) was rechromatographed on a silica gel 60 (1.2 kg) column (6 × 70 cm) eluted with chloroform–methanol–water (188:12:1). The first fractions consisted of mainly 16-acetylstrospeside, the middle fractions contained an overlap mixture, and the latter fractions contained honghelin.

Isolation of Dihydroifflaionic Acid—The air-dried residue of Fraction A was treated with petroleum ether. The resultant precipitate was filtered, washed with petroleum ether, dissolved in chloroform–petroleum ether (1:1), and stored at 5° overnight. The resultant powder was filtered and subsequently crystallized from methanol to yield stout white crystals of dihydroifflaionic acid, mp 259–260° [lit. (7) mp 260°]; mass spectrum: *m/e* 456 (M^+) and 248 (base); IR[4]: ν_{max} 3400–3500, 1700, 1450, 1390, 1280, 1020, and 990 cm^{-1}; PMR[4]: δ 4.50 (m, 1H), 3.42 (broad

[1] Identification was confirmed by Dr. Robert E. Perdue, Chief, Medicinal Plant Resources Laboratory, U.S. Department of Agriculture, Beltsville, Md., where a reference specimen (PR 26176) is maintained. The plant was collected in the Kilifi District of Kenya by R. W. Spjut and P. D. Ensor in November 1972.

[2] Data on the cytotoxic activity were provided through the courtesy of the Divison of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md.

[3] Carbon and hydrogen analyses were performed by Chemalytics, Inc., Tempe, Ariz. PMR, IR, UV, and mass spectral data were determined using a Varian T-60 spectrophotometer, a Beckman IR-33, a Beckman DB-G spectrophotometer, and a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), respectively. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

[4] These data were not reported previously.

t, 16 Hz, 1H), 1.23 (s, 6H), 1.01 (s, 3H), 1.10 (s, 6H), 1.00 (d, J = 5 Hz, 3H), and 0.80 (s, 3H) ppm.

Anal.—Calc. for $C_{30}H_{48}O_3$: C, 78.89; H, 10.59; mol. wt. 456. Found: C, 78.65; H, 10.58; *m/e* 456 (M^+).

Acetate of Dihydroifflaionic Acid—Acetylation of dihydroifflaionic acid with acetic anhydride in pyridine at room temperature for 48 hr yielded the acetate, which crystallized directly from water, mp 189–191°; mass spectrum: *m/e* 498 (M^+) and 207 (base); IR: ν_{max} 1735 and 1245 cm^{-1}.

Methyl Ester of Dihydroifflaionic Acid—Esterification of dihydroifflaionic acid with diazomethane yielded crystals from methanol, mp 209–210° [lit. (8) mp 207–209°]; mass spectrum: *m/e* 470 (M^+) and 262 (base); IR: ν_{max} 1730 and 1130–1140 cm^{-1}.

Methyl Ester of Acetyldihydroifflaionic Acid—Esterification of acetyldihydroifflaionic acid with diazomethane yielded crystals from methanol, mp 228–230°; PMR: δ 3.60 (s, 3H), 2.03 (s, 3H), and 4.50 (broad t, 16 Hz, 1H) ppm.

Isolation of 3,3′-Bis(*O*-methyl)quercetin—A chloroform solution of Fraction B was treated with an equal volume of petroleum ether. The resultant precipitate was filtered and separated into two flavonols on preparative TLC plates developed in dichloromethane–methanol (96:4). The upper spot was recovered and purified on preparative TLC plates developed in chloroform–methanol–water (188:12:1). The recovered material was crystallized from 95% ethanol to yield yellow needles, mp 256–259° [lit. (9) mp 258–259°]; IR[4]: ν_{max} 3100–3190, 1650, 880, and 810 cm^{-1}; PMR[4]: δ 12.73 (s, 1H), 10.80 (broad s, 1H), 10.40 (broad s, 1H), 7.62 (d, J = 2 Hz, 1H), 7.56 (d, J = 7 Hz, 1H), 6.97 (dd, J = 7 and 1 Hz, 1H), 6.45 (d, J = 2 Hz, 1H), 6.20 (d, J = 2 Hz, 1H), 3.87 (s, 3H), and 3.80 (s, 3H) ppm. The UV data were consistent with the literature data (10).

Anal.—Calc. for $C_{17}H_{14}O_7$: C, 61.82; H, 4.28; mol. wt. 330. Found: C, 62.14; H, 4.61; *m/e* 330 (M^+).

3,3′-Bis(*O*-methyl)quercetin Triacetate—Acetylation of 3,3′-bis(*O*-methyl)quercetin with acetic anhydride in pyridine at room temperature for 24 hr yielded the triacetate. Crystallization from methanol yielded white needles, mp 195° [lit. (11) mp 195°]; IR: ν_{max} 1760 and 1200 cm^{-1}.

Isolation of 3-*O*-Methylkaempferol—The lower spot from the previous preparative TLC plates was purified in the same manner. The recovered material was crystallized from methanol to yield yellow platelets, mp 270–272° [lit. (12) mp 270–272°]; PMR: δ 12.73 (s, 1H), 10.80 (broad s, 1H), 10.40 (broad s, 1H), 7.95 (dd, 2H), 6.88 (dd, 2H), 6.45 (d, J = 2 Hz, 1H), 6.20 (d, J = 2 Hz, 1H), and 3.80 (s, 3H) ppm. The UV and IR data were identical to the literature data (13, 14).

Anal.—Calc. for $C_{16}H_{12}O_6$: C, 64.00; H, 4.03; mol. wt. 300. Found: C, 64.21; H, 4.61; *m/e* 300 (M^+).

3-*O*-Methylkaempferol Triacetate—Acetylation of 3-*O*-methylkaempferol with acetic anhydride in pyridine at room temperature for 24 hr yielded the triacetate. Recrystallization from petroleum ether and ethyl acetate yielded yellow needles, mp 163° [lit. (12) mp 161–163°].

Isolation of Somalin—Somalin crystallized directly from Fraction C when dissolved in 20% aqueous methanol. Recrystallization from absolute methanol yielded fine white needles, mp 197–198° [lit. (15) mp 197°]; UV: λ_{max} 220 nm (log ϵ = 4.18); IR[4]: ν_{max} 1790, 1740, and 1620 cm^{-1}; PMR[4]: δ 5.87 (1H), 4.90 (2H), 4.70 (1H), 4.00 (1H), 3.43 (3H), 1.25 (d, J = 6 Hz, 3H), 0.93 (s, 3H), and 0.87 (s, 3H) ppm.

Anal.—Calc. for $C_{30}H_{46}O_7$: C, 69.47; H, 8.94; mol. wt. 518. Found: C, 69.47; H, 9.11; *m/e* 518 (M^+).

Mild Acid Hydrolysis of Somalin—The acid hydrolysis procedure was reported previously (16). The products isolated were digitoxigenin, mp 247–250° [lit. (15) mp 250°], and D-cymarose, mp 92–94° [lit. (17) mp 93–94°].

Isolation of Honghelosides A—Fraction D (2.5 g) was purified on a silica gel G (250-g) column (4 × 42 cm) eluted with dichloromethane–methanol (9:1). Fractions containing honghelosides A were purified further on preparative TLC plates developed in benzene–methanol (9:1). Honghelosides A was then crystallized from methanol and water, mp 209–211° [lit. (18) mp 208–211°]; mass spectrum: *m/e* 576 (M^+) and 355 (base); UV: λ_{max} 220 nm (log ϵ = 4.12); IR[4]: ν_{max} 1785, 1750, 1625, and 1245 cm^{-1}; PMR[4]: δ 5.93 (1H), 5.45 (m, 18 Hz), 4.90 (2H), 4.70 (dd, J = 9 and 1.5 Hz, 1H), 4.00 (1H), 3.43 (s, 3H), 1.98 (s, 3H), 1.25 (d, J = 6 Hz, 3H), and 0.93 (s, 6H) ppm.

Anal.—Calc. for $C_{32}H_{48}O_9$: C, 66.64; H, 8.39; mol. wt. 576. Found: C, 66.63; H, 8.38; *m/e* 576 (M^+).

Mild Acid Hydrolysis Product—The deacetylated derivative of honghelosides A was hydrolyzed with the same procedure as somalin into D-cymarose, mp 92–94°, and gitoxigenin, mp 225–227° [lit. (16) mp 224°].

Isolation of 16-Acetylstrospeside—The fractions containing 16-acetylstrospeside were purified on preparative TLC plates developed in ether–chloroform (85:15). The recovered material was subsequently crystallized from aqueous methanol to yield colorless platelets, mp 208–210° (mp 189–190° after drying under vacuum); mass spectrum: base at *m/e* 355; UV: λ_{max} 220 nm (log ϵ = 4.31); IR[4]: ν_{max} 1785, 1745, 1620, and 1245 cm^{-1}; PMR[4]: δ 5.93 (1H), 4.95 (2H), 5.50 (m, 18 Hz), 4.00 (1H), 3.60 (s, 3H), 1.27 (d, J = 6 Hz, 3H), and 0.93 (s, 6H) ppm.

Anal.—Calc. for $C_{32}H_{48}O_{10}$: C, 64.84; H, 8.16. Found: C, 64.31; H, 8.21.

Strospeside—16-Acetylstrospeside (100 mg) was dissolved in methanol (20 ml). After the addition of 0.25 *M* $KHCO_3$ (4 ml), the reaction mixture was stirred at room temperature for 3 days. The air-dried residue was purified on preparative TLC plates developed in benzene–ethyl acetate–acetone–ethanol (4:3:2:1). The recovered material was crystallized from methanol and water, mp 252–253° [lit. (15) mp 252°].

16-Anhydrostrospeside—16-Acetylstrospeside (30 mg) was absorbed on basic aluminum oxide, introduced on top of a small alumina column (1.5 × 15 cm) and displaced with benzene–methanol (9:1), and the stopcock was closed. The column remained at room temperature for 1 week. Then the stopcock was opened, and the column was eluted with chloroform–methanol (1:1). The recovered material was crystallized from methanol and water, mp 240–243° [lit. (18) mp 242–246°]; UV: λ_{max} 270 nm (log ϵ = 4.25).

Isolation of Honghelin—The fractions containing honghelin were purified on preparative TLC plates developed in ether–chloroform (9:1). The recovered material formed hydrate crystals from methanol and water, mp 135–137° [lit. (18) mp 133–136°]; mass spectrum: *m/e* 534 (M^+), 357, 339, and 203; UV: λ_{max} 220 nm (log ϵ = 4.25); IR[4]: ν_{max} 1780, 1740, and 1620 cm^{-1}; PMR[4]: δ 5.83 (1H), 4.90 (2H), 4.00 (1H), 3.60 (s, 3H), 0.93 (s, 3H), and 0.87 (s, 3H) ppm.

Honghelin Diacetate—Acetylation of honghelin with acetic anhydride in pyridine at room temperature for 24 hr yielded the diacetate. It was crystallized from methanol and water, mp 218–220° [lit. (19) mp 217–220°]; PMR[4]: δ 2.07 (s, 3H), 2.10 (s, 3H), 4.90 (m, 28 Hz, 4H), and 4.40 (d, J = 8 Hz) ppm.

Anal.—Calc. for $C_{34}H_{50}O_{12}$: C, 66.00; H, 8.14. Found: C, 66.29, H, 8.20.

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* E. Mead Johnson Memorial Fellow of the American Foundation for Pharmaceutical Education.

[x] To whom inquiries should be directed.

Perfusion and Molecular Modification of Idoxuridine to Alter Its Cerebrospinal Fluid Metabolism

PHILIP H. GUTIN *, LEON D. PROCKOP [‡], K. F. YIP, JAMES D. WEINSTEIN, and K. C. TSOU [x]

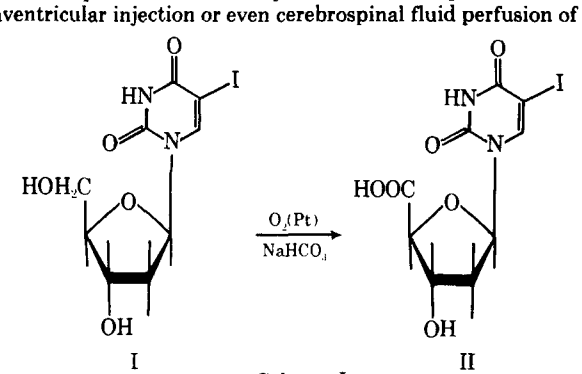
Abstract □ Two methods to deter the rapid intrathecal degradation of idoxuridine were investigated: (*a*) rapid drug perfusion through the ventricular system, and (*b*) modification of the molecule to its uronic acid derivative, 2′-deoxy-5-iodo-5′-uridinecarboxylic acid, to make it less susceptible to enzymatic digestion. Perfusion of idoxuridine through the ventricular system (ventriculocisternal) of dogs at 0.97 ml/min saturated the metabolic pathway so that the outflow solution yielded a single spot (R_f 0.76) on TLC indicative of the intact molecule. The ^{125}I-labeled uronic acid was synthesized from the ^{125}I-labeled parent compound, and the labeled compounds were compared after their individual intracisternal injection in dogs. Since there was no difference in the disappearance rates, the stability of the uronic acid was, in fact, no greater than that of the parent compound *in vivo*. Ventricular perfusion of idoxuridine, however, seems a suitable means for increasing the amount of active drug delivered to central nervous system tumors and viral infections.

Keyphrases □ Idoxuridine—metabolism in cerebrospinal fluid, effect of rapid perfusion and molecular modification, dogs □ Metabolism—idoxuridine in cerebrospinal fluid, effect of rapid perfusion and molecular modification, dogs □ Perfusion, rapid—idoxuridine through ventricular system, effect on metabolism in cerebrospinal fluid, dogs □ Molecular modification—idoxuridine to uronic acid derivative, effect on metabolism in cerebrospinal fluid, dogs □ Antiviral agents—idoxuridine, metabolism in cerebrospinal fluid, effect of rapid perfusion and molecular modification, dogs

Idoxuridine (I), an antimetabolite, is a useful topical antiviral agent in the treatment of *Herpes simplex* infections of the cornea (1, 2). This agent has also been administered systemically in cases of disseminated *H. simplex* and *H. simplex* encephalitis, but its value in this respect remains largely unproved (3–5).

BACKGROUND

The development of the Ommaya reservoir made possible the direct intraventricular injection or even cerebrospinal fluid perfusion of chemotherapeutic agents for central nervous system (CNS) viral infection or malignant disease (6). This subcutaneously implanted reservoir, with a catheter extending to the lateral ventricle, facilitates the delivery of high concentrations of potentially useful drugs to the brain. This route of administration is safe for I administration to patients with brain tumors or encephalitis (7). However, reservoir-delivered intraventricular therapy with I for progressive multifocal leukoencephalopathy in one patient (8) and for malignant brain tumors in four patients treated on this neurosurgical service was of no value[1].



I II

Scheme I

The lack of efficacy of intraventricular I therapy may be related to the very rapid metabolism of the drug by enzymes believed to be associated with the glial, ependymal, neural, or choroid plexus cells in or near the cerebral ventricles, where the drug is degraded to iodouracil and then deiodinated by the rate-limiting step to iodide and uracil (9, 10). Therefore, two methods of minimizing such enzymatic degradation were investigated: (*a*) rapid ventriculocisternal perfusion of I to maintain high levels of drug in the ventricular cerebrospinal fluid and, consequently, saturate the metabolic pathways; and (*b*) modification of the parent compound to its uronic acid derivative, 2′-deoxy-5-iodo-5′-uridinecarboxylic acid, in the hope that it would retain its antiviral and antitumor activity but be less susceptible to enzymatic breakdown. The uronic acid of floxuridine was synthesized previously and showed dramatic resistance to hydrolysis by acid or phosphorylase while remaining moderately toxic to tumor cells *in vitro* (11).

EXPERIMENTAL

Ventriculocisternal Perfusion—Mongrel dogs, 7–20 kg, were anesthetized with pentobarbital, 60 mg/kg iv, with supplements given as needed. The dog was secured in a stereotactic head frame, and an endotracheal tube was placed to ensure adequate ventilatory exchange. A skin incision was made, and a bur hole was drilled in the skull so that its center lay 22–24 mm forward from the ear bar and 7–8 mm lateral to the midline. A No. 18 needle was placed percutaneously into the cisterna magna.

With continuous pressure monitoring, a No. 19 needle in a stereotactic holder was introduced slowly through the bur hole vertically to a depth of 16–18 mm until the pulsations of the choroid plexus transmitted to the cerebrospinal fluid were noted on the pressure recording to assure ventricular placement. Cerebrospinal fluid pressure was measured on a pressure transducer[2] and transmitted for reading on a polygraph[3].

Artificial cerebrospinal fluid was prepared as a modified Krebs–Ringer bicarbonate solution that closely resembled the ionic composition of normal spinal fluid (12). Compound I[4] was diluted to 0.1 mg/ml in artificial cerebrospinal fluid and infused through the ventricular needle first at 0.39 ml/min and then at 0.97 ml/min by means of a constant-infusion pump[5].

Samples were collected from the cisternal needle over timed periods after a 30-min perfusion to ensure homogeneity in the ventricular system,

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[2] Stratham, Oxnard, Calif.
[3] Grass, Quincy, Mass.
[4] Schwarz/Mann, Orangeburg, N.Y.
[5] Harvard Apparatus, Millis, Mass.