Antitumor Agents V: Cytotoxic Cardenolides from *Cryptostegia grandiflora* (Roxb.) R. Br.

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Abstract ☐ An alcoholic extract of the aboveground parts of *Cryptostegia grandiflora* showed inhibitory activity against the KB cell culture and was fractionated through a series of partitions and column chromatography to yield five cardenolides: oleandrigenin, 16-propionylgitoxigenin—a new natural product, 16-anhydrogitoxigenin, gitoxigenin, and rhodexin B.

Keyphrases ☐ *Cryptostegia grandiflora* (Roxb.) R. Br.—isolation, identification of five cytotoxic cardenolides ☐ Cardenolides—isolation from *Cryptostegia grandiflora*, identification, cytotoxic activity ☐ Antitumor agents, potential—five cardenolides from *Cryptostegia grandiflora* ☐ Cytotoxicity—five cardenolides from *Cryptostegia grandiflora*

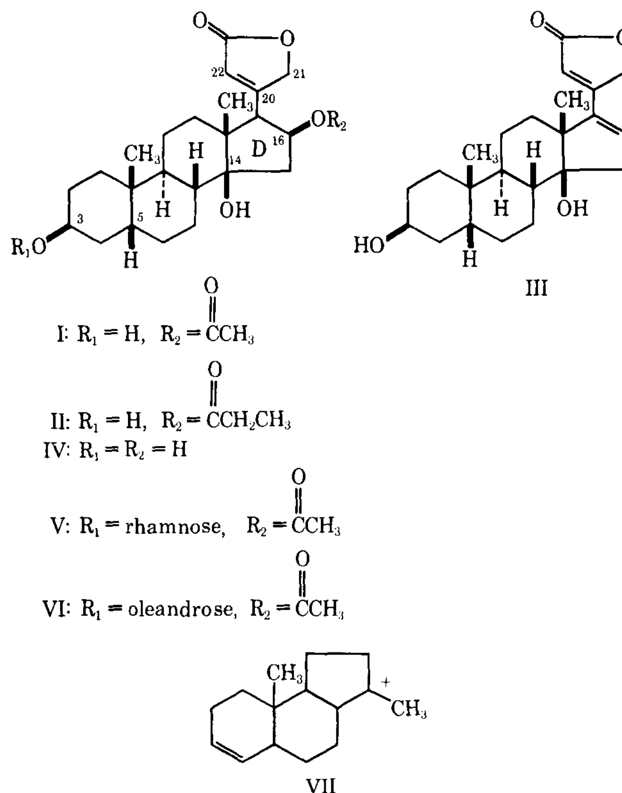
An alcoholic extract of the aboveground portion of *Cryptostegia grandiflora* (Roxb.) R. Br. (family Asclepiadaceae), a Madagascan plant naturalized in Mexico, was found to have inhibitory activity against the cell culture (KB) of a human carcinoma of the nasopharynx[1]. Systematic fractionation of the extract led to the isolation of five cardenolides; oleandrigenin (I), 16-propionylgitoxigenin (II), 16-anhydrogitoxigenin (III), gitoxigenin (IV), and oleandrigenin 3-rhamnoside (rhodexin B) (V), which are in part responsible for the cytotoxicity.

DISCUSSION

An outline of the fractionation procedure is given in Scheme I and the biological data are presented in Table I. The alcoholic residue (A) from percolation of the plant material with ethanol was partitioned between chloroform and water and resulted in concentration of the activity in the chloroform phase. Partitioning of the chloroform residue (C) between petroleum ether and 10% aqueous methanol gave an active methanol fraction (E) which, when carried through a countercurrent distribution procedure, was purified twofold. Chromatography of the countercurrent distribution fraction (F) on silicic acid yielded five main fractions pooled on the basis of dry weight and paper chromatographic analysis, once it was recognized that the cytotoxicity was regularly associated with material giving a positive Kedde's reaction (1). After adsorption, only column Fraction H directly yielded crystalline material, oleandrigenin. The other column fractions required additional purification on partition columns employing a Zaffaroni-type solvent system (2); the solvent composition was chosen by a screening method with paper chromatograms such that the desired substance had R_f 0.3–0.6. Details of this method were described previously (4).

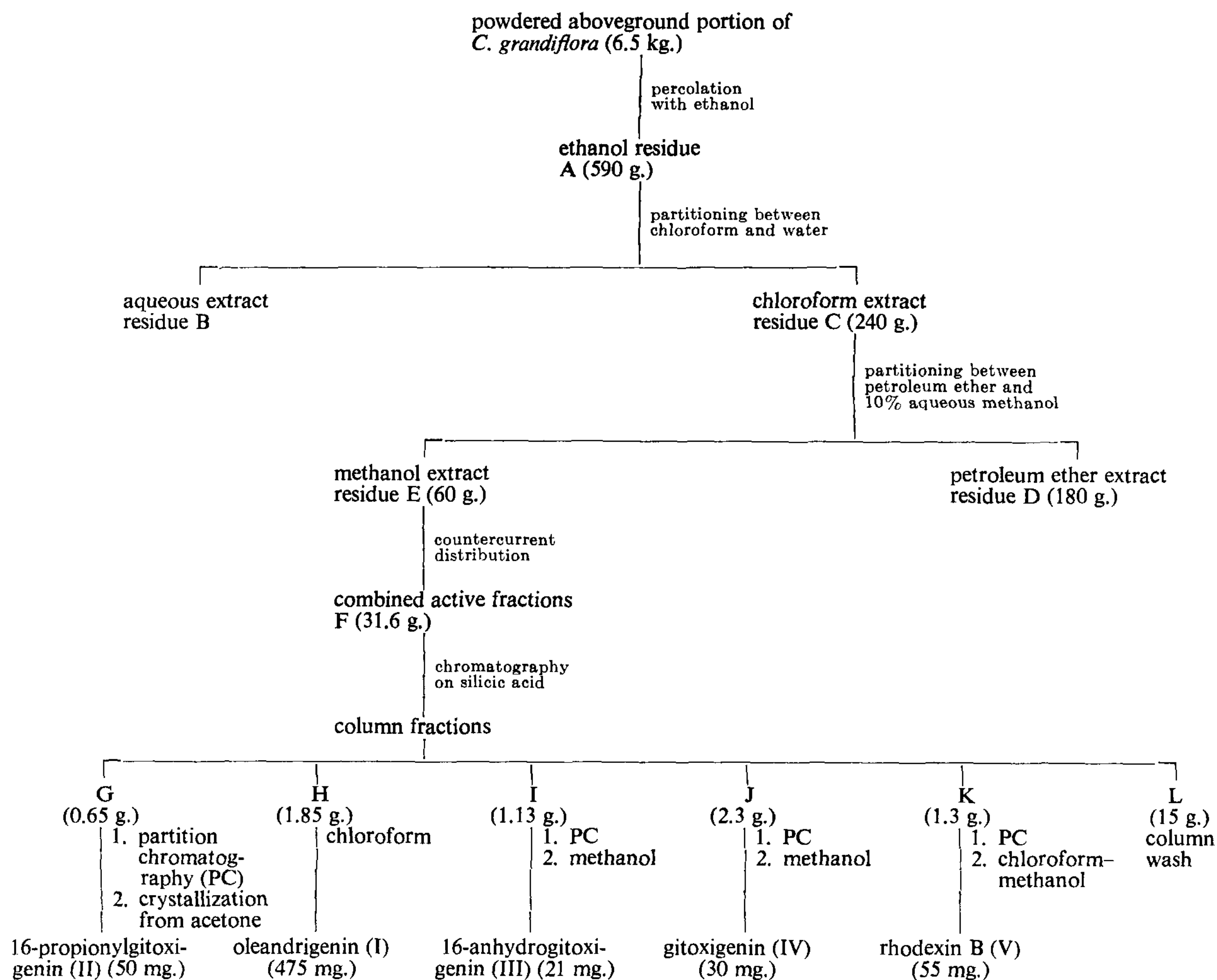
Oleandrigenin was identified from its physical and spectral properties and confirmed by direct comparison with an authentic sample obtained from oleandrin (VI) by hydrolysis (5). The second isolated cardenolide, 16-propionylgitoxigenin, came from Fraction G after partition chromatography and exhibited an NMR spectrum similar to oleandrigenin except for the lack of the acetate methyl group. The mass spectrum showed a weak molecular ion peak at *m/e* 446 (0.4%) in accord with the formula $C_{26}H_{38}O_6$, and that for the acetate derivative (3-acetyl 16-propionylgitoxigenin) appeared at *m/e* 448 (0.2%). Both spectra possessed a base peak at *m/e* 203 (VII), characteristic of 14β-hydroxycardenolides containing a hydroxy or ester substitution at the 3-position (6, 7). The additional 14 mass units above the molecular weight of oleandrigenin were traced to the methylene group of the propionate ester located at position 16 on the basis of: (*a*) the identification, chromatographically (8), of propionic acid as the ferric hydroxamate (9) in the hydrolysis mixture of the cardenolide; (*b*) the presence in the NMR spectrum ($CDCl_3$) of an A_2X_3 pattern (J = 7.4 Hz.) at δ 2.25 (quartet) and 1.07 (triplet); and (*c*) the mass spectrum containing peaks at *m/e* 74 ($CH_3CH_2COOH^{+\cdot}$) and 57 ($CH_3CH_2CO^+$). Confirmation of the structural assignment was obtained by direct comparison with a sample of 16-propionylgitoxigenin obtained on hydrolysis of 16-propionylgitoxin[2]. This is the first report of 16-propionylgitoxigenin as a natural product. It has, however, been obtained synthetically but its properties have not yet been published (10).

The third cardenolide was isolated from Fraction I after partition chromatography and was identified as 16-anhydrogitoxigenin on the basis of its spectral properties. The UV absorption maximum at 269 nm. (log ε 4.26) immediately suggested a 16-anhydro structure (11), and direct comparison of the acetate derivative (12, 13) with authen-



[1] The cell culture assay was performed through the auspices of the Cancer Chemotherapy National Screening Center (CCNSC) according to the procedure given in *Cancer Chemother. Rep.*, **25**, 22(1962). A purified substance is considered active if, on the average of two tests, it shows an $ED_{50} \leq 4$ mcg./ml., which is confirmable by another testing laboratory.

[2] The authors thank Dr. G. Baumgarten (Ysat Wernigerode, E. Germany) for a generous sample of 16-propionylgitoxin.



Scheme I—Flow diagram for fractionation of an alcoholic extract of C. grandiflora

tic 3-acetyl 16-anhydrogitoxigenin[3] confirmed its structure. Oleandrigenin was converted to 16-anhydrogitoxigenin by treatment with acid-washed alumina in chloroform at reflux temperature (13, 14), but oleandrigenin was recovered intact on standing for 10 days on a column of silicic acid. Although 16-anhydrogitoxigenin could be an artifact of isolation (13), it seems unlikely that it is formed during the adsorption chromatographic step.

Gitoxigenin, the fourth cardenolide, was isolated from Fraction J after partition chromatography. Its identity was established from the physical constants (15) and spectral properties and was confirmed by direct comparison with an authentic sample.

The fifth and most polar cardenolide was isolated from Fraction K and found to yield, on acid hydrolysis, oleandrigenin and a sugar identified chromatographically as rhamnose. A direct comparison of its properties with that of authentic rhodexin B[4] (16) indicated the two were identical. The original source of rhodexin B was *Rhodea japonica* Roth. (Liliaceae) and this report is a second finding.

Although all five cardenolides show cytotoxic properties, only oleandrigenin, gitoxigenin, rhodexin B, and possibly 16-propionylgitoxigenin could be considered significantly active by the protocol of the CCNSC[1] to merit further study. Other cardenolides that are cytotoxic are known (17–19), and a parallel appears to exist between cytotoxicity towards KB cells, a heart action, and the inhibition of the ATPase enzymes of the active transport of potassium and sodium ions (20).

The earlier investigation (13) of the cardiac glycosides of *C. grandiflora* resulted in the isolation of six compounds: cryptograndoside A (oleandrigenin-D-sarmentoside), cryptograndoside B (oleandrigenin-D-gluco-D-sarmentoside), the corresponding 16-desacetyl 16-anhydro derivatives as artifacts of alumina chromatography of the aforementioned glycosides, digitalinum verum (gitoxigenin-D-gluco-D-digitaloside) as the hexaacetate, and cryptograndoside C (an uncharacterized minor glycoside obtained as a crystalline acetate). The cardenolides from *C. grandiflora*, identified to date, are based on one genin, gitoxigenin, and more commonly on its 16-acetyl derivative (oleandrigenin).

EXPERIMENTAL[5]

Plant Material—The aboveground parts (leaves and stems) of *C. grandiflora* (Roxb.) R. Br. were collected[6] in Sonora, Mexico, received air dried, and then powdered through 80 mesh.

Extraction and Initial Separation—The powdered plant material (6.5 kg.) was percolated at room temperature with alcohol USP

[3] The authors are grateful to Professor T. Reichstein for a sample of this compound as well as cryptograndosides A and B, their 16-desacetyl 16-anhydro derivatives, and digitalinum verum. These latter samples were invaluable as chromatographic standards in the initial phases of the study.

[4] The authors thank Dr. K. Kitahashi of Takeda Chemical Industries, Ltd., Osaka, Japan, for the sample of rhodexin B.

[5] Melting points were determined on a Thomas-Hoover Uni-Melt capillary melting-point apparatus or a Fisher-Johns hot stage and are uncorrected. The IR spectra were obtained in $CHCl_3$ or as KBr pellets on a Perkin-Elmer model 237 or 257 spectrophotometer. UV spectra were taken in methanol on a Cary model 15 recording spectrophotometer. NMR spectra were recorded on a Varian A60-A instrument in the indicated solvent with tetramethylsilane as internal standard; chemical shifts are reported in δ (p.p.m.) units. Optical rotations were taken on a Jasco ORD/UV-5 spectropolarimeter or on a Zeiss polarimeter. Mass spectra were obtained on an AEI MS-9 double-focusing instrument *via* the direct inlet probe.

[6] The authors are indebted to Dr. R. E. Perdue, Jr., of the U. S. Department of Agriculture, Beltsville, Md., for these collections received in 1964, 1968, and 1971 as part of the agreement with the CCNSC. Voucher specimens are on deposit with Dr. Perdue.

until the extract was essentially colorless. Removal of the solvent by evaporation at reduced pressure and at 40° left 590 g. of a thick residue which was partitioned between 1 l. each of chloroform and water. The aqueous phase was extracted three additional times with chloroform, and the total chloroform extract residue after removal of solvent weighed 240 g. The solids from the aqueous phase were obtained by freeze drying.

The chloroform residue was partitioned between 1 l. of 10% aqueous methanol and four 1-l. portions of petroleum ether (b.p. 60–71°). The methanol phase after removal of solvent left a residue of 60 g. which was carried through a 10-stage countercurrent distribution (CCD) procedure using 1-l. separators containing 400 ml. of each phase of the solvent system of hexane–water–acetone–*tert*-butanol (5:2:4:4)[7]. The cytotoxic principles were contained in the first three funnels (Fraction F) and the residue weight was 31.6 g.

Adsorption Chromatography of the CCD Fraction—A column (8.6 cm. i.d.), slurry packed with 1.36 kg. (3 lb.) of silicic acid[8] in chloroform, was loaded with the 31.6-g. CCD fraction (F) in 125 ml. of solvent. Elution was with chloroform until the first dark-green band just emerged, and then 40-ml. column fractions were collected and the elution was continued with 2 l. each of 1, 2, 4, 6, 8, 10, and 12% methanol in chloroform. The column was finally washed with methanol–chloroform (1:1). The residue weight was determined for each fraction, and paper chromatographic analysis was performed on alternate ones. The solvent systems, saturated with formamide, were benzene–chloroform (6:4) (System A) and chloroform (System B), and they were used with Whatman No. 1 impregnated with formamide (3, 4). Pooled fractions were made from this analysis and are shown in Scheme I, with the bioassay results in Table I. Kedde's spray reagent was used (1).

Oleandrigenin (I)—Column Fraction H (1.85 g.) gave, from benzene, 958 mg. of crystals which were recrystallized from benzene and from chloroform to give material (475 mg.) with m.p. 224–225°. The combined mother liquors, when chromatographed on a partition column made from 200 g. of diatomaceous earth[9], 120 ml. of formamide, and Solvent A, yielded an additional 700 mg. of product. Effluent fractions (20 ml.) were monitored paper chromatographically. A direct comparison (IR, UV, NMR, and mixed melting point) of the isolated material with authentic oleandrigenin obtained from oleandrin[10] (13) showed the two to be indistinguishable. The R_f in Solvent A was 0.50.

16-Propionylgitoxigenin (II)—Fraction G (0.65 g.) was passed through the same partition column used to purify oleandrigenin and gave 50 mg. of a fraction that crystallized (m.p. 208–209°) from benzene. Recrystallization from acetone gave the colorless product, 16-propionylgitoxigenin, m.p. 212–214°; IR $\nu_{max.}$: 3615 (OH), 3490 (bonded OH), 1750 (γ-lactone), 1620, and 1640 cm.$^{-1}$ (olefin); NMR δ ($CDCl_3$): 5.98 (1 H, narrow triplet[11], H_{22}), 5.53 (1H, sextet, J = 3.0, 8.7, 9.6 Hz., H_{16}), 4.96 (2H, broadened triplet[11], H_{21}), 4.6 (1H, multiplet, $W^{1/2}$ = 6 Hz., H_3), 3.23 (1H, doublet, J = 8.7 Hz., H_{17}), 2.25 (2H, quartet, J = 7.4 Hz., methylene of propionate), 1.09 (3H, triplet, J = 7.4 Hz., methyl of propionate), and 0.96 (6H, singlet, H_{18} and H_{19}); mass spectrum m/e: 446 (molecular ion, 0.4%), 390 (4%), 372 (9), 354 (19), 336 (12), 249 (6), 228 (10), 221 (22), 203 (100), 147 (18), 124 (43), 74 (42), and 57 (31). The R_f in Solvent A was 0.65. Comparison (UV, IR, NMR, mixed melting point, and paper chromatography) of the isolated compound with 16-propionylgitoxigenin obtained from 16-propionylgitoxin[2] showed them to be identical.

Acetylation (acetic anhydride and pyridine at room temperature for 16 hr.) of 16-propionylgitoxigenin gave the crystalline 3-acetate derivative from acetone–ether–*n*-pentane, m.p. 214–216°; mass spectrum m/e: 488 (0.2%), 470 (0.2), 432 (3), 428 (4), 414 (6), 396 (7), 372 (5), 354 (23), 336 (12), 321 (5), 263 (6), and 203 (100).

Hydrolysis of 16-Propionylgitoxigenin (II)—About 1 mg. of 16-propionylgitoxigenin was treated according to *Reference 9* to form the ferric hydroxamate complex, which was identified by paper chromatography (8) as that of propionic acid. The complexes of

Table I—Cytotoxicity of Fractions from *C. grandiflora*

Fraction	ED_{50}, mcg./ml.
A	4.5
B	17
C	2.1
D	100
E	2.2
F	0.016
G	3.8
H	0.26
I	2.5
J	0.19
K	1.2
L	2.5
Oleandrigenin (I)	0.05 (0.18)
16-Propionylgitoxigenin (II)	3.7 (4.3)
16-Anhydrogitoxigenin (III)	4.8
Gitoxigenin (IV)	2.3 (2.0)
Oleandrigenin 3-rhamnoside (V)	0.025

propionic acid and acetic acid have R_f values of 0.45 and 0.29, respectively, in *n*-butanol–dimethylformamide–water (9:1:10).

Hydrolysis of 16-Propionylgitoxin—A 50-mg. sample of 16-propionylgitoxin was dissolved in 5 ml. of acetone containing 0.05 ml. of concentrated hydrochloric acid and allowed to stand at room temperature for 1 hr. Water was added, the acetone was evaporated, and the aqueous solution was extracted with chloroform. The chloroform residue (34 mg.) gave crystalline 16-propionylgitoxigenin from acetone, m.p. 211–212°.

16-Anhydrogitoxigenin (III)—Fraction I (1.13 g.) was chromatographed on a partition column utilizing Solvent A. Crystallization from acetone gave 21 mg. of 16-anhydrogitoxigenin, m.p. 240–244°; UV $\lambda_{max.}$: 269 nm. (log ϵ 4.26); IR (KBr) $\nu_{max.}$: 3490 (OH), 1725 (γ-lactone), and 1620 cm.$^{-1}$ (olefin); mass spectrum m/e: 372 (molecular ion 98%), 354 (96), 336 (42), 205 (65), and 192 (100); R_f 0.40 in Solvent A.

Acetylation of 11 mg. of 16-anhydrogitoxigenin with acetic anhydride and pyridine at room temperature for 18 hr. gave 8 mg. of the crystalline 3-acetyl derivative from acetone–ether–*n*-pentane, m.p. 199°. When compared (IR, mixed melting point, and paper chromatography, R_f 0.80 in Solvent B) with authentic 3-acetyl 16-anhydrogitoxigenin, the two compounds were indistinguishable.

Conversion of Oleandrigenin (I) to 16-Anhydrogitoxigenin (III)—Oleandrigenin (200 mg.) in 15 ml. of chloroform was refluxed in the presence of 10 g. acid-washed alumina[12] for 10 hr. From the filtered solution, 160 mg. of residue was recovered, showing only the one spot of 16-anhydrogitoxigenin on paper chromatography (System A). Two crystallizations from acetone–pentane gave 42 mg. of 16-anhydrogitoxigenin, m.p. 240–244°, identical with that obtained from the plant.

Gitoxigenin (IV)—Fraction J (2.3 g.) was separated on a partition column (3.7 × 68 cm.) made from 200 g. of diatomaceous earth[8], 120 ml. of formamide, and a mobile phase of benzene–chloroform (1:9). The column fraction crystallized from methanol to give 30 mg. of gitoxigenin, m.p. 232–235°. A comparison (IR, NMR, mixed melting point, and paper chromatography, R_f 0.40 in System B) with authentic gitoxigenin[10] showed the two to be identical.

Rhodexin B (Oleandrigenin 3-Rhamnoside) (V)—Fraction K (1.30 g.) was purified on a partition column employing Solvent System B and gave 55 mg. of crystalline (m.p. 273–274°) rhodexin B from chloroform–methanol. Hydrolysis of 1 mg. of the product with 0.1 ml. of 1% hydrochloric acid in acetone at room temperature for 3 days gave the products oleandrigenin and rhamnose, as identified with standard samples by paper chromatography. Solvent A was used for the genin; for rhamnose, *n*-butanol–acetone–10% NH_4OH–water (40:50:3:15) and toluene–*n*-butanol (1:2) on papers dipped in acetone–water (2:1) were used. Ethyl acetate–isopropanol–methanol (70:15:15) for TLC on silica gel G were employed. The spray reagent for the sugars was aniline phthalate reagent (22). A comparison [IR, NMR, TLC on silica gel G with 2-butanone–toluene–water–methanol–acetic acid (40:5:3:2.5:1) gave R_f 0.54, and paper chromatography with System B gave R_f 0.26] of the isolated compound with rhodexin B showed the two to be indistinguishable.

[7] This system was taken from a procedure utilized by Dr. M. E. Wall and his colleagues at the Research Triangle Institute, Durham, N. C., in their antitumor purification studies.
[8] Mallinckrodt, 100 mesh.
[9] Celite 545, Johns-Manville Corp., New York, N. Y., and purified as given in *Reference 3*.
[10] Purchased from Pfaltz and Bauer, Inc., Flushing, N. Y.
[11] Analysis of the H_{21} and H_{22} pattern was not made because it was not necessary for this study. It can, however, be found in *Reference 21*.
[12] Merck.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 4, 1971, from the *College of Pharmacy, Ohio State University, Columbus, OH 43210*
Accepted for publication December 29, 1971.
Supported by Public Health Service Research Grant CA-08133 from the National Cancer Institute, and Equipment Grant FR-00328 for the purchase of a Varian A60-A NMR spectrometer with accessories.
The authors thank Dr. R. L. Foltz of Battelle Memorial Institute and Mr. C. R. Weisenberger of the Chemistry Department, Ohio State University, for the mass spectral determinations.
Previous paper: R. W. Doskotch and F. S. El-Feraly, *J. Org. Chem.*, **35**, 1928(1970).
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Effects of Practolol, a New Adrenergic Receptor Blocking Agent on Cardiovascular Responses

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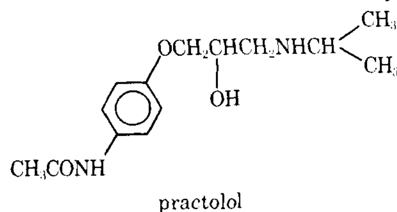
Abstract ☐ The claim that practolol, 4-(2-hydroxy-3-isopropylaminopropoxy)acetanilid, is a cardioselective β-adrenergic receptor blocking agent was investigated by testing this compound in cats and in hyperthyroid rabbits for its ability to block certain cardiovascular responses. Doses of 1, 2, 5, and 10 mg./kg. administered intravenously were ineffective in abolishing completely the pressor response to bilateral carotid occlusion. After the administration of either 2, 5, or 10 mg./kg. of this agent, the pressor response due to injected norepinephrine (3 mcg./kg.) was unaffected. However, these doses produced a moderate to marked depression of the increase in chronotropic response caused by the injection of norepinephrine (3 mcg./kg.) and isoproterenol (0.3 mcg./kg.) and the tachycardia attendant with hyperthyroid states. The blockade of the resting tachycardia in hyperthyroid rabbits was significant at all dose levels of practolol ($p \leqslant 0.01$ or 0.02). Only the 10-mg./kg. dose was found to produce a transient hypotension in cats.

Keyphrases ☐ Practolol—evaluation as cardioselective β-adrenergic blocking agent ☐ 4-(2-Hydroxy-3-isopropylaminopropoxy)acetanilid (practolol)—evaluation as cardioselective β-adrenergic blocking agent ☐ β-Adrenergic blocking agents, cardioselective—practolol evaluation

The introduction into pharmacology of the concept of α- and β-adrenergic receptors by Ahlquist (1) was greatly enhanced by the discovery and continued development of specific antagonists of these receptors. Some of these antagonists, such as dichloroisoproterenol, have been shown to possess an intrinsic β-receptor stimulant action (2–4). Others, such as pronetholol and propranolol, possess, in addition to β-adrenergic receptor blocking action, a cardiac depressant action (5–10). The blocking activity of these compounds is similar qualitatively in that they block all β-adrenergic receptors, but they differ quantitatively.

Recently, a new β-adrenergic receptor blocking agent, 4-(2-hydroxy-3-isopropylaminopropoxy)acetanilid[1] (AY-21,011), (I.C.I. 50,172), or practolol, was shown to possess cardioselective β-blocking activity (11–13). This investigation was undertaken to elucidate further the claim of cardioselective blockade by practolol



practolol

[1] Ayerst Laboratories, New York, N. Y.