Cytotoxic Cardenolides from *Acokanthera longiflora* Stapf. and Related Species

DAVID G. I. KINGSTON*x and T. REICHSTEIN‡

Abstract □ An alcoholic extract of the stems and twigs of *Acokanthera longiflora* Stapf. showed inhibitory activity against the KB cell culture and was fractionated through a series of partitions and column chromatography to yield three cardenolides: acovenoside A, opposide, and acolongifloroside K. Tests of these and other cardenolides from related species indicated that nearly all of the compounds tested had high *in vitro* activity but no *in vivo* activity.

Keyphrases □ *Acokanthera longiflora* Stapf.—isolation, identification of three cytotoxic cardenolides □ Cytotoxicity—cardenolides □ Cardenolides—isolated from *Acokanthera longiflora* Stapf., cytotoxicity

A random screening of botanical sources for anticancer activity showed that the aqueous alcoholic extract of the stems and twigs of *Acokanthera longiflora* Stapf. (family Apocynaceae) gave reproducible activity against the cell culture (KB) of a human carcinoma of the nasopharynx[1]. Systematic fractionation of the extract led to the isolation of three cardenolides: acovenoside A (I), opposide (II), and acolongifloroside K (III). In view of the cytotoxicity of these compounds, tests were also carried out on a number of cardenolides from related species, and several of these were found to be cytotoxic. The more readily available compounds were tested for their *in vivo* activity against L-1210 lymphoid leukemia[2]; the results of the tests indicated that these compounds had no activity in this system.

DISCUSSION

The alcoholic residue from percolation of the plant material with ethanol was partitioned between water and chloroform and resulted in a division of the activity between the two phases. The active chloroform fraction was partitioned between petroleum ether and 10% aqueous methanol to give an inactive petroleum ether fraction and an active methanol fraction. Chromatography of this active fraction on silicic acid yielded nine main fractions. Crystallization of the major active fraction yielded directly a material identified as acovenoside A (I) (1–3). The material was identified from its spectral properties and by direct comparison with authentic material[3] (IR, melting point, and mixed melting point).

The active aqueous extract from the original chloroform–water partition was subjected to partition between chloroform–ethanol (2:1) and water, and the aqueous residue was partitioned between butanol and water. Both organic extracts showed comparable activity, while the aqueous extract was essentially inactive. Analysis of the organic extracts by paper chromatography indicated that they both consisted of essentially identical mixtures of three materials giving a positive Kedde's reaction. The butanol fraction was subjected to partition column[4] chromatography with chloroform–benzene, half saturated with water, as the eluant. Combination of fractions on the basis of paper chromatographic analysis gave three fractions, each consisting of one component giving a positive Kedde's reaction together with several other fractions containing mixtures of Kedde-positive materials. The three fractions containing only one Kedde-positive component were each purified further by chromatography on silicic acid and, in two cases, by crystallization.

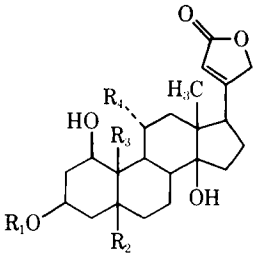
The first material was isolated in too small yield to allow complete characterization but was not identical with any of the known constituents of *A. longiflora*. Its characterization will be the subject of a subsequent report.

The second compound was identified as opposide (II) by comparison of its IR spectrum, color reaction with 84% H_2SO_4, paper chromatographic mobility, and melting point with literature values for authentic material (4, 5). This is the first reported isolation of opposide from *A. longiflora*.

The third compound was identified as acolongifloroside K (III) by paper chromatographic comparison with an authentic sample. The preparation available could not be induced to crystallize, although it was chromatographically homogeneous. Treatment with acetic anhydride and pyridine, however, yielded a crystalline acetate identical with acolongifloroside K pentaacetate (6).

The earlier investigation (7) of the cardiac glycosides of *A. longiflora* resulted in the isolation of eight compounds: acovenoside A; acolongiflorosides E, G, H, J, and K; and substances D and F. Paper chromatographic analysis of the extract of the present plant sample showed the absence of more than trace amounts of any other cardenolides; in particular, the acolongiflorosides G, H, and J were present in only trace amounts in their appropriate crude fractions. It may thus be concluded that the cytotoxicity of *A. longiflora* extracts is due largely to the presence of acovenoside A and acolongifloroside K, with lesser contributions from opposide and other cardenolides present in small quantities.

In view of the high cytotoxicity of the compounds isolated from *A. longiflora*, it was of interest to investigate the cytotoxicity of similar compounds from related species (Table I). With few exceptions, all investigated compounds had a moderately high cytotoxicity. The cytotoxicity of acovenosides A and B was previously reported (10). Other cardenolides that are cytotoxic are



I: R_1 = L-acovenose, R_2 = H, R_3 = CH_3, R_4 = H
II: R_1 = 6-desoxy-L-talose, R_2 = OH, R_3 = CH_3, R_4 = OH
III: R_1 = 6-desoxy-L-talose, R_2 = OH, R_3 = CH_2OH, R_4 = OH

[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 $ED_{50} \leq 4\ \mu g/ml$, which is confirmable by another testing laboratory.

[2] See Footnote 1. A purified substance is considered active if it shows a T/C ratio ≥125 on the average of two tests, which is confirmable by another testing laboratory.

[3] D. G. I. Kingston thanks Dr. G. J. Kapadia, Department of Pharmacognosy, Howard University, for an authentic sample of acovenoside A.

[4] Hyflo Supercel.

Table I—Cytotoxicity of Cardenolides from *Acokanthera* Species

Cardenolide	ED_{50}, μg/ml	Reference
Acovenoside A	0.025	This work
Acolongifloroside K	0.064, 0.40	This work
Opposide	0.045	This work
Acolongifloroside G	0.15	6
Acolongifloroside H	0.25	6
Acoschimperoside P	0.10	7
Acoschimperoside Q	0.25	7
Acoschimperoside N	>100	7
Diglucoacoschimperoside P	1.7	7
Diglucoacoschimperoside N	3.7	7
Acofrioside L	0.16	8
Acovenoside B	0.22	9

known (11-15), and a parallel appears to exist among cytotoxicity toward KB cells, a heart action, and the inhibition of the ATPase enzymes of the active transport of potassium and sodium ions (16).

Table II records the results of *in vivo* bioassays of the more readily available cardenolides in the L-1210 leukemia system. None of the compounds tested showed an *in vivo* activity high enough to suggest that it held promise as an antitumor drug, presumably because of high mammalian toxicities (3, 7).

EXPERIMENTAL[5]

Plant Material—The stems and twigs of *A. longiflora* Stapf. were collected[6] in Kenya, received air dried, and then ground in a hammermill to 0.31-cm (0.125-in.) mesh.

Extraction and Initial Separation—The powdered plant material (2.2 kg) was extracted with ethanol to give, after evaporation, 102 g of a thick residue which was partitioned between chloroform and water. The chloroform extract (43 g) was partitioned between 10% aqueous methanol and petroleum ether (bp 60–80°). The methanol phase, after removal of solvent, left a residue of 25 g.

The aqueous extract from the initial chloroform–water partitioning was extracted with chloroform–ethanol (2:1); the chloroform–ethanol extract weighed 7.5 g after evaporation of the solvent. The remaining aqueous solution was extracted with *n*-butanol; after evaporation and freeze drying, the *n*-butanol extract weighed 14 g, with the remaining aqueous extract accounting for approximately 36 g.

Adsorption Chromatography of Aqueous Methanol Fraction—Chromatography of the methanol fraction was carried out on silica gel with a gradient of chloroform to chloroform–12% methanol as eluant (8). Combination of fractions on the basis of dry weight and paper chromatography [chloroform–formamide system (4, 17-20)] yielded nine major fractions. No materials giving a purple color with the Kedde spray reagent (21) could be detected in fractions 1-5. Fraction 6 contained mainly acovenoside A, while fraction 7 contained small quantities of materials with the same R_f as authentic samples of the acolongiflorosides G and H (overlapping spots) together with other minor components. Fraction 8 showed the presence of a trace amount of Kedde-positive material near the origin, with the same R_f as acolongifloroside J.

Acovenoside A—Crystallization of fraction 6 from chloroform–benzene gave crystals, mp 220–225°. Recrystallization (three times) from acetone–petroleum ether yielded needles, mp 230–231° [lit. (1) mp 230–232°]; concentration of the mother liquors yielded rods with double melting points of 158–162° → 230–231° [lit. (1) 160–163° → 230–232°]. A mixed melting point with authentic material was undepressed, and the material had UV and IR spectra and optical rotation identical with those of authentic material.

Acetylation (acetic anhydride and pyridine at room temperature for 16 hr) of acovenoside A gave the crystalline diacetate as colorless needles from acetone–petroleum ether, mp 221–222° [lit. (1) mp 229–230°].

Partition Chromatography of Butanol Fraction—Partition column[4] chromatography was carried out on 6.0 g of the butanol fraction with benzene–butanol, half saturated with water, as eluant (4). A total of 100 fractions was obtained and combined on the basis of paper chromatography in the system of butanol–water (22).

Compound J′—Fractions 34-56 yielded a material which showed only one Kedde-positive spot on paper chromatography in butanol–water, R_f 0.54. The crude fraction was purified by adsorption chromatography on a silicic acid (80 g) column and elution with linear gradient of chloroform to chloroform–methanol (50:50). Fractions of 50 ml were collected, and fractions 18-20 yielded material homogeneous by paper chromatography (20 mg) and with a different R_f than any known constituent of *A. longiflora*.

Opposide—Fractions 62-71 showed only one Kedde-positive spot on paper chromatography. Adsorption chromatography on 90 g silicic acid with a chloroform to chloroform–methanol (50:50) gradient and collection of 50-ml fractions gave crude opposide (300 mg) in fractions 15-19. Crystallization from acetone followed by moist ether–methanol gave opposide (110 mg), mp 282–287° [lit. (4) mp 292–295°], undepressed when mixed with authentic material. The compound had an identical IR spectrum with that recorded for authentic material (5), an identical color reaction with 84% H_2SO_4, and identical chromatographic behavior to that of authentic material (4).

Acolongifloroside K—Fractions 74-87 were combined and submitted to adsorption chromatography on 100 g silicic acid. Elution with a gradient of chloroform to chloroform–methanol (50:50) and collection of 50-ml fractions gave acolongifloroside K (600 mg) in fractions 16-20. The chromatographically homogeneous material did not crystallize but behaved identically to authentic material on paper chromatography in butanol–water.

A sample was acetylated (acetic anhydride in pyridine) at room temperature for 16 hr. Usual workup gave acolongifloroside K pentaacetate, mp 290–291° [lit. (6) mp 286–292°].

Table II—*In Vivo* Activity of Various Cardenolides

Cardenolide	Tumor	Percent T/C
Acovenoside A	L-1210	111
Acolongifloroside K	L-1210	Toxic
Acovenoside C	L-1210	87

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[5] Melting points were determined on a Mel-Temp capillary melting-point apparatus and are corrected. The IR spectra were obtained as KBr disks on a Beckman IR-8 spectrophotometer. UV spectra were recorded on a Cary model 15 recording spectrophotometer. Optical rotation was recorded in methanol on a Bellingham and Stanley spectropolarimeter. Paper chromatography was carried out by the ascending technique.

[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 1969 as part of the agreement with the CCNSC. Voucher specimens are on deposit with Dr. Perdue.

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ˣ To whom inquiries should be directed.

Sterols of Marine Algae

L. M. SAFE, C. J. WONG, and R. F. CHANDLERˣ

Abstract □ The free and bound sterols of the marine algae *Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*, were isolated and identified by using a combination of TLC, GLC, and mass spectroscopic techniques. Preparative TLC enabled the fast, efficient separation of each sterol from the mixture for positive identification by GLC and mass spectroscopy. The chromatographic techniques employed permitted the detection and isolation of the more polar sterols in the presence of the more common 3β-monohydroxy sterols. To the authors' knowledge, this is the first recorded detailed sterol analysis of *L. saccharina* and the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. The mass spectra for these two sterols are presented.

Keyphrases □ Sterols, free and bound—isolated and identified from marine algae (*Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*) □ Algae, marine—isolation and identification of free and bound sterols □ Marine algae—isolation and identification of free and bound sterols □ *Laminaria saccharina*—isolation and identification of free and bound sterols

Marine algae have been the subject of numerous investigations in the fields of pharmacognosy and natural product chemistry for many years (1). Sterols found in algae (2, 3) and marine invertebrates (4) have been reported to exhibit antihypercholesterolemic activity in test animals (5–7), as has β-sitosterol in humans (8).

In most studies on the sterol content of marine and other organisms, the identification has been made on the basis of GLC retention times (9), occasionally accompanied by mass spectrometry of the mixture as a whole or of the individual GLC peaks when a GLC–mass spectrometer combined unit was available (10, 11). Column chromatography has also been employed extensively to separate simple mixtures of sterols (12), and preparative GLC can be used to complete purification that has been partially achieved by column chromatography (13).

Occasionally, new compounds have appeared when the natural sterol mixtures were analyzed by GLC. It is then necessary to isolate the individual component to characterize it fully (14, 15). Many sterols can be readily isolated from mixtures by preparative TLC in sufficient quantities to obtain NMR spectra and melting points as well as mass spectra (16). These physical data are necessary to give positive identification of unknown as well as known compounds (14, 15).

The preparative TLC methods used are simple techniques that can be employed along with GLC in analytical screening of algae as well as other biological material to determine sterol, triterpenoid, or other natural product content. These or other similar TLC methods have been in use for some time by workers studying sterol metabolism (17).

In the present work, the dried algae were extracted initially with a chloroform–methanol mixture to obtain the free sterols or their esters and subsequently with aqueous methanolic potassium hydroxide to obtain the water-soluble or bound sterols (18). In the two brown algae examined, the bound and unbound sterols were similar in composition and in approximately equal quantities, while the red algae showed the bound fraction to be present in approximately one-third the amount of free sterols. Isolation and separation were achieved completely by preparative

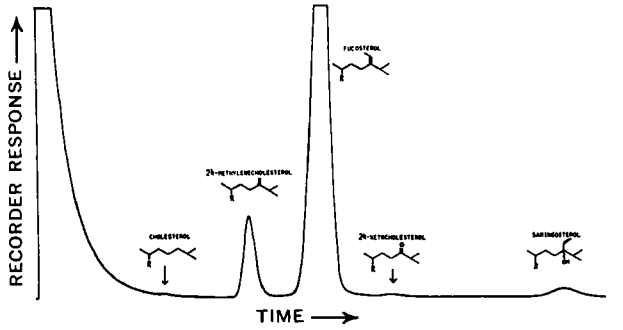

Figure 1—*GLC separation of* L. saccharina *sterols on 1.5% OV-17.*