RECENT STUDIES ON BIOACTIVE COMPOUNDS FROM PLANTS[1]

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Since 1974 we have been engaged in systematic isolation and characterization studies of bioactive compounds from tropical plants. The isolation is monitored by rapid in-house assays which currently consist of the following:

1) Insect antifeedant assays (1)
   Spruce bud-worm (*Choristoneura fumigerana*)
   Southern army-worm (*Spodeptera eridania*)
   Mexican bean beetle (*Epilachna varivestis*)
2) Helicocide (snail-killing assay)
   The South American snail *Biomphalaria pfeifferei* is used. This is the host for parasitic nematodes (schistosomes) which are responsible for schistosomiasis, a major tropical disease.
3) Plant growth regulatory assay with lettuce seeds, etc.

Most plants are collected on the basis of information gathered from literature survey and local folklore. The antifeedant portion of the project is an outgrowth of studies initiated by Dr. Isao Kubo[2] at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, a multi-disciplinary institute (where I was one of the research directors from 1969 to 1977). The plants dealt with in this article were collected by Isao Kubo in East Africa. More recently we are dealing with Mexican and North American plants.

The air-dried plant material is usually extracted with 40% aqueous methanol, the solvent is removed, and the residue is extracted successfully with hexane, ether, methanol and water. Each extract is submitted separately to the mentioned assays and the active principle(s) is(are) isolated. Since in most cases the compounds thus isolated, particularly the insect antifeedants, exhibit activities other than those monitored for, they are sent elsewhere for various further assays.

I. 4-HYDROXYISOXAZOLE (TRIUMFEROL), A SEED GERMINATION INHIBITOR (2). —This was isolated from the East African folk medicinal plant *Triumfetta rhombodea* (Triliaceae) following a lettuce seed antigermination assay. Triumferol, mp 67–68°, was obtained in 0.04% yield from the air-dried leaves (figs. 1 and 2). The physical constants given in the figures first led to the erroneous structure shown in square brackets. Except for the molecular weight, the novel dimeric structure would seem to satisfy all physical data. However, the sample was submitted to elementary analysis before an attempt was made to synthesize this unique heteroaromatic system, and to our surprise the results were completely off. It was noticed then that the sample vaporized very rapidly in the ms because of the low mp and that the nominal M^+ of 166 which we were observing (corresponding to dimeric structure $C_6H_6N_4O_2$) was due to a contaminant. A more careful high-resolution ms measurement after cooling of the sample gave the correct molecular formula.

A literature survey revealed that although 3,5-disubstituted 4-hydroxyisoxazoles had been prepared, the unsubstituted parent compound singularly was not known. The reason soon became clear when we embarked on its synthesis.

[1]Presented as a plenary lecture at the joint meeting of the American Society of Pharmacognosy and the Society for Economic Botany, Boston, Massachusetts, July 13–17, 1981.

[2]Dr. I. Kubo was at ICIPE as a Senior Research Scientist for the period of 1975/76. After spending 2 years at Columbia University, he joined the Division of Entomology and Parasitology, University of California, Berkeley, California, where he is currently an associate professor.

Seed germination inhibitor

Triumpheta rhomboidea bark

1) aq. MeOH (5.97g)
2) Et_2O (500mg)
3) SiO_2 column
4) Flash chromatography
Hexane/EtOAc (7/3)
or CH_2Cl_2/EtOAc (5/1)

210mg mp 67–68°

IR (CH_2Cl_2): 3560(sh.), 3180(br.) conc. dependent.
1640, 1625, 1410 arom.
1250 C–O stretch.
1090, 1000 arom. C–H bend.

UV (MeOH): 240 (ε 1,100) pKa: 7.75 (H_2O)
270nm in base.

FIG. 1. Extraction and physical properties of triumferol.

$C_3H_3NO_2$ 85.0163 (calcd. 85.0163)
58.0052 (M^+ – HCN calcd. 58.0035)

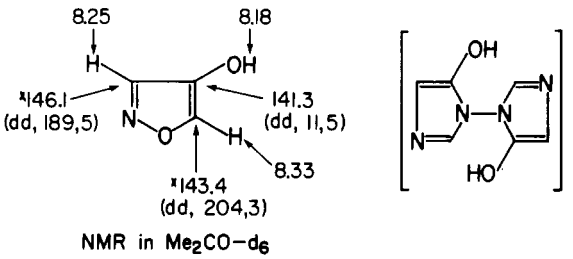


FIG. 2. Nmr data and structure of triumferol. The initial erroneous working structure is shown in square brackets.

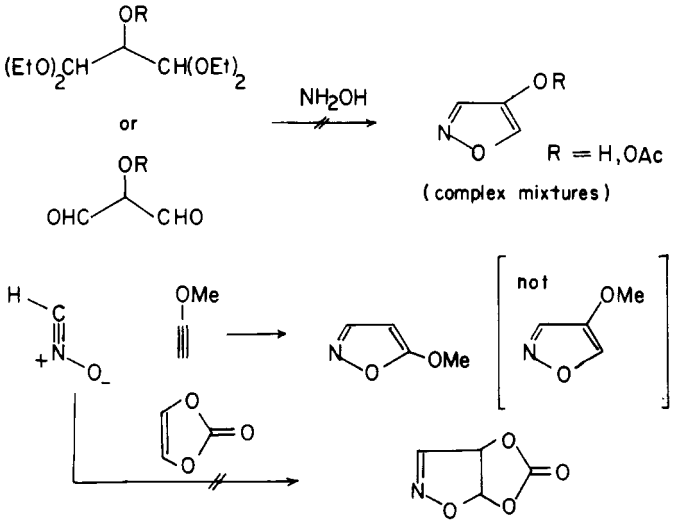


FIG. 3. Synthetic schemes for triumferol.

Namely, in our hands, the reported synthetic routes, including various modifications of the common scheme shown in figure 3 (upper half, most conventional route), reactions of 2-acetoxy-1,3-dienes and equivalents with hydroxylamine, metallation of 4-bromoisoxazole, diazo reactions of 4-amino-isoxazole, 1,3-dipolar addition to fulminic acid to vinylidene carbonate or to methoxy-acetylene (fig. 3, lower half) all failed to give the desired product. At this stage it was noticed that Baeyer-Villiger oxidation of the *tert*-alcohol shown in fig. 4 gave a trace of triumferol. A careful check of oxidation conditions and an improved synthesis of the starting 4-isoxazole carboxylic acid finally led to a satisfactory two-step synthesis in 70% yield of this elusive compound. The reason that we did not give up the synthesis of this simple molecule was that it was known to exist and to be reasonably stable.

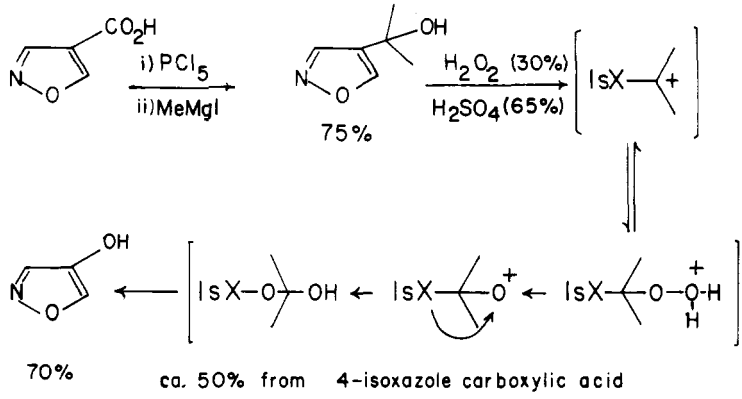


FIG. 4. Synthesis of 4-hydroxyisoxazole (triumferol).

II. TRICHILIN, A POTENT INSECT ANTIFEEDANT (3).—The common East African tree *Trichila roka* (Meliaceae) has yielded four antifeedants which together with azadirachtin (4) isolated from the Indian neem tree is the most potent and widely active of the various antifeedants we have isolated so far.

Although the isolation scheme summarized in fig. 5 is seemingly short and trivial, that was by no means the case. Although bioactive principles may be

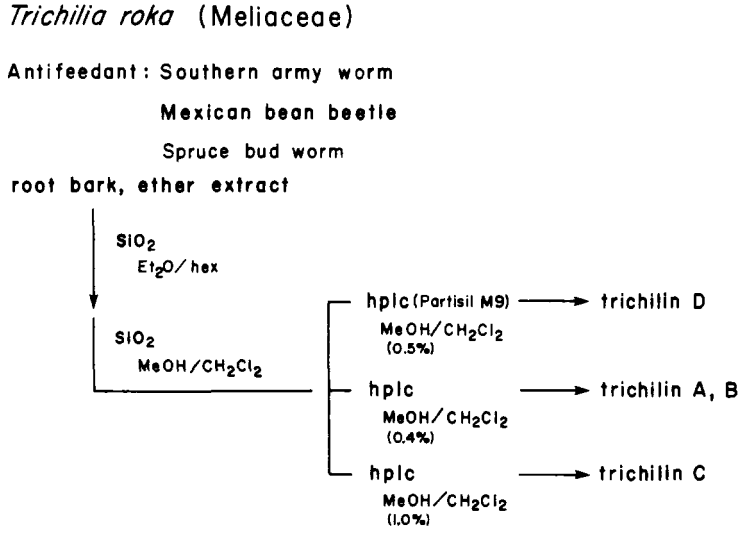
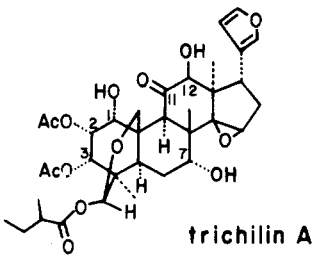


FIG. 5. Isolation and separation of trichilin congeners.

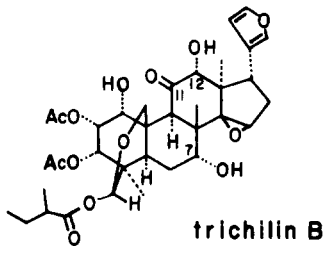
active as a crude mixture, no precise chemical studies or applications can be initiated until the compounds are isolated in pure form. Structural studies employing the most sophisticated recent techniques in spectroscopy are appealing and apparently intriguing; this is in contrast to, for example, subtle variations in solvent compositions and patient fractionations in an isolation scheme which in most instances are of no interest to others because they are only applicable to individual cases. They usually have no general appeal, and the audience may well snooze while the slides are flashed for a minute or so. However, the isolation is the crucial and necessary step as numerous examples have shown in the past. A failure or success in natural products studies, especially those dealing with sub-mg quantities, is often decided by the isolation step. In the case of trichilins, two experienced scientists spent considerable time in separating the congeners until success was achieved by Dr. M. Nakatani.

The four trichilins are new limonoids of complex structures (fig. 6), and a commercially practical synthesis is out of the question. Nevertheless, the crude extracts can readily be secured and it has since been found that they are present in other common Meliaceae. Thus, similar to the case of azadirachtin (5), which has a most complex structure, it may be worthwhile to consider the utility of the crude mixture.



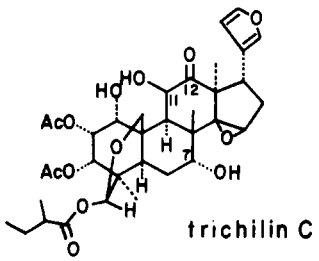
trichilin A

UV(MeOH) 213(ε 4,050)
CD(MeOH) 213(Δε +2.6), 304(Δε −3.7)



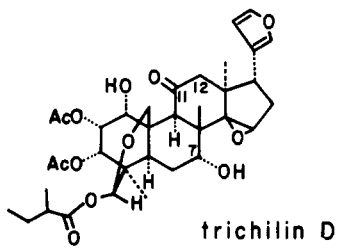
trichilin B

UV(MeOH) 209(ε 4,600)
CD(MeOH) 217(Δε +1.2), 306(Δε −1.9)



trichilin C

UV(MeOH) 214(ε 4,400),
CD(MeOH) 212(Δε +3.2), 292(Δε −1.0)



trichilin D

UV(MeOH) 215(ε 2,800)
CD(MeOH) 228(Δε +0.4), 298(Δε −3.1)

FIG. 6. The trichilins.

Structural studies involved the first application of the powerful technique of 2-dimensional J spectroscopy in ^{1}H-nmr (fig. 7), which clarified the coupling pattern of all pertinent protons. For example, three proton signals, 6β-H, 3'-Ha (proton in side-chain ester) and 16α-H, all overlap between 1.7–2.0 ppm in the ordinary 1-dimensional spectrum (fig. 7A), but the 6β-H and 3'-Ha peaks are resolved in trace D so that the J values could be measured. The 16α-H at 1.75 ppm indicated by the arrow in trace C was obscured by the more intense 3'-Ha, but it was feasible to estimate its chemical shift and J values (dd, J = 19 and 4 Hz) from

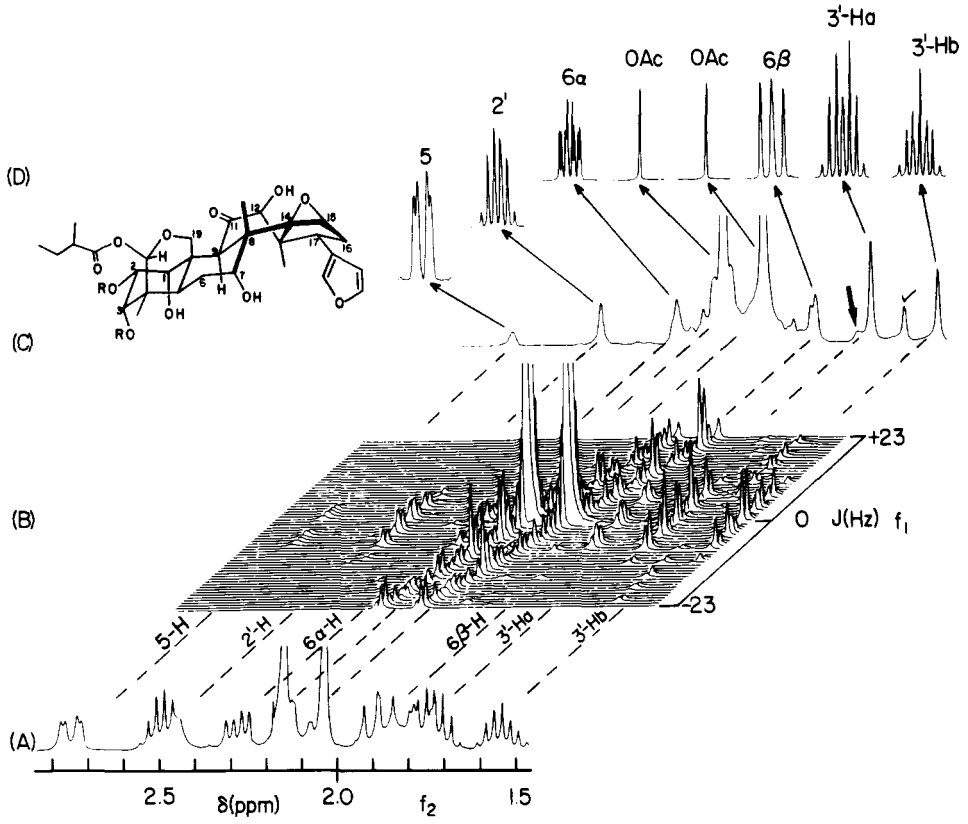


FIG. 7. Partial spectra of trichilin A measured in $CDCl_3$, 300 MHz. (A) Conventional nmr, (B) 2D–J spectrum (absolute value stacked plot, 64 traces shown), (C) "proton-decoupled-proton" spectrum from a projection of (B) into the horizontal axis, and (D) J spectra (cross sections of the stacked plot). Short thick arrow in trace C is the 16α-H signal (see text).

examination of a separate J spectrum; the 16β-H peak around 2.15 ppm could not be seen due to overlap of the intense acetate signal. The 2D-J spectrum of 6α-H is also resolved in trace D so that the J values can be measured, 2.28 ppm (dd, J = 14 and 4 Hz). It was gratifying to see the full structures of the trichilins confirmed when treatment of trichilin B with $Zn(BH_4)_2$ gave aphanastatin as a result of an unexpected migration of the acyl group from 2-OH to 1-OH. Aphanastatin was isolated by Polonsky and co-workers (6) as a cytotoxic agent, and the structure was determined by single-crystal X-ray analysis. Aphanastatin also turned out to be an extremely potent antifeedant of the spruce bud-worm (7). This is a further example of the frequently encountered parallel manifestation of insect antifeedant activity and tumor-inhibiting antileukemic activities (1,5c,8).

A preliminary structure-activity investigation of trichilins and derivatives showed some rather clear-cut results (fig. 8) (9). Namely, the substitution pattern on ring A appears to have little effect because the activity level against the Southern army-worm was similar for compounds in which R was OH or OAc and R' was H, OH or OAc. The 12α-OH was the most active followed by the 12β-OH analog; a 7-ene function destroys the activity.

III. 12β-ACETOXYHARRISON IN SOUTHERN ARMY-WORM ANTIFEEDANT (10).—The shrub *Harrisonia abyssinica* Oliv. (Simarubaceae) had previously yielded harrisonin, which is an African army-worm (*S. exempta*) antifeedant and which also exhibits antimicrobial, cytotoxic and plant-growth inhibitory activities (fig. 9)

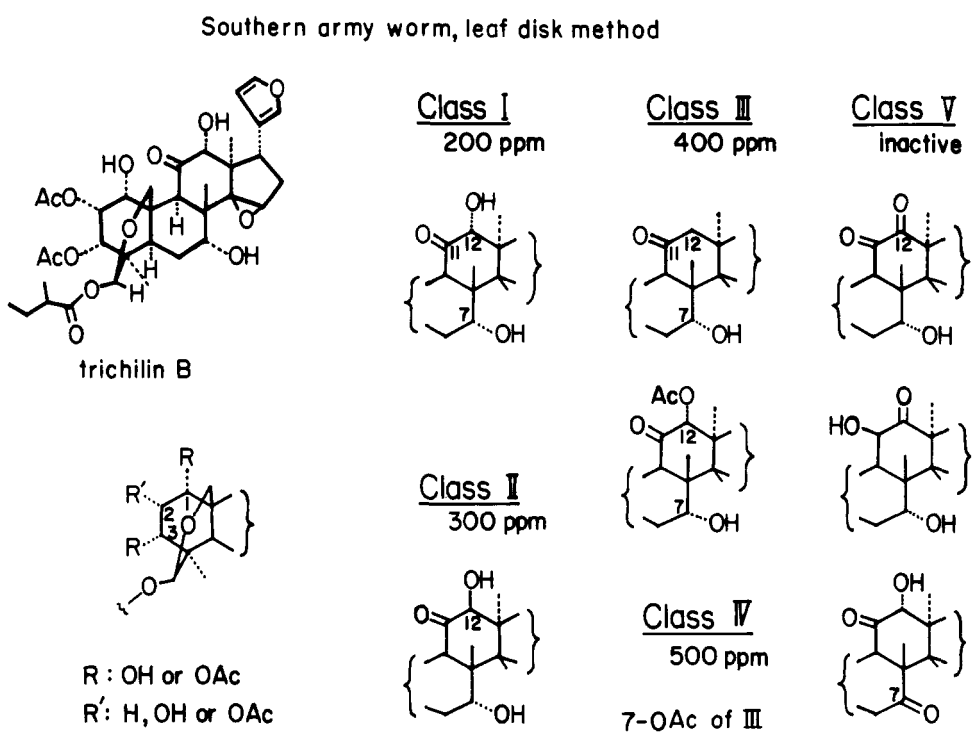
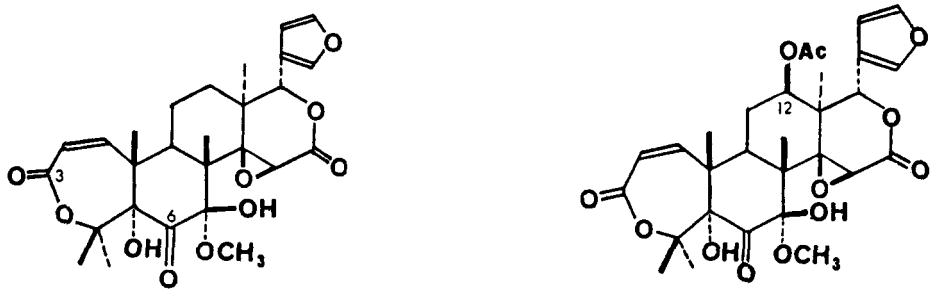


FIG. 8. Structure-activity correlation of trichilins and derivatives.

(11). Monitoring the fractionation of the ether extract of the air-dried root against the Southern army-worm led to the isolation of the 12β-acetoxy derivative (fig. 9). Since the entire extraction procedure was carried out without employment of methanol, the unusual 6-keto-7-hemiketal moiety is present as such in the plant and is not an artifact.

Harrisonia abyssinica (Simaroubaceae)

insect antifeedant



Heterocycles, 5 485 (1976)

FIG. 9. Obacunone and the two harrisonins.

Obacunone (see fig. 10 for structure), which coexists with the other two harrisonins, has no antifeedant activity. This may be due to the presence of the 7-ene function (see end of Section II, trichilin, above). However, another possibility is different ring A conformations between the inactive obacunone and the active harrisonins which may be related to attachment to binding sites in the taste buds, i.e., sensilla. Thus, analysis of the cd curves in the 220–260 nm region, where the Cotton effects due to the ring A ene-lactone appear, indicate that the signs of the 226–228 nm $\pi\pi^*$ and 250–257 nm $n\pi^*$ Cotton effects are opposite between obscunone and the two harrisonins (fig. 10). The sign of the $n\pi^*$ bands of ene-

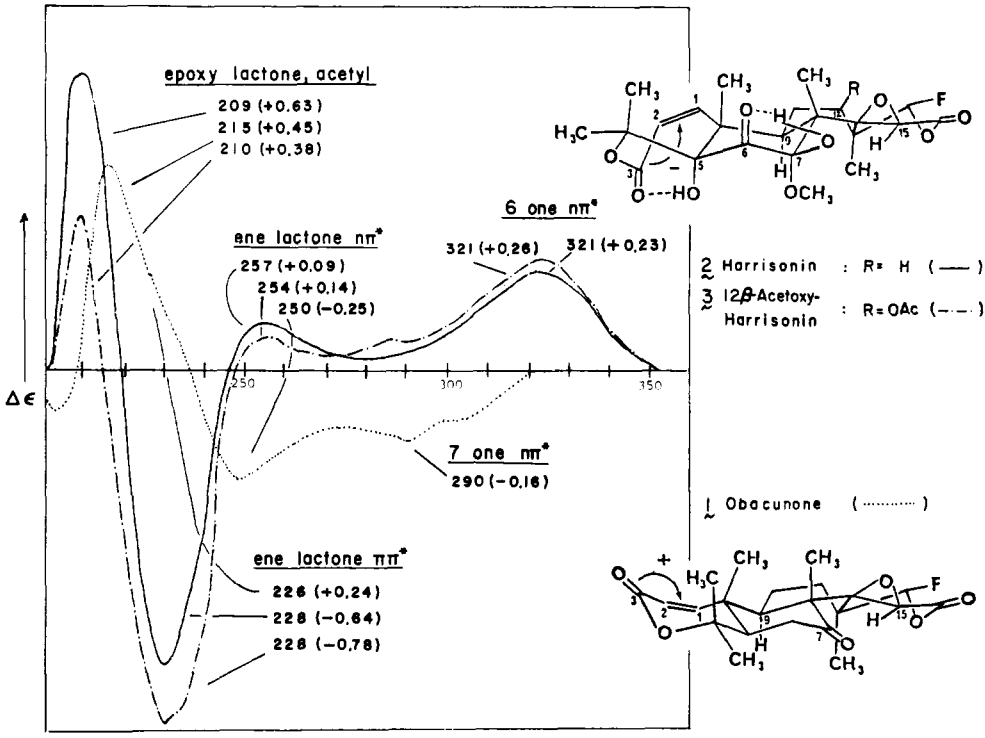


FIG. 10. Cd data, in MeOH. Intensities in parentheses are expressed in $\Delta\epsilon$.

lactones has been correlated with the double bond/carbonyl group chirality, i.e., the cd sign and chirality are opposite (12). The positive $n\pi^*$ bands of harrisonins suggest the chirality to be counter-clockwise, whereas the negative band of obacunone suggests the twist to be clockwise. The ir ($CHCl_3$) OH bands of 12β -acetoxyharrisonin are at 3480 and 3400 cm^{-1} and show that they are involved in intramolecular H-bonding. These data led to ring A conformations depicted in fig. 10.

IV. A COMMENT ON WARBURGANAL. Warburganal (13), a relatively simple sesquiterpene, is a potent antifeedant against the African army-worm but has little or no activity against other insects. It is also one of the most potent molluscicides and has a broad activity spectrum. Hence, as a defense substance for an African plant, it must be very effective. However, the broad spectrum, especially the strong cytotoxicity and hemolytic properties (upon injection), probably imposes serious restrictions on its use.

Biological Activity of Warburganal		
Insect antifeedant "choice test"		
Spodoptera exempta (oligophagous)		0.1 ppm
S. littoralis (polyphagous)		10 ppm
Schistocerca gregaria (polyphagous) and *Locusta migratoria* (gramnivorous)		
85–90% inhibition at 0.01% dry weight of fiber disc containing 5% sucrose		
[Dr. E. A. Bernays, COPR, London]		
weakly active or nonactive against		
S. eridania	*Epilachna varivestis*	
Schistocerca vaga	*Manduca sexta*	
Antimicrobial		
Saccharomyces cerevisiae		12.5 μg/ml
Candida utilis		3.1
Sclerotinia libertiana		50
Mucor mucedo		100
Molluscicidal		
Biompharis glabratus		5 ppm–2 hrs
B. pfeifferi		5 ppm–2 hrs
Lymnaca natalensis		10 ppm–2 hrs
Cytotoxicity		
KB test		0.01 μg/ml
Acute toxicity		
subcutaneous injection in mice, LD_{50} 20.4 mg/kg		

One aspect which is not clear is the fact that the bark of the tree *Warburgia ugandensis* from which warburganal was extracted is used popularly as a favorite hot spice by East Africans and, therefore, oral toxicity should not be pronounced (at least not acute toxicity). It is also known that the widely used insecticide pyrethroids are highly hemolylic when injected into animals.

V. CONSTITUENTS OF *Erythrina abyssinica* (LEGUMINOSAE) (14).—The ether extract of this East African medicinal plant has yielded, in addition to the two well-known phytoallexins, phaseollin and phaseollidin (pterocarpans), two ptero-

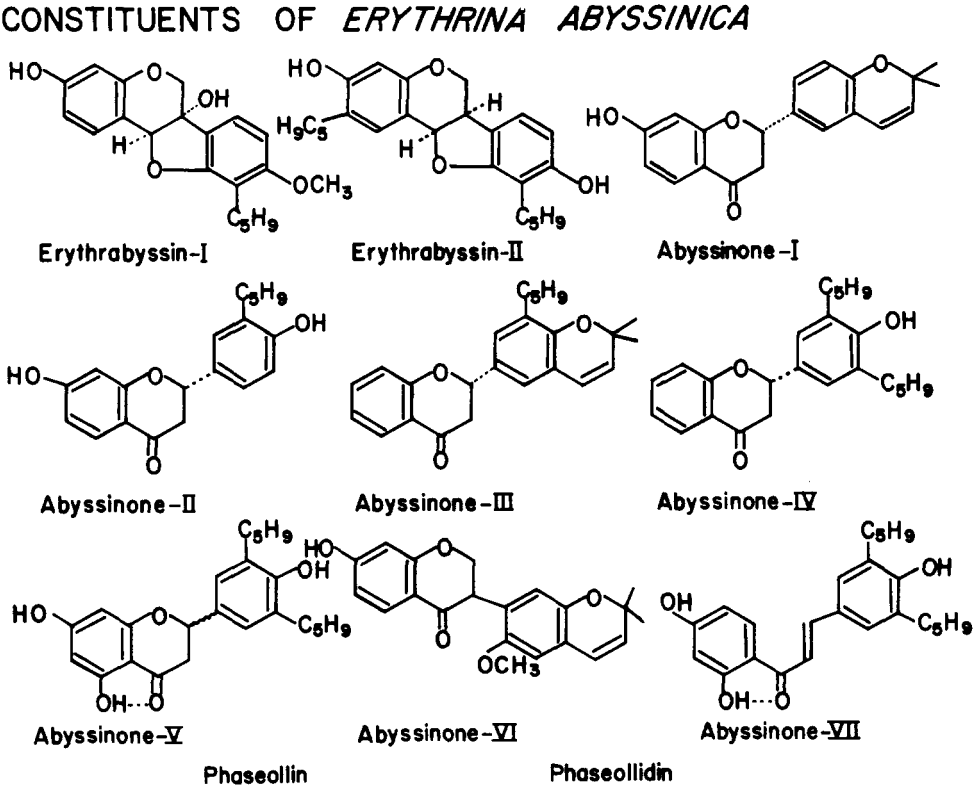


FIG. 11. New Antimicrobial Natural Phenolics from *E. absyssinica*.

carpans, five flavanones and one chalcone, all of which are new natural products (fig. 11). Some of them, particularly erythroabyssin-I and phaseollin, showed noteworthy antiyeast (25–50 μg/ml MIC *Saccharomyces cerevisiae, Candida utilis*) and antifungal activities (6–25 μg/ml MIC, *Sclerotinia lebertiana, Mucor mucedo, Rhizopus chinensis*). The so-called plant phenolics may be uninteresting from a structural viewpoint, but in terms of biological activity they indeed represent a promising and important compound class.

VI. BALANITINS, POTENT MOLLUSCICIDES (15,16).—*Balanites aegyptica* Del. (Balanitaceae), a popular East African folk medicinal plant, has given a group of molluscicidal saponins, balanitins-1, -2 and -3, which were efficiently separated by droplet counter-current chromatography (fig. 12) (17). In the following, the application of a micromethod for determining branching points in oligosaccharides based on cd is exemplified by balanitin-1.

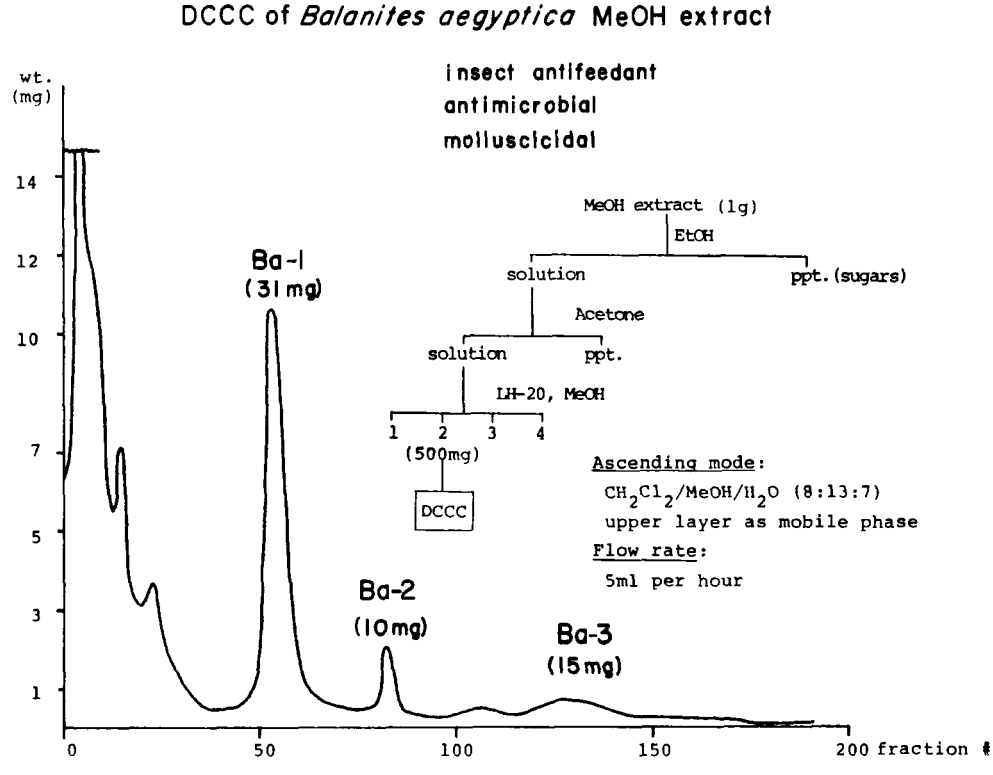


FIG. 12. Isolation scheme and droplet counter-current chromatograph (DCCC) trace of balanitins. The eluate from DCCC was fractionated into 3 ml aliquots of 200 fractions. Each fraction tube was evaporated to dryness and the residue weight was plotted against fraction number to give the chromatogram.

A recent systematic investigation of the cd of 50 pyranose di-, tri- and tetra-p-bromobenzoates has shown that:

(i) The A values, or difference in $\Delta\epsilon$ values between the extrema of split Cotton effects, depend solely on the spatial arrangements of the benzoate chromophores and are independent of the nonchromophoric substituents at other carbons; the A values shown in fig. 13 are, therefore, constants representative of the benzoate substituents.

(ii) There is an additivity relation in the case of tri- and tetrabenzoates, i.e., in the case of a molecule containing three interacting chromophores I, II

and III, the overall A value can be approximated by the summation of component A values: A≃A(I/II)+A(II/III)+A(III/I). For example, the A value of methyl-β-galactoside 2e,3e,4a-p-bromotetrabenzoate is +110, which is in good agreement with the sum of +62 (unit **1** in fig. 13; or 2e,3e)+16(unit **7**; 2e,4a)−4(unit **14**; 2e,6)+62(unit **3**; 3e,4a)−9(unit **13**; 3e,6)−15(unit **12**; 4a,6)=+112.

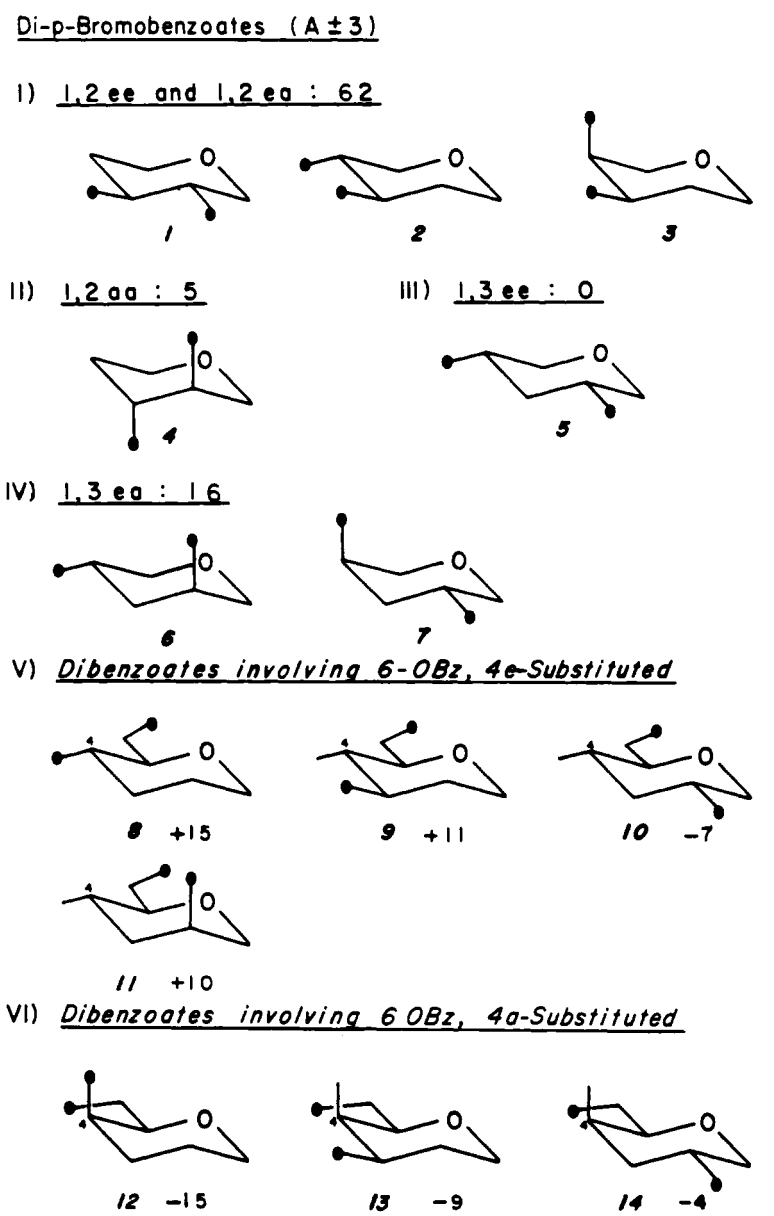


FIG. 13. The amplitudes ("A" values, in MeOH) of split cd curves of hexopyranose di-p-bromobenzoates (denoted by black dots). The signs of A values are dependent on chiralities of benzoate groups. The anomeric carbon is normally α-OMe but occasionally β-OMe; the "unsubstituted" nonanomeric carbons are either CH_2 or substituted with nonchromophoric groups such as OH, OAc, OMe, Me, etc. However, in the 6-benzoates, units **9-11/13/14**, the nonbenzoate substituents at C-4 are shown by eq or ax lines because the conformation of the 6-benzoate group *is* affected by the C-4 substituent.

The saponin (or oligosaccharide) is submitted to methanolysis with MeOH/HCl, and the mixture of methyl glycosides is per-p-bromobenzoylated and separated by hplc; this allows detection and characterization of various hexapyranoses at the level of 10 nanograms (as methyl glycoside per-p-bromobenzoates) (19). After identification of the sugar, the saponin, i.e., balanitin-1, 100 μg or 0.01 μmole, was permethylated with CH_3I/DMSO/NaH and methanolyzed, the solvent was removed and the residue was per-p-bromobenzoylated (fig. 14). The two uv-

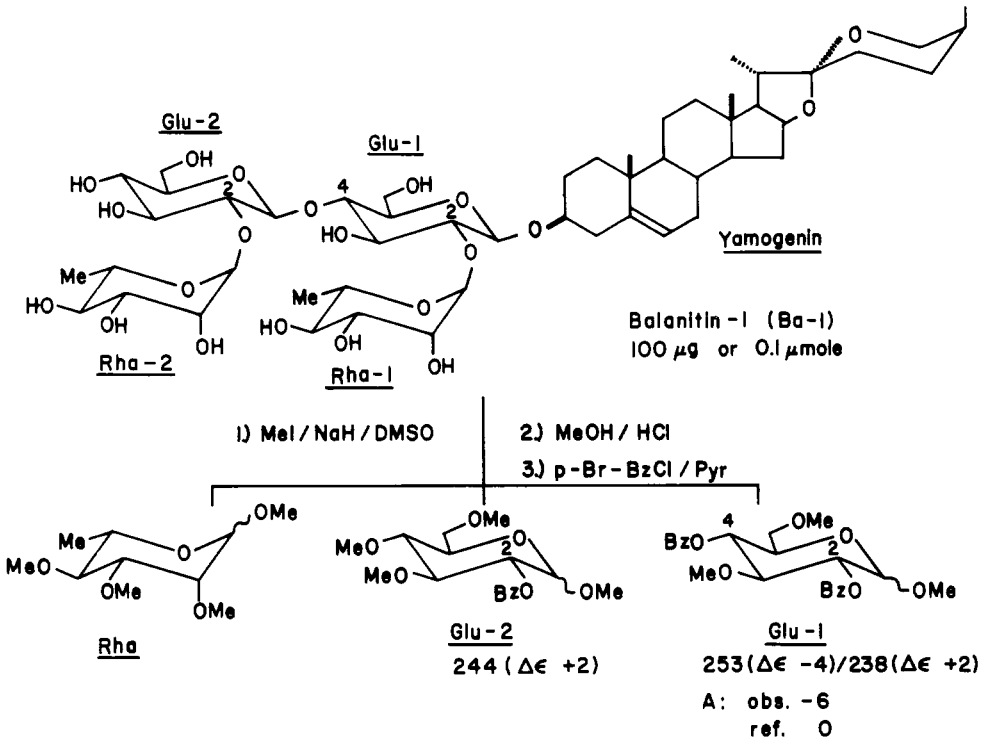


FIG. 14. Microscale determination of glycosidic linkage at branching point in balanitin-1.

absorbing products were separated by tlc; only the uv-absorbing species, i.e., the mono- and dibenzoates, need be collected since all terminal units become permethylated methyl glycosides and hence are "uv transparent" on tlc. The chemical ionization ms (CH_4 carrier gas) of the two spots indicated that they were a mono- and a dibenzoate. The amount of respective benzoates in the uv/cd cells can be estimated from the standard uv ϵ values at 244.5 nm[3] without weighing of samples, and from this the cd $\Delta\epsilon$ or A values can be obtained. The A values for the exciton-split cd curve of the dibenzoate at 253 nm/238 nm was -6, which compares well with the standard value 0 for unit **5** in fig. 13. The branching points in one of the glucose units, Glu-1, are thus at C–2 and C–4 (or 1,3-ee).

Methods such as those exemplified above constitute the basis for micromethods to determine oligosaccharide structures of glycoproteins, etc., which are currently under investigation.

ACKNOWLEDGEMENT

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[3]Standard ϵ values of para-brombenzoates (in MeOH): mono- 19,500, di- 38,200, tri- 57,200, and tetra- 76,400 (18).

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