SEARCHING FOR BIOACTIVE COMPOUNDS: VARIOUS STRATEGIES[1]

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ABSTRACT.—The Phytochemistry Research Laboratories of the University of Strathclyde have, as a primary goal, the generation of novel lead compounds from natural sources. In this lecture a number of recent studies with which we have been involved are reviewed. Each study reflects a different strategy that has led to the identification of compounds or extracts with interesting biological activity.

Recently in a major initiative the University of Strathclyde has established the Strathclyde Institute for Drug Research (SIDR). This new organization links pharmacists, pharmacologists, chemists, and biologists in a multidisciplinary research team capable of identifying and carrying out the early stages of research and development on bioactive compounds of pharmaceutical and agrochemical value. Within SIDR the Phytochemistry Research Laboratories (PRL) are viewed as a major source of novel lead compounds and the center for qualitative and quantitative analysis of xenobiotics. Not surprisingly the "generator" role SIDR has assigned to PRL has made us think long and hard about how best to find new bioactive compounds. The following examples, all of which were wholly or partly carried out at Strathclyde, have been selected to illustrate the diverse routes which have led us to extracts and compounds with biological activity.

THE ETHNOBOTANICAL ROUTE: AN ANTIBACTERIAL DITERPENE FROM *PREMNA SCHIMPERI*.—In 1987, a young Ethiopian biologist, Solomon Habtemariam, came to work with us. He brought with him the leaves of an Ethiopian species, *Premna schimperi* Engl. (Verbenaceae), the sap of which is used widely in the southern part of that country to treat cuts and skin infections. A preliminary screening of an EtOH extract confirmed significant antibacterial activity. Separation of a single active component was achieved by bioassay-guided vacuum liquid chromatography followed by cc of the active fraction (1).

The identification of the isolate as 12-oxo-10β,17α,19α,20α-cleroda-3,13(16)-dien-15-oic acid [1] rests on a full analysis of nmr and eims data. A major feature of the eims was the occurrence of the ions m/z 113 $[C_5H_5O_3]^+$, due to fission of the C-11/C-12 bond, and m/z 190 and 175 for the decalin nucleus (C-9/C-11 fission) and subsequent loss of a methyl radical. The isolated C-11 and C-14 methylene protons were observed in the ^{1}H-nmr spectrum (Figure 1) as an AB quartet and a singlet, respectively; the olefinic H-3 proton showed the expected long-range interaction with the vinylic 18-methyl (a on Figure 1), and the exomethylene protons were deshielded by the occurrence of a carbonyl on the β carbon. The relative stereochemistry was established by a series of difference nOe experiments (Figure 2), and absolute stereochemistry followed from the levorotatory nature of **1**.

Diterpene **1** is limited in its range of activity, which appears to be restricted to Gram-positive bacteria. However, against a number of important infective organisms such as *Staphylococcus aureus* its activity approaches that of streptomycin (Table 1).

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[1]Presented as a plenary lecture at the "Natural Products and the Disease Condition" Symposium of the Research Congress on Natural Products and the 30th Annual Meeting of the American Society of Pharmacognosy, San Juan, Puerto Rico, August 6–10, 1989.
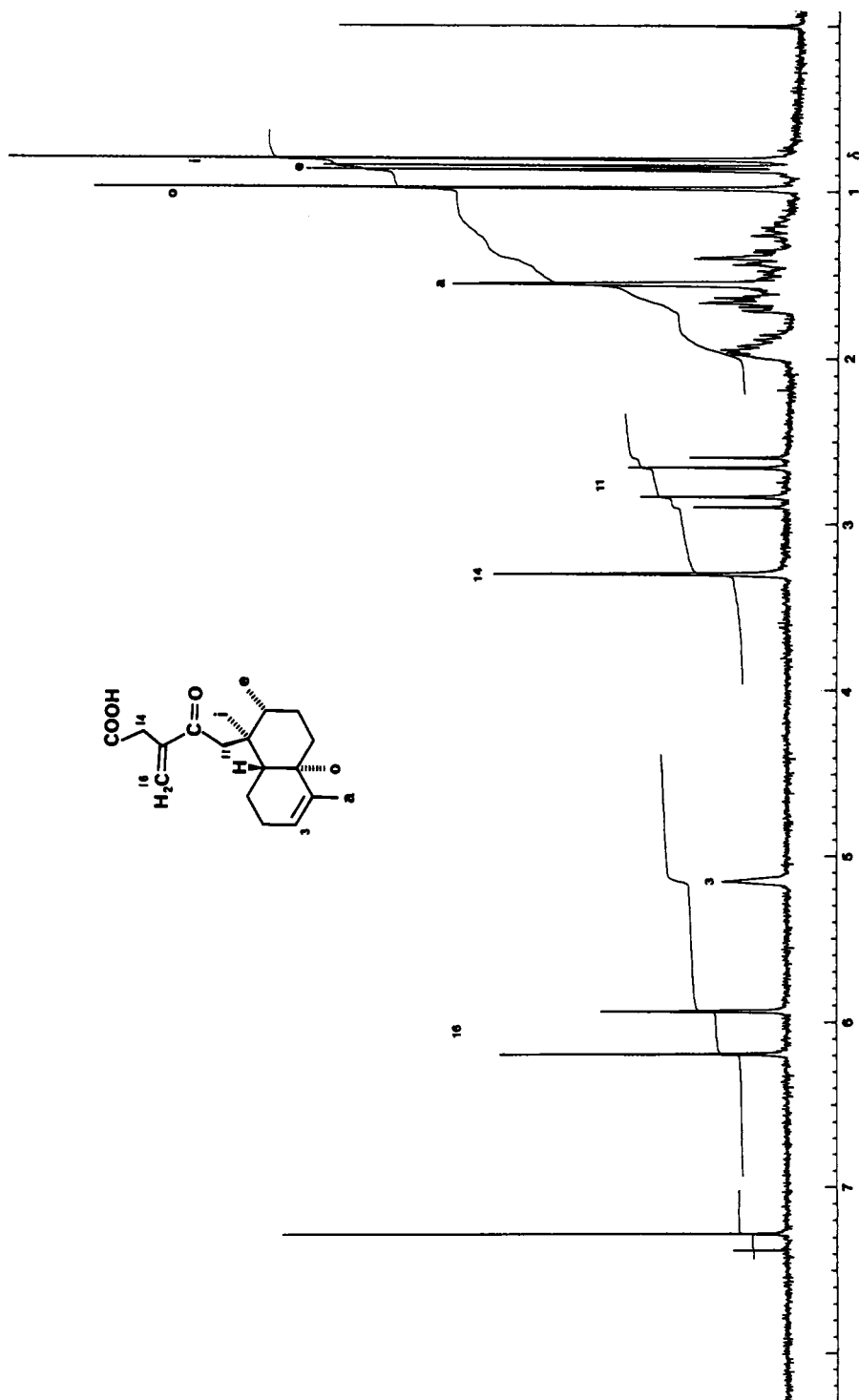
FIGURE 1. ^{1}H nmr spectrum of compound **1**, run at 250 MHz in CDCl$_3$.
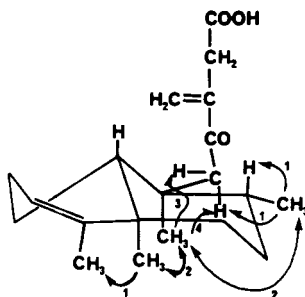
FIGURE 2.   NOe difference experiments on compound **1**, percentage enhancements.

While it is not a particularly exciting find, these results have verified ethnobotanical observations and thrown light on a new naturally occurring antibacterial.

BIOASSAY-GUIDED SERENDIPITY: YUEHCHUKENE.—The story of the isolation and characterization of the dimeric 3-prenylindole alkaloid yuehchukene [**2**-relative stereochemistry] has been recounted on a number of occasions (2–4). The compound was an unexpected product of the WHO-sponsored search for natural products that would control fertility; unexpected in the sense that the plant from which it was obtained, *Murraya paniculata* L. (Rutaceae), was not included in the ethnobotanically-driven selection of target plants but was tested in the participating laboratory in Hong Kong on the basis of a "hunch."

TABLE 1.   Minimum Inhibitory Concentration (MIC, µg/ml) of Compound **1** and Streptomycin Sulfate on *Staphylococcus aureus* and *Bacillus subtilis*.

Compound	MIC	
	S. aureus	*B. subtilis*
1	20.0	25.0
Streptomycin sulfate . . .	6.3	12.5

The identification of yuehchukene linked a very specific and sensitive (but time-consuming) bioassay, painstaking bioassay-guided separation by preparative hplc (Scheme 1), and what was then state-of-the-art nmr spectroscopy. During the phase in which identification took place there was never more than 5 mg of **2** available for analysis. This is just one of a number of examples where we have recently had success in unearthing compounds with significant bioactivity by running plant extracts more or less at random through specific screens; others, which confidentiality prevents me from dis-

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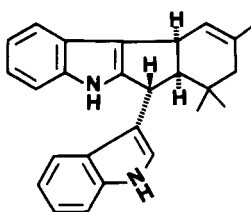
1. Cold extraction of fresh roots with C_6H_6 for 24 h.
2. Cc of concentrated extract on Si gel (elution with C_6H_6 plus increasing amounts of EtOAc) ↦ 10 fractions, each bioassayed.
3. A single active fraction ↦ further fractionation by preparative hplc [silica 5μ, solvent C_6H_6-CH_2Cl_2-iPrOH (18:2:1)]. Each fraction bioassayed.
4. Isolation of **2** as single active compound, yield 20 ppm.

SCHEME 1.   Procedure for the isolation of **2** from the roots of *Murraya paniculata*.

cussing at present, include discrete phytochemicals with anti-inflammatory and anti-protozoal activity.

STRAIGHT SERENDIPITY.—It is probably true to say that the majority of new natural products reported today are still the result of inquisitive chemists working, if they are honest, without any realistic goal beyond isolation and characterization. That has certainly been true for my research group, and despite the changed circumstances it is likely to remain so. We find that the exhaustive analysis of a plant sample (for all types of natural product) remains one of the best training grounds for post-graduate students. In selecting plants for such work the prime imperative is that they be chemically interesting, not necessarily that they have known bioactivity. As a consequence of this there is a fairly constant trickle of new molecules coming from our laboratories about which, in terms of bioactivity, we know nothing.

A recent example of this is a study performed on the Costa Rican plant *Zuelania guidonia* Sm. (Flacourtiaceae) (5). The Flacourtiaceae is not very well defined chemically, but there has been a report of limonoids and furocoumarins (6), metabolites that we normally regard as typical of the order Rutales, a taxonomic grouping with which the Flacourtiaceae has few affinities. This excited our interest in *Z. guidonia*, particularly when preliminary tlc analysis indicated a large number of petroleum-ether-soluble compounds in the stem bark. Through a combination of vacuum liquid chromatography, cc, and finally centrifugal preparative tlc, 17 compounds were isolated pure, of which 14 proved to be novel diterpenes based on the clerodane nucleus.

The individual compound that was most exhaustively studied in structure elucidation was identified as **3**. The empirical formula of $C_{33}H_{40}O_8$ for **3**, made up of a diterpene plus cinnamoyl and two acetoxy esters, was arrived at by correlation of uv, ir, eims, and ^{13}C-nmr data (Table 2). The ^{1}H-nmr spectrum (Figure 3) confirmed the presence of the three esters and a further free hydroxyl and by extensive homonuclear 2D-COSY and individual decoupling experiments allowed resolution of the C-9 side-chain, -CH_2-CH=C(Me)-CH=CH_2 (*m/z* 81), the sequence -CH-CH_2-CH(OH)-CH=C-CH-O- for C-10 to C-18 (clarified by C-2 oxidation to yield **4** and acetylation to yield **5**, Scheme 2), and -CH(OR)-CH_2CH$_{ax}$(Me)- for C-6 to C-8. The occurrence of the acetal through oxidation of the 18 and 19 methyl groups was substantiated by the

TABLE 2.   Characteristics of Compound **3** Important in Structure Elucidation.

Optical rotation	+79° (*c* = 1.0, $CHCl_3$)
Uv nm	max at 275
Ir cm^{-1}	3400 (OH), 1750, 1710 (esters), 1640 (α,β-unsaturated ester)
Eims	Apparent [M]$^+$ 546 = $C_{33}H_{38}O_7$, *m/z* 131 [C_9H_4O − cinnamoyl ester], 81 [C_6H_9], 43 [acetate]
^{1}H nmr	see Figure 3
^{13}C nmr	5 × q, 3 × t, 16 × d, 8 × s 39 H attached to C (+OH)
Empirical formula	$C_{33}H_{40}O_8$
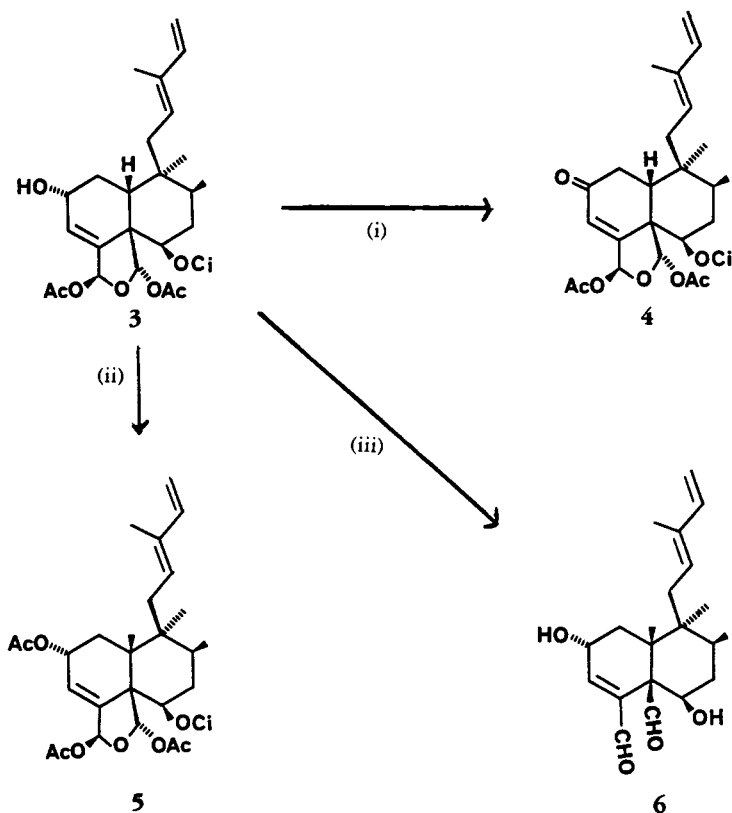
SCHEME 2.   Simple chemical modification of compound **3**: (i) Jones' Reagent;
(ii) Ac_2O in pyridine; (iii) saponification with 15% KOH in
EtOH.

highly deshielded ^{13}C-nmr resonances for C-18 (94.8 ppm) and C-19 (97.0 ppm) and
further confirmed by saponification to the clerodane 18,19-dial **6** (Scheme 2). The re-
maining problems relating to stereochemistry were resolved by extensive nOe investi-
gations (Figure 4).

When this work was approaching completion, similar diterpenes were reported
from two species of *Casearia* (7,8), a genus that is clearly allied to the Flacourtiaceae al-
though sometimes segregated in the family Samydaceae. It was noted (7) that **7**, from
C. sylvestris, was active against two cancer cell lines (e.g., LD_{50} 7.7 × 10^{-4} mmol/liter
against Chinese hamster lung cells). This prompted us to test **3** against tumor cell lines
available at Strathclyde. Although it showed some activity, results were disappointing
(ID_{50} ca. 35 μg/ml against ovarian small cell cancer). However, enough activity was
present to warrant some further investigation, and we are now beginning an extensive
analysis of ten of the *Z. guidonia* diterpenes, all of which have the oxygenation pattern of
3 but which differ in stereochemistry at C-2 and in the esterifying substituents at C-2
and C-6. This initial chance find has also been exploited in a promising search for simi-
lar compounds (see next section).

EXPLOITATION OF CHEMOTAXONOMIC KNOWLEDGE.—It is certainly true to
say that there is a degree of predictability in the distribution of natural products in na-
ture. Obviously, this may be used in the search for new sources of known compounds or
in trying to extend the range of structures of a given kind that are available for study.

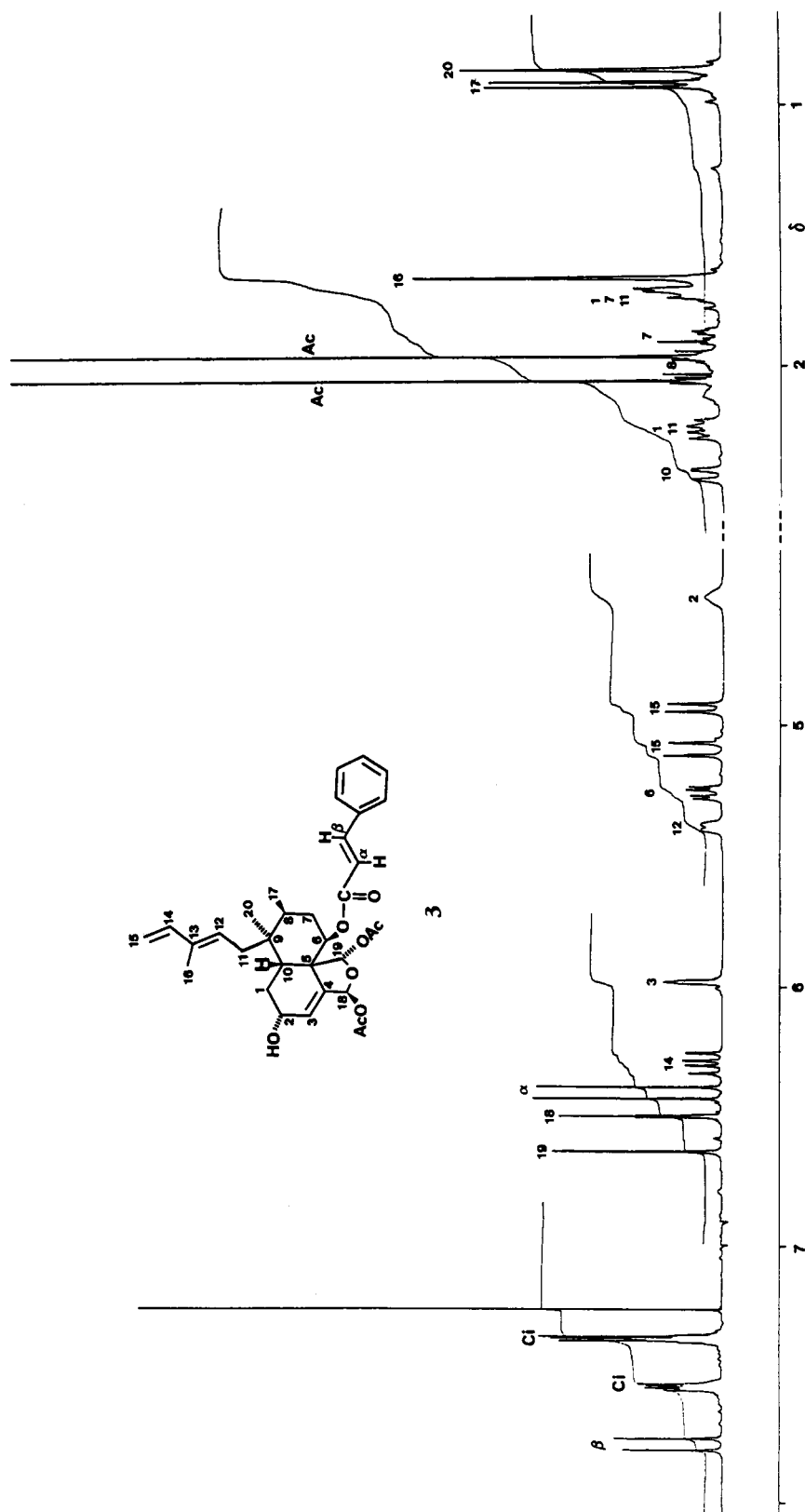The prerequisite for being able to exploit chemotaxonomic knowledge is to have rela-

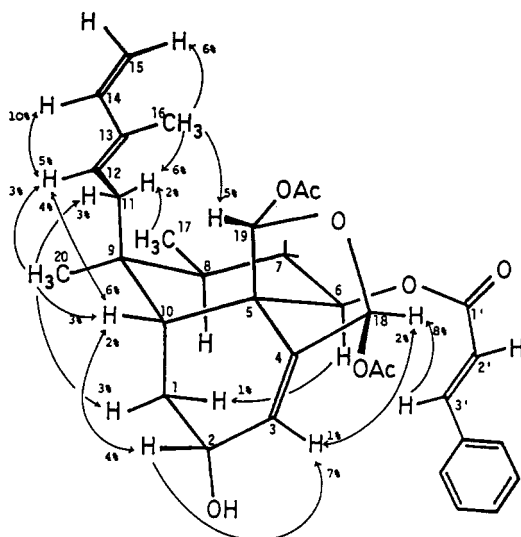FIGURE 3. ^{1}H-nmr spectrum of compound **3**, run at 360 MHz in CDCl$_3$.

FIGURE 4.   NOe difference experiments on compound **3**, percentage enhancements.

tively rapid access to plant species. Our own efforts make considerable use of the exten-sive research collaborations we have developed, notably in West Africa (9). This has, for example, made available to us through our colleagues at the Centre D'Etudes Plantes Medicinale in Cameroon as many as twelve further species of Flacourtiaceae. One of the first of these to be received shows more cytotoxic activity, as a crude petroleum ether ex-tract, than did pure **3**.

For a more well developed example I will return to the work on yuehchukene [**2**]. Subsequent to its isolation in low concentrations from *M. paniculata*, a systematic search of other species of the genus was undertaken (10). This revealed (Table 3) that **2** was restricted to only some species. A thorough analysis of these taxa showed that those containing **2** also contained an abundance of 8-prenylated coumarins (e.g., **8**), while those in which **2** was absent yielded many carbazoles (e.g., **9**). The dichotomy in al-kaloid biosynthesis appears to rest on the use of either 2-prenylindole (leading to car-bazoles) or 3-prenylindole (leading to **2**) as precursor, while 8-prenylcoumarin forma-tion appears to be centered in the **2**-forming group, without any alternative derivative being found in the rest of the genus. It was recognized (11) that this chemical division correlated perfectly with the proposal made by Tanaka, more than 50 years ago, that *Murraya* species formed two discrete taxonomic groups. Subsequently, an investigation of the volatile oils of the leaves of *Murraya* species (12) has revealed a corresponding dichotomy based on whether monoterpenes or sesquiterpenes predominate (Table 3).
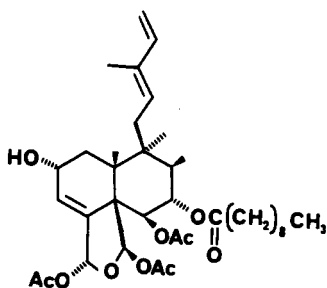
TABLE 3.   Distribution of Alkaloids, Coumarins, and Monoterpenes and Sesquiterpenes in Species of the Genus *Murraya* (10, 12).[a]

Species	Alkaloids[b]		8-Prenyl Coumarins	Terpenes	
	C	Y		C_{10}	C_{15}
Murraya alata	−	+	+	(+)	+
Murraya exotica	−	+	+	(+)	+
Murraya paniculata	−	+	+	(+)	+
Murraya glenei	?	?	+		
Murraya crenulata	+	−	−		
Murraya euchrestifolia	+	−	−		
Murraya koenigii	+	−	−	+	(+)
Murraya microphylla	+	−	−	+	(+)
Murraya siamensis	+	−	−		

  [a]+ = reported, − = looked for but not found, (+) = minor components of the volatile oil, ? = not yet investigated.
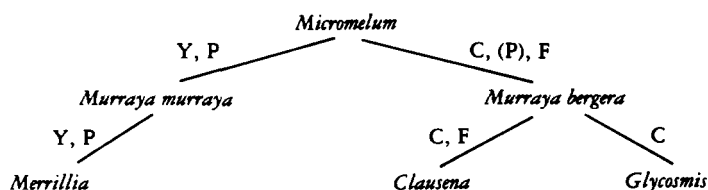 [b]C = carbazole alkaloids, Y = yuehchukene.

Murraya forms part of the tribe Clauseneae, a discrete group of the rutaceous sub-family Aurantioideae (Table 4). An examination of what was known about the chemistry of this group (13, 14) revealed that the monotypic genera *Merrillia* and *Micromelum* both produced 8-prenylcoumarins, while *Clausena* and *Glycosmis* were consistent and prolific producers of carbazoles but were not well known for 8-prenylcoumarins. In line with the chemotaxonomic prediction both *Merrillia* (15) and *Micromelum* (16) proved to

TABLE 4.   Taxonomy of the Rutaceae, Subfamily Aurantioideae, Tribe Clauseneae.

Subtribe	Genus
Micromelinae	*Micromelum*
Clauseninae	*Clausena*
	Glycosmis
	Murraya
Merrilliinae	*Merrillia*

be sources of **2**, although, as in *Murraya*, in very low concentration. In addition, *Micromelum* was also a source of carbazoles, making it the only taxon to produce yuehchukene, carbazoles, and 8-prenylcoumarins. Studies on several *Glycosmis* and *Clausena* species have so far confirmed the absence of **2** in these genera and the relative paucity of 8-prenylcoumarins which, in *Clausena*, seem to be replaced by 8-O-prenylfurocoumarins such as imperatorin [**10**]. As a result of these findings it seems appropriate to redefine the phylogeny of the Clauseneae as shown in Scheme 3.

SCHEME 3.   Suggested phylogeny of the genera of the Clauseneae based on the distribution of carbazole alkaloids (C), yuehchukene [**2**] (Y), 8-prenylcoumarins (P), and furocoumarins (F).
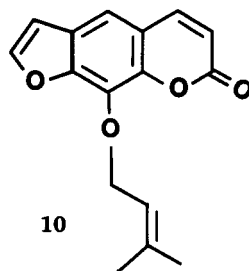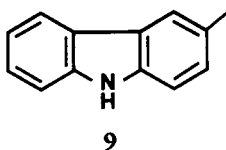
TAPPING A PLANT'S NATURAL DEFENSES.—Today it is widely held that the raison d'etre of many secondary metabolites is defending the producer against potential predators or pathogens, thus conferring some selective advantage. Study of this phenomenon can direct in a specific manner the search for compounds that will be of interest to the agrochemical industry or more generally as antibiotics. We have recently been studying the chemistry of the leaf of *Myrica gale* L. (Myricaceae), a shrub widespread on nutritionally poor wetlands in Scotland and which is notable for the relative absence of predation by herbivorous insects and lack of infestation by pathogenic fungi. For the last 3 years we have been analyzing the volatile oil and phenolic components of the leaf and attempting to correlate variation in composition with susceptibility to fungal necrosis that occurs as a result of damage to the leaf.

This has led to the isolation, using preparative hplc, of a new flavonoid that has proved to have considerable antifungal activity (MIC < 30 μg/ml against *Penicillium citrinum*, insufficient material yet available for accurate estimation). Where plants show enhanced resistance this compound **11** occurs at levels several times greater than in nonresistant plants. Its identification (17) as a kaempferol rhamnoside in which the rhamnose was further substituted by two acetoxy and one *p*-coumaroyl esters (at C-2, C-3, and C-4 of the sugar) followed from the ^{1}H-nmr spectrum and was confirmed by the fabms $[M + Na]^+$ 663. The problem of assigning the substitution positions of the esters was resolved by a heteronuclear long-range ^{1}H-^{13}C coupling experiment that indicated the placement of the *p*-coumaroyl ester at C-4 of the sugar and confirmed that the sugar was attached to the flavonol moiety through C-3.
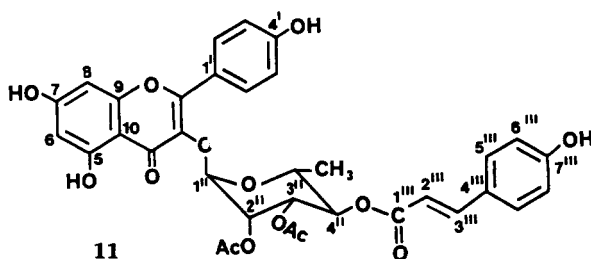
ACKNOWLEDGMENTS

LITERATURE CITED

1.   S. Habtemariam, A.I. Gray, G.W. Halbert, and P.G. Waterman, *Planta Med.*, in press.
2.   Y.C. Kong, K.F. Cheng, R.C. Cambie, and P.G. Waterman, *Chem. Commun.*, 47 (1985).

3.   Y.C. Kong, K.H. Ng, K.H. Wat, A. Wong, I.F. Saxena, K.F. Cheng, P.P.H. But, and H.T. Chang, *Planta Med.*, **304** (1985).

4.   P.G. Waterman, in: "Alkaloids. Chemical and Biological Perspectives." Ed. by S.W. Pelletier, Wiley Interscience, New York, 1986, p. 331.

5.   M.A. Khan, A.I. Gray, D. Reed, I.H. Sadler, and P.G. Waterman, *Phytochemistry*, in press.

6.   R. Hegnauer, "Chemotaxonomie der Pflanzen," Springer Verlag, Basel, 1988, Vol. 8, p. 487.

7.   H. Itokawa, N. Totsuka, K. Takeya, K. Watanabe, and E. Obata, *Chem. Pharm. Bull.*, **36**, 1585 (1988).

8.   E. Guittet, V. Stoven, J.-V. Lallemand, F. Ramiandrasoa, G. Kunesch, and C. Moretti, *Tetrahedron*, **44**, 2893 (1988).

9.   P.G. Waterman, *Phytochemistry*, **25**, 1 (1986).

10. Y.C. Kong, K.H. Ng, P.P.H. But, Q. Li, S.X. Yu, H.T. Zhang, K.F. Cheng, D.D. Soejarto, W.S. Kan, and P.G. Waterman, *J. Ethnopharmacol.*, **15**, 195 (1986).

11. P.P.H. But, Y.C. Kong, K.H. Ng, H.T. Chang, Q. Li, S.X. Yu, and P.G. Waterman, *Acta Phytotaxon. Sin.*, **24**, 186 (1986).

12. Q. Li, L.F. Zhu, P.P.H. But, Y.C. Kong, H.T. Chang, and P.G. Waterman, *Biochem. Syst. Ecol.*, **16**, 491 (1988).

13. I. Mester, in: "Chemistry and Chemical Taxonomy of the Rutales." Ed. by P.G. Waterman and M.F. Grundon, Academic Press, London, 1983, p. 31.

14. A.I. Gray, in: "Chemistry and Chemical Taxonomy of the Rutales." Ed. by P.G. Waterman and M.F. Grundon, Academic Press, London, 1983, p. 97.

15. Y.C. Kong, P.P.H. But, K.H. Ng, K.F. Cheng, K.L. Chang, K.M. Wong, A.I. Gray, and P.G. Waterman, *Biochem. Syst. Ecol.*, **16**, 47 (1988).

16. Y.C. Kong, P.P.H. But, K.H. Ng, Q. Li, K.F. Cheng, and P.G. Waterman, *Biochem. Syst. Ecol.*, **16**, 485 (1988).

17. R. Carlton, A.I. Gray, C. Lavaud, G. Massiot, and P.G. Waterman, *Phytochemistry*, in press.