The structures of phyllanthostatin 1 and phyllanthoside from the Central American tree *Phyllanthus acuminatus* Vahl[1]

GEORGE R. PETTIT,[2] GORDON M. CRAGG, DEVENS GUST, PETER BROWN,[3] AND JEAN M. SCHMIDT

Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, AZ 85287, U.S.A.

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A new antineoplastic glycoside, phyllanthostatin 1, and the related glycoside, phyllanthoside, have been isolated from the Central American tree, *Phyllanthus acuminatus* Vahl. The structures of phyllanthostatin (**1***a*) and phyllanthoside (**1***b*) were completely assigned by analyses of spectral data, principally by high resolution (400 MHz) nmr. In addition to exhibiting activity against the National Cancer Institute's P388 lymphocytic leukemia, phyllanthoside was found to possess a curative level of activity against the murine B16 melanoma.

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On a isolé d'un arbre de l'Amérique Centrale, le *Phyllanthus acuminatus* Vahl, un nouveau glycoside antinéoplastique, la phyllanthostatine 1, et le glycoside apparenté phyllanthoside. L'analyse des données spectrales obtenues principalement par la rmn à haute résolution (400 MHz) a permis d'établir complètement les structures de la phyllanthostatine (**1***a*) et du phyllanthoside (**1***b*). En plus de montrer une activité contre la leucémie lymphocytique P388 de l'Institut National du Cancer, on a trouvé que le phyllanthoside possède une certaine activité curative contre mélanoma B-16 de la murine.

[Traduit par le journal]

The Euphorbiaceae family is rich in plants with a long history of human medicinal applications (2, 3). The Euphorbiaceae genus *Phyllanthus* (about 600 species ranging from free floating aquatic forms to trees) contains several species that have been employed in the primitive treatment of cancer (4, 5). Because of the U.S. National Cancer Institute's (NCI) exploratory plant evaluation program, in collaboration with the U.S. Dept. of Agriculture (USDA), the roots of a tree believed[4] to be *P. brasiliensis* Muell were collected about a decade ago in Costa Rica. An ethanol extract of the original collection was found to inhibit growth of the NCI murine P388 lymphocytic leukemia (PS system), and the Kupchan group (6) isolated and partially characterized a PS active glycoside termed phyllanthoside from a 1974 re-collection. We now report the isolation of a new anticancer glycoside designated phyllanthostatin 1 (**1***a*, 52% life extension against the PS leukemia) and a substance spectroscopically identical to phyllanthoside (**1***b*)[5] from the roots of *P. acuminatus* Vahl,[4] re-collected (1978) in Costa Rica. Structure elucidation of these unusual 1 → 2 linked disaccharides (**1***a*, **1***b*) has been accomplished. Also we are pleased to note that phyllanthoside (**1***b*) has exhibited a curative level (at 8 mg/kg with an average 62–74% life extension over the dose range 4–16 mg/kg) of antineoplastic activity against the NCI murine B16 melanoma and is presently being considered for eventual human clinical trial.

The chipped roots (81.5 kg) of *P. acuminatus* were processed by a convenient new technique[6] for initial plant and animal extraction involving the use of methylene chloride – methanol (1:1) at ambient temperatures (7). After extraction enough water

[1]Antineoplastic Agents 73. For part 72 refer to ref. 1.

[2]Author to whom correspondence should be addressed.

[3]The study reported herein is dedicated to the memory of Professor P. Brown who passed away March, 1981.

[4]Later, information was uncovered by Drs. J. Duke and R. Spjut of the USDA indicating *P. brasiliensis* does not extend into Central America. A detailed study of the taxonomic problem by Dr. Spjut has revealed (private communication) that the plant collection provided for the original isolation of phyllanthoside was in actuality the very closely related Central American *P. acuminatus* Vahl. Thus the re-collections (about 150 kg of root from Costa Rica) of this plant, kindly provided by Dr. J. Duke and employed in the present investigation, have been identified as *P. acuminatus*.

[5]Unfortunately, no specimens of phyllanthoside or phyllanthocin remain from the original separations. Part of the large scientific and medical loss was due to Prof. M. Kupchan's untimely death in 1976.

[6]Developed by one of us (G.R.P.) with Drs. D. L. Doubek and D. L. Herald. Application of the safer (for personnel) methylene chloride (in place of chloroform) for most solvent partition and chromatographic procedures has been summarized with pertinent references as part of another study (7*a*).

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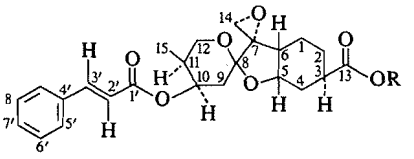
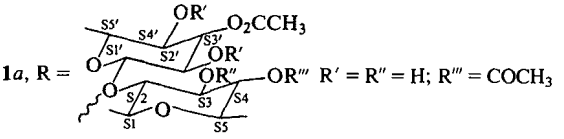




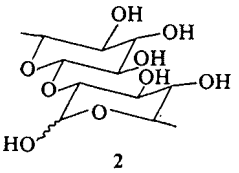
1*a*, R = OR' O_2CCH_3 OR' OR'' OR''' R′ = R″ = H; R‴ = $COCH_3$

b, **1***a*, R′ = R‴ = H; R″ = $COCH_3$

c, **1***a*, R′ = R″ = R‴ = $COCH_3$

d, R = CH_3

e, R = H



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was added to separate the methylene chloride phase. The latter fraction[7] (475 g) was partitioned using the sequence 9:1 → 4:1 → 3:2 methanol–water with ligroin → carbon tetrachloride → methylene chloride (7, 8). Combination of the carbon tetrachloride (44 g) and methylene chloride (210 g) fractions followed by gel permeation (Sephadex LH-20, methanol) and repeated silica gel (98:2:0.1 chloroform–methanol–water) column chromatography of a 38 g aliquot afforded 0.24 g of pure (by hplc, μ-Porasil column with 97:3.0:0.2 methylene chloride – methanol–water) phyllanthostatin 1 (**1***a*) as an amorphous solid, mp 125–126°C; $[\alpha]_D^{26}$ −3.6° (*c* 0.83, $CHCl_3$); λ_{max}(MeOH) (log ε): 216 (4.19), 222 (4.12), and 277 (4.29) nm; ir (KBr) ν_{max}: 3450, 1755, 1740, 1710, 1640, 1452, 1380, 1310, 1245, 1170, 1075, and 770 cm^{-1}; FD ms *m/e*: 805 (M^+ + H). Additional silica gel chromatography of PS active fractions yielded 0.6 g of pure (by hplc, as above) **1***b* as an amorphous solid with the following characteristics: mp 125–127°C; $[\alpha]_D^{22}$ +16.9° (*c* 0.71, $CHCl_3$); λ_{max}(MeOH) (log ε): 216 (4.25), 222 (4.19), and 277 (4.34) nm; ir (KBr) ν_{max}: 3475, 1750, 1735, 1710, 1640, 1452, 1380, 1311, 1253, 1173, 1080, and 770 cm^{-1}; FD ms *m/e*: 805 (M^+ + H). Comparison of spectral data with that recorded for authentic phyllanthoside (**1***b*) indicated that both are identical.[5]

The complete structures of phyllanthostatin 1 and phyllanthoside were deduced by interpreting the ^{13}C nmr (22.63 MHz, $CDCl_3$), ^1H nmr (400 MHz, $CDCl_3$), and EI high resolution and FD mass spectra.[8] Although the structure of glycoside **1***b* was not previously known, Kupchan *et al.* (6) reported structural studies of two degradation products which proved very useful in the present study. Methanolysis of glycoside **1***b* gave an aglycone (phyllanthocin (**1***d*) by X-ray crystallographic analysis) and a disaccharide, $C_{12}H_{22}O_9$. Acid hydrolysis of the disaccharide yielded 6-deoxy-D-glucose and spectral evidence suggested that the disaccharide portion contained two acetate groups.

Examination of the ^1H and ^{13}C nmr results for **1***a* and **1***b* indicated that these potent antineoplastic plant constituents have identical aglycones and differ only in the disaccharide portion. Both glycosides afforded identical peracetates (**1***c*, mp 122–126°C, $[\alpha]_D^{24}$ +26.3° (*c* 1.1, $CHCl_3$)) and were readily interconverted via an acetyl shift upon standing at room temperature for periods exceeding 24 h in 90% aqueous ethanol.[9] Thus, glycosides **1***a* and **1***b* differ only in the location of one acetyl group on the disaccharide unit and consist of aglycone **1***e* joined via an ester linkage to a diacetylated 6-deoxy-D-glucose disaccharide. In addition, the $^3J_{HH}$ coupling constants of ~8 Hz measured for both anomeric protons of glycosides **1***a* and **1***b* indicated β-linkages at the anomeric centers. At this point only location of the three 6-deoxy-D-glucose ester groups and position of the disaccharide linkage of **1***a* and **1***b* remained to be solved.

The ^1H nmr spectrum of phyllanthoside (**1***b*) exhibited two 6-deoxy-D-glucose ring proton resonances at δ 4.78 and 4.90 ppm. The ca. 1 ppm downfield shift of these resonances from the usual positions for such protons clearly indicated attachment to carbon atoms bearing ester groups (9). And ^1H nmr decoupling studies revealed that these two ester linkages were at S-3 and S-3′. The ^{13}C and ^1H nmr results showed that the third ester linkage was at S-1 (S-1: ^{13}C 92.06, ^1H 5.50, d, J = 8.1 Hz; S-1′: ^{13}C 103.84, ^1H 4.00, d, J = 7.8 Hz). Similar

[7]The NCI PS in vivo and in vitro lymphocytic leukemia bioassays were employed to guide each step of the separation.

[8]The spectral data were quite consistent with the assigned structures and will be summarized in a future complete report. Elemental analytical results were also consistent with structures **1***b* and *c*. The instrumental analyses were conducted using Varian-MAT 731, Bruker WH-90, and Bruker WH-400 instruments. We are pleased to thank Drs. R. R. Inners, P. D. Ellis, J. A. McCloskey, and J. Witschel, Jr. for expert assistance with these measurements.

[9]More polar products resulting from hydrolysis of the acetates are also formed. Similar interconversion occurs with solutions of the compounds in 0.9% saline containing 5% ethanol.

studies of phyllanthostatin (**1***a*) indicated that the ester linkages were at S-1, *S-4*, and S-3′. Because chemical (acetylation) evidence suggested that glycosides **1***a* and **1***b* differed only in the location of an acetate group, the ester at S-3 in phyllanthoside and S-4 in phyllanthostatin 1 must be acetate. Furthermore, treatment of phyllanthoside with cellulase in an acetate buffer (pH 5.0) yielded a monoacetyl derivative. Here ^{1}H nmr studies of this derivative showed that the S-3′ proton resonance was shifted upfield ca. 1 ppm, whereas resonances for the aglycone and the other glucose protons remained essentially unchanged. Accordingly, the second acetate group resides at S-3′ in both glycosides **1***a* and **1***b* and the aglycone must be linked to the disaccharide at S-1.

The final structural question concerned the nature of the disaccharide linkage. Because carbon atoms S-1 and S-3 in phyllanthoside (**1***b*) and S-1 and S-4 in phyllanthostatin (**1***a*) bear ester groups, the 6-deoxy-D-glucose units must, by elimination, be linked 1 → 2. This rather unusual linkage was confirmed by the following experiments. Methanolysis (0.1 *M* sodium methoxide in methanol) of phyllanthoside gave aglycone methyl ester **1***d* and disaccharide **2** (6), mp 217–219°C; $[\alpha]_D^{26}$ −3.3° (*c* 1.51, H_2O); ^{1}H nmr (100 MHz, D_2O) δ: 1.27 and 1.31 (6H, d, J = 6 Hz, 6,6′-CH_3), 3.06–4.12 (9H, m), 4.67 (d, J = 8 Hz), 5.4 (d, J = 4 Hz) ppm. Acetylation (acetic anhydride in pyridine) yielded a peracetate whose ^{1}H nmr spectrum featured two methyl group doublets at δ 1.18 and 1.22, acetate methyl resonances at 2.00–2.20, a three-proton multiplet at 3.40–4.05, a group of resonances from 4.55–5.6, and an α-anomeric proton ($^3J_{HH}$ = 3 Hz) at 6.29 ppm. The resonance at 6.29 ppm was assigned to S-1. The low-field resonances at 4.55–5.6 ppm were attributed to S-1′ plus the protons of the acetate-bearing ring carbon atoms which experienced an expected (9) downfield shift, relative to the parent disaccharide. The three protons appearing at 3.40–4.05 ppm correspond to S-5, S-5′, and the proton on the carbon involved in the anomeric linkage to the second sugar. Proton decoupling experiments showed resonances at 3.55 and 3.93 ppm which belong to S-5 and S-5′ and a doublet of doublets at 3.90 coupled to the resonance at 6.29 ppm which must arise from S-2. Therefore the anomeric linkage at S-2 was confirmed and the phyllanthostatin 1 (**1***a*) and phyllanthoside (**1***b*) structural assignments were completed.

We have also found that *P. acuminatus* contains a series of antineoplastic glycosides related to **1***a* and **1***b* and the structures of these new and potentially useful cancer chemotherapeutic agents are presently under investigation.

Acknowledgments

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