Phytochemistry, Vol. 28, No. 7, pp. 1827–1830, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

IDENTIFICATION OF PROANTHOCYANIDIN POLYMERS AS THE PISCICIDAL CONSTITUENTS OF *MAMMEA SIAMENSIS*, *POLYGONUM STAGNINUM* AND *DIOSPYROS DIEPENHORSTII*

FELIPE BALZA, ZYTA ABRAMOWSKI, G. H. NEIL TOWERS and PICHAET WIRIYACHITRA*

Botany Department, University of British Columbia, 6270 University Blvd., Vancouver, B.C., Canada, V6T 2B1; *Faculty of Pharmacy, Chiang Mai University, Chiang Mai 5002, Thailand

(*Received* 29 *September* 1988)

Key Word Index—*Mammea siamensis*; Guttiferae; *Polygonum stagninum*; Polygonaceae; *Diospyros diepenhorstii*; Ebenaceae; proanthocyanidins; piscicidal; molluscicidal; species from Thailand.

Abstract—Phytochemical investigations of piscicidal and molluscicidal plant species of Thailand has led to the isolation and characterization of proanthocyanidin polymers as the active compounds in the leaves of *Mammea siamensis*, *Polygonum stagninum* and *Diospyros diepenhorstii*. *Mammea* furnished a proanthocyanidin consisting exclusively of flavan 3-ol units with 2,3-*cis* stereochemistry and a number-average M_r of *ca* 2 100. *Polygonum* yielded a 2,3-*cis* procyanidin and *Diospyros* a 2,3-*cis* prodelphinidin polymer with a proportion of the monomeric units bearing a gallate group at C-3. Structural evidence for these compounds was obtained from ^{13}C NMR and IR analyses. Data on the marked piscicidal and molluscicidal activities of these compounds are presented.

INTRODUCTION

Although condensed tannins (proanthocyanidins or procyanidins) are commercially more important than hydrolysable tannins, it was only towards the end of the last decade that some of their structures were elucidated, presumably because of the difficulties in the isolation, purification and characterization of these compounds [1–3]. In addition to the identification of the anthocyanidins produced on heating these compounds with mineral acid in butanol, non-destructive techniques such as ^{13}C NMR spectroscopy as well as IR spectrophotometry have been employed with great success in the structural determination of these polymers [3, 4].

Lower-M_r proanthocyanidins are widely present in plant tissue in relatively low concentrations, the bulk of these most likely consisting of oligomers and perhaps polymers. Because of the potential usefulness of these phytochemicals as natural molluscicidal, piscicidal and insecticidal agents elucidation of their structures is of importance [5–7].

The biological effects of these polyphenols are believed to be linked to strong interactions with superficial glycoproteins and, it is thought that they render many plant tissues unacceptable as food sources to potential predators with either detrimental nutritional consequences or in many cases death.

RESULTS AND DISCUSSION

As part of a continuing collaborative study of piscicidal plants with potential use in the eradication of predatory fish in prawn farming as well as the killing of unwanted species appearing during pond preparation for fish culture in southeast Asia, we selected plant species with high activity. Among these was *Mammea siamensis* Miq. T. And. (Guttiferae, also previously known as *Ochrocarpus siamensis*) an evergreen tree native to Thailand (commonly known as the 'temple tree'), Burma, Laos, Cambodia and Viet Nam. The active constituent was identified as a proanthocyanidin, isolated in large yield from the leaves and shown to be lethal to fish and to snails.

The structure of the procyanidin **1** was derived from the analyses of its ^{13}C NMR and IR spectra, and from degradation studies. The polymer furnished only cyanidin when heated with 5% hydrochloric acid in *n*-butanol. The 75 MHz ^{13}C NMR spectrum [in Me_2CO-d_6–H_2O (1:1)] exhibited typical unsubstituted catechol ring-carbons at δ115, 116 and 119 assignable to C-2′, C-5′, and C-6′ respectively. A sharp absorption at 1523 cm^{-1}, attributed to the stretching modes of the aromatic ring in the IR spectrum (KBr) of **1** supported, as above, the degree of substitution for the B-ring of this polymer. The hydroxylation pattern of the B-ring was also reflected in the out-of-plane deformation of the hydrogen atoms, exhibiting a band at 770 cm^{-1}, typical of procyanidins [4]. The expanded heterocyclic ring-carbon region δ60–90, of the spectrum of **1** clearly shows the predominance of the *cis*-isomer indicated by the resonance at δ76 (C-2), and the absence of the corresponding downfield ^{13}C signal for the *trans*-isomer (δ84). These results suggest that **1** corresponds to a proanthocyanidin polymer with the flavan-3-ol units having a 2,3-*cis* configuration and a number-average molecular weight (M_n) of *ca* 2 100.

Polygonum stagninum Ham ex Meissn. (Polygonaceae), a common pond weed in Thailand, India and southeast Asia, grows abundantly in seasonally flooded roadside ditches and ponds. The ^{1}H decoupled ^{13}C NMR spectrum of **2**, the major active constituent of *P. stagninum*, exhibited the presence of a carbonyl group at δ166 and a strong resonance at δ110 attributable to the two

unsubstituted aromatic carbon signals (C-2″, C-6″) of a gallate ring, as shown in the APT experiment. An IR band at 1693 cm^{-1} in the spectrum of **2** also revealed a carbonyl group of an aromatic ester. Catechol ring-carbon atoms at δ115, 116 and 119 assigned to C-2′, C-5′ and C-6′ respectively, were observed as well.

An upfield shift of the C-4 carbon signal at the point of inter-flavanoid linkage (δ34) in addition to the corresponding C-4 resonances in related C-3 unsubstituted polymers (δ36) suggested the presence of a galloyl moiety attached to the C-3 hydroxyl group of **2** [8], and a 2:1 ratio of non-galloylated procyanidin to 3-*O*-galloyl substituted units. The introduction of a galloyl group at C-3 brings a decrease in linewidth of the C-2′, C-5′ and C-6′ signals of the catechol rings. It also contributes to the greater signal separation between C-2′ and C-5′, in the spectrum of **2** compared with **1** (Fig. 1). Conceivably, the catechol ring-carbons experience more interference owing to the proximity of the galloyl moiety in **2** whereas in **1**, lacking substitution at C-3, free rotation of the B-ring appears to be virtually unaffected.

The C-3 resonance of the chain-terminating flavan-3-ol in **1** was clearly defined at δ 66, as well as the corresponding downfield ^{13}C signal, δ 69, of the flavan-3-*O*-galloyl in **2**. An integral for the C-3 signal of proanthocyanidin monomer units at δ73 and that of the C-3 terminating unit at δ69, provided a ratio of 6:1 for **2**, which translated into a number-average M_r (M_n) of *ca* 2 500. As in **1**, the relative upfield position for the C-2 signal indicated that **2** possessed a 2,3-*cis* stereochemistry [2].

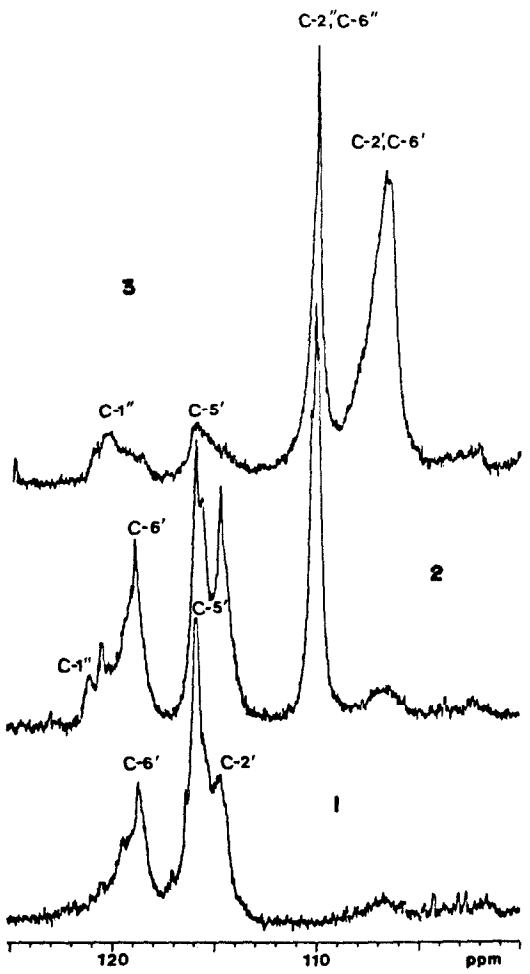


Fig. 1. 75 MHz ^{13}C NMR spectra [Me_2CO-d_6–H_2O (1:1)] of the aromatic C-H region for proanthocyanidins **1**–**3**.

Diospyros diepenhorstii. Miq. (Ebenaceae), a common tree in southern Thailand, west Malaysia, Indonesia, Phillipines is a member of a large genus. The major active component (**3**) against fish and snails from the leaves afforded only delphinidin when treated with hot 5% hydrochloric acid in *n*-butanol.

IR studies have suggested that double peaks in the 1540–1520 cm^{-1} region are observed only in the spectra of gallocatechin or prodelphinidin polymers, and in proanthocyanidins with a minimum of ~60 per cent of prodelphinidin units [4]. IR spectral data for the 3-*O*-galloyl procyanidin (**2**) and the 3-*O*-galloyl prodelphinidin (**3**) polymers revealed double peaks at 1517, 1524 cm^{-1}, and 1524, 1539 cm^{-1}, respectively.

The characterization of **3** followed readily from the previous ^{13}C NMR analyses for **1** and **2**. In their spectra the ^{13}C chemical shift patterns of the unsubstituted aromatic ring-carbons at C-2 and C-3 (δ105-120) are features diagnostic of pyrogallol, catechol and gallate rings. Carbon signals at δ107 (C-2′, C-6′) arising from pyrogallol, and δ110 (C-2″, C-6″) from gallate were displayed in the ^{13}C NMR spectrum of **3** (Fig. 1). The low intensity resonance at δ166, typical of a gallate carboxyl carbon, and a residual ^{13}C signal derived from the unsubstituted C-5′ of a catechol ring appeared as well (δ116).

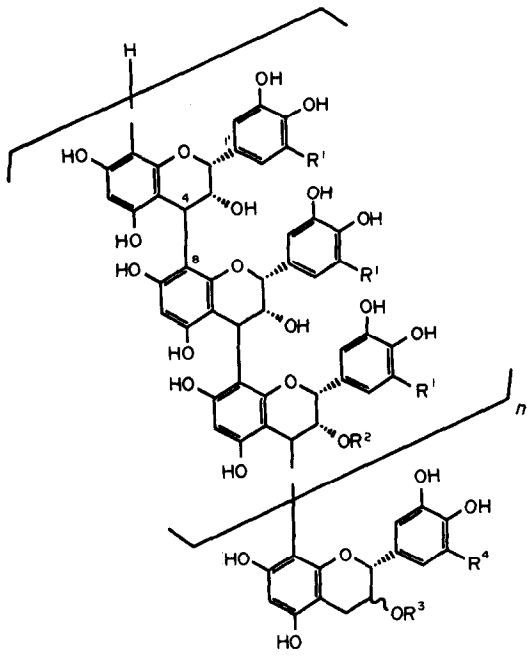
Although *M. siamensis* and *P. stagninum* leaves afforded homogeneous procyanidin and 3-*O*-galloyl procyanidin polymers, as estimated from their ^{13}C NMR spectra, leaves of *D. diepenhorstii* yielded a 3-*O*-galloyl prodelphinidin polymer with a 2,3-*cis* stereochemistry and a chain-terminating flavan-3-ol unit displaying a catechol B-ring. The identity of this polymer terminal unit was deduced from the presence of an extra ^{13}C signal due to C-5′ (δ116), and the absence of cyanidin after degradation of the polymer with alcoholic hydrochloric acid. Oligomer and polymer chains are built up by addition of further 'upper' units and only these may yield a carbocation, and be capable of producing anthocyanidins [9, 10].

Examination of the heterocyclic region provided evidence for a 2,3-*cis* configuration in **3**, indicated by an upfield signal at δ76 (C-2) of the *cis*-isomer, and no observable resonance for the *trans*-isomer. A ratio of 2:1 for prodelphinidin and the corresponding 3-*O*-galloyl substituted units was obtained. An evaluation of the integral for the C-3 resonances of **3** suggested a 6:1 ratio between the prodelphinidin and the terminal flavan-3-ol units, which accounted for a number-average M_n of *ca* 2 500.

Piscidal and molluscicidal activity. As mentioned earlier, the need for a safe and biodegradable natural product to eradicate predatory fish as well as unwanted species, led to the isolation and characterization of these biocides. Their toxicity to fish is comparable to saponins, as shown in Table 1, with an LC_{50} of *ca* 0.3 ppm for proanthocyanidins and another *ca* 10-fold higher for the crude extracts with the exception of *M. siamensis* (LC_{50} = 0.7 ppm). A long term experiment, to evaluate its stability, was carried out with 2 l of a 5.0 ppm stock solution of **1**, and a control

Table 1. Piscicidal and molluscicidal activities of crude extracts and purified compounds from plant species

Plant	[Piscicidal activity (LC_{50}) 24 hr] Crude extract	Proanthocyanidin
Mammea siamensis	0.7 ppm	0.3 ppm (**1**)
Polygonum stagninum	2.5 ppm	0.3 ppm (**2**)
Diospyros diepenhorstii	2.3 ppm	0.2 ppm (**3**)
Plant	[Molluscicidal activity (LC_{50}) 24 hr] Crude extract	Proanthocyanidin
Mammea siamensis	30 ppm	15 ppm
Polygonum stagninum	$\leq$50 ppm	35 ppm
Diospyros diepenhorstii	>50 ppm	40 ppm



1 $R^1 = R^2 = R^3 = R^4 = H$

2 $R^1 = R^4 = H, R^2 = R^3 =$ galloyl

3 $R^1 = OH, R^2 =$ galloyl, $R^3 = R^4 = H$

of distilled water kept for over a period of six weeks. The survival rates for tilapia (*Oreochromis mossambicus*), observed daily in this solution, remained unchanged (100% mortality) over a four week period. After this it gradually dropped to 40% mortality.

During the course of our study several other phytochemicals related to proanthocyanidins were tested for activity including gallic acid, (+)-catechin and (−)-epicatechin. These were found to be inactive. However, 5,3′-dihydroxy-7,4′-dimethoxyflavanone, isolated from *Artemisia dracunculus* L. [11], despite its low solubility in water, was very toxic to fish (LC_{50} = 1.0 ppm). On the other hand 5,7,4′-trihydroxyflavanone (naringenin) was not active at concentrations in the range 10–20 ppm, or even higher. Similarly, two dihydroflavonols from *A. dracunculus*, 3,5,4′-trihydroxy-7-methoxyflavanone and 3,5,4′-trihydroxy-7,3′-dimethoxyflavanone [12] were found to be inactive.

The molluscicidal activity of these naturally occurring proanthocyanidins (**1**–**3**), although not very high (LC_{50} = 15–40 ppm), may be useful in the control of snails which are indirectly responsible for schistosomiasis in rural areas of Thailand, China, as well as other countries in Asia.

EXPERIMENTAL

^{13}C NMR spectra were determined at 75 MHz in Me_2CO-d_6–H_2O (1:1). Chemical shifts are referenced to the partly deuterated signal of Me_2CO-d_6 (δ_c = 29.8 ppm). Ft IR spectra were obtained in KBr pellets.

Extraction and isolation. Leaf material (~1.0 kg) of *Mammea siamensis*, *Polygonum stagninum* and *Diospyros diepenhorstii* was collected in the southern provinces of Thailand, identified, and voucher specimens deposited in the Herbarium at the Department of Biology, Prince of Songkla University. Leaves were finely milled and extracted with 95% EtOH (3 × 2 l) for 48 hr. The combined extract was concd *in vacuo* and redissolved in a minimum amount of MeOH–H_2O (4:1), extracted with petrol (3 × 250 ml) and concd to dryness (100–250 g). A portion of this freeze-dried extract (4.0 g) was poured onto a column of Sephadex LH-20 (40 × 4.0 cm) and eluted with MeOH. The collected fractions were checked with a long-wavelength UV-lamp (360 nm).

Proanthocyanidin polymers. Concn under red. pres. of the final fractions, eluted after fluorescent phenolic compounds, and further purification on Sephadex LH-20 column (40 × 2.0 cm) with MeOH followed by freeze-drying of the residue gave the polymers as a glassy light-brown powder (375–550 mg). A sample (5 mg) was heated at 80° with 5 ml of 5% HCl in *n*-butanol for 2 hr. Cyanidin and delphinidin were determined by measurement of their absorptions at 535 and 546 nm, respectively. Chromatography on Merck cellulose-precoated plastic sheets with BAW (6:1:2) solvent, confirmed the presence of cyanidin (R_f 0.48) and delphinidin (R_f 0.34).

Bioassays. Extracts for toxicity to fish and snails were weighed and redissolved in 95% EtOH to a final concn of 10 mg crude extract per ml EtOH and tested against tilapia *Oreochromis mossambicus* fry (standard fork length ~1 cm), and a planorbid snail, *Biomphalaria havanensis*. Similar procedures were followed for the pure proanthocyanidins **1**–**3**, albeit with a final concn not larger than 100 ppm. Bioassays were performed with 10 fish placed in 200 ml of dist. H_2O for 24 hr at 20° [13]. Fish were considered dead when there was no movement or any response to gentle prodding. Tests were carried out in duplicate and the 24 hr LC_{50} values obtained by plotting concentrations of extract (or proanthocyanidins) vs percentage of fish killed on probability-log paper. Duplicates of 5 snails of uniform size in 500 ml of H_2O were tested. After an exposure period of 24 hr, followed by a recovery period of 24 hr, death was indicated by discoloration, lack of muscle contraction, leakage of haematological fluid, deterioration of body tissues and the absence of heart-beat checked under a microscope [14, 15].

Proanthocyanidin **1**. Isolated from *M. siamensis* (Found: C, 55.4; H, 5.0. $C_{15}H_{12}O_6 \cdot 2\,H_2O$ requires C, 55.6; H, 4.9%), $[\alpha]_{578}^{20}$ +19° (MeOH; *c* 0.5). ν_{max}^{KBr} cm^{-1}: 3100–3600, 1610, 1523, 1443, 1360, 1284, 1248, 1159, 1060, 824, 770 and 614. ^{13}C NMR (Me_2CO-d_6: H_2O, 1:1): 76.0 (C-2), 71.9 (C-3), 36.4 (C-4), 102.0

(C-4a), 156, (C-5), 96.8 (C-6), 156 (C-7), 107 (C-8), 156 (C-5), 96.8 (C-6), 156 (C-7), 107 (C-8), 156 (C-8a), 132 (C-1'), 115 (C-2'), 145 (C-3', C-4'), 116 (C-5') and 119 (C-6').

Proanthocyanidin **2**. Isolated from *P. stagninum* (Found: C, 54.3; H, 4.8. $C_{15}H_{12}O_6 \cdot 2\frac{1}{2}\ H_2O$ ($\frac{2}{3}$), $C_{22}H_{16}O_{10} \cdot 2\frac{1}{2}\ H_2O$ ($\frac{1}{3}$) requires C, 54.2; H, 4.9%), $[\alpha]_{578}^{20} + 25°$ (MeOH; *c* 0.5). ν_{max}^{KBr} cm^{-1}: 3100–3600, 1693, 1612, 1524, 1517, 1449, 1368, 1342, 1285, 1230, 1158, 1030, 819, 803 (w), 764 and 732 (w). ^{13}C NMR (Me_2CO-d_6: H_2O, 1 : 1): 75.3 (C-2), 73.0 (C-3), 34.0 (C-4), 102 (C-4a), 155 (C-5, C-7, C-8a), 96.6 (C-6), 107 (C-8), 130.5 (C-1'), 121 (C-1''), 115 (C-2'), 144.7 (C-3', C-4'), 116 (C-5'), 119 (C-6'), 110 (C-2'', C-6''), 145 (C-3'', C-5''), 139 (C-4''), and 166 (C=O).

Proanthocyanidin **3**. Isolated from *D. diepenhorstii* (Found: C, 56.0; H, 4.4. $C_{15}H_{12}O_7 \cdot H_2O$ ($\frac{2}{3}$), $C_{22}H_{16}O_{11} \cdot H_2O$ ($\frac{1}{3}$) requires C, 55.8; H, 4.2%). $[\alpha]_{578}^{20} + 89°$ (MeOH; *c* 0.4). ν_{max}^{KBr} cm^{-1}: 3100–3600, 1697, 1613, 1539, 1524, 1449, 1343, 1210, 1147, 1103, 1031, 832, 800, 761, and 734. ^{13}C NMR (Me_2CO-d_6: H_2O, 1 : 1): 76 (C-2), 73 (C-3), 37 (C-4), 102 (C-4a), 156 (C-7, C-8a), 96 (C-6), 132 (C-1'), 120 (C-1''), 107 (C-2', C-6'), 146 (C-3', C-5'), 116 (C-5'), 110 (C-2'', C-6''), 145 (C-3'', C-5''), 139 (C-4''), and 166 (C=O).

Acknowledgements—We express our gratitude to the International Development Research Centre, Ottawa, Canada, for their generous financial support and interest throughout this collaborative project between Universities in Thailand and in British Columbia. We also wish to thank the Department of Chemistry, the University of British Columbia for the use of the NMR facility, and James F. Maxwell (Herbarium, Department of Biology, Prince of Songkla University, Thailand) for the collection and identification of the plant material.

REFERENCES

1. Fletcher, A. C., Porter, L. J., Haslam, E. and Gupta, R. K. (1977) *J. Chem. Soc. Perkin Trans. I* 1628.
2. Czochanska, Z., Foo, L. Y., Newman, R. H. and Porter, L. J. (1980) *J. Chem. Soc. Perkin Trans I* 227.
3. Sun, D., Wong, H. and Foo, L. Y. (1987) *Phytochemistry* **26**, 1825.
4. Foo, L. Y. (1981) *Phytochemistry* **20**, 1397.
5. Schaufelberger, D. and Hostettman, K. (1983) *Planta Med.* **48**, 105.
6. Hostettman, K. (1984) *Naturwissenschaften* **71**, 247.
7. Scalbert, A. and Haslam, E. (1987) *Phytochemistry* **26**, 3191.
8. Nonaka, G., Muta, M. and Nishioka, I. (1983) *Phytochemistry* **22**, 237.
9. Hemingway, R. W., McGraw, G. W., Karchesy, J. J., Foo, L. Y. and Porter, L. J. (1983) *J. Appl. Polymer Sci. Appl. Polym. Sym.* **37**, 967.
10. Porter, L. J., Hrstich, L. N. and Bock, G. C. (1986) *Phytochemistry* **25**, 223.
11. Balza, F., Jamieson, L. and Towers, G. H. N. (1985) *J. Nat. Prod.* **48**, 339.
12. Balza, F. and Towers, G. H. N. (1984) *Phytochemistry* **23**, 2333.
13. Sprague, J. B. (1973) *The ABC's of Pollutant Bioassay using Fish* in *Biological Methods for the Assessment of Water Quality* (Cairns, J. and Dickson, K. L., eds). ASTM.
14. Nakanishi, K. and Kubo, I. (1978) *Isr. J. Chem.* **16**, 28.
15. Dossaji, S. F., Kairu, M. G., Gondwe, A. T. and Ouma, J. H. (1977) *Lloydia* **40**, 290.