FURTHER STUDIES ON PHORBOL ESTER BIOACTIVITY IN THE EUPHORBIACEAE[1]

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ABSTRACT

Six hundred and thirty-four organic extracts, including 36 previously untested genera of the Euphorbiaceae, were examined in a phorbol dibutyrate receptor binding assay. Phorbol bioactivity was newly detected in the genera *Anthostema, Blachia, Borneodendron, Dichostemma, Spirostachys, Tapoides, Trigonostemon,* and *Wetria.*

The activity of phorbol esters in the National Cancer Institute (NCI) primary anti-HIV screen (Gustafson et al., 1992; Erickson et al., 1995) prompted us to investigate the taxonomic distribution of phorbol esters, as measured by a simple competition binding assay. We have extended our previous study (Beutler et al., 1989) to detect the presence of phorbol esters in crude organic extracts of Euphorbiaceae. Our aim has been to examine as many tribes and genera as possible to map the distribution of phorbol ester bioactivity in the family.

MATERIALS AND METHODS

SAMPLES

The plant samples for this study were collected under the auspices of the NCI Developmental Therapeutics Program. Critical plant determinations were made by Michael Huft, Missouri Botanical Garden, D. Doel Soejarto, Field Museum, and John Burley, Arnold Arboretum. The taxonomic framework adopted was that of Webster (1994), except for the distinction of two species of *Wetria*, as maintained by Soejarto. Voucher specimens (see Appendix 1) are located at the Field Museum (Southeast Asia), the Missouri Botanical Garden (Africa), or the New York Botanical Garden (Americas) except where indicated in the appendix. Specimens without numbers (s.n.) are not vouchered. Dried plant materials were processed by percolation in a 1:1 mixture of methylene chloride : methanol, and the solvent evaporated in vacuo at 40°C. All crude extract samples were dissolved in DMSO at a concentration of 10–20 mg/ml for initial examination, then stored at −20°C. For dose response studies, these samples were further diluted in DMSO.

PHORBOL DIBUTYRATE BINDING ASSAY

The assay was performed as previously reported (Beutler et al., 1989). Each extract was tested at an initial concentration of 100 μg/ml. If displacement at this concentration was greater than 60 percent, it was taken as a positive result. The definition was increased from the value of 50 percent displacement used in our previous report, due to examination of nonspecific binding effects of the crude extracts. Initial examination and dose response were performed in duplicate, with at least four concentrations used for dose response. The incubation was terminated by filtration over Whatman GF/B glass fiber filters in a Brandel cell harvester. A recent modification of the assay used for a minority of samples involved a reduction of the assay volume to 250 μl in 96-well polypropylene microtiter plates, with harvest onto GF/B glass fiber paper, which was then dried *in vacuo* and counted on a Betaplate counter.

RESULTS AND DISCUSSION

The cumulative total of active genera including our previous results (Beutler et al., 1989) are tab-

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Table 1. Distribution of phorbol bioactivity by genus (Webster, 1994), including previous results (Beutler et al., 1989).

Taxon	Samples active/ samples tested		
I. Phyllanthoideae			1/199
6. Antidesmeae		1/88	
Antidesma	1/42		
II. Oldfieldioideae			0/14
III. Acalyphoideae			4/169
30. Acalypheae		4/76	
Wetria	4/4		
IV. Crotonoideae			65/118
34. Manihoteae		1/3	
Cnidoscolus	1/2		
38. Jatropheae		8/8	
Jatropha	8/8		
39. Codiaeae		10/13	
Dimorphocalyx	2/2		
Codiaeum	6/6		
Blachia	2/2		
40. Trigonostemoneae		9/9	
Trigonostemon	9/9		
42. Crotoneae		26/40	
Croton	23/37		
Eremocarpus	1/1		
Fahrenheitia	2/2		
44. Aleuritideae		11/21	
Aleurites	1/1		
Borneodendron	1/1		
Cyrtogonone	5/5		
Crotonogyne	2/2		
Tapoides	2/2		
V. Euphorbioideae			83/134
45. Stomatocalyceae		1/10	
Plagiostyles	1/3		
46. Hippomaninae		46/77	
Duvigneaudia	2/3		
Excoecaria	8/8		
Maprounea	5/8		
Omalanthus	6/15		
Sapium	19/25		
Spirostachys	4/5		
Stillingia	2/2		
49. Euphorbieae		36/47	
Anthostema	4/4		
Dichostemma	6/6		
Euphorbia	25/33		
Synadenium	1/1		

ulated (Table 1) according to Webster's (1994) arrangement of genera. Thirty-six genera that we had not previously examined were tested (*Amanoa, Anthostema, Austrobuxus, Blachia, Borneodendron, Cephalomappa, Chaetocarpus, Chamaesyce, Cleidion, Clutia, Conceveiba, Dichostemma, Elateriospermum, Erythrococca, Koilodepas, Lingelsheimia, Monadenium, Neoscortechinia, Omphalea, Pausandra, Pera, Pimelodendron, Plukenetia, Pseudolachnostylis, Ptychopyxis, Sampantaea, Sauropus, Savia, Sebastiania, Senefeldera, Strophioblachia, Synadenium, Tapoides, Trigonostemon, Wetria,* and *Zimmermanniopsis*), bringing the total number of genera tested to 111. Further species and plant parts of previously tested genera were also examined. Representatives of a total of 39 of the 49 tribes distinguished (Webster, 1994) have been examined. In total, 106 species in the subfamily Phyllanthoideae, and 2 species of the Oldfieldioideae have been examined with negative results. A single positive test of *Antidesma nigricans* Tul. (Phyllanthoideae) seeds (Beutler et al., 1989) prompted us to test a sample from each of the 26 species of *Antidesma* in the NCI repository. The lack of further positive results with these samples leads us to suspect that the single positive sample may have been misidentified. Seventy-six species of Acalyphoideae were tested, with 2 species of *Wetria* being positive (see below). Thirty-two of the 51 species of Crotonoideae examined appear to contain phorbol esters (63%), while 43 of the 63 species of Euphorbioideae tested are active (68%).

While the results of the ^{3}H-PDBu binding assay reflect only the presence of phorbol esters which bind to protein kinase C, there appears to be a good correlation between the bioactivity data and the chemical literature on phorbol diterpene distribution. Our laboratory has demonstrated in three specific instances (*Homalanthus nutans* (Forster) Pax (Gustafson et al., 1992), *Excoecaria agallocha* L. (Erickson et al., 1995), and *Maprounea* spp. (Beutler et al., 1995)) that the activity detected in our samples is due to conventional phorbol esters. Isolation of the compounds responsible for ^{3}H-PDBu binding activity in the newly identified genera (*Anthostema, Blachia, Borneodendron, Dichostemma, Spirostachys, Tapoides, Trigonostemon,* and *Wetria*) should be pursued to positively identify the compounds responsible for the observed bioactivity.

These results indicate that phorbol ester bioactivity is primarily limited to subfamilies IV and V in Webster's (1994) scheme. The only exception found in our data is that for two species of *Wetria*, which is placed in the Acalyphoideae. The overall pattern of distribution is consistent with previous chemotaxonomic data (Kinghorn, 1979) and lends biochemical support to Webster's arrangement of genera. Further, our data are consistent with embryological data (Jensen et al., 1994), and legumin-like protein distribution (Kapil & Bhatnagar, 1994), which both separate the Phyllanthoideae and Oldfieldioideae from the other three subfamilies.

The observation of ^{3}H-PDBu binding activity in *Wetria insignis* and *W. macrophylla*, and the isolation of phorbol esters from *Pycnocoma* by Bergquist et al. (1989) may appear anomalous, but would be consistent with the proposed derivation of Crotonoideae and Euphorbioideae from Acalyphoideae if the biosynthetic capability to form phorbol esters arose in a common ancestor of the Acalyphoideae, Crotonoideae, and Euphorbioideae. Hecker and Adolf have perceived a similar chemotaxonomic pattern to that which we observed (Adolf & Hecker, 1977). It must be emphasized that no definitive work has been done on the biosynthesis of any phorbol ester in any plant. Such a study would facilitate interpretation of the chemical data for taxonomic or phylogenetic purposes. Alternative explanations for our pattern of results are that *Wetria* and *Pycnocoma* have been misclassified or misidentified; however, we believe the identifications to be correct. It is notable, however, that *Wetria* and *Pycnocoma* are placed in different tribes, and that their close relatives such as *Cleidion* or *Sampantaea*, and *Ptychopyxis*, respectively, have not displayed ^{3}H-PDBu displacing activity.

Patterns of diterpene occurrence and irritancy have been previously examined by Schmidt (1986), who analyzed the occurrence of all diterpene hydrocarbon skeletons (e.g., crotofolane, lathyrane, casbane, etc.) in the Euphorbiaceae. Since that review, other novel diterpene skeletal types have been discovered, and known types have been found in previously unexamined genera (e.g., Kashman et al., 1993; Bernart et al., 1993).

We conclude that phorbol bioactivity and phorbol ester biosynthesis are more widespread throughout the genera of the Euphorbiaceae than has been previously reported. The chemotaxonomy of this family can be explored by application of the ^{3}H-PDBu binding method to more taxa, and by bioassay-guided fractionation to determine the chemical structures responsible for these results. The ^{3}H-PDBu binding assay allows rapid testing, generating semiquantitative data that can be used to prioritize isolation of the compounds responsible for the bioactivity. It is more selective than such assays as mouse ear irritancy and does not require the use of whole animals. The distribution of ^{3}H-PDBu binding activity may also be of use in assessing the potential toxicology of Euphorbiaceae used as phytomedicines.

Literature Cited

Adolf, W. & E. Hecker. 1977. Diterpenoid irritants and cocarcinogens in Euphorbiaceae and Thymelaeaceae: Structural relationships in view of their biogenesis. Israel J. Chem. 16: 75–83.

Bergquist, K. E., H. Obianwu & B. Wickberg. 1989. Isolation and structure determination of a novel phorbol derivative in an intramolecular diester macrolide. J. Chem. Soc., Chem. Comm. 183–184.

Bernart, M. W., Y. Kashman, M. Tischler, J. H. Cardellina II & M. R. Boyd. 1993. Bershacolone, an unprecedented diterpene cyclobutene from *Maprounea africana*. Tetrahedron Lett. 34: 4461–4464.

Beutler, J. A., A. B. Alvarado, T. G. McCloud & G. M. Cragg. 1989. Distribution of phorbol ester bioactivity in the Euphorbiaceae. Phytother. Res. 3: 188–192.

———, Y. Kashman, M. Tischler, J. H. Cardellina II, G. N. Gray, M. Currens, M. E. Wall, M. C. Wani & M. R. Boyd. 1995. A reinvestigation of *Maprounea* triterpenes. J. Nat. Prod. 58: 1039–1046.

Erickson, K. L., J. A. Beutler, J. H. Cardellina II, J. B. McMahon, D. J. Newman & M. R. Boyd. 1995. A novel phorbol ester from *Excoecaria agallocha*. J. Nat. Prod. 58: 769–772.

Gustafson, K. R., J. H. Cardellina II, J. B. McMahon, R. J. Gulakowski, J. Ishitoya, Z. Szallasi, N. E. Lewin, P. M. Blumberg, O. S. Weislow, J. A. Beutler, R. W. Buckheit, G. M. Cragg, P. A. Cox, J. P. Bader & M. R. Boyd. 1992. A nonpromoting phorbol from the Samoan medicinal plant *Homalanthus nutans* inhibits cell killing by HIV-1. J. Med. Chem. 35: 1978–1986.

Jensen, U., I. Vogel-Bauer & M. Nitschke. 1994. Leguminlike proteins and the systematics of the Euphorbiaceae. Ann. Missouri Bot. Gard. 81: 160–179.

Kapil, R. N. & A. K. Bhatnagar. 1994. The contribution of embryology to the systematics of the Euphorbiaceae. Ann. Missouri Bot. Gard. 81: 145–159.

Kashman, Y., M. W. Bernart, M. Tischler, J. H. Cardellina II & M. R. Boyd. 1993. Koumbalones A and B, new casbane diterpenes from *Maprounea africana*. J. Nat. Prod. 57: 426–430.

Kinghorn, A. D. 1979. Cocarcinogenic irritant Euphorbiaceae. *In:* A. D. Kinghorn (editor), Toxic Plants. Columbia Univ. Press, New York.

Schmidt, R. J. 1986. Biosynthetic and chemosystematic aspects of the Euphorbiaceae and Thymelaeaceae. *In:* F. J. Evans (editor), Naturally Occurring Phorbol Esters. CRC Press, Boca Raton.

Webster, G. 1994. Synopsis of the genera and suprageneric taxa of Euphorbiaceae. Ann. Missouri Bot. Gard. 81: 33–144.

Appendix 1. Specimens of Euphorbiaceae examined. The characters + and − refer to the ^{3}H-PDBu screening results for the specimen.

Acalypha lancetillae Standley, *Balick 1924* (NY) (−); *Alchornea cordifolia* (Schum. & Thonn.) Muell. Arg., *Fay 8575* (MO) (−); *Amanoa bracteosa* Planch., *Jongkind 2147* (MO) (−); *Amanoa caribaea* Krug & Urb., *Tuxill 106* (NY) (−); *Amanoa strobilacea* Muell. Arg., *McPherson 15094* (MO) (−); *Anthostema aubryanum* Baillon, *McPherson 15212*, *Wilks 2626* (MO) (+); *Antidesma bunius* (L.) Spreng., *Burley 68* (F) (−); *Antidesma celebicum* Miq., *Burley 3558* (F) (−); *Antidesma coriaceum* Tul., *Soepadmo 318* (F) (−); *Antidesma cumingii* Muell. Arg., *Soejarto 7375* (F) (−); *Antidesma cuspidatum* Muell. Arg., *Soepadmo 110* (F) (−); *Antidesma ghaesembilla* Gaertn., *Varadarajan 1528* (F) (−); *Antidesma gymnogyne* Pax & K. Hoffm., *Soejarto 5878* (F) (−); *Antidesma leptocladum* Tul., *Burley 173* (F) (−); *Antidesma leucocladum* Hook. f., *Soepadmo 111* (F) (−); *Antidesma leucopodum* Miq., *Soepadmo 96* (F) (−); *Antidesma luzonicum* Merr., *Soejarto 7790* (F) (−); *Antidesma membranaceum* Muell. Arg., *Schmidt 1137* (MO) (−); *Antidesma menasu* Miq., *Bhaduri 35* (a) (−); *Antidesma montanum* Bl., *Soepadmo 59* (F) (−); *Antidesma neurocarpum* Miq., *Burley 371* (F) (−); *Antidesma nitidum* Tul., *Varadarajan 1501* (F) (−); *Antidesma pendulum* Hook. f., *Soepadmo 210* (F) (−); *Antidesma pentandrum* (Blanco) Merr., *Soejarto 7937* (F) (−); *Antidesma petiolare* Tul., *Miller 3741* (MO) (−); *Antidesma petiolatum* Airy Shaw, *Takeuchi 4444* (F) (−); *Antidesma polyanthum* K. Schum. & Laut., *Takeuchi 4388* (F) (−); *Antidesma salicinum* Ridl., *Soepadmo 261, Ong 172* (F) (−); *Antidesma sarcocarpum* Airy Shaw, *Takeuchi 7048* (F) (−); *Antidesma tomentosum* Bl., *Burley 53* (F) (−); *Antidesma venosum* Tul., *Schmidt 1236* (MO) (−); *Aporusa arborea* (Bl.) Muell. Arg., *Burley 1727* (F) (−); *Aporusa planchoniana* Baill., *Soejarto 5950* (F) (−); *Aporusa prainiana* King ex Gage, *Burley 1722* (F) (−); *Austrobuxus nitidus* Miq., *McDonald 4055, Burley 4589* (F) (−).

Baccaurea puberula Merr., *Meijer 122596* (F) (−); *Bischofia javanica* Bl., *Soejarto 5842* (F) (−); *Borneodendron aenigmaticum* Airy Shaw, *Meijer 109845* (F) (+).

Cephalomappa sp., *Frodin 2069* (F) (−); *Chaetocarpus castanicarpus* (Roxb.) Thw., *Soepadmo 308* (F) (−); *Chaetocarpus globosus* (Sw.) Fawc. & Rend., *Zanoni 45133, Garcia 2635* (NY) (−); *Chrozophora* sp., *Ghafoor 4510* (F) (−); *Claoxylon longifolium* (Bl.) Miq., *Burley 382* (F) (−); *Cleidion lanceolatum* Merr., *Soejarto 7975* (F) (−); *Cleidion spiciflorum* (Burm. f.) Merr., *Soejarto 7797* (F) (−); *Cleistanthus insignis* Airy Shaw, *Takeuchi 4634* (F) (−); *Clutia abyssinica* Jaub. & Spach, *Gereau 3027* (MO) (−); *Codiaeum luzonicum* Merr., *Soejarto 6195* (F) (+); *Codiaeum variegatum* (L.) A. Juss., *Harini 135* (F) (+); *Conceveiba rhytidocarpa* Muell. Arg., *Beck 1049* (NY) (−); *Croton argyratus* Bl., *Soejarto 6190, Burley 1468* (F) (+); *Croton lechleri* Muell. Arg., *Boom 7792, Bennett 4531* (NY) (+); *Croton leiophyllus* Muell. Arg., *Soejarto 6455* (F) (+); *Croton macrostachyus* Del., *Gereau 2576* (MO) (+); *Croton monathgynus* Michx., *Spjut 11129* (b) (−); *Croton palawanensis* Merr., *Soejarto 6476* (F) (+); *Croton punctatus* Jacq., *Beutler s.n.* (+); *Croton* spp., *Daly 5617* (+), *Williams 656* (+), *Takeuchi 4508* (−) (F); *Cyrtogonone argentea* (Pax) Prain, *McPherson 13735* (MO) (+).

Dichostemma glaucescens Pierre, *McPherson 15198, Dibata 935* (MO) (+); *Drypetes longifolia* (Bl.) Pax & K. Hoffm., *Burley 755* (F) (−); *Drypetes madagascariensis* (Lam.) Humbert & Leandri, *Zarucchi 7459* (MO) (−); *Drypetes parvifolia* (Muell. Arg.) Pax & K. Hoffm., *Fay 8260* (MO) (−); *Drypetes paxii* Hutch., *Martin 509* (MO) (−); *Drypetes sibuyanensis* (Elmer) Pax & K. Hoffm., *Burley 3029* (F) (−); *Drypetes similis* Hutch., *Nemba 551* (MO) (−).

Elateriospermum tapos Bl., *Burley 3175, Soepadmo 193, Frodin 2022* (F) (−); *Endospermum diadenum* (Miq.) Airy Shaw, *Burley 1450* (F) (−); *Erythrococca ulugurensis* A. R.-Sm., *Gereau 2949* (MO) (−); *Euphorbia laurifolia* Juss., *Mena 61* (NY) (+); *Euphorbia maculata* L., *Beutler s.n.* (−); *Euphorbia tirucalli* L., *Harini 150* (F) (+); *Excoecaria phillipensis* Merr., *Soejarto 6553* (F) (+).

Flueggea virosa (Willd.) Voigt, *Rulangaranga 7* (MO) (−).

Glochidion pomiferum Airy Shaw, *Takeuchi 4076* (F) (−).

Homonoia riparia Lour., *Ong 170* (F) (−); *Hyeronima clusioides* (Tul.) Muell. Arg. *Acevedo 2163* (NY) (−).

Koilodepas brevipes Merr., *Burley 707* (F) (−).

Lingelsheimia frutescens Pax, *McPherson 15084* (MO) (−).

Macaranga conifera (Zoll.) Muell. Arg., *Meijer 119104* (F) (−); *Macaranga denticulata* (Bl.) Muell. Arg., *Stone 15911* (F) (−); *Macaranga lowii* King ex Hook. f., *Burley 1397* (F) (−); *Macaranga pleioneura* Airy Shaw var. *velutina* Whitmore, *Takeuchi 4499* (F) (−); *Macaranga ramiflora* Elm., *Stone 15849* (F) (−); *Mallotus cuneatus* Ridl., *Soejarto 5791* (F) (−); *Mallotus philippinensis* (Lam.) Muell. Arg., *Soejarto 6230* (F) (−); *Mallotus ricinoides* (Pers.) Muell. Arg., *Burley 79* (F) (−); *Margaritaria discoidea* (Baill.) Webster, *Gereau 2574* (MO) (−); *Monadenium laeve* Stapf., *Kayombo 1027* (MO) (−).

Neoschortechinia kingii (Hook. f.) Pax & K. Hoffm., *Burley 1380* (F) (−); *Neoschortechinia nicobarina* Hook. f., *Burley 2478* (F) (−); *Neotrewia cumingii* (Muell. Arg.) Pax & K. Hoffm., *Madulid 6798, Burley 3587* (F) (−).

Omalanthus acuminatus (Muell. Arg.) Pax, *Cox 1021* (BRY) (+); *Omalanthus megaphyllus* Merr., *Nicholson 1* (c) (+); *Omalanthus nervosus* J. J. Sm., *Takeuchi 4176* (F) (−); *Omalanthus novaguinensis* (Warb.) K. Sch., *Takeuchi 4004* (F) (−); *Omalanthus leschenaultianus* A. Juss., *Nicholson 3* (c) (+); *Omalanthus rotundifolius* Merr., *Nicholson 4* (c) (−).

Pausandra sp., *Daly 6034* (MO) (−); *Pera arborea* Mutis, *Balick 3036, Balick 3066* (NY) (−); *Pera benesis* Rusby, *Betella 109* (NY) (−); *Pimelodendron amboinicum* Hassk., *Takeuchi 4236* (F) (−); *Plukenetia* sp., *Acevedo 1691* (NY) (−); *Pseudolachnostylis maprouneifolia* Pax var. *maprouneifolia*, *Gereau 2749* (MO) (−); *Ptychopyxis philippina* Croiz., *McDonald 3745* (F) (−).

Sampantaea amentiflora Airy Shaw, *Soejarto 8202* (F) (−); *Sapium biloculare* (S. Wats.) Pax, *Wirt s.n.* (d) (+); *Sapium laurocerasus* Desf., *Acevedo 2181* (NY) (+); *Sapium marmieri* Huber, *Daly 5634* (MO) (+); *Sapium* sp., *Soejarto 6193* (F) (+); *Sauropus androgynous* Merr., *Harini 35* (F) (−); *Savia platyrhachis* Baill., *Harder 1620* (MO) (−); *Sebastiania brasiliensis* Spreng., *Saldias 745* (NY) (−); *Securinega virosa* (Roxb.) Baill., *Fay 8060* (MO) (−); *Senefeldera* cf. *macrophylla* Ducke, *Beck 1056* (NY) (−); *Spirostachys africana* Sond., *Muhoro 6252* (MO) (+); *Stillingia sylvatica* L., *Spjut s.n.* (b) (+); *Strophioblachia fimbricalyx* Boerl., *Soejarto 6321* (F) (−); *Synadenium glaucescens* Pax, *Rulangaranga 179* (MO) (+).

Tapoides villamilii (Merr.) Airy Shaw, *Meijer 128772* (F) (+); *Thecacoris madagascariensis* A. Juss. var. *montana* Leandri, *Zarucchi 7384* (MO) (−); *Trigonostemon sumatranus* Pax & K. Hoffm., *Burley 689* (F) (+); *Trigonostemon viridissimus* (Kurz) Airy Shaw, *McDonald 3401* (F) (+).

Wetria insignis (Steud.) Airy Shaw, *Takeuchi 6386* (F) (+); *Wetria macrophylla* (Bl.) J. J. Sm., *Soejarto 7770* (F) (+).

Zimmermanniopsis uzungwae A. R.-Smith (ined.), *Kayombo 610* (MO) (−).

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