Bisnorsesquiterpenoids from *Euphorbia resinifera* Berg. and an Expeditious Procedure to Obtain Resiniferatoxin from Its Fresh Latex

Ernesto Fattorusso,*[a] Virginia Lanzotti,[b] Orazio Taglialatela-Scafati,[a] Gian Cesare Tron,[c] and Giovanni Appendino*[c]

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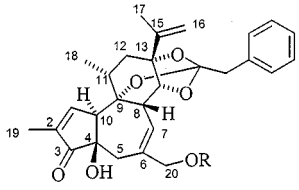
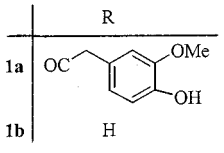
The fresh latex of cultivated *E. resinifera* Berg. is a convenient source of the ultrapotent vanilloid resiniferatoxin, and a transesterification-reesterification procedure that substantially alleviates the hazards associated with the direct isolation of this obnoxious diterpenoid has been developed. Various known phorboid constituents of the diterpenoid fraction were obtained in pure form and have been spectroscopically characterized for the first time, while the non-diterpenoid fraction of the latex gave, besides some common triterpenoids, three new bisnorsesquiterpenes of the dihydroionol type. The structures of these compounds were established through a combination of spectroscopic data and chemical reactions.

Introduction

The daphnane diterpene resiniferatoxin (RTX, **1a**), first isolated in 1975 from the fresh latex of the succulent spurge *Euphorbia resinifera* Berg., a plant endemic to Morocco, is the most potent irritant known.[1] The dried latex of this plant has long been an article of commerce under the name of Euphorbium, and is one of the oldest drugs in the Western medicinal tradition.[2]





[a] Dipartimento di Chimica delle Sostanze Naturali
Via Montesano 49, 80131 Napoli, Italy
Fax: (internat.) + 39-081/678-552
E-mail: fattoru@unina.it
[b] Dipartimento STAAM,
Via De Sanctis, 86100 Campobasso, Italy
[c] Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche
V.le Ferrucci 33, 28100 Novara, Italy
E-mail: appendin@pharm.unito.it

RTX was discovered thanks to its extraordinary activity in the mouse-ear erythema assay, in which it is approximately a thousandfold more potent than tetradecanoyl phorbolacetate (TPA), the most powerful tumour-promoting phorbol ester.[1a] RTX was later also recognised as an ultrapotent vanilloid, outperforming capsaicin in binding assays by a factor of up to ten thousand.[3,4] No compound structurally unrelated to RTX is known to approach its potency in these assays, and the extraordinary biological activity of this diterpenoid and its complex molecular architecture have fostered intense research activity. Thus, RTX is currently undergoing human clinical trials for the treatment of bladder hyperriflexia and diabetic neuropathy,[5] and its total synthesis has been achieved.[6] Surprisingly, neither these studies nor the long and compelling history of medicinal use of Euphorbium[2] reawakened interest in *E. resinifera*, the source both of RTX and of Euphorbium. In addition to RTX, *E. resinifera* contains a complex mixture of irritant and tumour-promoting ingenol and 12-deoxyphorbol esters, as well as anticancer ingol esters.[7] The isolation of RTX from *E. resinifera* was never described in detail, and most of the accompanying phorboids were never obtained as single compounds and characterized as such. Furthermore, apart from some common triterpenoids, the composition of the non-phorboid fraction of the latex also remained essentially unknown. Despite these enticing clues, the obnoxious nature of the plant and its potent skin irritancy appear to have deterred further investigations. Indeed, no study on *E. resinifera* has been published since the pioneering reports by Hecker in the late 1970s, in which fresh latex collected in Morocco was employed.[7]

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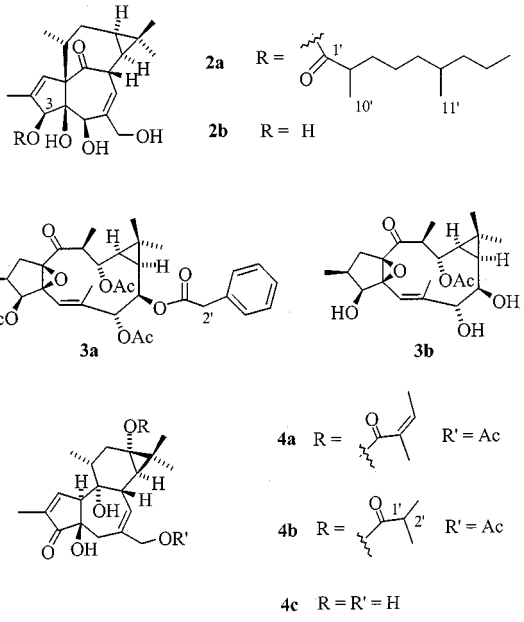
E. resinifera is a common indoor plant,[8] and Euphorbium is commercially available and cheap. These observations prompted us to start a systematic investigation into this prolific producer of biologically interesting compounds. Our aim was to investigate further the composition of *E. resinifera* and to develop a straightforward isolation procedure for RTX, currently an expensive rarity available commercially only in amounts insufficient to sustain a significant medicinal chemistry effort.[9]

Here we report an RTX isolation procedure that substantially alleviates the hazards associated with the manipulation of the diterpenoid fraction from the plant, together with the discovery that, in addition to phorboids and triterpenoids, *E. resinifera* also produces unusual and novel bisnorsequiterpenes of the dihydroionol type. These are also among the major constituents of commercial Euphorbium.



Results and Discussion

RTX is a trace component of *E. resinifera*, and it was clear from the outset that a quick, nonchromatographic procedure to separate the diterpenoid fraction from the much more abundant gums and triterpenoids would be pivotal to expeditious isolation of RTX. To this end, fresh latex obtained from plants growing in nurseries was extracted with EtOAc and filtered through a pad of TLC-grade silica gel to remove the gummy fraction, affording the resinous fraction as an amorphous, semisolid material. This was crystallised from acetonitrile to afford a white precipitate of triterpenoids and norsesquiterpenoids (see below), while concentration of the mother liquors gave the diterpenoid fraction as an extremely irritant yellowish gum. Solvents other than acetonitrile resulted in less efficient removal of the triterpenoids and/or in a substantial degree of retention of RTX in the crystalline mass. Column chromatography of the diterpene fraction gave RTX contaminated with ingenol and 12-deoxyphorbol esters, removal of which required careful HPLC purification. RTX was eventually obtained in 0.0020% yield from the fresh latex.[10]

In addition to RTX, the diterpene fraction also gave the ingenol monoester **2a**, the ingol ester **3a**, and the 12-deoxyphorbol esters **4a** and **4b**. With the possible exception of **3a** (see below), all of these compounds had already been reported from *E. resinifera*, but without structural characterization; nor had some of them been obtained in a pure state.

Compound **2a** is a 3-monoacyl ingenol derivative, described by Hecker as a component of a the so-called RL3 mixture, from which, however, it was never obtained in pure form.[11] MS and NMR measurements confirmed the original structural assignment, while the constitution of the unusual acyl moiety was unambiguously established by acid methanolysis followed by GC-MS analysis. In biological assays, **2a** showed powerful apoptotic activity as well as PKC activating properties:[12] the hallmark of 3-monoacyl ingenol esters.[13]

The ^{1}H NMR spectrum of **3a** showed the resonances typical of an ingol tetraester, with three acetyl moieties and one phenylacetyl unit. Location of ester functions and specific assignment of the carbon resonances of the ester groups were accomplished with the aid of the HMBC spectrum. The ^{13}C NMR resonances of the carbonyl groups were quite close, but the splitting was sufficient to locate the acetyl groups at C(3), C(7), and C(12) [cross-peaks of the acetyl carbonyl atoms at δ = 170.8, 170.5, and 170.7 with protons at C(3), C(7), and C(12), respectively], while the detection of a cross-peak between the carbonyl group at δ = 170.1 with both H(8) and a benzylic methylene group confirmed the location of the phenylacetyl group at C(8). Recent studies have highlighted that configurational isomers of ingol occur in spurges, casting doubts on many early structural assignments within these compounds.[14] The excellent fit of all scalar couplings with those originally reported for ingol,[15] and the substantial agreement of the dipolar couplings [e.g., H(1α)/H(2); H(2)/H(3); H(7)/H(8); H(8)/H(19); H(12)/H(19)] with the overall geometry established by the X-ray analysis of ingol tetraacetate[16] support the view that compound **3a** and ingol share the same configuration. A positional isomer of **3a** (ingol 3,8,12-triacetate 7-phenylacetate) has been reported as a constituent of *E. resinifera*,[11a] but the lack of physical and spectroscopic data did not allow comparison with **3a**.[17]

The 12-deoxyphorbol esters **4a** and **4b** were both reported as constituents of *E. resinifera*,[11a] but their full spectroscopic characterization was not reported. Compound **4a** was later isolated from *E. poisonii*,[18] and characterized by ^{1}H and ^{13}C NMR spectroscopy. Corresponding data for **4b** are given in the Exp. Sect.

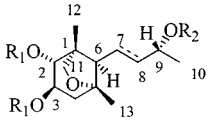
The high irritancy of the diterpene mixture made the chromatographic steps required for the isolation of RTX inconvenient for scaling up under normal laboratory conditions. This prompted us to explore an alternative and less hazardous approach, based on the hydrolysis of RTX to

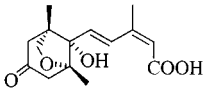
ROPA [resiniferonol orthophenyl acetate (**1b**) a compound devoid of vanilloid activity and only a weak irritant and tumour-promoter] and its reconversion into RTX under controlled conditions. We reasoned that the acyl groups of deoxyphorbol and ingenol esters should be stripped from their polyols by basic hydrolysis, while the orthoester group of RTX should be expected to survive basic conditions. A mixture of ROPA, a relatively apolar compound,[1a] and the polar parent polyols ingenol, 12-deoxyphorbol and ingol (or partially hydrolysed forms of them) would eventually be obtained. The benign nature of ingenol and deoxyphorbol, compared to their esters, and the difference in polarity between ROPA and the phorboid polyols should translate into a much easier separation and substantially reduced hazards than those inherent in the isolation of RTX from the native diterpenoid mixture. This strategy was implemented through a short (1 h) subjection of the diterpenoid fraction to pH = 10. A mixture of ROPA (**1b**), 12-deoxyphorbol 13-esters, 12-deoxyphorbol (**4c**), ingenol (**2b**), and ingol 12-acetate (**3b**) was obtained and easily separated by column chromatography. More strongly basic conditions or longer reaction times resulted in extensive degradation of the phorboid mixture.

Conversion of ROPA into RTX has been accomplished by a variety of methods, based either on the nucleophilic displacement of a C(20)-activated ROPA derivative with homovanillic acid,[11b,19] or, alternatively, on the esterification of ROPA with homovanillic acid protected at the phenolic hydroxy group and activated by various means.[6,19b] Surprisingly, the Mitsunobu esterification[20] has never been employed for this purpose. We found this method to be high-yielding and convenient, directly affording RTX from ROPA without any further deprotection step. The use of di-*tert*-butyl azodicarboxylate was mandatory, since more common azodicarboxylates (diethyl, diisopropyl) gave dihydro derivatives difficult to remove from the final product.

Attempts to obtain RTX from commercial Euphorbium failed, since no RTX could be isolated from the diterpenoid fraction obtained from two distinct commercial samples of the drug. These results support anecdotal information concerning the stability of RTX in the latex, as well as the long-standing observation that Euphorbium is unsuitable for use in human and veterinarian medicine until aged,[2] presumably to allow extensive degradation of RTX.

The crystalline mass obtained by treatment of the EtOAc-soluble portion of the latex with acetonitrile contained compounds with a wide range of polarities, and was fractionated by MPLC on silica gel with a gradient solvent system from *n*-hexane to methanol. The triterpenoids euphol and euphorbol[7] were obtained by elution with *n*-hexane/EtOAc (8:2 and 7:3), while the novel bisnorsesquiterpenoids glycosides euphorbioside A (**5a**) and B (**5b**) as well as the aglycone of **5a** (compound **5c**) were obtained from more polar fractions. These compounds, displaying much higher stabilities than phorboids, could also be obtained from commercial Euphorbium.





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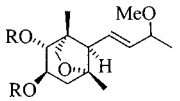
	R_1	R_2	
5a	β-D-Glu	H	$\Delta^{7,8}$
5b	β-D-Glu	H	7,8-dihydro
5c	H	H	$\Delta^{7,8}$
5d	(*S*)-MTPA	(*S*)-MTPA	$\Delta^{7,8}$
5e	(*R*)-MTPA	(*R*)-MTPA	$\Delta^{7,8}$



	R
7a	H
7b	*p*-BrC_6H_4CO

Euphorbioside A (**5a**) was obtained as an amorphous powder with $[\alpha]_D^{25} = -8$ (MeOH) and molecular formula $C_{19}H_{32}O_9$ (HRMS). The carbon atoms were sorted out by DEPT experiments, into three methyl, three methylene, eleven methine groups and two quaternary carbon atoms, one of these oxygenated (δ = 83.0). The resonances of protonated carbon atoms were associated with those of the directly attached hydrogen atoms through the 2D ^{1}H-detected HMQC experiment (Table 1).

The presence of a hexose moiety was evident from the loss of a 180 amu ($C_6H_{12}O_6$) fragment in the FAB-MS spectrum, and by the presence of an anomeric carbon atom (signal at δ = 106.0). The sugar was identified as β-glucopyranose on the basis of the chemical shift and splitting pattern of the oxymethine protons, identified with the aid of the COSY spectrum and by dipolar couplings (Figure 1).

The aglycone moiety of **5a** displayed high-field ^{1}H NMR resonances (δ values in CD_3OD) attributable to three methyl (δ = 1.28, doublet; δ = 1.15 and 1.19, singlets), one methylene (δ = 1.65 and 1.99), and one methine group (δ = 2.19), while the lower-field region of the spectrum showed the signals of three oxymethine, one isolated oxymethylene (δ = 3.35 and 4.07, J = 8.1) and two mutually coupled *trans*-olefinic protons (δ = 5.80 and 5.65, J = 15.7 Hz). The COSY spectrum of **5a** sorted out these resonances into three distinct spin systems, shown in Figure 1. The first one (fragment A) encompassed a vicinal diol flanked by a methylene group [H(2) to H_2(4)], the second (fragment B) a five-carbon moiety spanning C(6) to C(10), while the third constituted an isolated oxymethylene group.

These spin domains were combined with the aid of the long-range correlations observed in the ^{1}H-^{13}C HMBC spectrum. The HMBC cross-peaks exhibited by H_2(11) and by H(6) were diagnostic for assembling the spin systems into an oxygen-bridged bicyclic dihydroionol structure. Thus, H_2(11) was coupled with C(2) (δ = 88.1), C(1) (δ = 50.0), C(12) (δ = 18.5), C(5) (δ = 83.0), and C(6) (δ = 61.4), while H(6) exhibited key correlation peaks with C(13) (δ = 23.8), C(4) (δ = 42.4), and C(2). The detection of an HMBC cross-peak between the relatively downfield shifted C(2) and H(1′) signals indicated the location of the glycosidic linkage.

The relative configuration of the bicyclic core of **5a** was determined by combined analysis of the scalar and the dipolar couplings of the carbinol protons H(2) and H(3) (Figure 1). Thus, the value of $J_{2,3}$ (7.9 Hz) was diagnostic of a

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectroscopic data of euphorbioside A (**5a**) and euphorbioside B (**5b**) in CD_3OD

Position	**5a** δ_C (mult.)	δ_H (int., mult., *J* in Hz)	**5b** δ_C (mult.)	δ_H (int., mult., *J* in Hz)
1	50.0 (C)		49.5 (C)	
2	88.1 (CH)	3.57 (d, 1 H, 7.9)	87.9 (CH)	3.51 (d, 1 H, 7.9)
3	71.6 (CH)	3.88 (ddd, 1 H, 10.6. 7.9, 6.6)	71.5 (CH)	3.82 (ddd, 1 H, 10.6. 7.9, 6.6)
4ax	42.4 (CH_2)	1.65 (dd, 1 H, 13.6, 10.6)	42.3 (CH_2)	1.63 (dd, 1 H, 13.6, 10.9)
4eq		1.99 (dd, 1 H, 13.6, 6.6)		1.97 (dd, 1 H, 13.6, 6.6)
5	83.0 (C)		82.8 (C)	
6	61.4 (CH)	2.19 (1 H, bd, 10.1)	56.9 (CH)	1.50 (1 H, bt, 3.1)
7a	123.5 (CH)	5.65 (dd, 1 H, 15.7, 10.1)	22.0 (CH_2)	1.30 (m, 1 H)
7b				1.73 (m, 1 H)
8	142.7 (CH)	5.80 (dd, 1 H, 15.7, 5.8)	39.8 (CH_2)	1.60 (q, 2 H, 6.3)
9	68.7 (CH)	4.32[a]	68.0 (CH)	3.75 (q, 1 H, 6.3)
10	24.3 (CH_3)	1.28 (d, 3 H, 6.3)	24.0 (CH_3)	1.22 (d, 3 H, 6.3)
11a	74.6 (CH_2)	3.35[a]	74.0 (CH_2)	3.35[a]
11b		4.07 (d, 1 H, 8.1)		3.97 (d, 1 H, 8.1)
12	18.5 (CH_3)	1.15 (s, 3 H)	18.9 (CH_3)	1.20 (s, 3 H)
13	23.8 (CH_3)	1.19 (s, 3 H)	25.0 (CH_3)	1.28 (s, 3 H)
1′	106.0 (CH)	4.32 (d, 1 H, 7.9)	106.1 (CH)	4.35 (d, 1 H, 7.9)
2′	74.9 (CH)	3.28 (t, 1 H, 7.9)	74.9 (CH)	3.28 (t, 1 H, 7.9)
3′	74.5 (CH)	3.35[a]	74.5 (CH)	3.35[a]
4′	71.4 (CH)	3.32 (dd, 1 H, 8.5, 7.2)	71.3 (CH)	3.32 (dd, 1 H, 8.5, 7.2)
5′	77.8 (CH)	3.35[a]	77.8 (CH)	3.35[a]
6′a	62.3 (CH_2)	3.92 (dd, 1 H, 11.8, 2.1)	62.1 (CH_2)	3.92 (dd, 1 H, 11.8, 2.1)
6′b		3.68 (dd, 1 H, 11.8, 6.2)		3.68 (dd, 1 H, 11.8, 6.2)

[a] Overlapped by other signals.

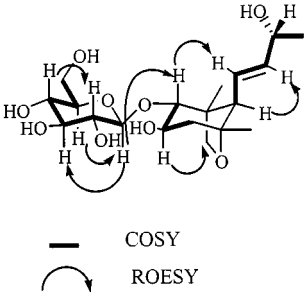
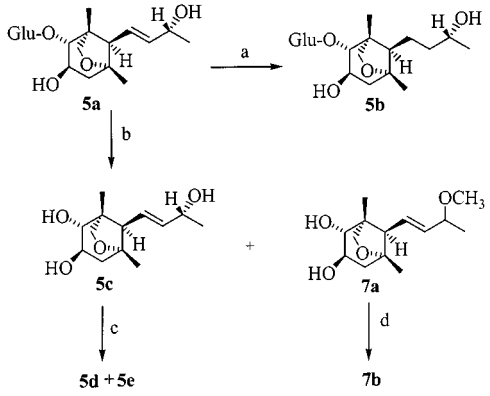


Figure 1. Partial structures by COSY and key ROESY correlations of compound **5a**



Scheme 1. a: H_2/10% Pd on charcoal; b: HCl in MeOH; c: (*R*)- and (*S*)-MTPA chloride; d: *p*-Br-benzoyl chloride and DMAP

trans orientation within a bicyclo[3.2.1]octane framework,[21] while the dipolar coupling of H(3) with H(11a) and that of H(2) with H(7) (Figure 1) related the configuration of the vicinal oxymethine groups to that of the remaining stereocenters within the bicyclic core. Finally, the dipolar coupling between H(2) and the anomeric proton confirmed the location of the sugar moiety at C(2). Euphorbioside A is therefore the C(2)-glycosylated derivative of a novel bisnorsesquiterpene with a 5,11-epoxymegastigmane skeleton, structurally related to phaseic acid (**6**),[22] a metabolite of the plant hormone abscisic acid.

Euphorbioside B (**5b**) showed close spectroscopic similarities to **5a**, and the differences could easily be explained in terms of saturation of the double bond. This conclusion was supported by catalytic hydrogenation (H_2/10% Pd on charcoal) of **5a**, which afforded a compound identical to **5b** (Scheme 1).

Together with major quantities of the glucosides **5a** and **5b**, small amounts of a further megastigmane derivative (**5c**) were obtained. This compound was obtained as an amorphous solid, of molecular formula $C_{13}H_{22}O_4$ (HRMS). Inspection of COSY, HMQC, and HMBC spectra identified it as the aglycone of euphorbioside A. To confirm this assignment, euphorbioside A (**5a**) was subjected to acid methanolysis with 1 N HCl in 85% MeOH for 2 h at 70 °C (Scheme 1), to afford a mixture of **5c** and its 9-*O*-methyl derivative **7a**, the result of a nucleophilic substitution with methanol at C(9). The stereochemistry at this carbon atom of **7a** has been left undetermined. The availability of this compound, in which the allylic hydroxy moiety is blocked, turned out to be crucial for unambiguous deter-

mination of the absolute configuration of the *vic*-diol system. Thus, **7a** was treated with *p*-bromobenzoyl chloride in dry pyridine and catalytic amounts of 4-(dimethylamino)pyridine (DMAP), to afford the bis(bromobenzoate) **7b**. This compound displayed a single CD-active system, and its spectrum indicated negative chirality in the diaroyloxy system, corresponding to a (2*R*,3*S*) configuration.[23] The absolute configurations at C(2) and C(3) having been established, the absolute configuration at C(9) was next determined by the Mosher method. Thus, treatment of **5c** with (−)-(*R*)- and (+)-(*S*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) chloride in dry pyridine afforded the (*S*)-MTPA triester **5d**, and the (*R*)-MTPA triester **5e**, respectively. Analysis of the Δδ ($\delta_S - \delta_R$) values according to the modified Mosher method for secondary alcohols (Figure 2) determined an (*R*) configuration for C(9).[24]

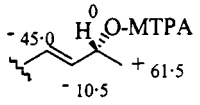


Figure 2. Application of the modified Mosher method for determination of the absolute stereochemistry at C(9) of **5a**; Δδ ($\delta_S - \delta_R$) values are given in Hz

Euphorbiosides and related compounds are the first sesquiterpenoids isolated from *Euphorbia resinifera*, and represent only the second case of occurrence of sesquiterpenoids in spurges,[25] a type of plants, the secondary metabolism of which is biased toward the production of di- and triterpenoids.[26] One may therefore speculate on whether euphorbiosides are true sesquiterpenoids or, rather, higher isoprenoids that have undergone metabolic fragmentation.

Conclusion

E. resinifera can provide access to molecular diversity of great biomedical relevance. Current interest focuses on RTX, and the procedure that we have developed for its isolation substantially alleviates the hazards associated with the direct isolation of this obnoxious compound from a highly poisonous matrix like the fresh latex of the plant. Furthermore, the diterpenoid fraction of the latex is a convenient source of other basic *Euphorbia* polyols of biomedical interest (ingol, 12-deoxyphorbol, and ingenol), while structurally unique compounds, named euphorbiosides, have been obtained from side-cuts of the diterpenoid fraction.

While evaluation of the biological activity and chemotaxonomic significance of euphorbiosides awaits further studies, the observation that *E. resinifera* substantially maintains its phorboid profile during greenhouse cultivation stands in sharp contrast with what has been reported for other African euphorbias, in which phorboid production was totally shut down by domestication,[27] and should pave the way to in-depth investigation of the structure-activity relationships of resiniferonoids.

Experimental Section

Caution: The latex of *E. resinifera* and RTX are both strongly irritant to skin and mucous membranes, and the latex displays tumour-promoting activity. Handling of these substances should be carried out wearing latex gloves and face protection, and avoiding contact with the skin. The use of disposable plastic "glassware" is advisable for all operations involving either the latex or Euphorbium.

General Remarks: Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates, which were viewed by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Merck silica gel was used for column chromatography (CC). Optical rotations (MeOH or $CHCl_3$): Perkin−Elmer 192 polarimeter, equipped with a sodium lamp (λ = 589 nm) and a 10-cm microcell. IR (KBr): Bruker model IFS-48 spectrophotometer. UV spectra (CH_3CN): Beckman DU70 spectrophotometer. CD spectra (CH_3CN): JASCO 500A polarimeter. Low- and high-resolution EI (70 eV) and FAB mass spectra (CsI ions, glycerol matrix): VG Prospec (FISONS) mass spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra: Bruker AMX 500 spectrometer; chemical shifts are referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.34$, $\delta_C = 49.0$; $CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$). The multiplicities of ^{13}C resonances were determined by DEPT experiments. Homonuclear ^1H connectivities were determined by using COSY experiments. One-bond heteronuclear ^1H-^{13}C connectivities were determined with the 2D HMQC pulse sequence, using a BIRD pulse 0.50 s before each scan in order to suppress the signals originating from protons not directly bonded to ^{13}C (interpulse delay set for $^1J_{CH} = 125$ Hz). During the acquisition time, ^{13}C broad-band decoupling was performed by use of the GARP sequence. Two- and three-bond ^1H-^{13}C connectivities were determined by 2D HMBC experiments optimized for a $^{2,3}J$ value of 9 Hz. Medium-pressure liquid chromatography (MPLC): Büchi 861 apparatus. High performance liquid chromatography (HPLC) separations in isocratic mode: Varian apparatus equipped with an RI-4 refractive index detector and with LUNA SI60 (250 × 4 mm) or Hibar LiChrospher SI60, RP18, or μ-Bondapack C18 columns. GC-MS experiments: Hewlett−Packard 5890 gas chromatograph with a mass-selective detector MSD HP 5790 MS. A fused silica column, (25 m × 0.20 mm HP-5; cross-linked 25% Ph-Me-silicone, 0.33 mm film thickness) was used with a helium carrier flow of 10 mL/min. The temperature of the column was varied, after a delay of 5 min from the injection, from 130 to 300 °C with a slope of 5 °C·min^{-1}. *Euphorbia* diterpenoids retain solvents strongly and are not amenable to elemental analysis.

Plant Material: Fresh *E. resinifera* Berg. latex was obtained by pricking commercial samples of the plant [purchased from Vivai Crova, Chieri (TO), Italy and Botaniké, Baveno (VR), Italy] with a needle, and collecting the latex on preweighed strips of filter paper, which were stored at 4 °C until extraction. Alternatively, a wad of cotton was wrapped around the stem, which was then pricked, letting the latex soak the cotton wad. Each puncture provided ca. 100 mg of latex, and ca. 20 g of latex was taken from each plant. Euphorbium was either purchased from Thomas Friedrich, Euskirchen, Germany (sample A) or was a gift from Farmacia Appendino (sample B), Carmagnola, Italy.

Extraction and Isolation

1. Direct Isolation of RTX: Filter paper strips on which 25 g of latex had been adsorbed (= fresh latex) were magnetically stirred with EtOAc (3 × ca. 100 mL). The pooled solutions were filtered through a short bed of TLC-grade silica gel (ca. 20 g), and the clear filtrate was taken to dryness, to afford a pasty, yellowish residue (=

resinous fraction). Trituration with acetonitrile gave a precipitate, which was collected on a sintered glass funnel and washed three times with acetonitrile, eventually affording a white powder (1.56 g, 6.2%). The mother liquors (0.7 g) were purified by open column chromatography, using a hexane/EtOAc gradient. Fractions eluted with hexane/EtOAc (6:4) gave a complex mixture of phorboids (72 mg) and 12.4 mg resiniferatoxin still contaminated by other phorboids. Further purification by HPLC (Microporasil column; hexane/EtOAc, 5:5) eventually afforded resiniferatoxin (**1a**; 6.7 mg, 0.027%), identified by comparison (^{1}H NMR, MS, HPLC) with an authentic commercial (Alexis Corporation) sample. The phorboid fraction could only be partially separated with this solvent system, to afford crude **2a**, **3a** and **4a/4b**. For satisfactory purification of these compounds, a further HPLC step was required [LUNA SI60 column with *n*-hexane/EtOAc (1:1) for **2a**, and hexane/EtOAc (7:3) for **4a** and **4b**]; Hibar LiChrospher SI60 [7.8 ×300 mm column with *n*-hexane/EtOAc (1:1) for **3a**]. The overall yields were 14 mg (0.056%) for **2a**, 7 mg (0.028%) for **3a**, 12 mg (0.048%) for **4a**, and 9 mg (0.036%) for **4b**.

Ingenol 3-(2,6-Dimethylnonanoate) (2a): Amorphous solid. $[\alpha]_D^{25}$ = −4 (*c* = 0.003, $CHCl_3$). IR (KBr): $\tilde{\nu}$ = 3644, 1640, 1455 cm^{-1}. FABMS (positive ions): *m*/*z* = 517 $[M + H]^+$. HRFABMS: found *m*/*z* = 517.3555 $[M + H]^+$, calcd. *m*/*z* = 517.3531 for $C_{31}H_{49}O_6$. ^{1}H NMR ($CDCl_3$): δ = 0.69 (1 H, bq, *J* = 8.8 Hz, 13-H); 0.83 (d, *J* = 6.6 Hz, 3 H, 11′-H_3); 0.87 (t, *J* = 7.3 Hz, 3 H, 9′-H_3); 0.93 (dd, *J* = 11.8, 8.8 Hz, 1 H, 14-H); 0.97 (d, *J* = 6.6 Hz, 3 H, 18-H_3); 1.05 (s, 3 H, 16-H_3); 1.08 (s, 3 H, 17-H_3); 1.18 (d, *J* = 6.5 Hz, 3 H, 10′-H_3); 1.25 (6 H, overl., 5′-H_2, 7′-H_2, 8′-H_2); 1.30 (m, 1 H, 4′b-H); 1.33 (m, 1 H, 4′a-H); 1.37 (m, 1 H, 6′-H); 1.45 (m, 1 H, 3′b-H); 1.67 (m, 1 H, 3′a-H); 1.73 (ddd, 1 H, *J* = 16.2, 8.8, 5.9 Hz, 12b-H); 1.77 (d, *J* = 1.2 Hz, 3 H, 19-H_3); 2.26 (ddd, 1 H, *J* = 16.2, 8.8, 2.2 Hz, 12a-H); 2.51 (1 H, overl., 2′-H); 2.53 (1 H, overl., 11-H); 4.03 (s, 1 H, 5-H); 4.14 (2 H, overl., 20-H_2); 4.15 (1 H, overl., 8-H); 5.43 (s, 1 H, 3-H); 6.03 (d, *J* = 1.2 Hz, 1 H, 1-H); 6.06 (1 H, bd, *J* = 4.4 Hz, 7-H). ^{13}C NMR ($CDCl_3$): δ = 12.4 (C-19); 13.3 (C-10′); 14.2 (C-18); 14.5 (C-9′); 16.1 (C-17); 16.2 (C-11′); 23.8 (C-14); 24.8 (C-13); 25.0 (C-5′, C-7′, C-8′); 25.1 (C-15, C-4′); 26.1 (C-16); 29.2 (C-3′); 31.5 (C-12); 32.1 (C-6′); 35.8 (C-2′); 39.4 (C-11); 40.6 (C-8); 64.9 (C-20); 72.5 (C-10); 74.4 (C-5); 83.2 (C-3); 86.5 (C-4); 126.6 (C-7); 129.7 (C-1); 136.0 (C-2); 170.5 (C-1′); 208.6 (C-9). For methanolysis and GC-MS analysis, a sample of **2a** (2 mg) was dissolved in 1 N HCl (1 mL) in MeOH/H_2O (85:15) and the obtained solution was kept for about 12 h at 70 °C in a sealed tube. Once cooled, the reaction mixture was neutralised with Ag_2CO_3 and centrifuged. The supernatant was dried under nitrogen and then analysed by GC-MS. The presence of methyl 2,6-dimethylnonanoate was established by comparison of the retention times and mass spectrum with those of an authentic sample.

Ingol 3,7,12-Triacetate 8-(Phenylacetate) (3a): Amorphous solid. $[\alpha]_D^{25}$ = +15 (*c* = 0.002, $CHCl_3$). IR (KBr): $\tilde{\nu}_{max}$ 1723, 1705, 1650, 812 cm^{-1}. EIMS: *m*/*z* (%) = 610 (100), 551 (30), 490 (22), 432 (28), 294 (35), 165 (80). ^{1}H NMR ($CDCl_3$): δ = 0.81 (s, 3 H, 19-H_3); 0.92 (d, *J* = 7.3 Hz, 3 H, 16-H_3); 1.03 (3 H, overl., 20-H_3); 1.03 (1 H, overl., 11-H); 1.06 (s, 3 H, 18-H_3); 1.09 (dd, *J* = 10.5, 9.0 Hz, 1 H, 9-H); 1.68 (1 H, bd, *J* = 15.0 Hz, 1β-H); 1.98 (s, 3 H, CH_3CO); 2.05 (3 H, br. s, 17-H_3); 2.06 (s, 3 H, CH_3CO); 2.10 (s, 3 H, CH_3CO); 2.50 (m, 1 H, 2-H), 2.78 (dd, *J* = 15.0, 8.8 Hz, 1 H, 1α-H); 2.88 (m, 1 H, 13-H); 3.72 (s, 2 H, COCH_2Ph); 4.52 (1 H, bd, *J* = 10.5 Hz, 8-H); 4.82 (dd, *J* = 11.0, 2.5 Hz, 1 H, 12-H); 5.14 (d, *J* = 8.5 Hz, 1 H, 3-H); 5.15 (1 H, br. s, 7-H); 5.40 (1 H, bs., 5-H); 7.32−7.24 (5 H, overlapped, $COCH_2Ph$). ^{13}C NMR ($CDCl_3$): δ = 13.1 (C-20); 16.1 (C-19); 17.2 (C-16); 17.5 (C-17); 19.5 (C-10); 20.4 (*C*H_3CO); 20.8 (*C*H_3CO); 21.0 (*C*H_3CO); 25.0 (C-9); 29.0 (C-18); 29.4 (C-11); 29.5 (C-2); 31.6 (C-1); 42.7 (C-13); 70.3 (C-12); 70.7 (C-15); 71.6 (C-8); 72.8 (C-4); 76.5 (C-3); 76.7 (C-7); 117.5 (C-5); 138.5 (C-6); 127.6 ($COCH_2Ph$); 128.0 ($COCH_2Ph$); 128.3 ($COCH_2Ph$); 133.0 ($COCH_2Ph$); 170.1 (*C*OCH_2Ph); 170.5 (CH_3CO); 170.7 (CH_3CO); 170.8 (CH_3CO); 207.8 (C-14).

12-Deoxyphorbol 13-Isobutyrate 20-Acetate (4b): $[\alpha]_D^{25}$ = +121 (*c* = 0.003, $CHCl_3$). IR (KBr): $\tilde{\nu}_{max}$ 3420, 2920, 1708, 1649, 1455 cm^{-1}. FABMS (positive ions): *m*/*z* = 461 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 0.78 (d, *J* = 5.1 Hz, 1 H, 14-H); 0.89 (d, *J* = 6.6 Hz, 3 H, 18-H_3); 1.08 (s, 3 H, 16-H_3); 1.16 (d, *J* = 6.6 Hz, 6 H, 3′,4′-H_3); 1.18 (s, 3 H, 17-H_3); 1.63 (dd, *J* = 15.0, 11.8 Hz, 1 H, 12b-H); 1.78 (s, 3 H, 19-H_3); 1.96 (m, 1 H, 11-H); 2.07 (s, 3 H, CH_3CO); 2.08 (1 H, overl., 12a-H); 2.39 (d, *J* = 19.0 Hz, 1 H, 5b-H); 2.52 (ept., *J* = 6.6 Hz, 1 H, 2′-H); 2.53 (d, *J* = 19.0 Hz, 1 H, 5a-H); 3.03 (dd, *J* = 6.0, 5.1 Hz, 1 H, 8-H); 3.29 (1 H, br. s, 10-H); 4.48 (2 H, AB q, *J* = 12.5 Hz, 20-H_2); 5.75 (1 H, bd, *J* = 6.0 Hz, 7-H); 7.63 (1 H, br. s, 1-H). ^{13}C NMR ($CDCl_3$): δ = 10.1 (C-19); 13.8 (C-3′,-4′); 15.3 (C-17); 18.8 (C-18); 21.0 (*C*H_3CO); 22.9 (C-15); 23.6 (C-16); 31.9 (C-12); 32.8 (C-14); 35.5 (C-2′); 36.4 (C-11); 39.0 (C-5); 39.6 (C-8); 55.5 (C-10); 62.8 (C-13); 69.7 (C-20); 73.5 (C-4); 76.0 (C-9); 132.7 (C-2); 134.1 (C-7); 134.8 (C-6); 161.7 (C-1); 169.8 (C-1′); 170.9 (CH_3CO); 209.2 (C-3).

2. Isolation of RTX by a Saponification-Reesterification Procedure. − a) Isolation of ROPA: Fresh latex (100 g) of *E. resinifera* was extracted with EtOAc, filtered on a pad of TLC grade silica gel, and treated with acetonitrile as described above. The crude phorboid mixture (2.4 g) was treated with NaOMe (0.1 M, 25 mL). After it had been stirred for 1 h at room temp., the reaction mixture was worked up by neutralisation with HOAc, extraction with EtOAc, and washing with brine. After removal of the solvent, the residue was purified by gravity column chromatography. Elution with hexane/EtOAc (6:4) gave ROPA [**1b**, 20 mg, 0.01%, identified by comparison (NMR, MS, HPLC) with a commercial sample (Alexis Corporation)], together with a mixture of 12-deoxyphorbol and its 13-esters (36 mg). Further elution with hexane/EtOAc (2:8) afforded ingenol (**2b**, 15.2 mg, 0.076%, identified by comparison with the NMR spectroscopic data of an authentic sample, available from previous studies[28]) and ingol 12-acetate (**3b**, 31.2 mg, 0.016%).[15] − **b) Mitsunobu Esterification of ROPA:** Homovanillic acid (13.8 mg, 0.076 mmol, 2 mol-equiv.), triphenylphosphane (TPP, 20 mg, 0.076 mmol, 2 mol-equiv.) and di-*tert*-butyl azodicarboxylate (17.5 mg, 0.076 mmol, 2 mol-equiv.) were added sequentially to a cooled (0 °C) solution of ROPA (18 mg, 0.038 mmol) in dry THF (0.30 mL). The reaction mixture was stirred at room temp. for 1h, and then worked up by addition of silica gel (70−123 mesh, ca. 1 g) and removal of the solvent. The residue was directly loaded onto a short (3 g) column of silica gel and eluted, first with hexane/EtOAc (9:1) to remove the excess unchanged TPP, di-*tert*-butyl azodicarboxylate and di-*tert*-butyl hydrazodicarboxylate, and next with hexane/EtOAc (8:2), to give 16 mg (67%) RTX as an amorphous gum.

3. Fractionation of the Acetonitrile Precipitate: A sample of the acetonitrile precipitate from the fresh latex (12.5 g) was column-chromatographed by MPLC on silica gel (230−400 mesh) with a gradient solvent system from *n*-hexane to MeOH, through EtOAc. Fractions obtained by elution with *n*-hexane/EtOAc (8:2 to 7:3) principally contained a mixture of the triterpenoids euphol and euphorbol (ca. 3 g). Fractions eluted with EtOAc/MeOH (1:1) were purified by HPLC on a Hibar LiChrospher Si60 (10μm) (3.9 × 300 mm) column with a mobile phase EtOAc/MeOH (8:2) to give pure **5c** (1.5 mg). Fractions eluted with MeOH were further chromato-

graphed by MPLC on an RP-18 column with a gradient solvent system from H_2O to MeOH, and fractions obtained by elution with H_2O/MeOH (7:3) were finally purified by HPLC on a μ-Bondapack C-18 (10 mm) (3.9 × 300 mm) column with a mobile phase H_2O/MeOH (8:2) to afford pure euphorbioside A (**5a**, 170 mg, 1.4%) and euphorbioside B (**5b**, 62 mg, 0.50%).

Euphorbioside A (5a): Amorphous solid. $[\alpha]_D^{25} = -8$ (c = 0.005, MeOH). IR (KBr): $\tilde{\nu}_{max} = 3699\ cm^{-1}$. FABMS (positive ions): m/z = 427 $[M + Na]^+$, 405 $[M + H]^+$, 225. HRFABMS: found m/z = 405.2134 $[M + H]^+$, calcd. m/z = 405.2126 for $C_{19}H_{33}O_9^{\cdot}$. ^1H and ^{13}C NMR (CD_3OD): see Table 1.

Euphorbioside B (5b): Amorphous solid. $[\alpha]_D^{25} = -3$ (c = 0.004, MeOH). IR (KBr): $\tilde{\nu}_{max} = 3699\ cm^{-1}$. FABMS (positive ions): m/z = 429 $[M + Na]^+$, 407 $[M + H]^+$. HRFABMS: found m/z = 407.2296 $[M + H]^+$, calcd. m/z = 407.2283 for $C_{19}H_{35}O_9^{\cdot}$. ^1H and ^{13}C NMR (CD_3OD): see Table 1.

Deglucosyl Euphorbioside A (5c): Amorphous solid. $[\alpha]_D^{25} = -3$ (c = 0.001, MeOH). IR (KBr): $\tilde{\nu}_{max} = 3710\ cm^{-1}$. FABMS (positive ions): m/z = 243 $[M + H]^+$. HRFABMS: found m/z = 243.1567 $[M + H]^+$, calcd. m/z = 243.1526 for $C_{13}H_{23}O_4^{\cdot}$. ^1H NMR (CD_3OD): δ = 1.03 (s, 3 H, 12-H_3); 1.17 (s, 3 H, 13-H_3); 1.28 (d, J = 6.6 Hz, 3 H, 10-H_3); 1.67 (dd, J = 13.6, 10.6 Hz, 1 H, 4ax-H); 1.93 (dd, J = 13.6, 6.6 Hz, 1 H, 4eq-H); 2.19 (d, J = 10.1 Hz, 1 H, 6-H); 3.34 (1 H, obscured by residual solvent signal, 11b-H); 3.53 (d, J = 7.9 Hz, 1 H, 2-H); 3.73 (ddd, 1 H, J = 10.6, 7.9, 6.6 Hz, 3-H); 4.01 (d, J = 8.5 Hz, 1 H, 11a-H); 4.31 (dq, J = 6.3, 5.8 Hz, 1 H, 9-H); 5.69 (dd, J = 15.7, 10.1 Hz, 1 H, 7-H); 5.79 (dd, J = 15.7, 5.8 Hz, 1 H, 8-H). ^{13}C NMR (CD_3OD): δ = 18.5 (C-12); 23.0 (C-13); 24.3 (C-10); 42.1 (C-4); 50.0 (C-1); 61.5 (C-6); 68.8 (C-9); 70.5 (C-3); 74.3 (C-2); 75.0 (C-11); 82.8 (C-5); 123.5 (C-7); 142.1 (C-8).

Catalytic Hydrogenation of Euphorbioside A: Palladium on charcoal catalyst (10%; 1 mg) was added to **5a** (6.0 mg) in dry EtOH (1 mL). The solution was stirred at room temperature under hydrogen for 20 min. The catalyst was then removed by filtration and the solvent was evaporated to obtain a mixture, which, after purification by HPLC on an RP18 column (eluent H_2O/MeOH, 8:2), afforded **5b** (3.0 mg, 50%).

Methanolysis of Euphorbioside A: Euphorbioside A (10 mg) was dissolved in 4 mL of 1 N HCl in MeOH/H_2O (85:15) and the solution was stirred for 2 h at 70 °C. The reaction mixture was worked up by neutralisation with Ag_2CO_3, and centrifugation and the supernatant was dried under nitrogen and partitioned between H_2O and EtOAc. The apolar layer (5 mg) was purified by HPLC (SI60, EtOAc/MeOH, 85:15) and afforded, as main products, **5c** (2.5 mg, 42%) and **7a** (1.8 mg, 28%).

(9ζ)-9*O*-Methyl Deglucosyl Euphorbioside A (7a): Amorphous solid. $[\alpha]_D^{25} = -5$ (c = 0.001, MeOH). IR (KBr): $\tilde{\nu}_{max}$ 3710 cm^{-1}. FABMS (positive ions): m/z = 257 $[M + H]^+$. HRFABMS: found m/z = 257.1767 $[M + H]^+$ calcd. m/z = 257.1755 for $C_{14}H_{25}O_4^{\cdot}$. ^1H NMR (CD_3OD): δ = 1.02 (s, 3 H, 12-H_3); 1.20 (s, 3 H, 13-H_3); 1.27 (d, J = 6.5 Hz, 3 H, 10-H_3); 1.68 (dd, J = 13.5, 10.5 Hz, 1 H, 4ax-H); 1.95 (dd, J = 13.5, 6.5 Hz, 1 H, 4eq-H); 2.23 (d, J = 10.0 Hz, 1 H, 6-H); 3.30 (s, 3 H, OCH_3); 3.34 (1 H, obscured by residual solvent signal, 11b-H); 3.50 (d, J = 7.5 Hz, 1 H, 2-H); 3.75 (m, 1 H, 3-H); 3.84 (m, 1 H, 9-H); 4.02 (d, J = 8.5 Hz, 1 H, 11a-H); 5.60 (dd, J = 15.7, 7.0 Hz, 1 H, 8-H); 5.75 (dd, J = 15.7, 10.0 Hz, 1 H, 7-H).

***p*-Bromobenzoylation of 7a:** A sample of **7a** (1.5 mg) was dissolved in dry pyridine (1.0 mL) and treated with *p*-bromobenzoyl chloride (20 mg) and catalytic amounts of 4-(dimethylamino)pyridine. The mixture was stirred overnight at 60 °C and chilled water was then added to the solution, which, after 30 min, was extracted with $CHCl_3$. The obtained extract was separated on an HPLC LUNA SI60 column (eluent *n*-hexane/EtOAc, 85:15) to yield **7b** (1.0 mg, 26%) as an amorphous solid. $[\alpha]_D^{25} = -2$ (c = 0.001, $CHCl_3$). FABMS: m/z = 621, 623, 625 (1:2:1) $[M + H]^+$. UV (CH_3CN): λ_{max} 244 nm. CD (CH_3CN): λ_{max} = 250 nm (Δε = −12.5), 242 nm (Δε = 0.0), 234 nm (Δε = +6.2). ^1H NMR ($CDCl_3$): δ = 1.00 (s, 3 H, 12-H_3); 1.13 (s, 3 H, 13-H_3); 1.28 (d, J = 6.5 Hz, 3 H, 10-H_3); 1.55 (dd, J = 13.5, 10.5 Hz, 1 H, 4ax-H); 2.16 (d, J = 10.0 Hz, 1 H, 6-H); 2.20 (dd, J = 13.5, 6.5 Hz, 1 H, 4eq-H); 3.27 (s, 3 H, OCH_3); 3.38 (d, J = 8.5 Hz, 1 H, 11b-H); 3.74 (m, 1 H, 9-H); 3.99 (d, J = 8.5 Hz, 1 H, 11a-H); 4.98 (d, J = 7.0 Hz, 1 H, 2-H); 5.05 (m, 1 H, 3-H); 5.62 (2 H, overlapped, 7-H and 8-H); 7.59 (d, J = 6.5 Hz, 4 H, benzoate protons); 7.89 (d, J = 6.5 Hz, 4 H, benzoate protons).

(*S*)-MTPA Ester 5d: A sample of **5a** (1.5 mg) was dissolved in dry pyridine (3 mL) and (−)-(*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) chloride (5 μL; purchased from Sigma Aldrich) and DMAP (spatula tip) were added to the solution, which was then stirred overnight at room temperature. After dilution with EtOAc, the solution was concentrated in vacuo and the residue was purified by HPLC on silica gel (SI60 column, LUNA) with EtOAc/*n*-hexane (1:1) as eluent. The (*S*)-MTPA ester **5d** (1.4 mg, 50%) was obtained as an amorphous solid. IR (KBr): $\tilde{\nu}_{max} = 1715\ cm^{-1}$. ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.09 (s, 3 H, 12-H_3); 1.19 (s, 3 H, 13-H_3); 1.49 (d, J = 6.7 Hz, 3 H, 10-H_3); 2.09 (d, J = 10.1 Hz, 1 H, 6-H); 2.14 (1 H, overl., 4ax-H); 2.15 (1 H, overl., 4eq-H); 3.24 (d, J = 8.0 Hz, 1 H, 11b-H); 3.55 (s, 9 H, 3 × OMe); 4.01 (d, J = 8.0 Hz, 1 H, 11a-H); 5.08 (d, J = 7.2 Hz, 1 H, 2-H); 5.13 (dq, J = 6.7, 2.1 Hz, 1 H, 9-H); 5.14 (m, 1 H, 3-H); 5.60 (dd, J = 15.0, 10.1 Hz, 1 H, 7-H); 5.67 (dd, J = 15.0, 2.1 Hz, 1 H, 8-H); 7.41 (m, 9 H, MTPA phenyl); 7.53 (m, 6 H, MTPA phenyl).

(*R*)-MTPA Ester 5e: A sample of **5a** (1.5 mg) was treated as above with (+)-(*S*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) chloride to give the (*R*)-MTPA ester **5e** (1.2 mg, 39%) as an amorphous solid. IR (KBr): $\tilde{\nu}_{max} = 1715\ cm^{-1}$. ^1H NMR ($CDCl_3$): δ = 1.11 (s, 3 H, 12-H_3); 1.22 (s, 3 H, 13-H_3); 1.37 (d, J = 6.7 Hz, 3 H, 10-H_3); 1.54 (m, 1 H, 4ax-H); 2.07 (d, J = 9.6 Hz, 1 H, 6-H); 2.32 (m, 1 H, 4eq-H); 3.38 (d, J = 8.0 Hz, 1 H, 11b-H); 3.57 (s, 9 H, 3 × OMe); 4.06 (d, J = 8.0 Hz, 1 H, 11a-H); 5.21 (d, J = 7.5 Hz, 1 H, 2-H); 5.42 (dq, J = 6.7, 2.1 Hz, 1 H, 9-H); 5.23 (m, 1 H, 3-H); 5.69 (1 H, overl., 7-H); 5.69 (1 H, overl., 8-H); 7.39 (m, 9 H, MTPA phenyl); 7.53 (m, 6 H, MTPA phenyl).

4. Analysis of Commercial Euphorbium (Sample A as Representative): Euphorbium (120 g) was triturated with EtOAc (2 × 200 mL) to give a milky suspension, which was filtered through a pad of TLC-grade silica gel and then crystallized from acetonitrile as described previously. A white precipitate (26 g) was obtained, while the mother liquors gave a reddish gum (8 g). The latter was treated with 0.1 M NaOMe as described for the fresh latex. The ingol derivative **3b** (18 mg, 0.015%) was the only phorboid that could be obtained in pure form. The ^{13}C NMR spectrum of the crude hydrolysate, and also that of the original phorboid fraction, lacked the diagnostic orthoester resonance (at δ ≈ 110) typical of RTX and its derivatives.

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