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Triphenylphosphonium Cations of the Diterpenoid Isosteviol: Synthesis and Antimitotic Activity in a Sea Urchin Embryo Model

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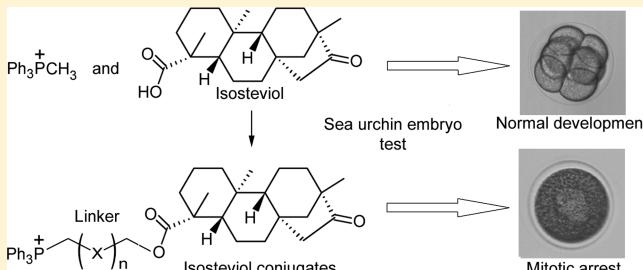
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S Supporting Information

ABSTRACT: A series of novel triphenylphosphonium (TPP) cations of the diterpenoid isosteviol (**1**, 16-oxo-*ent*-beyeran-19-oic acid) have been synthesized and evaluated in an *in vivo* phenotypic sea urchin embryo assay for antimitotic activity. The TPP moiety was applied as a carrier to provide selective accumulation of a connected compound into mitochondria. When applied to fertilized eggs, the targeted isosteviol TPP conjugates induced mitotic arrest with the formation of aberrant multipolar mitotic spindles, whereas both isosteviol and the methyltriphenylphosphonium cation were inactive.

The structure–activity relationship study revealed the essential role of the TPP group for the realization of the isosteviol effect, while the chemical structure and the length of the linker only slightly influenced the antimitotic potency. The results obtained using the sea urchin embryo model suggested that TPP conjugates of isosteviol induced mitotic spindle defects and mitotic arrest presumably by affecting mitochondrial DNA. Since targeting mitochondria is considered as an encouraging strategy for cancer therapy, TPP-isosteviol conjugates may represent promising candidates for further design as anticancer agents.

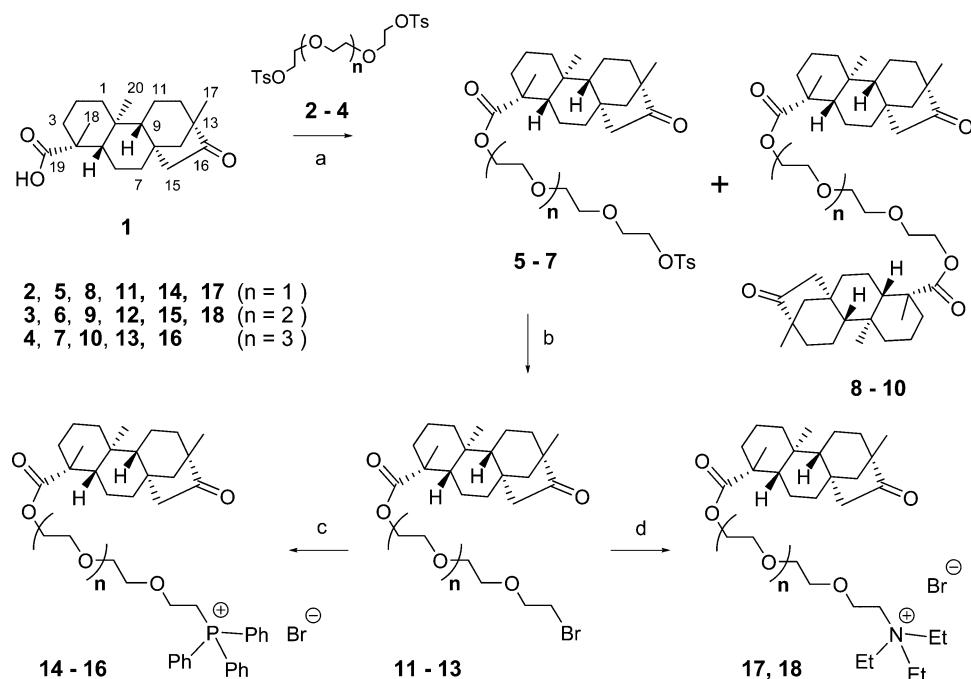


Numerous natural terpenoids, such as sesquiterpenoids,¹ diterpenoids of the abietane,^{2–4} pimarane,^{2,5} and *ent*-kaurene⁶ series, tetracyclic triterpenoids of the lanostane⁷ and cucurbitane⁸ series, and pentacyclic triterpenoids of the lupane,⁹ oleanane,^{7,10,11} ursane,⁷ and friedelane¹ series, affect the *in vitro* growth and viability of human tumor cells, suggesting their potential as anticancer agents. Typically, these molecules are extracted from the respective natural sources in small quantities, thereby restricting their further investigation for the treatment of cancer. The diterpenoid abietic acid^{7,12} and triterpenoid acids, including betulinic,⁹ oleanolic,⁷ glycyrrheticin,^{7,10,11} and ursolic⁷ acids, are readily extracted from higher plants in high yields and represent favorable exceptions. These natural products display cytotoxicity against panels of human cancer cell lines.^{7,13} In *in vivo* preclinical trials, betulinic acid showed a pronounced antitumor effect together with the absence of systemic toxicity.¹⁴ Notably, betulinic acid induces apoptosis via a direct effect on mitochondria, leading to cytochrome *c* release into the cytosol.¹⁴

Isosteviol (**1**, 16-oxo-*ent*-beyeran-19-oic acid¹⁵) is a tetracyclic natural product hydrolytic derivative related structurally to the diterpenoid acids (Scheme 1). It can be easily obtained by

acid hydrolysis of the *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae) glycoside stevioside,¹⁶ a component of various nonsugar low-calorie sweeteners manufactured on a large scale in a number of countries. Isosteviol markedly inhibited *in vivo* chemically induced mouse skin carcinogenesis, suggesting its potential chemopreventive properties.¹⁷ However, *in vitro* biological evaluation of isosteviol in human cancer cell lines failed to reveal cytotoxic effects,^{18–23} except for very weak cytotoxicity against HL60 human leukemia cells ($\text{IC}_{50} = 75 \mu\text{M}$),²⁴ MOLT-4 T-lymphoblastic leukemia cells ($\text{LD}_{50} = 84 \mu\text{M}$),²⁵ BALL-1 B-lymphoblastic leukemia cells ($\text{LD}_{50} = 87 \mu\text{M}$),²⁵ and NUGC-3 human gastric cells ($\text{LD}_{50} = 167 \mu\text{M}$).²⁵ The cytotoxicity of **1** was considered to be associated with the inhibition of mammalian DNA metabolic enzymes topoisomerase II and DNA polymerases α , β , and λ .²⁵ In spite of the negligible antiproliferative potency of **1**, chemical modifications of the isosteviol ring D,^{18–22,26,27} the synthesis of 16,19-dihydroxy-*ent*-beyerane and cinnamic acid esters,²⁴ and a coupling of two isosteviol molecules by a diamide linker²³

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Scheme 1.^a

^aReagents and conditions: (a) MeCN, K_2CO_3 , reflux, 10–12 h. (b) LiBr, acetone, reflux, 14–16 h. (c) PPh_3 , toluene, reflux, 37–75 h. (d) NEt_3 , CH_3CN , reflux, 17–23 h.

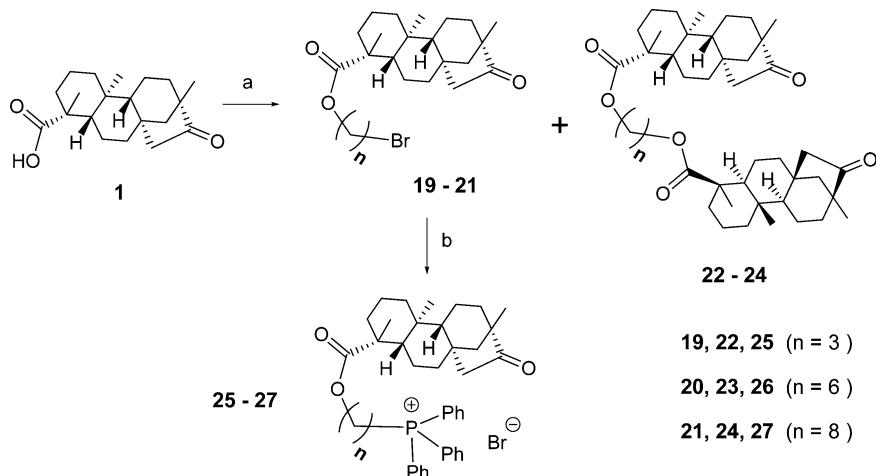
furnished a series of derivatives that demonstrated cytotoxic activity against a panel of human cancer cell lines with IC_{50} values in the range 1–7 μM .^{18–24,26,27} The mechanisms of action and cellular targets of isosteviol derivatives remain unclear. Only for C-19 O-acylated isosteviol analogues has an apoptosis-inducing effect in HL60 leukemia cells been reported.²⁴

Generally, DNA-damaging agents initiate the mitochondria-related intrinsic apoptotic pathway, which involves an increase of mitochondrial outer membrane permeability and the release of cytochrome *c* into the cytosol, thereby triggering downstream apoptotic events including caspase activation.²⁸ Therefore, the effective intracellular trafficking and targeted delivery to mitochondria may improve the cytotoxicity of a given molecule. It was found that tetraphenylphosphonium and methyltriphenylphosphonium bromides easily penetrate into mitochondria due to a highly negative membrane potential and a pH gradient across the mitochondrial inner membrane.²⁹ Moreover, a markedly higher mitochondrial transmembrane potential in malignant cells in comparison to that in normal epithelial cells provides the selective accumulation of the phosphonium cations inside cancer cell mitochondria.²⁹ Hence, the bulky lipophilic triphenylphosphonium (TPP) cations are assumed to be promising carriers for the predominant drug accumulation in mitochondria.²⁹ Notably, a conjugation of several well-known antioxidants and anticancer agents with TPP has resulted in both the targeting of mitochondria in cancer cells^{29–40} and the increase of cytotoxicity.^{13c,38–40} The application of mitochondrion-targeting TPP-conjugated mesotetraphenylporphyrin derivatives³⁴ essentially improved capabilities of photodynamic therapy in oncology.³⁵ In addition, ⁶⁴Cu-labeled triphenylphosphonium cations containing 1,4,7,10-tetraazacyclododecane moieties were used successfully as novel radiotracers for imaging tumors by positron-emission tomography.^{36,37}

It is worth noting that among natural products only 20,29-dihydrobetulinic acid and 3-*epi*-20,29-dihydrobetulinic acid have been conjugated with the TPP cation through a trimethylene linker, yielding derivatives with a markedly increased cytotoxicity against human cancer cells.^{13c} A lipophilic polymethylene linker is the only one reported to connect the TPP cation to a small molecule,^{13c,30–40} whereas for a water-soluble isosteviol triethylammonium salt, a hydrophilic poly(ethylene glycol) (PEG) linker binds together the diterpenoid skeleton and the N^+Et_3 moiety.⁴¹ It was found that this amphiphilic isosteviol derivative readily integrated with a lipid bilayer of dipalmitoylphosphatidylcholine liposomes and formed micelle-like aggregates in water, mediating DNA transfer into bacterial cells.⁴¹ Considering such evidence, it was supposed that partially water-soluble amphiphilic TPP conjugates of isosteviol containing a long hydrophilic PEG linker might also form micelles, facilitating their penetration into the mitochondrial matrix.

This hypothesis was also based on the observation that the incorporation of the diterpenoid paclitaxel in liposomes modified with stearyl triphenyl phosphonium enhanced its accumulation in mitochondria, triggering apoptosis in the paclitaxel-resistant OVCAR-3 ovarian cancer cell line.⁴² Hence, it was aimed to synthesize a series of novel biologically active isosteviol derivatives, with the isosteviol scaffold conjugated with a TPP cation by polymethylene or PEG linkers.

All synthesized isosteviol derivatives were evaluated for antimitotic activity using the sea urchin embryo model. Due to the abundance and accessibility of these gravid test animals, their straightforward artificial spawning, fertilization, and rearing, the relatively large size of the eggs and embryos, and their rapid synchronous development, optical transparency, and high penetrability to different chemicals, this simple organism model has been applied extensively in a wide range of biological and ecological tests of natural products and synthetic

Scheme 2.^a

^aReagents and conditions: (a) $\text{Br}(\text{CH}_2)_n\text{Br}$, MeCN, K_2CO_3 , reflux, 3–13 h. (b) PPh_3 , toluene, reflux, 37–75 h.

Table 1. Effects of Methyltriphenylphosphonium Bromide (MTPPB), Isosteviol (1), and Its TPP (14–16, 25–27) and Ammonium (17, 18) Conjugates on Sea Urchin Embryos

compound	linker length (number of atoms)	cleavage alteration	full mitotic arrest	embryo spinning	posthatching lethality ^b
isosteviol		>4	>4	>4	>4
MTPPB		>4	>4	>4	>4
14	8	1	2	>10	2
15	11	1	2	>10	2
16	14	1	4	>10	4
17	8	>100	>100	>100	>100 40 ^c
18	11	>100	>100	>100	>100 100 ^c
25	3	2	4	>4	4
26	6	2	4	>4	1
27	8	2	4	>4	0.5
etoposide		0.5	>68	>68	40 4 ^c
nocodazole		0.005	0.01	0.1	>0.5 0.02 ^d
paclitaxel		0.5	5	>10	2

^aThe sea urchin embryo assay was conducted as described previously.⁴⁴ Fertilized eggs and hatched blastulae were exposed to 2-fold decreasing concentrations of compounds. Duplicate measurements showed no differences in effective threshold concentration (EC) values. ^bDeath of embryos exposed to compounds at the hatched blastula stage. ^cInhibition of skeletal spiculae growth. ^dDevelopmental arrest at the late gastrula stage.

compounds, especially in the study of antiproliferative/antimitotic activity. The well-known development of the sea urchin embryos facilitates the interpretation of the aberrant morphogenetic events caused by a given test molecule. Moreover, the easily observed *in vivo* phenotypic abnormalities of eggs and embryos exposed to a test compound can provide information about its mode of action and a putative cellular target.⁴³ The *in vivo* phenotypic sea urchin embryo assay used in the present study provided information on antiproliferative, antimitotic, cytotoxic, and microtubule-destabilizing activities of the molecules tested along with their solubility and permeability potential.⁴⁴ The assay yields highly reproducible results that correlate well with values obtained by conventional cell-based and *in vitro* tubulin polymerization assays.⁴⁵ Using the above animal model, the preliminary structure–activity relationship of the resulting compounds was used to reveal the contributions of the diterpenoid *ent*-beyerane moiety, the linker

structure, and the TPP group to the resultant antimitotic effects.

RESULTS AND DISCUSSION

The targeted amphiphilic TPP conjugates of isosteviol (14–16) with PEG as a linker were obtained by three steps (Scheme 1). Reaction of isosteviol (1) with a double excess of the ditosylates of various polyethylene glycols (2–4), which were obtained by interaction of appropriate polyethylene glycols with tosyl chloride,⁴⁶ gave a mixture of tosylates 5–7 and diketones 8–10. Tosylates 5–7 were further converted into the corresponding bromides 11–13 in boiling acetone in the presence of LiBr. In the final step, bromides 11–13 were refluxed with triphenylphosphine in toluene to obtain the TPP cations 14–16. The isosteviol derivatives 17 and 18 with a triethylammonium group instead of a triphosphonium moi-

ety—the analogues of TPP conjugates **14** and **15**—were synthesized according to a reported procedure.⁴¹

Conjugates of isosteviol (**25–27**) with the TPP group attached to the diterpenoid *ent*-beyerane skeleton by hydrophobic polymethylene linkers were synthesized via a reported procedure (Scheme 2).⁴⁷ Isosteviol (**1**) was refluxed in CH₃CN with a 2-fold excess of dibromoalkanes in the presence of K₂CO₃ to afford products of monoalkylation (**19–21**) and dialkylation (**22–24**). Further interaction of compounds **19–21** with Ph₃P in toluene under reflux yielded the TPP conjugates **25–27**.

Since numerous structurally diverse antimitotic agents have been found to target microtubules,⁴⁸ the possible involvement of microtubules in the antiproliferative effects of isosteviol derivatives was investigated. Microtubules are cytoskeletal organelles involved in cell shape maintenance, intracellular transport, cell adhesion and migration, and ciliary and flagellar motility. An essential role for microtubules in cell division is the formation of the mitotic spindle, responsible for the correct chromosomal orientation and separation into daughter cells.⁴⁹

Antiproliferative effects of isosteviol (**1**), methyltriphenylphosphonium bromide (MTPPB), TPP-isosteviol conjugates **14–18** and **25–27**, and dialkylation products **23** and **24** were studied using the sea urchin embryo model. The assay includes (i) a fertilized egg test for antimitotic activity and (ii) a means of assessing embryo viability, morphogenesis, and swimming pattern after exposure to a compound just after hatching, ~9 h postfertilization. Specific embryo motility changes, namely, the lack of forward movement, settlement to the bottom of the culture vessel, and rapid spinning around the animal–vegetal axis, indicate the microtubule-destabilizing activity caused by a molecule.⁴⁴ The test results are shown in Table 1. An inhibitor of tubulin polymerization, nocodazole, a microtubule stabilizer, paclitaxel, and a topoisomerase II inhibitor, etoposide, served as reference compounds.

In addition, throughout the study, mitotic spindle patterns in fertilized eggs arrested by the test molecules were analyzed. Importantly, sea urchin embryos offer the experimental possibility for direct *in vivo* observation of the mitotic apparatus using conventional light microscopy (Figure 1). Mitotic phenotypes of fertilized eggs exposed to a compound can be easily monitored, yielding the opportunity to suggest a mechanism of action. During the first division of intact eggs, normal bipolar mitotic spindles can be seen as two large light spots (Figure 1A). Tubulin-targeting agents interfere with microtubule assembly, resulting in diverse mitotic spindle defects and cleavage arrest. Microtubule stabilizers cause the formation of multipolar mitotic asters,^{44,50} as shown for paclitaxel-treated eggs (Figure 1C). In contrast, inhibitors of tubulin polymerization, e.g., nocodazole, completely block spindle assembly, producing eggs devoid of mitotic apparatus, with small clear spots corresponding to nuclei⁴³ in the center of arrested eggs (Figure 1D).

As shown in Table 1, both isosteviol (**1**) and methyltriphenylphosphonium bromide were inactive up to a 4 μM concentration, whereas the TPP conjugates of isosteviol (**14–16** and **25–27**) caused cleavage alteration, cleavage arrest, and posthatching embryo death in the low micromolar concentration range. Interestingly, compounds **14–16** and **25–27** generated a distinct arrested egg phenotype with a mitotic apparatus as a centrally located large light spot (Figure 1E).

Preliminary structure–activity relationship studies showed that the TPP moiety is essential for antimitotic activity.

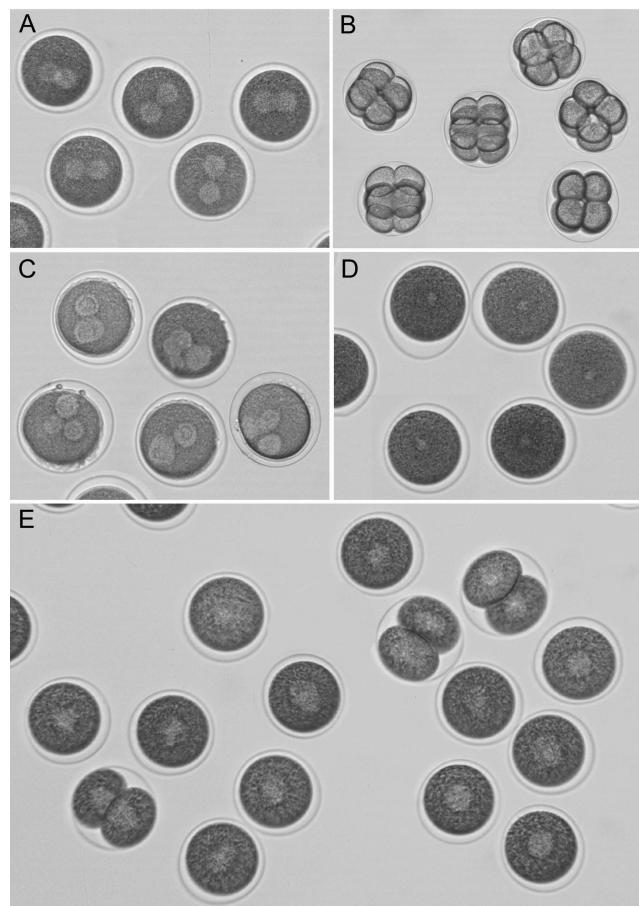


Figure 1. Mitotic spindle defects in the arrested sea urchin eggs exposed to isosteviol derivative **26** and microtubule-targeted agents. (A) Intact eggs with normal bipolar mitotic apparatus at the first cleavage anaphase, 1 h postfertilization. (B–G) At 2.5 h postfertilization. (B) Control eight-cell embryos. (C) Multipolar mitotic spindles caused by the microtubule stabilizer paclitaxel (5 μM). (D) Eggs devoid of mitotic apparatus in the presence of the microtubule destabilizer nocodazole (50 nM). (E) Formation of aberrant mitotic spindles initiated by **26** (2 μM). Samples were incubated at 21 °C. The average egg/embryo diameter was 115 μm.

Specifically, the replacement of TPP in **14** and **15** with triethylamine afforded the inactive conjugates **17** and **18**, which caused only weak effects, namely, retardation of skeletal spiculae growth at the pluteus stage. Although compounds with PEG and polymethylene linkers displayed closely comparable effects, the PEG-containing conjugates **14–16** were slightly more active when applied to fertilized eggs. Alternatively, molecules with polymethylene fragments (**25–27**) affected posthatching development in a more potent manner than **14–16**, especially compound **27**, with the longest octamethylene linker (Table 1). The antimitotic activity of all TPP conjugates was independent of linker length.

It is worth noting that compounds **14–16** and **25–27** failed to block mitotic spindle assembly or to induce embryo spinning typical of microtubule destabilizers. In contrast to isosteviol derivatives, nocodazole was unable to kill embryos at posthatching stages even at a concentration 1000 times higher than that sufficient for cleavage alterations. Multipolar mitotic spindles were observed in eggs treated by microtubule-stabilizing agents, but not in eggs exposed to TPP-isosteviol conjugates. Therefore, the differences in both developmental

alterations and mitotic spindle patterns caused by TPP conjugates in comparison with the tubulin-targeting compounds paclitaxel and nocodazole suggested that the antimitotic effect of the synthesized isosteviol derivatives is not mediated by the direct interaction with tubulin/microtubules.

Considering the ability of isosteviol (**1**) to inhibit the DNA-related enzymes topoisomerase II and DNA polymerase α ,²⁵ the effects of TPP-isosteviol conjugates and DNA targeting molecules were compared. In the assay system used, the topoisomerase II inhibitor etoposide displayed cleavage alterations but failed to induce mitotic arrest. When applied to hatched blastulae, etoposide caused embryo death at a concentration significantly higher than that for cleavage alteration (Table 1). According to literature data, a specific inhibitor of DNA polymerase α , the tetracyclic diterpene aphidicolin,⁵¹ and an inhibitor of DNA replication, tribromoindole carbaldehyde, initiated the formation of arrested eggs with homogeneous cytoplasm, lacking both mitotic spindle and condensed chromatin.^{52,53} In contrast to these DNA-targeting agents, compounds **14–16** and **25–27** blocked cell division, inducing the formation of aberrant mitotic spindles (Figure 1E). These data suggest that the antimitotic effects of TPP-isosteviol conjugates are unlikely to be related to the inhibition of either topoisomerase II or DNA polymerase α .

The conjugation of anticancer agents with TPP leads to their selective accumulation in cancer cell mitochondria, thereby consistently enhancing cytotoxicity. Specifically, this was reported for TPP derivatives of the DNA-alkylating agent chlorambucil,³⁹ the proapoptotic natural product curcumin,⁴⁰ and an inhibitor of DNA topoisomerases I and II, dihydrobetulinic acid.^{13c,54} The present study demonstrated that isosteviol (**1**) did not affect sea urchin embryo development at any stage up to a 4 μ M concentration, while TPP-isosteviol conjugates **14–16** and **25–27** each exhibited strong antimitotic activity. The data suggested the predominant contribution of the diterpenoid *ent*-beyerane moiety to the antimitotic properties of TPP-isosteviol conjugates, since the TPP cation itself was inactive, as determined from MTPPB tests (Table 1). In addition, the accumulation in mitochondria ensured by a TPP transporter could play a key role in the response of the sea urchin embryos to isosteviol derivatives.

It was reported recently that mitochondria are implicated in the regulation of centrosome duplication and mitotic spindle assembly.⁵⁵ Specifically, the depletion of mitochondrial DNA induces the formation of aberrant multipolar spindles followed by mitotic arrest in the G₂/M phase. Considering these literature data, it was decided to analyze the mitotic spindle morphology in sea urchin eggs arrested by isosteviol conjugates. At first glance, the test compounds initiated the formation of monopolar mitotic spindles (Figure 1E). However, a detailed microscopic study using the phase-contrast imaging technique revealed irregular multipolar asters in a certain number of eggs (Figure 2), suggesting the possible interaction of isosteviol derivatives with mitochondrial DNA.

Therefore, a series of TPP conjugates of the diterpenoid isosteviol (**1**) were synthesized, where TPP was introduced as a carrier for the preferential delivery of the diterpenoid *ent*-beyerane moiety into mitochondria. The targeted molecules exhibited pronounced antimitotic effects in the sea urchin embryo model. A preliminary structure–activity relationship study revealed the essential role of the TPP group for the realization of the isosteviol effect, whereas the chemical structure and the length of the linker had only limited impact

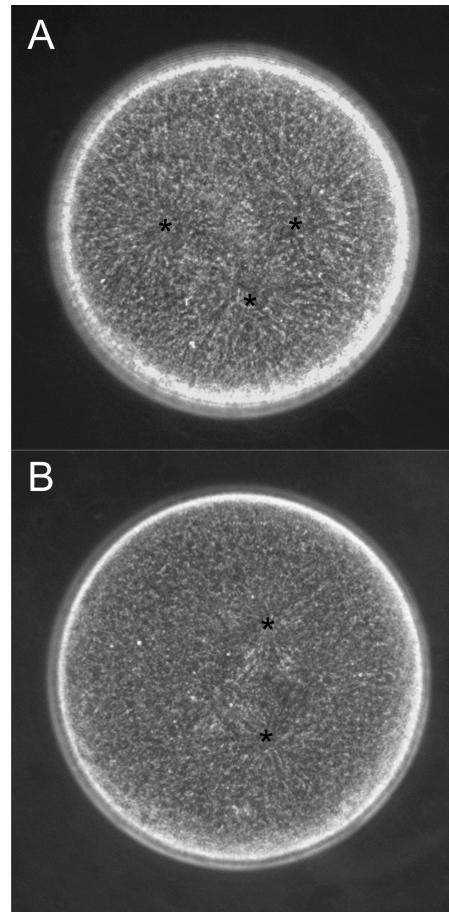


Figure 2. Aberrant mitotic spindles (asterisks) in the eggs arrested by **26** (2 μ M). Phase-contrast imaging at higher magnification.

on antimitotic activity. The mechanism of action and cellular target(s) of isosteviol derivatives require further clarification. Nevertheless, the results obtained in the assay system used suggest that TPP conjugates of isosteviol might affect mitochondrial DNA, thereby inducing mitotic spindle defects and mitotic arrest. As targeting mitochondria is an encouraging strategy for cancer therapy,⁵⁶ TPP-isosteviol conjugates represent a promising starting point for their further design as possible anticancer agents.

EXPERIMENTAL SECTION

General Experimental Procedures. Compound melting points were determined on a Boetius compact heating table. Optical rotations were determined on a PerkinElmer 341 polarimeter (concentration *c* is given as g/mL). ¹H and ³¹P NMR spectra were collected on Bruker Avance-400 and Bruker Avance-500 NMR spectrometers. Mass spectra were obtained on an UltraFlex III TOF/TOF time-of-flight mass spectrometer (Bruker Daltonik, Bremen, Germany). Positively charged ions were registered. High-resolution MALDI mass spectra were acquired in the reflectron mode with Triton X-100 as a reference. Solutions of analyte samples in CHCl₃ at a concentration of 1 mg/mL were used. A mixture of Triton X-100 solution and analyte sample (1:1 v/v) was used for internal calibration. 2,5-Dihydroxybenzoic acid (5 mg/mL in methanol) was used as matrix. The dried-droplet spotting technique was applied in preparing a matrix and analyte solutions. After application of 0.5 μ L of each solution to the target plate MTP AnchorChip (Bruker Daltonik GmbH, Bremen, Germany), the solvent was allowed to evaporate under ambient conditions, and the plate was inserted into the mass spectrometer for analysis. The data were processed using the FlexAnalysis 3.0 software (Bruker Daltonik,

Bremen, Germany). Elemental analysis was accomplished with the automated EuroVector EA3000 CHNS-O elemental analyzer (EuroVector, Milano, Italy). The progress of reactions and the purity of products were monitored by TLC on Sorbfil plates (IMID Ltd., Krasnodar, Russian Federation). The TLC plates were visualized by treatment with 5% H_2SO_4 , followed by heating to 120 °C. The targeted compounds were isolated using dry-column flash chromatography on KSKG silica gel (<0.063 mm, Crom-Lab Ltd., Lyubertsy, Russian Federation).

All solvents were dried according to standard protocols. Isosteviol (**1**) was prepared from the sweetener Sweta (Stevian Biotechnology Corp., Labu, Negeri Sembilan, Malaysia) as described previously:⁵⁷ mp 234 °C (lit.⁵⁷ mp 234–235 °C); $[\alpha]^{20}_D$ –72.3 (c 1.02, EtOH) (lit.⁵⁸ $[\alpha]^{20}_D$ –72, c 0.12, MeOH). Triethylene glycol, tetraethylene glycol, and dibromoalkanes were purchased from Vekton Closed Corporation (Krasnodar, Russian Federation), ABCR GmbH & Co. (Karlsruhe, Germany), and Alfa Aesar (Heysham, United Kingdom), respectively. Ditosylates of polyethylene glycols **2–4** were synthesized according to a literature procedure.⁴⁶ Ditosylate of triethylene glycol **2**: mp 80 °C (lit.⁴⁶ mp 80–81 °C); ditosylate of tetraethylene glycol **3**: oil; MALDIMS m/z 502.1 (calcd for $C_{22}H_{30}O_9S_2$, 502.133); ditosylate of pentaethylene glycol **4**: oil, MALDIMS m/z 546.2 (calcd for $C_{24}H_{34}O_{10}S_2$, 546.159). MTPPB was synthesized as previously described:⁵⁹ mp 238–240 °C (lit.⁵⁹ mp 230–233 °C).

General Procedure for the Synthesis of Tosylates 5–7 and Diketones 8–10. Poly(ethylene glycol) ditosylate (2 mmol) was added to a stirred warm solution (50 °C) of isosteviol (**1**, 1 mmol) in dry CH_3CN . The reaction mixture was cooled to room temperature, and K_2CO_3 (2 mmol) was added. The resulting mixture was heated at 70 °C for 10–12 h, the precipitate was filtered off, and CH_3CN was removed under reduced pressure. The crude oily syrup obtained was purified by dry-column flash chromatography (step gradient of 6:1 to 1:2.5 petroleum ether/ethyl acetate) to afford the targeted tosylates (**5–7**) and diketones (**8–10**).

8'-p-Toluenesulfonyloxy-3',6'-dioxaoctan-1'-yl 16-oxo-ent-beyeran-19-oate (5): viscous oil; 5.0 g, 53% yield; $[\alpha]^{20}_D$ –52.3 (c 0.55, MeOH); 1H NMR ($CDCl_3$, 400 MHz) δ 0.62–2.14 (18H, m, ent-beyerane skeleton), 0.70 (3H, s, H-20), 0.96 (3H, s, H-17), 1.18 (3H, s, H-18), 2.17 (1H, d, J = 13.2 Hz, H-3), 2.44 (3H, s, CH_3C_{ar}), 2.60 (1H, dd, J = 3.4, 18.6 Hz, H_{α} -15), 3.56 (4H, br s, OCH_2CH_2O), 3.64 (2H, t, J = 4.8 Hz, CH_2O), 3.68 (2H, t, J = 4.8 Hz, CH_2O), 4.08–4.24 (4H, m, C(O)OCH₂ and CH_2OTs), 7.33 (2H, d, J = 8.0 Hz, H- C_{ar}), 7.78 (2H, d, J = 7.9 Hz, H- C_{ar}); MALDIMS, m/z 627.4 [M + Na]⁺, 643.4 [M + K]⁺; anal. C 65.56; H 8.24; S 5.16%, calcd for $C_{33}H_{48}O_8S$, C 65.54; H 8.0; S 5.30%.

11'-p-Toluenesulfonyloxy-3',6',9'-trioxaundecan-1'-yl 16-oxo-ent-beyeran-19-oate (6): viscous oil; 1.75 g, 43% yield; $[\alpha]^{20}_D$ –52.0 (c 0.58, MeOH); 1H NMR ($CDCl_3$, 400 MHz) δ 0.82–1.90 (18H, m, ent-beyerane skeleton), 0.71 (3H, s, H-20), 0.97 (3H, s, H-17), 1.19 (3H, s, H-18), 2.18 (1H, d, J = 13.2 Hz, H-3), 2.44 (3H, s, CH_3C_{ar}), 2.61 (1H, dd, J = 3.8, 18.6 Hz, H_{α} -15), 3.58 (4H, s, OCH_2CH_2O), 3.60 (4H, s, OCH_2CH_2O), 3.65–3.70 (4H, m, 2 \times CH_2O), 4.13–4.19 (4H, m, C(O)OCH₂ and CH_2OTs), 7.33 (2H, d, J = 8.5 Hz, H- C_{ar}), 7.79 (2H, d, J = 8.4 Hz, H- C_{ar}); MALDIMS m/z 671.3 [M + Na]⁺, 687.3 [M + K]⁺; anal. C 64.87; H 8.05; S 5.01%, calcd for $C_{35}H_{52}O_9S$, C 64.79; H 8.08; S 4.94%.

14'-p-Toluenesulfonyloxy-3',6',9',12'-tetraoxatetradecan-1'-yl 16-oxo-ent-beyeran-19-oate (7): viscous oil; 0.18 g, 41% yield; $[\alpha]^{20}_D$ –51.5 (c 1.85, CH_2Cl_2); 1H NMR ($CDCl_3$, 500 MHz) δ 0.82–1.90 (18H, m, ent-beyerane skeleton), 0.71 (3H, s, H-20), 0.96 (3H, s, H-17), 1.19 (3H, s, H-18), 2.18 (1H, d, J = 13.3 Hz, H-3), 2.44 (3H, s, CH_3C_{ar}), 2.61 (1H, dd, J = 3.7, 18.6 Hz, H_{α} -15), 3.57 (4H, s, OCH_2CH_2O), 3.59–3.65 (8H, m, 2 \times OCH_2CH_2O), 3.65–3.70 (4H, m, 2 \times CH_2O), 4.13–4.19 (4H, m, C(O)OCH₂ and CH_2OTs), 7.33 (2H, d, J = 7.9 Hz, H- C_{ar}), 7.79 (2H, d, J = 8.2 Hz, H- C_{ar}); MALDIMS m/z 715.3 [M + Na]⁺; anal. C 64.25; H 8.10; S 4.56%, calcd for $C_{37}H_{56}O_{10}S$, C 64.14; H 8.15; S 4.63%.

3',6'-Dioxaoctane-1',8'-dylbis(16-oxo-ent-beyeran-19-oate) (8): amorphous powder; 1.63 g, 28% yield; mp 127–129 °C; $[\alpha]^{20}_D$ –76.0 (c 0.19; MeOH+ CH_2Cl_2); 1H NMR ($CDCl_3$, 400 MHz) δ 0.71 (6H, s,

2 \times H-20), 0.97 (6H, s, 2 \times H-17), 1.19 (6H, s, 2 \times H-18), 0.87–1.93 (36H, m, ent-beyerane skeleton), 2.18 (2H, d, J = 13.3 Hz, 2 \times H-3), 2.62 (2H, dd, J = 3.7, 18.6 Hz, 2 \times H_{α} -15), 3.59–3.62 (4H, br s, OCH_2CH_2O), 3.65–3.71 (4H, m, 2 \times C(O)OCH₂CH₂), 4.13–4.23 (4H, m, 2 \times C(O)OCH₂); MALDIMS m/z 773.66 [M + Na]⁺, 789.62 [M + K]⁺; anal. C 73.58; H 9.42%; calcd for $C_{46}H_{70}O_8$, C 73.56; H 9.39%.

3',6',9'-Trioxaundecane-1',11'-dylbis(16-oxo-ent-beyeran-19-oate) (9): amorphous powder; 0.57 g, 23% yield; mp 85–88 °C; $[\alpha]^{20}_D$ –78.3 (c 0.69; CH_2Cl_2); 1H NMR ($CDCl_3$, 400 MHz) δ 0.71 (6H, s, 2 \times H-20), 0.97 (6H, s, 2 \times H-17), 1.20 (6H, s, 2 \times H-18), 0.85–1.92 (36H, m, ent-beyerane skeleton), 2.19 (2H, d, J = 13.5 Hz, 2 \times H-3), 2.62 (2H, dd, J = 3.7, 18.6 Hz, 2 \times H_{α} -15), 3.59–3.65 (8H, m, 2 \times OCH_2CH_2O), 3.66–3.70 (4H, m, 2 \times C(O)OCH₂CH₂), 4.14–4.23 (4H, m, 2 \times C(O)OCH₂); MALDIMS m/z 817.52 [M + Na]⁺; anal. C 72.58; H 9.40%; calcd for $C_{48}H_{74}O_9$, C 72.51; H 9.38%.

3',6',9',12'-Tetraoxatetradecane-1',14'-dylbis(16-oxo-ent-beyeran-19-oate) (10): viscous oil; 0.05 g, 19% yield; $[\alpha]^{20}_D$ –75.5 (c 0.64; CH_2Cl_2); 1H NMR ($CDCl_3$, 400 MHz) δ 0.70 (6H, s, 2 \times H-20), 0.95 (6H, s, 2 \times H-17), 1.18 (6H, s, 2 \times H-18), 0.86–1.90 (36H, m, ent-beyerane skeleton), 2.17 (2H, d, J = 13.3 Hz, 2 \times H-3), 2.60 (2H, dd, J = 18.6, 3.7 Hz, 2 \times H_{α} -15), 3.60–3.62 (8H, m, 2 \times OCH_2CH_2O), 3.62–3.64 (4H, m, OCH_2CH_2O), 3.64–3.69 (4H, m, 2 \times C(O)OCH₂CH₂), 4.12–4.21 (4H, m, 2 \times C(O)OCH₂); MALDIMS m/z 861.50 [M + Na]⁺; anal. C 71.60; H 9.40%; calcd for $C_{50}H_{78}O_{10}$, C 71.56; H 9.37%.

General Procedure for the Synthesis of Bromides 11–13. Lithium bromide (1.1 mmol) was added to a stirred solution of each tosylate (1 mmol) in acetone, and the mixture was refluxed for 14–16 h. The resulting precipitate was filtered off, and acetone was removed under reduced pressure. The separated oily syrup was purified by dry-column flash chromatography on silica gel (petroleum ether/ethyl acetate, 5:1–1:5) to afford pure bromides 11–13.

8'-Bromo-3',6'-dioxaoctan-1'-yl 16-oxo-ent-beyeran-19-oate (11): viscous oil; 3.7 g, 88% yield; $[\alpha]^{20}_D$ –60.2 (c 1.42, MeOH); 1H NMR ($CDCl_3$, 400 MHz) δ 0.82–2.0 (18H, m, ent-beyerane skeleton), 0.71 (3H, s, H-20), 0.97 (3H, s, H-17), 1.19 (3H, s, H-18), 2.18 (1H, d, J = 13.5 Hz, H-3), 2.62 (1H, dd, J = 3.7, 18.6 Hz, H_{α} -15), 3.46 (2H, t, J = 6.3 Hz, CH_2Br), 3.61–3.67 (4H, m, OCH_2CH_2O), 3.67–3.71 (2H, m, CH_2O), 3.80 (2H, br t, J = 6.2 Hz, CH_2O), 4.16–4.21 (2H, m, C(O)OCH₂); MALDIMS m/z 535.2 [M + Na]⁺; anal. C 60.27; H 8.09; Br 15.45%; calcd for $C_{26}H_{41}BrO_5$, C 60.81; H 8.05; Br 15.56%.

11'-Bromo-3',6',9'-trioxaundecan-1'-yl 16-oxo-ent-beyeran-19-oate (12): viscous oil; 0.99 g, 78% yield; $[\alpha]^{20}_D$ –59.3 (c 1.02, MeOH); 1H NMR ($CDCl_3$, 400 MHz) δ 0.81–1.89 (18H, m, ent-beyerane skeleton), 0.69 (3H, s, H-20), 0.94 (3H, s, H-17), 1.17 (3H, s, H-18), 2.16 (1H, d, J = 13.5 Hz, H-3), 2.59 (1H, dd, J = 3.7, 18.6 Hz, H_{α} -15), 3.43 (2H, t, J = 6.3 Hz, CH_2Br), 3.58–3.69 (10H, m, CH_2O), 3.77 (2H, t, J = 6.3 Hz, CH_2O), 4.13–4.17 (2H, m, C(O)OCH₂); MALDIMS, m/z 579.2 [M + Na]⁺, 597.2 [M + K]⁺; anal. C 60.37; H 8.19; Br 14.45%; calcd for $C_{28}H_{45}BrO_6$, C 60.32; H 8.14; Br 14.33%.

14'-Bromo-3',6',9',12'-tetraoxatetradecan-1'-yl 16-oxo-ent-beyeran-19-oate (13): 0.10 g, 67% yield; viscous oil; $[\alpha]^{20}_D$ –39.0 (c 2.27, CH_2Cl_2); 1H NMR ($CDCl_3$, 500 MHz) δ 0.82–1.90 (18H, m, ent-beyerane skeleton), 0.71 (3H, s, H-20), 0.96 (3H, s, H-17), 1.19 (3H, s, H-18), 2.18 (1H, d, J = 13.3 Hz, H-3), 2.62 (1H, dd, J = 3.7, 18.6 Hz, H_{α} -15), 3.46 (2H, t, J = 6.3 Hz, CH_2Br), 3.60–3.71 (14H, m, CH_2O), 3.80 (2H, t, J = 6.3 Hz, CH_2O), 4.14–4.21 (2H, m, C(O)OCH₂); MALDIMS, m/z 623.2 [M + Na]⁺, 639.2 [M + K]⁺; anal. C 60.01; H 8.19; Br 13.34%; calcd for $C_{30}H_{49}BrO_7$, C 59.89; H 8.21; Br 13.28%.

General Procedure for the Synthesis of TPP Conjugates of Isosteviol (14–16, 25–27). Triphenylphosphine (1.5–3 mmol) was added to the solution of bromide (11–13 or 19–21) (1 mmol) in dry toluene, and the mixture was stirred under reflux for 37–75 h. Toluene was removed under reduced pressure, and the precipitate was washed with hot petroleum ether (3 \times 5 mL) and dissolved in ethyl acetate. Petroleum ether (5 mL) was added to the reaction mixture. The

resulting precipitate was washed with diethyl ether (3 mL) and dried in vacuo to give the pure TPP conjugate.

19-(9'-Triphenylphosphonium-1',4',7'-trioxanonyl)-16,19-dioxo-ent-beyerane bromide (14): amorphous powder; 0.01 g, 1% yield; mp 60–66 °C; $[\alpha]^{20}_D$ –44.0 (*c* 0.47, MeOH); ^{31}P NMR (CDCl_3) δ –26.05; ^1H NMR (CDCl_3 , 400 MHz) δ 0.68–1.90 (18H, m, *ent*-beyerane skeleton), 0.67 (3H, s, H-20), 0.96 (3H, s, H-17), 1.18 (3H, s, H-18), 2.16 (1H, d, *J* = 13.5 Hz, H-3), 2.56 (1H, dd, *J* = 3.5, 18.4 Hz, H_{α} -15), 3.25–3.30 (2H, m, OCH_2), 3.31–3.35 (2H, m, OCH_2), 3.37–3.44 (2H, m, $\text{OCH}_2\text{CH}_2\text{P}$), 3.89–4.00 (2H, m, CH_2P), 4.01–4.06 (2H, m, C(O) $\text{OCH}_2\text{CH}_2\text{O}$), 4.18–4.25 (2H, m, C(O) OCH_2), 7.61–7.68 (6H, m, H-C_{ar}), 7.72–7.78 (3H, m, H-C_{ar}), 7.80–7.88 (6H, m, H-C_{ar}); HRMS *m/z* 695.3870 (calcd for $\text{C}_{44}\text{H}_{56}\text{O}_5\text{P}$, 695.3860).

19-(12'-Triphenylphosphonium-1',4',7',10'-tetraoxadodecyl)-16,19-dioxo-ent-beyerane bromide (15): amorphous powder; 0.04 g, 2% yield; mp 65–70 °C; $[\alpha]^{20}_D$ –29.5 (*c* 0.41, MeOH); ^{31}P NMR (CDCl_3) δ –26.29; ^1H NMR (CDCl_3 , 500 MHz) δ 0.69 (3H, s, H-20), 0.96 (3H, s, H-17), 1.18 (3H, s, H-18), 0.84–1.92 (18H, m, *ent*-beyerane skeleton), 2.16 (1H, d, *J* = 13.4 Hz, H-3), 2.60 (1H, dd, *J* = 3.6, 18.4 Hz, H_{α} -15), 3.27–3.35 (4H, m, 2 \times OCH_2), 3.35–3.40 (2H, m, OCH_2), 3.48–3.51 (2H, m, OCH_2), 3.60–3.65 (2H, m, OCH_2), 3.89–3.99 (2H, m, CH_2P), 4.12–4.16 (2H, m, C(O) $\text{OCH}_2\text{CH}_2\text{O}$), 4.18–4.25 (2H, m, C(O) OCH_2), 7.61–7.67 (6H, m, H-C_{ar}), 7.72–7.78 (3H, m, H-C_{ar}), 7.82–7.88 (6H, m, H-C_{ar}); HRMS *m/z* 739.4124 (calcd for $\text{C}_{46}\text{H}_{60}\text{O}_6\text{P}$, 739.4122).

19-(15'-Triphenylphosphonium-1',4',7',10',13'-pentaoxapentadecyl)-16,19-dioxo-ent-beyerane bromide (16): viscous oil; 0.01 g, 7% yield; $[\alpha]^{20}_D$ –27.3 (*c* 0.37, MeOH); ^{31}P NMR (CDCl_3) δ –25.68; ^1H NMR (CDCl_3 , 500 MHz) δ 0.70 (3H, s, H-20), 0.97 (3H, s, H-17), 1.19 (3H, s, H-18), 0.80–1.90 (18H, m, *ent*-beyerane skeleton), 2.17 (1H, d, *J* = 13.5 Hz, H-3), 2.60 (1H, dd, *J* = 13.8, 18.6 Hz, H_{α} -15), 3.26–3.35 (4H, m, 2 \times OCH_2), 3.37–3.40 (2H, m, OCH_2), 3.50–3.54 (2H, m, OCH_2), 3.55–3.62 (4H, m, OCH_2CH_2), 3.63–3.67 (2H, m, OCH_2), 3.90–3.99 (2H, m, CH_2P), 4.13–4.19 (2H, m, C(O) $\text{OCH}_2\text{CH}_2\text{O}$), 4.21–4.28 (2H, m, C(O) OCH_2), 7.62–7.68 (6H, m, H-C_{ar}), 7.72–7.78 (3H, m, H-C_{ar}), 7.83–7.90 (6H, m, H-C_{ar}); HRMS *m/z* 783.4413 (calcd for $\text{C}_{48}\text{H}_{64}\text{O}_7\text{P}$, 783.4384).

19-(3'-Triphenylphosphoniumpropyloxy)-16,19-dioxo-ent-beyerane bromide (25): powder; 0.28 g, 18% yield; mp 114–117 °C; $[\alpha]^{20}_D$ –41.5 (*c* 1.05, CHCl_3); ^{31}P NMR (CDCl_3) δ –24.69; ^1H NMR (CDCl_3 , 400 MHz) δ 0.44 (3H, s, H-20), 0.97 (3H, s, H-17), 1.11 (3H, s, H-18), 0.82–2.01 (20H, m, *ent*-beyerane skeleton and CH_2 fragment of linker), 2.11 (1H, d, *J* = 13.5 Hz, H-3), 2.42 (1H, dd, *J* = 3.8, 18.6 Hz, H_{α} -15), 3.90–4.04 (1H, m, CH_2P), 4.12–4.25 (1H, m, CH_2P), 4.40–4.51 (2H, m, C(O) OCH_2), 7.67–7.73 (6H, m, H-C_{ar}), 7.77–7.83 (3H, m, H-C_{ar}), 7.83–7.91 (6H, m, H-C_{ar}); HRMS *m/z* 621.3511 (calcd for $\text{C}_{41}\text{H}_{50}\text{O}_3\text{P}$, 621.3492).

19-(3'-Triphenylphosphoniumpropyloxy)-16,19-dioxo-ent-beyerane bromide (26): powder; 0.3 g, 20% yield; mp 72–74 °C; $[\alpha]^{20}_D$ –32.7 (*c* 1.09, CHCl_3); ^{31}P NMR (CDCl_3) δ –24.51; ^1H NMR (CDCl_3 , 400 MHz) δ 0.64 (3H, s, H-20), 0.97 (3H, s, H-17), 1.15 (3H, s, H-18), 0.81–1.91 (22H, m, *ent*-beyerane skeleton and $(\text{CH}_2)_4$ fragment of linker), 2.15 (1H, d, *J* = 13.3 Hz, H-3), 2.54 (1H, dd, *J* = 3.6, 18.5 Hz, H_{α} -15), 3.85–4.03 (4H, m, CH_2P and C(O) OCH_2), 7.65–7.73 (6H, m, H-C_{ar}), 7.74–7.81 (3H, m, H-C_{ar}), 7.83–7.92 (6H, m, H-C_{ar}); HRMS *m/z* 663.3511 (calcd for $\text{C}_{44}\text{H}_{56}\text{O}_3\text{P}$, 663.3492).

19-(8'-Triphenylphosphoniumoctyloxy)-16,19-dioxo-ent-beyerane bromide (27): powder; 0.56 g, 37% yield; mp 83–84 °C; $[\alpha]^{20}_D$ –37.2 (*c* 1.03, CHCl_3); ^{31}P NMR (CDCl_3) δ –24.63; ^1H NMR (CDCl_3 , 500 MHz) δ 0.68 (3H, s, H-20), 0.96 (3H, s, H-17), 1.16 (3H, s, H-18), 0.82–1.91 (24H, m, *ent*-beyerane skeleton and $(\text{CH}_2)_6$ fragment of linker), 2.16 (1H, d, *J* = 13.7 Hz, H-3), 2.59 (1H, dd, *J* = 3.8, 18.5 Hz, H_{α} -15), 3.82–3.90 (2H, m, CH_2P), 3.91–4.01 (2H, m, C(O) OCH_2), 7.67–7.72 (6H, m, H-C_{ar}), 7.76–7.81 (3H, m, H-C_{ar}), 7.83–7.90 (6H, m, H-C_{ar}); HRMS *m/z* 691.4275 (calcd for $\text{C}_{46}\text{H}_{66}\text{O}_3\text{P}$, 691.4271).

19-(9'-Triethylammonium-1',4',7'-trioxanonyl)-16,19-dioxo-ent-beyerane bromide (17): This was synthesized as previously described:⁶⁰ mp 90–93 °C (lit.⁶⁰ mp 90–93 °C).

19-(12'-Triethylammonium-1',4',7',10'-tetraoxadodecyl)-16,19-dioxo-ent-beyerane bromide (18): This was prepared according to a

literature procedure.⁶⁰ Triethylamine (1.5 mL, 10 mmol) was added to a stirred solution of bromide 12 (0.56 g, 1 mmol) in CH_3CN (12 mL). The reaction mixture was refluxed for 23 h, CH_3CN was removed under reduced pressure, and the resulting oily syrup was purified by column chromatography on silica gel (0.060–0.200 mm, 60 Å (Acros Organics, NJ, USA), step gradient of 30:1 to 10:1 CH_2Cl_2 /methanol), to isolate ammonium compound 18 in 27% yield (0.18 g, 0.27 mmol) as a viscous oil: $[\alpha]^{20}_D$ –49.0 (*c* 1.31, MeOH); ^1H NMR (CDCl_3 , 400 MHz) δ 0.83–1.90 (18H, m, *ent*-beyerane skeleton), 0.70 (3H, s, H-20), 0.95 (3H, s, H-17), 1.18 (3H, s, H-18), 1.39 (9H, t, *J* = 7.2 Hz, 3 \times NCH_2CH_3), 2.16 (1H, d, *J* = 13.7 Hz, H-3), 2.59 (1H, dd, *J* = 3.7, 18.5 Hz, H_{α} -15), 3.52–3.62 (12H, m, 3 \times NCH_2CH_3 , $\text{CH}_2\text{CH}_2\text{N}$ and $\text{OCH}_2\text{CH}_2\text{O}$), 3.63–3.68 (4H, m, $\text{OCH}_2\text{CH}_2\text{O}$), 3.78–3.80 (2H, m, $\text{OCH}_2\text{CH}_2\text{N}$), 3.94–3.98 (2H, m, C(O) $\text{OCH}_2\text{CH}_2\text{O}$), 4.12–4.20 (2H, m, C(O) OCH_2); HRMS *m/z* 578.4488 (calcd for $\text{C}_{34}\text{H}_{60}\text{NO}_6$, 578.4415).

General Procedure for the Synthesis of Bromides 19–21. K_2CO_3 (2 mmol) was added to a solution of α,ω -dibromoalkane (2–4 mmol) in dry CH_3CN ; then a warm solution (30 °C) of isosteviol (1) (1 mmol) in dry CH_3CN was added dropwise, and the reaction mixture was stirred under reflux for 3–13 h. A solid residue was filtered off, CH_3CN was removed under reduced pressure, and the resulting oily syrup was purified by dry-column flash chromatography on silica gel (step gradient of 50:1 to 4:1 petroleum ether/ethyl acetate) to afford the target bromides 19–21. Dialkylation byproducts 23–24 were detected in the reaction mixtures without further isolation.

3'-Bromo-n-propyl 16-oxo-ent-beyeran-19-oate (19): powder; 2.04 g, 46% yield; mp 97–99 °C; $[\alpha]^{20}_D$ –61.7 (*c* 1.10, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) δ 0.70 (3H, s, H-20), 0.96 (3H, s, H-17), 1.18 (3H, s, H-18), 0.82–1.90 (18H, m, *ent*-beyerane skeleton), 2.13–2.19 (3H, m, H-3 and CH_2 fragment of linker), 2.61 (1H, dd, *J* = 3.9, 18.6 Hz, H_{α} -15), 3.46 (2H, t, *J* = 6.5 Hz, CH_2Br), 4.08–4.14 (1H, m, C(O) OCH), 4.18–4.23 (1H, m, C(O) OCH); anal. C 62.68; H 8.32; Br 18.02%; calcd for $\text{C}_{23}\text{H}_{35}\text{BrO}_3$, C 62.87; H 8.03; Br 18.18%.

6'-Bromo-n-hexyl 16-oxo-ent-beyeran-19-oate (20): viscous oil; 1.91 g, 40% yield; $[\alpha]^{20}_D$ –46.6 (*c* 1.52, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) δ 0.70 (3H, s, H-20), 0.96 (3H, s, H-17), 1.18 (3H, s, H-18), 0.80–1.90 (22H, m, *ent*-beyerane skeleton and $(\text{CH}_2)_4$ fragment of linker), 2.17 (1H, d, *J* = 13.5 Hz, H-3), 2.62 (1H, dd, *J* = 3.7, 18.6 Hz, H_{α} -15), 2.40 (2H, t, *J* = 6.8 Hz, CH_2Br), 3.95–4.07 (2H, m, C(O) OCH_2); anal. C 64.88; H 8.62; Br 16.42%, calcd for $\text{C}_{26}\text{H}_{41}\text{BrO}_3$, C 64.85; H 8.58; Br 16.59%.

8'-Bromo-n-octyl 16-oxo-ent-beyeran-19-oate (21): viscous oil; 2.62 g, 51% yield; $[\alpha]^{20}_D$ –48.2 (*c* 1.67, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) δ 0.71 (3H, s, H-20), 0.98 (3H, s, H-17), 1.19 (3H, s, H-18), 0.80–1.93 (24H, m, *ent*-beyerane skeleton and $(\text{CH}_2)_6$ fragment of linker), 2.19 (1H, d, *J* = 13.1 Hz, H-3), 2.63 (1H, dd, *J* = 3.7, 18.5 Hz, H_{α} -15), 3.41 (2H, t, *J* = 6.8 Hz, CH_2Br), 3.96–4.06 (2H, m, C(O) OCH_2); anal. C 66.08; H 8.82; Br 15.58%, calcd for $\text{C}_{28}\text{H}_{45}\text{BrO}_3$, C 66.00; H 8.90; Br 15.68%.

Sea Urchin Embryo Assay.⁴⁴ Adult sea urchins, *Paracentrotus lividus* L. (Echinidae), were collected from the Mediterranean Sea on the Cyprus coast in March–April and November 2014 and kept in an aerated seawater tank. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered seawater and fertilized by adding drops of diluted sperm. Embryos were cultured at room temperature under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered seawater. The embryos were observed with a Biolam light microscope (LOMO, St. Petersburg, Russian Federation). For treatment with the test compounds, 5 mL aliquots of embryo suspension were transferred to six-well plates and incubated as a monolayer at a concentration up to 2000 embryos/mL. Stock solutions of MTTPPB, isosteviol (1), and compounds 23–27 were prepared in DMSO at 10 mM concentration followed by a 10-fold dilution with 96% EtOH. This procedure enhanced the solubility of the test compounds in the salt-containing medium (seawater), as evidenced by microscopic examination of the samples. The maximal tolerated concentrations of DMSO and EtOH in the *in vivo* assay were determined to be 0.05% and 1%, respectively. Higher concentrations

of either DMSO ($\geq 0.1\%$) or EtOH ($>1\%$) caused nonspecific alterations and retardation of the sea urchin embryo development independent of the treatment stage. Stock solutions of compounds **14–16** (5 mM) and **17** and **18** (both 10 mM) were prepared in distilled water. Etoposide (LANS, Veropharm, Moscow, Russian Federation, 20 mg/mL in 96% EtOH), nocodazole, and paclitaxel (both Sigma-Aldrich Co., St. Louis, MO, USA) served as reference compounds. Stock solutions of nocodazole and paclitaxel, both at 5 mM, were prepared in DMSO and 96% ethanol, respectively. The antimitotic activity was assessed by exposing fertilized eggs (8–15 min postfertilization, 45–55 min before the first mitotic cycle completion) to 2-fold decreasing concentrations of the compound. Cleavage alteration and arrest were clearly detected at 2.5–5.5 h after fertilization. The effects were estimated quantitatively as an effective threshold concentration, resulting in cleavage alteration and embryo death before hatching or full mitotic arrest. At these concentrations, all tested compounds caused 100% cleavage alteration and embryo death before hatching, whereas at 2-fold lower concentrations the compounds failed to produce any effect. For microtubule-destabilizing activity and developmental defects, the compounds were tested on free-swimming blastulae just after hatching (8–9 h postfertilization), which originated from the same embryo culture. Embryos were observed until the four-arm pluteus stage (34–36 h postfertilization). The specific changes in swimming pattern, namely, both spinning on the bottom of the well and lack of forward movement, were interpreted to be the result of the microtubule-destabilizing activity of a molecule. Microphotographs were obtained using an AmScope binocular microscope with an MU500 digital camera (United Scopes LLC, Irvine, CA, USA). Video illustrations are available at <http://www.chemblock.com>. The sea urchin embryo assay data are available free of charge via the Internet at <http://www.zelinsky.ru>.

Experiments with the sea urchin embryos fulfill the requirements of biological ethics. The artificial spawning does not cause animal death, embryos develop outside the female organism, and both postspawned adult sea urchins and the excess of intact embryos are returned to the sea, their natural habitat.

ASSOCIATED CONTENT

Supporting Information

¹H NMR spectra of compounds **5–16**, **18–21**, and **25–27**; ³¹P NMR spectra of compounds **14–16** and **25–27**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00124.

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Notes

The authors declare no competing financial interest.

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