FULL PAPER

Identification of Two New Phosphorylated Polyketides from a Brazilian *Streptomyces* sp. Through the Use of LC-SPE/NMR

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Two new polyketide phosphate monoesters, phosdiecin A (**1**) and phosdiecin B (**2**), were isolated from a culture of the marine *Streptomyces* sp. SS99BA-2 using the hyphenated technology LC-SPE/NMR. The compounds showed to be new representatives of an important class of antitumor and antibiotic metabolites known as fostriecins. Their structures, including relative configuration attribution, were fully elucidated through extensive analyses of NMR and MALDI-TOF/TOF HR-MS data. Herein, the application of this system to isolate and identify the new compounds is described.

Keywords: *Streptomyces*, Polyketides, Phosdiecin A and B, Marine drugs

Introduction

Polyketides are complex natural products produced by many kinds of living beings and constitute a large class of secondary metabolites grouped together based on their biosynthetic origin. They are all biologically produced from acyl CoA precursors by polyketide synthases (PKSs). From a pharmacological point of view, polyketides are an important source of novel therapeutics as they have been used as antiparasitics antibiotics, immunosuppressants, cytotoxic agents, and cholesterol-lowering agents [1].

A particular class of polyketides exclusively produced by the *Streptomyces* species, called the fostriecin family, has recently drawn the attention of researchers because of its pronounced antitumor and antibiotic activities. In 1983, fostriecin (**3**) was reported by *Stampwala et al.* [2] as the first member of this class with the unique structure composed of an α,β-unsaturated-γ-lactone, a conjugated (*Z*,*Z*,*E*)-triene, and a central phosphate functionality, which have later been proved to be key structural features for its activity [3]. Few years later, phospholine (**4**) was isolated from the fermentation of *S. hygroscopicus* and showed strong activities against L1210 murine leukemia cells, P388, and EL-4 murine leukemia cells [4]. Subsequently, further related compounds, leustroducsin (**5**) [5] and the phoslactomycins (**6**) [6] were described. Sultriecin (**7**) was obtained from a culture of *S. roseiscleroticus* L827-2 (ATCC 53903) and it was the only member of this class with the sulfate functionality instead of the phosphate found in the others [7]. A potent *in vitro* activity against P388 and L1210 leukemia, and B16 melanoma cells was observed. Phostriecin (**8**) is a further phosphate monoester derivative described along with the total synthesis of **7** [3]. Finally, cytostatin (**9**) was obtained from a culture of *Streptomyces* sp. MJ654-NF4 also showed cytotoxic activity [8]. It could be shown that all these compounds (**1** – **9**) (*Fig. 1*) act by inhibition of the serine/threonine protein phosphatase 2A (PP2A), a protein responsible for a complex mechanism of intracellular regulation [9].

The investigation of the BuOH extract from a fermentation broth of *Streptomyces* sp. SS99BA-2 by the hyphenated technique LC-SPE/NMR led to the isolation and characterization of two new metabolites, phosdiecin A (**1**) and phosdiecin B (**2**), whose structures are shown in *Fig. 2*. Herein, the structural elucidation of these new members of this important class of polyketides is described.

Results and Discussion

The bacterium designated as SS99BA-2 (GenBank AJ560629.1) is an isolated species of marine streptomycete found in samples of sediments collected at depths between 12 and 15 m in the São Sebastião Channel (northern coast of São Paulo State, Brazil) [10]. In the present investigation, SS99BA-2 was cultivated in shake flasks, and the culture filtrate was successively extracted

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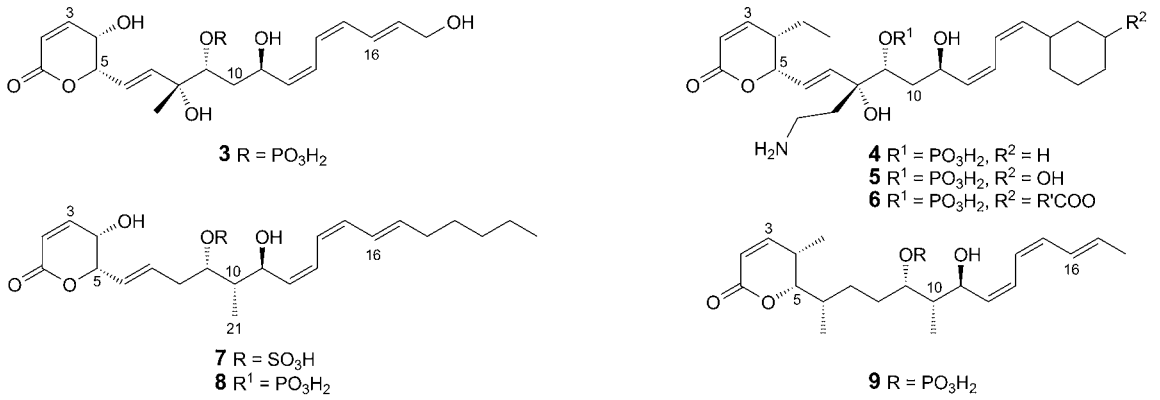

Fig. 1. Chemical structures of known polyketides of the fostriecin family produced by *Streptomyces* sp.

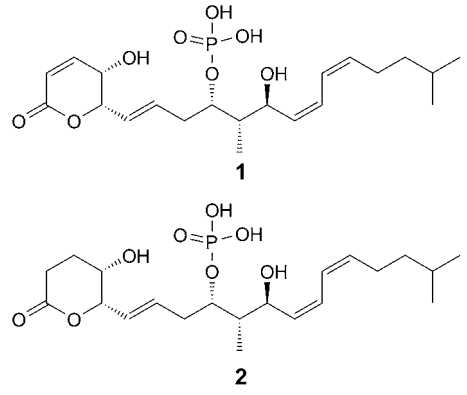

Fig. 2. Chemical structures of Phosdiecin A (**1**) and Phosdiecin B (**2**)

with AcOEt and BuOH. Analyses of the BuOH extract revealed the presence of two new metabolites, whose structures were fully elucidated by NMR spectroscopy and high-resolution mass spectrometry (HR-MS). The new secondary metabolites were isolated by using an automated coupled LC-SPE system, with monitoring of the UV-DAD response at 210 nm. The two compounds (segregated according to their chromatographic peaks) were adsorbed in polydivinylbenzene SPE cartridges during 20 sequential chromatographic runs. The compounds were subsequently eluted from the SPE cartridges with CD_3OD before NMR spectroscopic and high-resolution mass spectrometric analyses.

MALDI-TOF/TOF HR-MS of **1** indicated a pseudomolecular ion at *m/z* 443.1820 ($[M - H]^-$) consistent with a molecular formula of $C_{21}H_{33}O_8P$ (calcd for 443.1829). A total of 29 unexchangeable H-atoms were detected in the ^{1}H-NMR spectrum (*Fig. 3*), including nine Me H-atoms, six CH_2 H-atoms, six CH H-atoms, and eight olefinic H-atoms, which were completely determined by HSQC correlation map. The ^{13}C-NMR spectrum of **1** showed 21 C-atom signals related to three Me groups, three CH groups, six CH groups, six olefinic C-atoms, and one ester C-atom. The structure shown in *Fig. 2* was determined through extensive analyses of ^{1}H,^{1}H-COSY, HSQC, and HMBC correlation maps. Two vicinal olefinic H-atoms appearing as a *doublet* (H–C(2)) at δ(H) 6.06 and a double *doublet* (H–C(3)) at δ(H) 7.04 presented both HMBC correlation with C(1), (δ(C) 167,1), suggesting the presence of a α,β-unsaturated ester moiety. The coupling constant of 9.80 Hz between H–C(2) and H–C(3) is a strong evidence of a (*Z*) double bond system. The HMBC correlation between H–C(3) and the sp^3 C-atoms C(4) (δ(C) 64.8) and C(5) (δ(C) 84.0) leads to the 4-hydroxy-α,β-unsaturated lactone ring.

Other two olefinic H-atom signals at δ(H) 5.84 (H–C(6)) and δ(H) 5.91 (H–C(7)) showed to be part of an (*E*) double bond system through the coupling constant value (J = 16.0 Hz). The HMBC correlation and other coupling constant analyses showed that C(6) was directly attached to the C(5) position of the electrophilic α,β-unsaturated-δ-lactone. A sequence of one CH_2 C-atom (C(8), δ(C) 38.9) and three CH C-atoms (C(9), δ(C) 77.7; C(10), δ(C) 44.8; and C(11), δ(C) 69.6) was assigned by ^{1}H- and ^{13}C-NMR data as well as with 2D-NMR data. A P–C coupling (δ(C) 77.7, ^2J(P,C) = 5.0 Hz) detected in ^{13}C-NMR spectrum of **1** verified a phosphoryl ester at C(9) position. The presence of phosphate was confirmed by a ^{31}P-NMR spectrum in CD_3OD in which a signal appearing as a singlet at δ(P) 0.44 was attributed to the phosphate group. Furthermore, the CH(11) C-atom was concluded to be connected to a OH group (*Fig. 4*).

Towards the end of the side chain, a (*Z*,*Z*)-diene was detected. This was further linked to a terminal isopentyl group. A combination of COSY, HSQC, and HMBC-NMR data allowed to identify the direct connection between two Me C-atoms (C(19) and C(20), δ(C) 23.9), one CH C-atom (C(18), δ(C) 29.8) and two CH_2 C-atoms (C(16), δ(C) 27.4 and C(17), δ(C) 41.0). The geometries of the conjugated diene system were established as (*Z*,*Z*) on the basis of their coupling constants (J(H–C(12),H–C(13)) = 10.15 Hz, J(H–C(14),H–C(15)) = 11.37 Hz), respectively (*Table*).

The relative configuration of **1** was deduced from the NOESY spectrum as well as the coupling constant values between key H-atom nuclei. The *syn* H–C(4) and

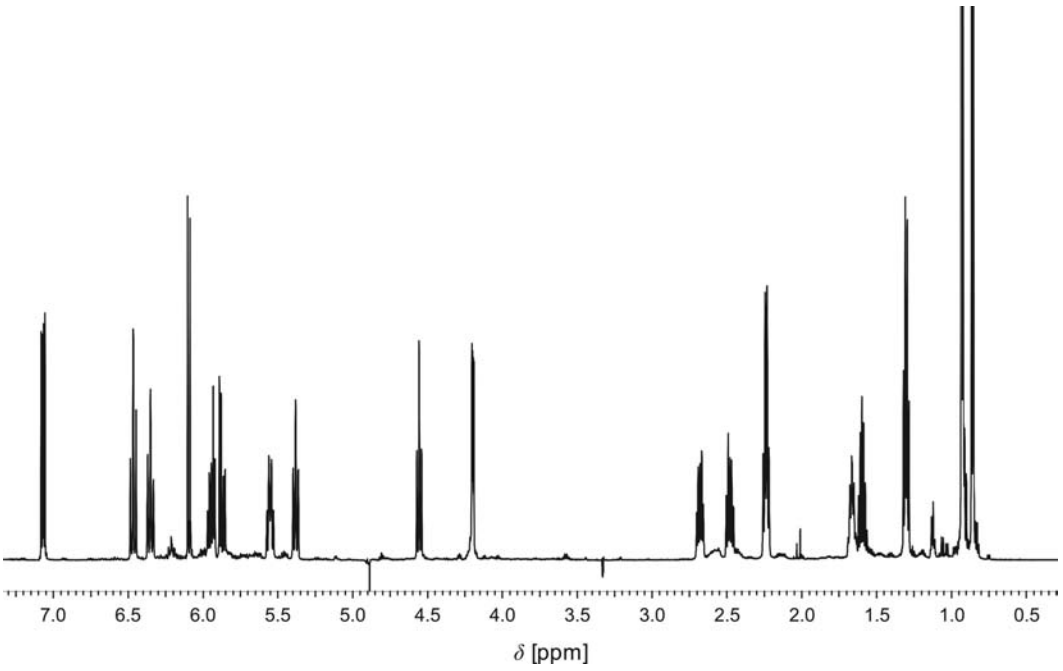

Fig. 3. ^{1}H-NMR spectrum (600 MHz; CD_3OD) of Phosdiecin A (**1**)

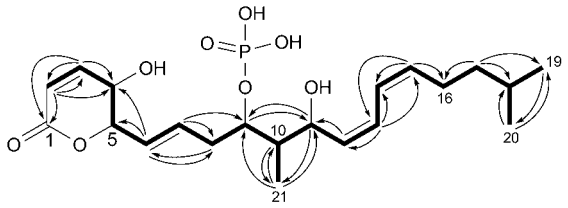

Fig. 4. COSY and important HMBC correlations in the structure of **1**

H–C(5) configuration was determined through the coupling constant observed for H–C(4) and H–C(5) (J = 3.0 Hz) [3][9]. This coupling constant is similar to that observed for the others compounds already known with the same cyclic system: sultriecin (**7**) (J = 2.9 Hz) and cytostatin (**9**) (J = 2.7 Hz). The H–C(10) and H–C (11) coupling constant (J = 9.54 Hz) is a strong indicative of an *anti*-relationship and it is in good agreement with the coupling constant observed for fostriecin (**3**) (J = 9.6 Hz), sultriecin (**7**) (J = 10.2 Hz) and cytostatin (**9**) (J = 9.4 Hz). It has already been shown that there is a preferential twist-boat conformation due to an intramolecular H-bond between the OH group at C(11) and the O-atom at C(9). This was proven by total synthesis and analyses of NOESY and coupling constant data [11]. A correlation between the Me at position 10 with H–C(11) was observed in the NOESY spectrum, and no correlation was observed between this H-atom with H–C(9). These results also support the existence of an intramolecular H-bond between the OH group of C(11) and the O-atom of C(9) phosphate resulting in a rigid twist-boat cyclic structure as already found in fostriecin (**3**), sultriecin (**7**), phostriecin (**8**), and cytostatin (**9**) (*Fig. 5*).

Although there is no direct evidence to correlate the relative configuration of the α,β-unsaturated ester moiety with the one of the polyketide chain, we tentatively proposed the configuration shown in *Fig. 2*, given that the configuration of the related compounds, fostriecin (**3**) and sultriecin (**7**), has been proven by total synthesis [3].

Analysis of MALDI-TOF/TOF HR-MS of compound **2** ($[M - H]^-$ *m/z* 445.1981) led to the molecular formula of $C_{21}H_{35}O_8P$ (calc. 445.1985) which was concluded to have one double bond equivalent less than **1**. This suggested the saturation of one of the four double bonds present in the structure of compound **1**, as the ring opening of the α,β-unsaturated lactone would require another O-atom as the hydrolyzes of the ester would lead to a carboxyl acid and an alcohol.

^{1}H- and ^{13}C-data revealed the saturation of the C(2)/C(3) C=C bond in **2**. While the NMR spectra of **1** and **2** were similar, the CH groups H–C(3) and H–C(4) in **1** were replaced by two CH_2 groups in **2** at δ(H) 2.51 – 2.55, δ(C) 30.5 (C(3)), and δ(H) 2.00 – 2.13 and 2.20 – 2.70, δ(C) 25.8 (C(4)) in **2**. The analyses of the HSQC and HMBC spectral data led to the tetrahydropyran-2-one cyclic system instead of the α,β-unsaturated lactone. All remaining spectral data obtained indicate that there is no additional structural difference between the two isolated polyketides. The relative configuration was established as the same as in **1** on the basis of the NOESY spectrum and the coupling constant values of **2** which were approximately the same as for **1**.

The structure of compounds **1** and **2** are closely related to sultriecin (**3**) and phostriecin (**4**), except when it comes to the hydrophobic tail at the end of the

Table. NMR Spectroscopic data (^{13}C, 150 MHz; ^{1}H, 600 MHz) for phosdiecins A (**1**) and B (**2**). δ in ppm, J in Hz

Position	**1**[a])		**2**[a])	
	δ(H)	δ(C)	δ(H)	δ(C)
1		167.1		181.6
2	6.06 (*d*, *J* = 9.80)	123.8	2.51 – 2.55 (*m*)	30.5
3a	7.04 (*dd*, *J* = 5.49, 9.80)	148.3	2.00 – 2.13 (*m*)	25.8
3b			2.20 – 2.27 (*m*)	25.8
4	4.17 (*dd*, *J* = 3.00, 5.49)	64.8	4.49 (*dt*, *J* = 5.00, 6.90)	85.9
5	4.88 (*dd*, *J* = 3.00, 7.00)	84.0	4.09 (*dd*, *J* = 6.45, 6.90)	76.3
6	5.84 (*dd*, *J* = 7.00, 16.00)	129.4	5.61 (*dd*, *J* = 6.45, 15.3)	133.1
7	5.84 – 5.91 (*m*)	133.8	5.76 – 5.83 (*m*)	132.0
8a	2.44 (*ddd*, *J* = 6.72, 6.72, 13.45)	38.9	2.32 – 2.41 (*m*)	38.7
8b	2.65 (*ddd*, *J* = 6.72, 6.72, 13.45)	38.9	2.56 – 2.63 (*m*)	38.7
9	4.66 – 4.74 (*m*)	77.7	4.66 – 4.72 (*m*)	77.3
10	1.60 – 1.64 (*m*)	44.8	1.53 – 1.64 (*m*)	44.6
11	4.53 (*AB*, *J* = 9.54)	69.6	4.53 (*AB*, *J* = 9.07)	69.8
12	5.35 (*AB*, *J* = 10.15)	134.1	5.34 (*AB*, *J* = 9.94)	134.3
13	6.43 (*AB*, *J* = 11.52)	127.7	6.42 (*AB*, *J* = 11.38)	127.7
14	6.32 (*AB*, *J* = 11.37)	125.7	6.32 (*AB*, *J* = 11.08)	125.9
15	5.52 (*dt*, *J* = 7.72, 11.17)	136.0	5.50 (*dt*, *J* = 7.63, 10.8)	135.9
16	2.18 – 2.24 (*m*)	27.4	2.18 – 2.24 (*m*)	27.4
17	1.24 – 1.30, (*m*)	41.0	1.24 – 1.30 (*m*)	41.0
18	1.55 – 1.64 (*m*)	29.8	1.55 – 1.64 (*m*)	29.8
19, 20	0.90 (*d*, *J* = 6.67)	23.9	0.89 (*d*, *J* = 6.71)	24.0
21	0.82 (*d*, *J* = 6.98)	10.2	0.80 (*d*, *J* = 6.79)	10.2

[a]) Recorded in CD_3OD.

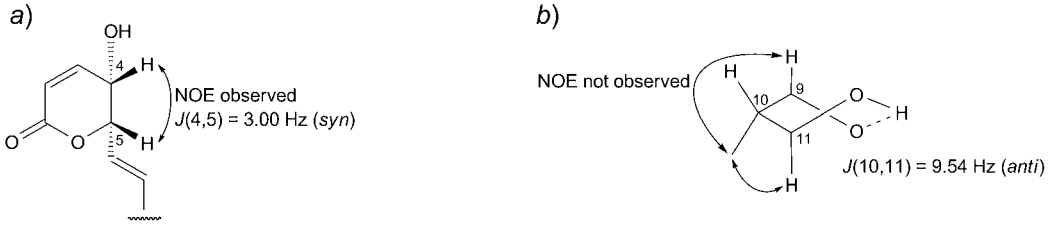

Fig. 5. *a*) Relative configuration of the α,β-unsaturated lactone system. *b*) Relative configuration at C(9), C(10), and C(11)

structure. The reported compounds possess an (*Z*,*Z*,*E*)-triene with a pentyl group, instead of a (*Z*,*Z*)-diene with an isopentyl group in the compounds found in this work. Also, compound **2** is the first report of this class of secondary metabolites with a tetrahydropyran-2-one instead of the α,β-unsaturated lactone.

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Experimental Part

General

Analyses were performed on a chromatograph (*1200* series; *Agilent GmbH*) fitted with a *G1311A* quaternary pump, a *G1322A* degasser, a *G1315D* variable wavelength diode array detector, and a *G1329A* autosampler. *Hystar* 2.3 software (*Bruker*). The chromatograph was directly coupled to an automatic cartridge exchanger (*Bruker Biospin GmbH*) equipped with a range of cartridges containing different stationary phases, to which the flow was directed automatically. IR Spectra: IR_*Prestige-21* spectrometer (*Fourier* transform infrared spectrometer; *Shimadzu*); $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker Avance III* instrument (14.1 Tesla/600 MHz) equipped with a TCI 5-mm triple resonance ($^{1}H/^{13}C/^{15}N$) z-field gradient cryoprobe; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-MS: samples were mixed with matrix solution and applied onto target spots of a *MTP 384* ground steel TF target plate (*Bruker Daltonics*, Bremen, Germany). The samples were air dried. The matrix solution contained 5 mg/ml of α-cyano-4-hydroxycinnamic acid (CHCA) in MeCN. The peptide calibration standard (~ 1000 – 3200 Da; *BrukerDaltonics*) was applied nearest of analyzed samples spot and used for the internal calibration of the mass spectrometer. MALDI-TOF analyses

were performed in the reflectron mode acquiring negative-charged ions produced by using a *Smartbeam-II* laser technology with adjustable repetition rate of up to 1 kHz (355 nm) mounted in a *BrukerAutoflex Speed* spectrometer. Acquisition was controlled by the *FlexControl* software package (version 3.3; *Bruker Daltonics*) and mass spectra were acquired in the mass range m/z 360 – 3500 kDa.

Fermentation of Streptomyces sp. SS99BA-2 and Workup

The marine *Streptomyces* sp. SS99BA-2 was cultivated in GYM culture medium (glucose 4 g/l, malt extract 10 g/l, yeast extract 4 g/l, pH = 7.2). For the microorganism reactivation procedure, a 500 ml *Erlenmeyer* flask containing 50 ml of reactivation medium were inoculated with the microorganism preserved in 3.5 ml *CryoTube* vials. The flask was kept in a shaker at 200 rpm and a temperature of 28 °C. After 24 h of cultivation, growth could be observed visually from the changes in turbidity and color of the culture broth, as well as the formation of a biofilm on the walls of the flask. The suspension in the flask was used as inoculum for 4500 ml *Erlenmeyer* flasks containing 40 ml of culture medium (GYM). These flasks were inoculated with 10 ml (20%) of suspension. This gave a 200 ml final volume of inoculum medium. Twenty *Erlenmeyer* flasks containing 40 ml of production culture medium (GYM) were inoculated with 10 ml of inoculum suspension, resulting in a final volume of 1000 ml of production medium. The culture lasted 72 h. The resulting broth was centrifuged at 10,000 rpm for 20 min to separate the cells. The supernate was successively extracted with AcOEt and BuOH. The solvent was dried by speed-vacuum (*Centrivap* Concentrator; *Labconco Corp.*) to yield 10 mg of dry BuOH extract.

Isolation of Phosdiecins A (**1**) *and B* (**2**)

A 3 mg portion of the dry BuOH extract was dissolved in 1.5 ml of a 1:1 mixture of *Milli-Q* H_2O and MeOH. The solution was filtered through a PVDF membrane syringe filter (25 mm, 0.45 μm; *Tedia Brazil*) before HPLC analysis. A *Eurobond Prontosil C18* column (125 × 4.0 mm, 5 μm) was used for the chromatographic separation. Gradient elution was performed using a combination of 0.05% TFA-HPLC (*Tedia Brazil*) in *Milli-Q* H_2O (eluent *A*) and 0.05% TFA-HPLC in MeCN (eluent *B*). A isocratic elution of 50% eluent *B* for 5 min, followed by a linear gradient to 60% of eluent *B*, over 2 min, leading to a total run time of 7 min, was used to resolve the two chromatographic peaks of interest which were detected using the UV response at 210 nm (retentions times (t_R): 6.23 and 6.63 min) The corresponding compounds were adsorbed on solid-phase extraction cartridges (*HySphere* Resin GP, 10 mm × 2 mm, 10 μm spherical polydivinylbenzene stationary phase) using an automatic cartridge exchanger (*Bruker Biospin GmbH*). Twenty consecutive chromatographic runs were performed, with 20 μl injections and a flow rate of 1.0 ml/min. After the adsorption process, the cartridges were dried with nitrogen for 30 min to remove residual solvent. CD_3OD (99.8% D) was used to elute the compounds from the SPE cartridges directly into NMR tubes (*Bruker*, 3 mm o.d.) to afford phosdiecin A (500 μg) and phosdiecin B (300 μg).

Phosdiecin A (= **(1*E*,4*S*,5*S*,6*S*,7*Z*,9*Z*)-6-Hydroxy-1-[(2*S*,3*S*)-3-hydroxy-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]-5,13-dimethyltetradeca-1,7,9-trien-4-yl Dihydrogen Phosphate**; **1**). IR (KBr): 3437, 2926, 2860, 2368, 1751, 1633, 1462, 1381, 1192, 1018. ^{1}H- and ^{13}C-NMR: see the *Table*. MALDI-TOF/TOF HR-MS: 443.1820 ($[M - H]^-$, $C_{21}H_{32}O_8P^-$; calc. 443.1829).

Phosdiecin B (= **(1*E*,4*S*,5*S*,6*S*,7*Z*,9*Z*)-6-Hydroxy-1-[(2*S*,3*S*)-3-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]-5,13-dimethyltetradeca-1,7,9-trien-4-yl Dihydrogen Phosphate**; **2**). IR (KBr): 3435, 2927, 2864, 2530, 2374, 2235, 2077, 1649, 1452, 1211, 1116, 972. ^{1}H- and ^{13}C-NMR: see the *Table*. MALDI-TOF/TOF HR-MS: 445.1981 ($[M - H]^-$, $C_{21}H_{34}O_8P^-$; calc. 445.1985).

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