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One-pot synthesis of functionalized 4,5-dihydroisoxazole derivatives via nitrile oxides and biological evaluation with plant cells

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Abstract—1,3 Dipolar cycloadditions of nitrile oxides generated in situ in the presence of a variety of olefins provided 4,5-dihydroisoxazoles. The whole procedure could be performed in a practical and efficient one-pot operation. The products are of excellent purity (95%) and are isolated in 60–83% yields. Some of them enhanced the accumulation of indole alkaloids in periwinkle cell cultures.
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1. Introduction

Recently, the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway, firstly discovered in bacteria, was demonstrated to be present in algae, plants and several Apicomplexa species including *Plasmodium falciparum*, the causal agent of malaria. The MEP pathway provides isopentenyl diphosphate (IPP), the common precursor of all isoprenoids, and it was proven that the natural antibiotic fosmidomycin [3-(*N*-formyl-*N*-hydroxyamino)propylphosphonic acid] is an efficient inhibitor of the second enzyme of the MEP pathway, that is 1-deoxy-D-xylulose-5-phosphate (DOXP) reductoisomerase that converts DOXP into 2-C-methyl-D-erythritol 5-phosphate (MEP).[1] Since the MEP pathway is vital in plants and Apicomplexa but does not occur in animals, enzymes of this pathway represent targets for the search of new classes of herbicides and antimalarial drugs. Indeed, fosmidomycin and its derivative FR-900098 (Fig. 1) have herbicide activities[2] and protect mice against *Plasmodium vinckei* infection.[3] This first successful application of herbicides acting on the MEP pathway as antibacterial drugs[4] has been recently confirmed and Fosmidomycin was shown to be an effective treatment for malaria.[5]

Our aim was to synthesise new drugs with herbicide and/or antimalarial properties from Fosmidomycin taken as a lead compound. Isoxazolines and isoxazoles are versatile intermediates for the synthesis of a wide variety of complex natural products[6] and important pharmacophores in medicinal chemistry. Δ^2-Isoxazolines are found in a number of pharmaceutical agents for example GPIIb/IIIa inhibitors[7] and human leukocyte elastase inhibitors.[8] The isoxazol(in)es with biogenic amines could serve as a part of 'privileged structures' for various receptors.[9]

In the present study, we synthesised two series of compounds with the 4,5-dihydroisoxazole structure (Fig. 2) and tested their biological effects on a plant model system, that is cell suspensions of *Catharanthus roseus*, an Apocynaceae that accumulates several therapeutically valuable monoterpenoid indole alkaloids (MIA) originating from the MEP pathway.[10] Previous studies have shown a strong correlation between the MEP pathway gene expression and MIA accumulation in such cell suspensions.[11,12] We give here the effect of fosmidomycin and fosmidomycin analogues on both *C. roseus* cell

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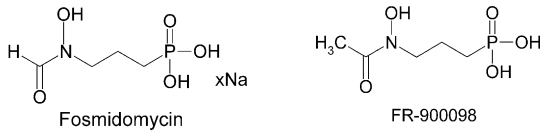


Figure 1. Structures of fosmidomycin and FR900098.

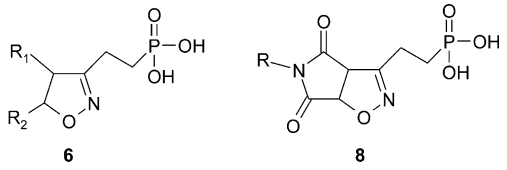


Figure 2. Structure of 4,5-dihydroisoxazole derivatives **6** and **8**.

growth and MIA accumulation. Results with Apicomplexa will be presented elsewhere.

2. Chemistry: results and discussion

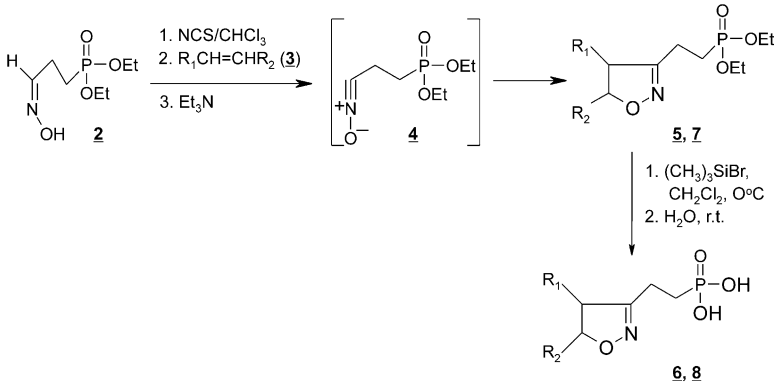
It is known that nitrile oxides undergo [3 + 2] cycloaddition with olefins and acetylenes to provide isoxazolines and isoxazoles, respectively.[13] The major limitation of the chemistry of isoxazol(in)es is the propensity of nitrile oxides to undergo rapid dimerization to furoxan *N*-oxide.[14] We circumvent this problem by generating the nitrile oxide species in situ under high dilution conditions in the presence of small excess of the olefinic trap. Purification and isolation of the desired product become an issue.

Synthesis of functionalized 4,5-dihydroisoxazole derivatives via α-nitroalkenes in a one-pot reaction has been reported by Li.[15] In the present study, we synthesised 4,5-dihydroisoxazoles through a one-pot synthesis as described in Scheme 1. The dipolarophiles **3** ($R_1CH{=}CHR_2$) were commercially available and the oximes were prepared from aldehydes with hydroxylamine in presence of sodium hydrogen carbonate in EtOH/H_2O at 60 °C.[16] The oxime **2** was chlorinated with 1.0 equiv *N*-chlorosuccinimide in $CHCl_3$ for 2 h to provide chloro oxime,[17,18] a precursor to the nitrile oxides. To this was added a 1.25-fold excess of dipolarophile (olefin) as a chloroform solution before generating the nitrile oxide **4** by slow addition of triethylamine over a period of 15 min.[17,19] The resulting mixture was shaken overnight.

The reaction products were purified by column chromatography. A compilation of cycloadducts obtained in this manner is listed in Table 1 (Fig. 2). Using the described procedure, a variety of Δ^2-isoxazolines were synthesised in good yield (60–83%) with purities ranging from 95 to 100%. The purity and the identity of the products were confirmed by ^1H and ^{13}CNMR and MS. Nitrile oxide cycloadditions to terminal alkenes proceed regioselectively to give 5-substituted Δ^2-isoxazolines as single isomeric products (**5a–d**, **7a**, **7b**, Table 1). On the other hand, reactions with 1,2-disubstituted internal alkenes lead to a mixture regioisomers of the 2-isoxazoline[13c] (each as a pair of enantiomers) in approximately a 1:1 ratio (**5e**, **6e**, Table 1). The relative configuration between the 4- and 5-substituents is determined from the geometry of the alkene. Generally, nitrile oxides react with terminal alkenes to give the 5-isomer of the isoxazoline.[13c]

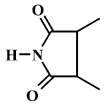
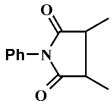
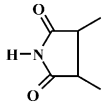
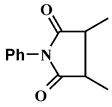
We present here the synthesis of succinimide based 2-isoxazolines **8**. The structures are illustrated in Figure 2. All these compounds were prepared according to the general route described in Scheme 1, starting from maleimide or *N*-phenyl maleimide, which were coupled to nitrile oxide **4** in good yield and high purity (Table 1).

The phosphonic acid **6** can be prepared by hydrolysing the corresponding diethylphosphonates **5** (Scheme 1). The hydrolysis includes a combination method comprising transformation of the ester excepting silyl ester of compounds **5** into a silyl ester and subsequent hydrolysis of the residue silyl ester in water.[20] The hydrolysis of the diethylphosphonates **5** is usually carried out in the present of or absence of solvents under anhydrous condition under cooling.[16,21] The silyl compounds is preferably used in an amount of **5** or more molar equivalents to 1 mol of the diethylphosphonates. Trimethylbromosilane smoothly and quantitatively converts a variety of dialkyl phosphonates to the corresponding bis(trimethylsilyl)esters under exceedingly mild conditions, typically 1–2 h at 25 °C.[20,21] The corresponding trimethylsilyl phosphonates were hydrolysed by H_2O to the expected phosphonic acids **6a–e** (Table 1). The same hydrolysis procedure was carried out to afford



Scheme 1. Synthesis of functionalized 4,5-dihydroisoxazole derivatives.

Table 1. Results for 4,5-dihydroisoxazoles **5**, **6**, **7** and **8** synthesis

Product	R_1	R_2	Yield (%)
5a	H	$COOCH_3$	64
5b	H	CH_2OH	60
5c	H	CH_2Ph	76
5d	H	SO_2CH_3	70
5e	CH_3	$COOCH_3$	82
6a	H	COOH	70
6b	H	CH_2OH	73
6c	H	CH_2Ph	79
6d	H	SO_2CH_3	75
6e	CH_3	COOH	77
7a			65
7b			73
8a			83
8b			80

the phosphonic acids **8a** and **8b** using the corresponding succinimide phosphonates **7a** and **7b** (Scheme 1, Table 1).

3. Biological results and discussion

To begin with, we investigated the effect of fosmidomycin used as control. We found that the drug had no effect on periwinkle cell growth but inhibited the production of ajmalicine (chosen as a marker of MIA production) in a dose-dependent manner with an IC_{50} value of 10 μM (Fig. 3). This is consistent with the idea that MIAs originate from IPP synthesised through the MEP pathway (also called the non-mevalonate pathway) and that the well-known mevalonate pathway has few or no effect on MIA biosynthesis.

Then, we studied the effects of fourteen fosmidomycin analogues (Table 2). Up to the 125 μM concentration,

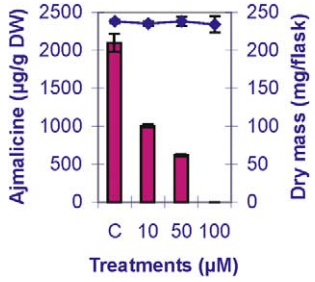


Figure 3. Effect of fosmidomycin on alkaloid production in periwinkle cells (■).C: control cells. Growth cells are not affected (—◆—). Data represent average values from three replicates ± SE. DW, dry weight.

Table 2. Effects of molecules on ajmalicine content of *Catharanthus* cells

Compd	Concentration (μM)	Ajmalicine production (mg g DW^{-1})[a]
5a	50–125	0 ± 0.03 (+70)
5b	50–125	2.66 ± 0.02 (+33)
5c	50–125	3.48 ± 0.02 (+74)
5d	50–125	3.32 ± 0.01 (+66)
5e	50–125	3.40 ± 0.02 (+70)
7a	50–125	2.00 ± 0.01
7b	50–125	2.00 ± 0.03
6a	50–125	2.00 ± 0.02
6b	50–125	2.00 ± 0.02
6c	50–125	2.00 ± 0.01
6d	50–75[b]	2.50 ± 0.03 (+25)
6e	50–75	3.20 ± 0.02 (+60)
8a	50–125	2.00 ± 0.01
8b	50–100[c]	3.20 ± 0.03 (+60)

Ajmalicine production in control cells: 2.00 ± 0.03 mg g DW^{-1}. Values are the mean of three determinations ± SE.
[a] DW, dry weight.
[b] Toxic at higher concentrations.
[c] Growth is decreased at 125 μM. Percentage of modification of alkaloid accumulation is given in parentheses.

we observed no effect on cell growth with the exception of compounds **6d** and **8b** which decreased the cell growth at 75–100 μM. Surprisingly, none of the drugs can inhibit the capacity of periwinkle cells to produce MIAs. Compounds **6a**, **6b**, **6c**, **8a** (phosphonic acid derivatives) and **7a**, **7b** showed no effect on MIA accumulation but the corresponding diethylphosphonates **5a**, **5b**, **5c**, **5d**, **5e** and acids **6e**, **8b** were able to increase MIA accumulation.

Since Fosmidomycin inhibits MIA biosynthesis, results obtained with some of its derivatives were rather unexpected. Taking in account the fact that several fosmidomycin analogues with enhancing effect on MIA accumulation are esters **5**, we tested the effect of fosmidomycin ester [diethyl 3-(*N*-formyl-*N*-hydroxyamino)propylphosphonate] and found that it also increased the production of MIAs (Fig. 4).

We do not know by which mechanism MIA production is enhanced but, as periwinkle cell suspensions have long been considered to be potential sources of medicinally important MIAs, any treatment that can increase MIA production is of potential interest.

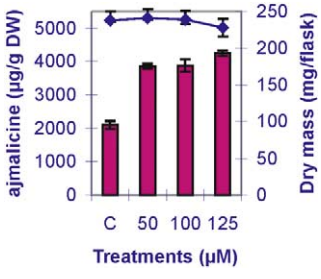


Figure 4. Effect of fosmidomycin ester on alkaloid production in periwinkle cells (■). C: control cells. Growth cells are not affected (—◆—). Data represent average values from three replicates ± SE. DW, dry weight.

4. Conclusion

In summary, one-pot synthesis of functionalized 4,5-dihydroisoxazole derivatives through a 1,3-dipolar cycloaddition of nitrile oxide with dipolarophiles has been developed. Some of them increased the production of MIAs in periwinkle cell cultures.

5. Experimental

5.1. Chemistry

^{1}H and ^{13}C NMR spectra for all compounds were recorded on a Bruker DPX 200 at 200.131 and 50.32 MHz, respectively, in deuteriochloroform and D_2O. The coupling constants are recorded in hertz (Hz) and the chemical shifts are reported in parts per million (δ, ppm) downfield from tetramethylsilane (TMS), which was used as an internal standard. Mass spectra were recorded on a R 10-10 C Nermag (70 eV) apparatus. Organic solvents were purified when necessary according to literature methods[22] or purchased from Aldrich Chimie. All solutions were dried over anhydrous magnesium sulfate and evaporated on a Buchi rotatory evaporator. Analytical thin-layer chromatography (TLC) was carried out on precoated plates (silica gel, 60 F 254, Merck), and spots were visualised under UV light or by iodine vapour. Flash chromatography was performed with Kieselgel 60 (230–400 mesh) silica gel (Merck). All anhydrous reactions were performed in over-dried glassware under an atmosphere of argon. The column chromatography solvents employed were distilled and solvent mixtures were reported as volume to volume ratios. When analyses are indicated by symbols of the elements, analytical results obtained for those elements were ±0.4% of the theoretical values.

Starting materials were obtained from commercial sources—Aldrich, Avocado and Acros. Diethyl ester of [2-(1,3-dioxolan-2-yl)ethyl]phosphonic acid was prepared by method of Arbusov from 2-(2-bromoethyl)-1,3-dioxolane in 76% according to published procedure.[23] This cyclic acetal was hydrolysed to afford the phosphorylated aldehyde **1**: diethyl(3-oxopropyl)phosphonate,[23,24] which was converted into the corresponding oxime **2** as a mixture of the *E*- and *Z*-isomeres in 65% yield by treatment with hydroxylamine.[16] The oxime **2** prepared in this manner did not require further purification for conversion to the hydroximinoyl chloride[17,18] and corresponding nitrile oxide **4** generated by addition of triethylamine.[17,19] 2-Isoxazolines **5** are obtained by 1,3-dipolar addition of nitrile oxides to olefins **3**.[25] Fosmidomycin and fosmidomycin ester were prepared according to published procedure.[25]

5.2. General procedure for the synthesis of 2-isoxazolines 5a–5e, 7a,b

N-Chlorsuccinimid (NCS, 167 mg, 1.25 mmol) was stirred in a flask containing dry chloroform (2 mL) and pyridine (0.006 mL). The oxime (260 mg, 1.25 mmol) was added at 25 °C in one portion. The chlorination was usually over in ca. 2 h as observed by the disappearance of the suspended NCS. The olefin (1.56 mmol) was added at 25 °C and the triethylamine (131 mg, 1.31 mmol) in $CHCl_3$ (0.5 mL) was added drop by drop over ca. 15 min. The reaction mixture was stirred at room temperature overnight under an argon atmosphere. The solution was washed with water (2 × 2 mL), dried and evaporated in vacuum. The resulting oil was purified by flash chromatography on a silica gel column with CH_2Cl_2/MeOH (95:5) as eluent to gave the pure 2-isoxazolines.

5.2.1. Methyl-3-[2-(diethoxyphosphoryl)ethyl]-4,5-dihydroisoxazole-5-carboxylate (5a). The title compound was prepared from methyl acrylate according to the general procedure; yellow oil; yield: 64%. ^{1}H NMR ($CDCl_3$) δ 1.36 (6H, t, J = 7.0 Hz, C$\underline{H}_3$CH$_2$O), 2.11 (2H, m, PC$\underline{H}_2$), 2.66 (2H, m, C$\underline{H}_2$CH$_2$P), 3.28 (2H, m, 4-H), 3.83 (3H, s, C$\underline{H}_3$O), 4.14 (4H, q, J = 7.1 Hz, C$\underline{H}_2$O), 5.06 (1H, m, 5-H). ^{13}C NMR ($CDCl_3$) δ 16.77, 16.89 (J_{PC} = 6.0 Hz, POCH$_2$C$\underline{H}_3$), 21.30, 24.16 (J_{PC} = 143.9 Hz, P$\underline{C}$H$_2$), 21.58, 21.65 (J_{PC} = 3.5 Hz, PCH$_2\underline{C}$H$_2$), 41.39 (4-$\underline{C}$H$_2$), 53.16 (O$\underline{C}$H$_3$), 62.20, 62.33 (J_{PC} = 6.5 Hz, PO$\underline{C}$H$_2$CH$_3$), 77.67 (5-$\underline{C}$H), 157.43, 157.76 (J_{PC} = 16.6 Hz, $\underline{C}$=N), 171.08 ($\underline{C}$=O). MS (CI with NH_3) m/z 294 (M + 1). Anal. $C_{11}H_{20}NO_6P$ (C, H, N). **5a** is a known compounds: it was prepared via α-nitroalkenes.[15]

5.2.2. 3-[2-(Diethoxyphosphoryl)ethyl]-5-hydroxymethyl-4,5-dihydroisoxazole (5b). The title compound was prepared from allyl alcohol according to the general procedure; yellow oil; yield: 60%. ^{1}H NMR ($CDCl_3$) δ 1.38 (6H, t, J = 7.0 Hz, C$\underline{H}_3$CH$_2$O), 2.10 (4H, m, CH$_2$ + PC$\underline{H}_2$), 2.64 (2H, m, C$\underline{H}_2$CH$_2$P), 2.98 (2H, m, 4-H), 3.63 (1H, m, CH$_2$O$\underline{H}$), 4.15 (4H, q, J = 7.0 Hz, C$\underline{H}_2$O), 4.72 (1H, m, 5-H). ^{13}C NMR ($CDCl_3$) δ 16.80, 16.91 (J_{PC} = 5.8 Hz, POCH$_2$C$\underline{H}_3$), 21.39, 24.24 (J_{PC} = 143.7 Hz, P$\underline{C}$H$_2$), 21.83 (PCH$_2\underline{C}$H$_2$), 38.87 (4-$\underline{C}$H$_2$), 62.36 (PO$\underline{C}$H$_2$CH$_3$), 64.02 ($\underline{C}$H$_2$OH), 80.87 (5-$\underline{C}$H), 158.55, 159.00 (J_{PC} = 16.6 Hz, $\underline{C}$=N). MS (IC with NH_3) m/z 266 (M + 1). Anal. $C_{10}H_{20}NO_5P$ (C, H, N).

5.2.3. 5-Benzyl-3-[2-(diethoxyphosphoryl)ethyl]-4,5-dihydroisoxazole (5c). The title compound was prepared from allylbenzene according to the general procedure; yellow oil; yield: 76%. ^{1}H NMR ($CDCl_3$) δ 1.34 (6H, t, J = 6.9 Hz, C$\underline{H}_3$CH$_2$O), 2.00 (2H, m, PC$\underline{H}_2$), 2.50–3.10 (6H, m, CH$_2$ + 4-H + C$\underline{H}_2$CH$_2$P), 4.10 (4H, q, J = 7.2 Hz, C$\underline{H}_2$O), 4.86 (1H, m, 5-H), 7.25–7.29 (5H, m, arom. H). ^{13}C NMR ($CDCl_3$) δ 16.77, 16.88 (J_{PC} = 5.5 Hz, POCH$_2$C$\underline{H}_3$), 21.27, 24.11 (J_{PC} = 142.9 Hz, P$\underline{C}$H$_2$), 21.89, 21.95 (J_{PC} = 3.0 Hz, PCH$_2\underline{C}$H$_2$), 41.28 (4-$\underline{C}$H$_2$), 41.78 (Ph$\underline{C}$H$_2$), 62.16, 62.28 (J_{PC} = 6.0 Hz, PO$\underline{C}$H$_2$CH$_3$), 81.36 (5-$\underline{C}$H), 127.09, 128.92, 129.79, 137.21 ($\underline{C}_{arom}$), 157.71, 158.05 (J_{PC} = 17.1 Hz, $\underline{C}$=N). MS (IC with NH_3) m/z 326 (M + 1). Anal. $C_{16}H_{24}NO_4P$ (C, H, N).

5.2.4. 3-[2-(Diethoxyphosphoryl)ethyl]-5-methylsulfonyl-4,5-dihydroisoxazole (5d). The title compound was prepared from methyl vinyl sulfone according to the

general procedure; yellow oil; yield: 70%. ^{1}H NMR ($CDCl_3$) δ 1.36 (6H, t, J = 7.1 Hz, $C\underline{H}_3CH_2O$), 2.15 (2H, m, $PC\underline{H}_2$), 2.70 (2H, m, $C\underline{H}_2CH_2P$), 3.02 (3H, s, SCH_2), 3.54 (2H, m, 4-H), 4.14 (4H, q, J = 7.6 Hz, $C\underline{H}_2O$), 5.41 (1H, m, 5-H). ^{13}C NMR ($CDCl_3$) δ 16.74, 16.86 (J_{PC} = 6.0 Hz, $POCH_2\underline{C}H_3$), 21.22, 21.30 (J_{PC} = 4.0 Hz, $PCH_2\underline{C}H_2$), 24.07 ($P\underline{C}H_2^*$), 37.21, 38.25 ($S\underline{C}H_3$), 62.31, 62.42 ($PO\underline{C}H_2CH_3$), 91.69 (5-$\underline{C}$H), 158.85, 159.17 ($\underline{C}$=N). MS (IC with NH_3) m/z 314 (M + 1). Anal. $C_{10}H_{20}NO_6PS$ (C, H, N). (*) One line of the $P\underline{C}H_2$-doublet disturbed by the $POCH_2CH_3$ signal.

5.2.5. Methyl-3-[2-(diethoxyphosphoryl)ethyl]-4-methyl-4,5-dihydroisoxazole-5-carboxylate (5e). The title compound was prepared from methyl crotonate according to the general procedure; yellow oil; the mixture of two regioisomers was obtained, yield: 82%. ^{1}H NMR ($CDCl_3$) δ 1.36 (9H, m, $CH_3 + C\underline{H}_3CH_2O$), 2.11 (2H, m, $PC\underline{H}_2$), 2.77 (2H, m, $C\underline{H}_2CH_2P$), 3.82 (3H, s, $C\underline{H}_3O$), 4.14 (4H, q, $C\underline{H}_2O$), 4.60 (1H, m, 4-CH), 5.06 (1H, m, 5-CH). MS (IC with NH_3) m/z 308 (M + 1). Anal. $C_{12}H_{22}NO_6P$ (C, H, N).

5.2.6. [2 - (Diethoxyphosphoryl)ethyl]dihydroisoxazolo[4,5-*c*]succinimide (7a). The title compound was prepared from maleimide according to the general procedure; yellow oil; yield: 65%. ^{1}H NMR ($CDCl_3$) δ 1.33 (6H, t, J = 7.0 Hz, $C\underline{H}_3CH_2O$), 2.18 (2H, m, $PC\underline{H}_2$), 2.75 (2H, m, $C\underline{H}_2CH_2P$), 4.12 (4H, q, J = 7.0 Hz, $C\underline{H}_2O$), 4.40 (1H, d, J = 9.4 Hz, CH), 5.34 (1H, d, J = 9.4 Hz, CH), 10.99 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 16.74, 16.86 (J_{PC} = 6.0 Hz, $POCH_2\underline{C}H_3$), 20.58 ($PCH_2\underline{C}H_2$), 20.75, 23.61 (J_{PC} = 143.9 Hz, $P\underline{C}H_2$), 58.40 ($\underline{C}$H), 62.72, 62.84 (J_{PC} = 6.0 Hz, $PO\underline{C}H_2CH_3$), 80.92 ($\underline{C}$H), 154.07, 154.39 (J_{PC} = 16.1 Hz, $\underline{C}$=N), 172.38, 174.09 ($\underline{C}$=O). MS (IC with NH_3) m/z 305 (M + 1). Anal. $C_{11}H_{17}N_2O_6P$ (C, H, N).

5.2.7. *5.2.7.N*-Phenyl-[2-(diethoxyphosphoryl)ethyl]dihydroisoxazolo[4,5-*c*]succinimide (7b). The title compound was prepared from *N*-phenylmaleimide according to the general procedure; yellow oil; yield: 73%. ^{1}H NMR ($CDCl_3$) δ 1.36 (6H, t, J = 6.9 Hz, $C\underline{H}_3CH_2O$), 2.23 (2H, m, $PC\underline{H}_2$), 2.90 (2H, m, $C\underline{H}_2CH_2P$), 4.15 (4H, q, J = 7.1 Hz, $C\underline{H}_2O$), 4.62 (1H, d, J = 9.6 Hz, CH), 5.53 (1H, d, J = 9.6 Hz, CH), 7.31–7.53 (5H, m, arom. H). ^{13}C NMR ($CDCl_3$) δ 16.78, 16.89 (J_{PC} = 5.5 Hz, $POCH_2\underline{C}H_3$), 20.89 ($PCH_2\underline{C}H_2$), 21.02, 23.89 (J_{PC} = 144.4 Hz, $P\underline{C}H_2$), 57.24 ($\underline{C}$H), 62.39, 62.48 (J_{PC} = 4.5 Hz, $PO\underline{C}H_2CH_3$), 79.73 ($\underline{C}$H), 120.28, 126.57, 129.74, 131.23 ($\underline{C}_{arom}$), 154.07, 154.35 (J_{PC} = 14.1 Hz, $\underline{C}$=N), 170.47, 171.80 ($\underline{C}$=O). MS (IC with NH_3) m/z 381 (M + 1). Anal. $C_{17}H_{21}N_2O_6P$ (C, H, N).

5.3. General procedure for the hydrolysis of the diethoxyphosphonates to phosphonic acids

Trimethylsilyl bromide (536 mg, 3.5 mmol) was added to a solution of the corresponding diethoxyphosphonates (0.5 mmol) in dry methylene chloride (3 mL) under ice-bath cooling. The mixture was stirred at room temperature overnight and then concentrated under reduced pressure to leave an oil. This oil was dissolved in water (3 mL) and stirred at room temperature for 1 h. The solution was washed with chloroform (2×2 mL) and treated with activated charcoal. After removal of the charcoal by filtration, the filtrate was concentrated under reduced pressure. The residue was dissolved in water (2 mL) and adjusted to pH 4.0–5.0 with 1 N NaOH (0.5 mmol). The mixture was concentrated under reduced pressure to give a residue, which was dissolved in methanol, and ethanol was added to the solution. The whole was stirred overnight at room temperature, then the precipitated crystalline solids were collected and recrystallized from methanol-ethanol to give the corresponding phosphonic acid as the monosodium salt.

5.3.1. [2-(5-Carboxy-4,5-dihydroisoxazol-3-yl)ethyl]phosphonic acid (6a). Yellow oil; yield: 70%. ^{1}H NMR (D_2O) δ 1.91 (2H, m, PCH_2), 2.57 (2H, m, $C\underline{H}_2CH_2P$), 3.42 (2H, m, 4-H), CH—overlapping with D_2O). ^{13}C NMR (D_2O) δ 21.13 ($PCH_2\underline{C}H_2$), 22.23, 24.96 (J_{PC} = 137.4 Hz, $P\underline{C}H_2$), 41.40 (4-$\underline{C}H_2$), 77.05 (5-$\underline{C}$H), 161.04, 161.35 (J_{PC} = 15.6 Hz, $\underline{C}$=N), 174.93 ($\underline{C}$=O). MS (IC with NH_3) m/z 246 (M + Na). Anal. $C_6H_{10}NO_6P$ (C, H, N).

5.3.2. [2-(5-Hydroxymethyl-4,5-dihydroisoxazol-3-yl)ethyl)]phosphonic acid (6b). Yellow oil; yield: 73%. ^{1}H NMR (D_2O) δ 1.97 (2H, m, PCH_2), 2.56–2.83 (4H, m, CH_2 + 4-H), 3.16 (1H, m, $CH_2O\underline{H}$), 3.57 (2H, m, $C\underline{H}_2OH$), CH—overlapping with D_2O). ^{13}C NMR (D_2O) δ 21.42 ($PCH_2\underline{C}H_2$), 22.27, 24.99 (J_{PC} = 136.9 Hz, $P\underline{C}H_2$), 38.44 (4-$\underline{C}H_2$), 62.92 ($\underline{C}H_2OH$), 80.89 (5-$\underline{C}$H), 162.04 ($\underline{C}$=N). MS (IC with NH_3) m/z 210 (M + 1). Anal. $C_6H_{12}NO_5P$ (C, H, N).

5.3.3. [2-(5-Benzyl-4,5-dihydroisoxazol-3-yl)ethyl)]phosphonic acid (6c). Yellow oil; yield: 79%. ^{1}H NMR (D_2O) δ 1.76 (2H, m, PCH_2), 2.38 (2H, m, $C\underline{H}_2CH_2P$), 2.65–3.10 (4H, m, $PhCH_2$ + 4-H), 4.87 (1H, m, 5-H), 7.17–7.29 (5H, m, arom. H). ^{13}C NMR (D_2O) δ 21.27 ($PCH_2\underline{C}H_2$), 22.16, 24.88 (J_{PC} = 136.9 Hz, $P\underline{C}H_2$), 40.19 (4-$\underline{C}H_2$), 41.03 ($Ph\underline{C}H_2$), 81.40 (5-$\underline{C}$H), 127.18, 128.96, 129.97, 137.42 ($\underline{C}_{arom}$), 161.90, 162.23 (J_{PC} = 16.6 Hz, $\underline{C}$=N). MS (IC with NH_3) m/z 270 (M + 1). Anal. $C_{12}H_{16}NO_4P$ (C, H, N).

5.3.4. [2-(5-Methylsulfonyl-4,5-dihydroisoxazol-3-yl)-ethyl]phosphonic acid (6d). Yellow oil; yield: 75%. ^{1}H NMR (D_2O) δ 1.95 (2H, m, PCH_2), 2.60 (2H, m, $C\underline{H}_2CH_2P$), 2.93 (3H, s, S–CH_3), 3.52 (2H, m, 4-H), 5.64 (1H, m, 5-H). ^{13}C NMR (D_2O) δ 19.44 ($PCH_2\underline{C}H_2$), 21.87, 24.62 (J_{PC} = 138.4 Hz, $P\underline{C}H_2$), 36.27 ($S\underline{C}H_2$), 38.15 (4-$\underline{C}H_2$), 91.65 (5-$\underline{C}$H), 161.68, 162.01 (J_{PC} = 16.6 Hz, $\underline{C}$=N). MS (IC with NH_3) m/z 258 (M + 1). Anal. $C_6H_{12}NO_6PS$ (C, H, N).

5.3.5. [2-(5-Carboxy-4-methyl-4,5-dihydroisoxazol-3-yl)ethyl]phosphonic acid (6e). Yellow oil; the mixture of two regioisomers was obtained, yield: 77%. ^{1}H NMR (D_2O) δ 1.19 (3H, m, CH_3), 1.91 (2H, m, PCH_2), 2.60 (2H, m, $C\underline{H}_2CH_2P$), 4-CH and 5-CH—overlapping with D_2O. MS (IC with NH_3) m/z 260 (M^+Na). Anal. $C_7H_{12}NO_6P$ (C, H, N).

5.3.6. 2-[(Dihydroisoxazol[4,5-*c*]succinimide)-3-yl)]ethylphosphonic acid (8a). Yellow oil; yield: 83%. ^{1}H NMR (D_2O) δ 2.01 (2H, m, PCH_2), 2.60 (2H, m, $C\underline{H_2}CH_2P$), 4.52 (1H, d, $J = 8.7$ Hz, CH), 5.36 (1H, d, $J = 9.0$ Hz, CH). ^{13}C NMR (D_2O) δ 20.39 ($PCH_2\underline{C}H_2$), 21.50, 24.24 ($J_{PC} = 137.9$ Hz, $P\underline{C}H_2$), 58.61 (<u>C</u>H), 80.94 (<u>C</u>H), 155.90, 156.21 ($J_{PC} = 15.6$ Hz, <u>C</u>=N), 174.56, 177.00 (<u>C</u>=O). MS (IC with NH_3) *m*/*z* 249 (M + 1). Anal. $C_7H_9N_2O_6P$ (C, H, N).

5.3.7. 2-[*N*-Phenyl(dihydroisoxazol[4,5-*c*]succinimide)-3-yl)]ethylphosphonic acid (8b). Yellow oil; yield: 80%. ^{1}H NMR (D_2O) δ 1.58 (2H, m, PCH_2), 2.51 (2H, m, $C\underline{H_2}CH_2P$), 4.34 (1H, d, $J = 9.0$ Hz, CH), 5.00 (1H, d, $J = 9.0$ Hz, CH), 7.21–7.37 (5H, m, arom. H). ^{13}C NMR (D_2O) δ 22.46 ($PCH_2\underline{C}H_2$), 25.11, 27.68 ($J_{PC} = 129.3$ Hz, $P\underline{C}H_2$), 83.29 (<u>C</u>H), 122.59, 126.57, 129.68, 136.70 ($\underline{C}_{arom}$), 159.58, 159.94 ($J_{PC} = 18.1$ Hz, <u>C</u>=N), 169.24, 176.82 (<u>C</u>=O). MS (IC with NH_3) *m*/*z* 325 (M + 1). Anal. $C_{13}H_{13}N_2O_6P$ (C, H, N).

5.4. Biological evaluation

5.4.1. Plant material. Periwinkle (*C. roseus* [L.] G. Don) cells suspensions (line C20) were subcultured every 7 days (dilution rate 1:10) in the B5 Gamborg et al.'s medium[26] containing 58 mM sucrose and 4.5 μM 2,4-dichlorophenoxacetic acid (2,4-D).[27] They were grown in 250 mL Erlenmeyer flasks (with 50 mL culture medium) on a rotary shaker (100 rpm) at 24 °C, in darkness.[28]

5.4.2. Treatments. Periwinkle cells were subcultured in a MIA-inducing medium (i.e., 2,4-D free B5 medium to which 4.5 μM zeatin were added at day 3).[29] The molecules to be tested were added at day 3 to the medium (final concentrations 50, 75, 100 and 125 μM). The cells were harvested at day 7 (vacuum filtration) and freeze dried for ajmalicine and dry mass quantitations.

5.4.3. Alkaloid quantitation. MIA were extracted from 25 mg freeze dried cells with methanol. Ajmalicine, the chosen marker of alkaloid accumulation, was quantified by spectrofluorodensitometry[28] (TLC scanner 3, Camag, λ_{em}: 365 nm).

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