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Phytochemistry 66 (2005) 1797–1803
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Fosmidomycin analogues as inhibitors of monoterpenoid indole alkaloid production in *Catharanthus roseus* cells

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Received 10 February 2005; received in revised form 2 June 2005

Abstract

Substituted 3-[2-(diethoxyphosphoryl)propyl]oxazolo[4,5-*b*]pyridine-2(3*H*)-ones were obtained by functionalization at 6-position with various substituents (aryl, vinyl, carbonyl chains) via reactions catalysed with palladium. We found that these new fosmidomycin analogues inhibited the accumulation of ajmalicine, a marker of monoterpenoid indole alkaloids production in plant cells. Some of them have greater inhibitory effect than fosmidomycin and fully inhibit alkaloid accumulation at the concentration of 100 μM.
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Keywords: *Catharanthus roseus*; Apocynaceae; Madagascar periwinkle; Monoterpenoid indole alkaloids; Methylerythritol phosphate pathway; Synthesis; Substituted oxazolo[4,5-*b*]pyridine-2(3*H*)-ones; Fosmidomycin analogues; Pd-catalysed reactions

1. Introduction

The methylerythritol phosphate (MEP) pathway is a newly discovered alternative biosynthetic route to isopentenyl diphosphate (IPP) found to be present in many bacteria, algae, plants and some protozoa (Rohmer, 1999; Rodríguez-Concepción and Boronat, 2002). The second enzyme of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267), converts 1-deoxy-D-xylulose-5-phosphate (DXP) into the branched compound 2C-methyl-D-erythritol-4-phosphate (MEP) (Takahashi et al., 1998). DXR is a molecular target for fosmidomycin (Kuzuyama et al., 1998; Altincicek et al., 2000; Mueller et al., 2000; Proteau, 2004), and the crystal structure of *E. coli* DXR as a complex with fosmidomycin in the presence of NADPH and manganese has been elucidated (Steinbacher et al., 2003; Mac Sweeney et al., 2005).

Catharanthus roseus (Madagascar periwinkle, Apocynaceae) produces pharmaceutically valuable monoterpenoid indole alkaloids (MIAs). Among them, the bisindole alkaloids vinblastine and vincristine are powerful antitumor drugs whereas ajmalicine and serpentine are used in the treatment of hypertension and circulatory disorders. MIAs were recently proven to originate from the MEP pathway (Contin et al., 1998). Treating *C. roseus* hairy roots or suspensions cells with fosmidomycin had no effect on the cell growth, but inhibited the accumulation of MIA (Hong et al., 2003; Mincheva et al., 2004). Similarly, some functionalized 4,5-dihydroisoxazole derivatives (substrate analogues based on fosmidomycin structure) obtained by one-pot synthesis via nitrile oxides were shown to inhibit MIA production without affecting the cell growth (Mincheva et al., 2004).

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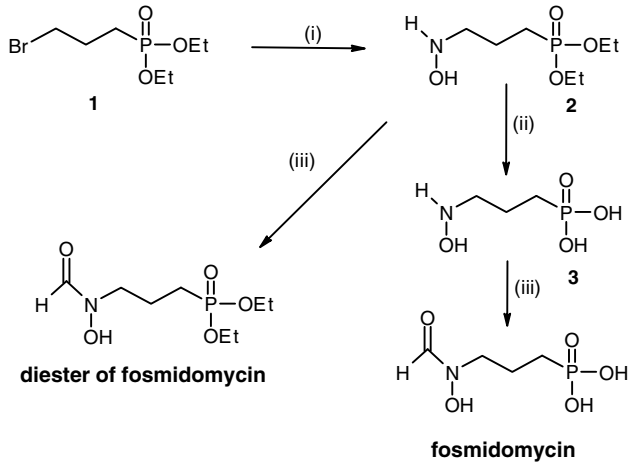
doi:10.1016/j.phytochem.2005.06.002

Oxazolopyridinones and their derivatives have been investigated in numerous studies for their applications as drugs and pesticides (Viaud et al., 1995; Viaud et al., 1996). Investigating such compounds could lead to the discovery of new representatives of this class having valuable pharmacological properties. In another study, we prepared fosmidomycin analogues containing a benzoxazolone or oxazolopyridinones ring: none of the 13 studied phosphonic esters and phosphonic acids had effect on *C. roseus* cell growth, but four of the esters exerted a significant inhibition of ajmalicine accumulation: 45–85% at 125 μM (Courtois et al., 2004). It is known that the activities of oxazolopyridinones depend mainly on the nature of the substituents on the basic heterocyclic framework. This paper deals with the synthesis of new fosmidomycin analogues via Pd-catalysed reactions and their biological evaluation on the growth of *C. roseus* cells and contents in MIAs.



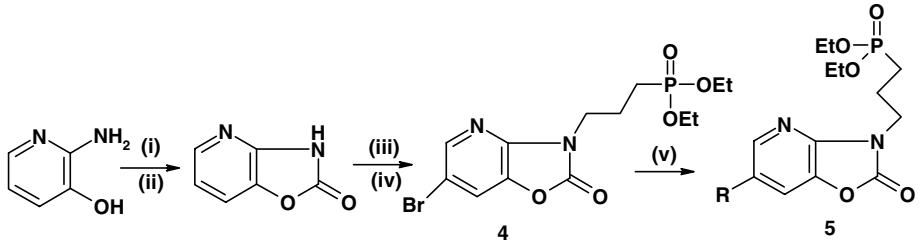
Scheme 1. Synthesis of precursors of the fosmidomycin. Reagents and conditions: (i) $NH_2OH \cdot HCl/H_2O$–MeOH/NaOH, 0 °C; reflux 40–45 °C. (ii) CH_3COOH/HCl, reflux. (iii) HCOOH/$(CH_3CO)_2O$, 0 °C, reflux 45 °C.

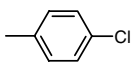
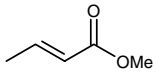
2. Results

2.1. Synthesis

In a first step, we prepared the following compounds: fosmidomycin [3-(*N*-formyl-*N*-hydroxyamino)propylphosphonic acid], diester of fosmidomycin [diethyl-3-(*N*-formyl-*N*-hydroxy-amino)propylphosphosphonate] and two precursors in the synthesis of fosmidomycin, i.e. [diethyl-3-(*N*-hydroxyamino)propylphosphosphonate] (**2**) and 3-(*N*-hydroxyamino)propyl phosphonic acid (**3**), (see Scheme 1) (Kamiya et al., 1980; Hemmi et al., 1981; Hemmi et al., 1982). The synthesis of **2** was carried out by starting from diethyl-3-bromopropylphosphonate (**1**). The ethyl groups of **2** were removed by hydrolysis with concentrated hydrochloric acid–acetic acid under reflux to give the hydroxylamine **3**. Formylation of 3 with acetic–formic anhydride at room temperature gave 70% yield of fosmidomycin as the monosodium salt. Similarly, **2** was also formylated to give diester of fosmidomycin.

In a second step, the inhibitory effect of compound **4** on the MIA production in *C. roseus* cells (see below) led us to investigate the effects of the substituted 3-[2-(diethoxyphosphoryl)-propyl]oxazolo[4,5-*b*]pyridine-2(3*H*)-ones at 6-position (**5a**–**d**). We chose to investigate the palladium catalysed cross-coupling (Tietze et al., 2004) of 6-bromo-3-[2-(diethoxyphosphoryl)propyl]oxazolo [4,5-*b*]pyridine-2(3*H*)-one (**4**) with the commercially available boronic acids using Suzuki methodology (Suzuki, 1998; Miyaura and Suzuki, 1995). Compound **4** was coupled directly with phenylboronic acid or 4-chlorophenyl boronic acid under anhydrous conditions [3% $Pd(PPh_3)_4$, 1:1 *N*,*N*-dimethylformamide (DMF)–triethylamine (Et_3N), reflux for 24 h], (Thompson and Gaudino, 1984) to provide the corresponding Suzuki coupled products **5a** and **5b** in



Compounds	5a	5b	5c	5d
R				

Scheme 2. Synthesis of fosmidomycin analogues via palladium-catalysed reactions. Reagents and conditions: (i) CS_2, reflux, EtOH/KOH/H_2O; (ii) $KMnO_4$, CH_3COOH; (iii) Br_2/DMF; (iv) EtONa, $Br(CH_2)_3PO(OEt)_2$, DMF, reflux; (v) **5a**: $PhB(OH)_2$, $Pd(PPh_3)_4$, DMF/Et_3N, reflux 90 °C; **5b**: $ClPhB(OH)_2$, $Pd(PPh_3)_4$, DMF/Et_3N, reflux 90 °C; **5c**: $CH_2{=}CHSnBu_3$, $(Ph_3P)_2PdCl_2$, toluene, 110 °C; **5d**: $CH_3OCOCH{=}CH_2$, $Pd(OAc)_2$, P(*o*-tol)$_3$, DMF/Et_3N, 130 °C.

high yield (Scheme 2). Synthesis of compound **5c** was performed according to Stille's reaction by using as key reaction palladium (II) catalysed coupling of arylstannane [tributyl(vinyl)tin] with **4** (Stille, 1986; Echavarren and Stille, 1987). The Heck reaction has shown great versatility in the construction of carbon–aryl bond (Heck, 1991). Although generally utilized in the formation of cyclic or linear carbon-based systems, the Heck reaction has been efficiently applied to heterocyclic ring system (Link and Overman, 1998). Synthesis of compound **5**d was performed in good yield according to Heck methodology using palladium (II) acetate and tri-*o*-tolylphosphine in DMF with Et_3N as base (Viaud et al., 1995).

2.2. Biological evaluation

Fosmidomycin inhibited ajmalicine production (chosen as a marker of MIA production) in a dose-dependent manner (IC_{50} value, 10 μM) whereas the diester of fosmidomycin doubled the accumulation of ajmalicine (Fig. 1(a)). So, in a first time, we investigated the biological effect of the precursors, ester **2** and acid **3** (Scheme 1). These two compounds had no effect on cell growth: compound **2** did not affect MIA accumulation (up to 125 μM), but **3** increased the alkaloid production

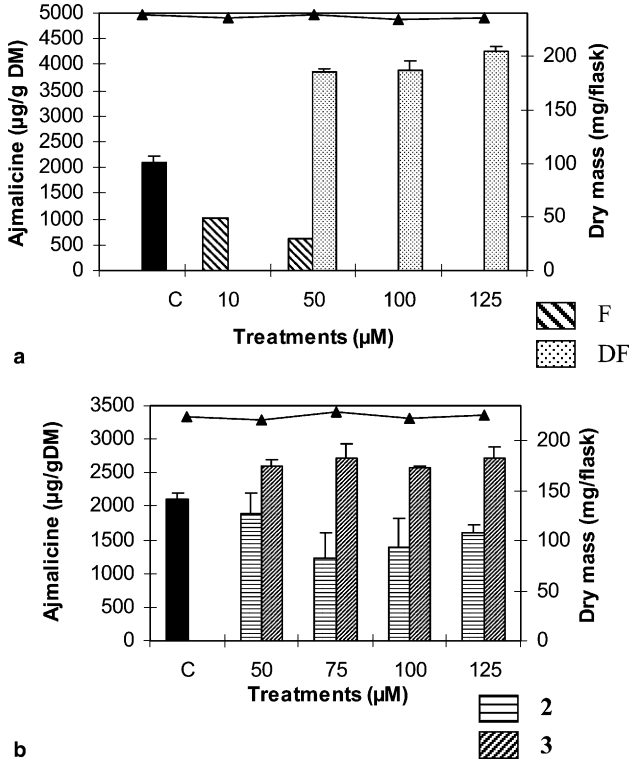


Fig. 1. (a) Effects of fosmidomycin (**F**) and diester of fosmidomycin (**DF**) on alkaloid accumulation in periwinkle cells. (b) Effects of intermediates **2** and **3** on alkaloid accumulation in periwinkle cells. Standard errors of the mean of three assays. C: control cells (■), DM: dry mass, growth cells (-▲-).

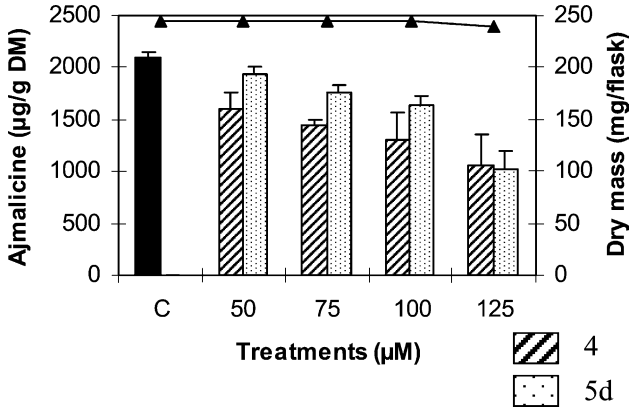


Fig. 2. Effects of initial product **4** and the product **5d** on alkaloid accumulation in periwinkle cells. Standard errors of the mean of three assays. C: control cells (■), DM: dry mass; growth cells (-▲-).

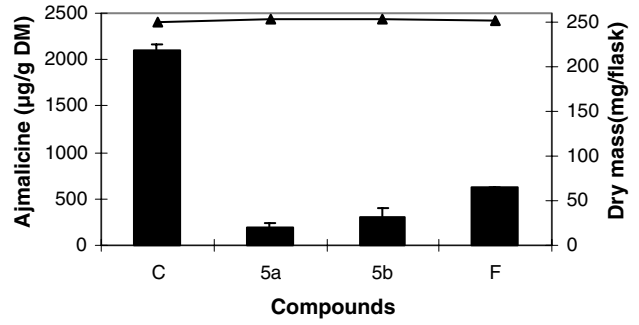


Fig. 3. Inhibition of alkaloid accumulation by compounds **5a**, **5b** and fosmidomycin (**F**) at 50 μM. Standard errors of the mean of three assays. C: control cells (■), DM: dry mass, growth cells (-▲-).

up to 38% (Fig. 1(b)). These responses were not surprising in reference to our previous report (Mincheva et al., 2004).

Then, four new products **5a**–**d**, were synthesized (Scheme 2) from **4**, (Courtois et al., 2004). Compound **4** is a fosmidomycin analogue containing an oxazolopyridinone ring. It inhibited alkaloid production by about 50% (IC_{50}: 125 μM; Fig. 2).Compound **5d** did not possess a better inhibitory activity than **4** (Fig. 2) but **5a** and **5b** were more efficient (Fig. 3) with inhibitory effects of 91% and 87%, respectively. We observed a complete inhibition of ajmalicine accumulation at a concentration of 100 μM. Clearly, the inhibitory activities of **5a** and **5b** were better than the one fosmidomycin (inhibition 69%: Fig. 3). Compound **5c** was not tested due to its non-solubility in culture medium.

3. Discussion and conclusions

Fosmidomycin is a phosphonic acid derivative originally isolated from *Streptomyces lavandulae*. This antibiotic had formerly been shown to have anti-bacterial activity against most gram-negative bacteria (Mine et al., 1980). Then, it was found to specifically inhibit

the plant and bacteria DXR which catalyses the second step of the MEP pathway (see references in Section 1), and it is now extensively used as a tool to inhibit the biosynthesis of natural compounds coming from this pathway, for example carotenoids (Laule et al., 2003), taxol (Palazon et al., 2003) or various isoprenoids (Rodriguez-Concepcion et al., 2004a). In agreement with data of labelling studies showing that MIA derivate from the MEP pathway (Contin et al., 1998), MIA production is inhibited by fosmidomycin in both *C. roseus* hairy roots (Hong et al., 2003) and suspension cells (Mincheva et al., 2004, and this study).

The Suzuki, Heck and Stille reactions used in the present study to prepare new fosmidomycin analogues were found to be efficient methods for generating compounds **5a**–**d**. It was reported that inhibiting the MEP pathway of plants could be useful in the search of novel antibiotics, antimalarials and herbicides (Lichtenthaler et al., 2000). Using *C. roseus* suspension cells, we found that the derivatives **5a** and **5b** are powerful inhibitors of ajmalicine production. Such esters were more active than fosmidomycin, and the presence of phenyl moiety seems to be responsible of their strong inhibitory effect. However, a definite conclusion cannot be drawn. The MIA pathway is highly complex and we cannot exclude that other steps downstream of the MEP pathway, or even the regulation of the alkaloid biosynthesis might be affected. Indeed, only the effect of the inhibitors on DXR activity would give a definitive answer to this question. Since we previously have characterized a cDNA clone encoding DXR from *C. roseus* (Veau et al., 2000) and that *E. coli* DXR cDNA is also available (Takahashi et al., 1998), we are presently preparing recombinant proteins to test the effects of the inhibitors and those previously synthesized (Mincheva et al., 2004; Courtois et al., 2004). If a positive response is obtained, the inhibitors could be of pharmacological interest because the MEP pathway is functional in the Apicomplexa *Plasmodium falciparum*. Fosmidomycin and analogues prepared from this antibiotic are becoming promising drugs against malaria (Jomaa et al., 1999; Missinou et al., 2002; Lell et al., 2003; Phaosiri and Proteau, 2004; Rodriguez-Concepcion, 2004b).

4. Experimental

4.1. General

^{1}H and ^{13}C NMR spectra for all compounds were recorded on a Bruker DPX 200 at 200.131 and 50.32 MHz, in $CDCl_3$. 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. Analytical thin-layer chromatography (TLC) was carried out on precoated plates (silica gel, 60 F254, Merck), and spots were visualized under UV light or by iodine vapor. Flash chromatography was performed with Kieselgel 60 (230–400 mesh) silica gel (Merck).

4.2. Reagents and chemicals

Organic solvents were purified when necessary according to literature methods or purchased from Aldrich Chimie. All solutions were dried over anhydrous magnesium sulfate and evaporated on a Buchi rotatory evaporator. All anhydrous reactions were performed in over-dried glassware under an argon atmosphere. The column chromatography solvents employed were distilled and solvent mixtures were reported as volume to volume ratios. Starting materials were purchased from Aldrich Chimie (St. Quentin-Fallavier, France) or Acros (Noisy-le-Grand, France).

$(Ph_3P)_2PdCl_2$, $Pd(PPh_3)_4$, $Pd(OAc)_2$, P(*o*-tol)$_3$ were furnished by Aldrich. Oxazolo[4,5-*b*]pyridin-2(3*H*)-one (Kalcheva and Peshakova, 1989) and 6-bromooxazolo[4,5-*b*]pyridin-2(3*H*)-one (Viaud et al., 1995) were prepared by following the reported procedures. Diethyl-3-bromo-propylphosphonate was prepared by method of Arbusov according to published procedure (Germanaud et al., 1988). Literature procedures were used for the preparation of 6-bromo-3-[2-(diethoxyphosphoryl)propyl]oxazolo[4,5-*b*]pyridine-2(3*H*)-one (Courtois et al., 2004). Fosmidomyn, diester of fosmidomycin and two precursors of the fosmidomycin **2** and **3** were prepared according to published procedure (Kamiya et al., 1980; Hemmi et al., 1981, 1982).

4.3. Synthesis of new fosmidomycin analogues

4.3.1. General procedure for Suzuki coupling

A stirred mixture of 150 mg (0.38 mmol) of 6-bromo-3-[2-(diethoxyphosphoryl)propyl]oxazolo[4,5-*b*] pyridine-2(3H)-one (**4**), 1.14 mmol phenylboronic acid (or 4-chlorophenyl boronic acid), 22 mg (5 mol%) of $Pd(PPh_3)_4$ and 6 ml DMF–Et_3N (1:1) was refluxed (90 °C) overnight under a nitrogen atmosphere. The mixture was cooled to room temperature and the solvents were distilled off under reduced pressure. The residue was partitioned between EtOAc and brine. The organic extracts were dried with $MgSO_4$ and concentrated under reduced pressure. Purification of the crude product by column chromatography on silica gel with CH_2Cl_2–MeOH (95:5) as eluent afforded the desired compound **5a** (or **5b**) as a yellow oil.

*4.3.1.1. 6-Phenyl-3-[2-(diethoxyphosphoryl)propyl]oxazolo [4,5-b]pyridine-2(**3H**)-one(**5a**).* (132.0 mg, 88.6%). For ^{1}H NMR (200 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz,

$CDCl_3$) assignments, see Tables 1 and 2; MS (IC with NH_3) *m/z*: 390.8 $[M]^+$ for $C_{19}H_{23}N_2O_5P$.

*4.3.1.2. 6-(4-Chlorophenyl)-3-[2-(diethoxyphosphoryl) propyl]oxazolo[4,5-b]pyridine-2(**3H**)-one (**5b**).* (125.0 mg, 77.2%). For ^1H NMR (200 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) assignments, see Tables 1 and 2; MS (IC with NH_3) *m/z*: 425.3 $[M + 1]^+$ for $C_{19}H_{22}ClN_2O_5P$.

*4.3.2. Synthesis of 6-vinyl-3-[2-(diethoxyphosphoryl) propyl]oxazolo[4,5-b]pyridine-2(3H)-one (**5c**)*

To a stirred solution of 6-bromo-3-[2-(diethoxyphosphoryl)propyl]oxazolo[4,5-*b*]pyridine-2(3*H*)-one (**4**), (130 mg, 0.33 mmol) in toluene (5 ml) vinyltributyltin (0.21 ml, 0.73 mmol) and dichlorobis(triphenylphosphine)palladium (II) (10 mg, 0.014 mmol) were successively added and the mixture was refluxed at 110 °C under argon overnight. After cooling, the solvent was removed under pressure and residue obtained was hydrolysed and extracted with EtOAc. The organic layers were dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography on a silica gel column with CH_2Cl_2–MeOH (95:5) as eluent to give the desired compound **5c** as yellow oil (79.5 mg, 70.6%). For ^1H NMR (200 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) assignments, see Tables 1 and 2; MS (IC with NH_3) *m/z*: 341.5 $[M + 1]^+$ for $C_{15}H_{21}N_2O_5P$.

*4.3.3. Synthesis of 3[[2-(diethoxyphosphoryl)propyl] oxazolo[4,5-b]pyridin-6-yl]acrylic acid methyl ester (**5d**)*

To a stirred solution of 6-bromo-3-[2-(diethoxyphosphoryl)propyl]oxazolo[4,5-*b*]pyridine-2(3*H*)-one (**4**), (713 mg, 1.81 mmol) in DMF (15 ml) methyl acrylate (0.23 ml, 2.62 mmol), triethylamine (0.36 ml, 2.62 mmol), palladium diacetate (5 mg, 0.02 mmol) and tri-*o*-tolyl-phosphine (26 mg, 0.08 mmol) were successively added. The mixture was stirred at 130 °C under argon overnight. After cooling, the residue obtained after concentration under vacuum was hydrolysed and extracted with EtOAc. The organic layers were dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography on a silica gel column with CH_2Cl_2–MeOH (95:5) as eluent to give the desired compound

Table 1
^1H NMR spectral data for compounds **5a**–**d** (in $CDCl_3$)

Position	5a	5b	5c	5d
2-(Diethoxyphosphoryl)propyl moiety				
$POCH_2CH_3$	1.28 *t* (7.00)	1.34 *t* (7.04)	1.29 *t* (7.04)	1.34 *t* (6.70)
PCH_2	1.85 *m*	1.81 *m*	1.81 *m*	1.81 *m*
PCH_2CH_2	2.15 *m*	2.20 *m*	2.14 *m*	2.14 *m*
(NCH_2, $POCH_2CH_3$)[a]	4.05 *m*	4.10 *m*	4.04 *m*	4.08 *m*
Oxazolopiridinone moiety				
Aromatic H_7	[b]	[b]	7.50 *s*	7.61 *s*
Aromatic H_5	8.28 *s*	8.30 *d* (1.78)	8.03 *s*	8.20 *s*
R moiety				
	7.39–7.69	7.53–7.74	5.32 *d* (10.91), H_{vinyl}	3.75 *s*, OCH_3
	H_{phenyl}	H_{phenyl}	5.71 *d* (17.57), H_{vinyl}	6.41 *d* (16.00), CH
			6.70 *dd* (10.91; 17.57), H_{vinyl}	7.68 *d* (16.03), CH

[a] Overlapped signals.
[b] Pyridin lying between phenyl protons.

Table 2
^{13}C NMR spectral data for compounds **5a**–**d** (in $CDCl_3$)

Position	5a	5b	5c	5d
2-(Diethoxyphosphoryl)propyl moiety				
$POCH_2CH_3$	16.77, 16.88 (5.5)	16.82, 16.93 (5.5)	16.73, 16.85 (6.0)	16.79, 16.91 (6.0)
PCH_2CH_2	21.69	21.73, 21.82 (4.5)	21.63, 21.72 (4.5)	21.64, 21.73 (4.5)
PCH_2	22.13, 24.98 (143.4)	22.19, 25.04 (143.4)	22.08, 24.93 (143.4)	22.12, 24.97 (143.4)
NCH_2	41.79, 42.17 (19.12)	41.88, 42.27 (19.6)	41.76, 42.14 (19.1)	41.96, 42.34 (19.1)
$POCH_2CH_3$	62.09, 62.21 (6.0)	62.16, 62.29 (6.5)	62.10, 62.23 (6.5)	62.11, 62.23 (6.04)
Oxazolopiridinone moiety				
Aromatic C	115.47–145.88	115.35–145.36	112.78, 115.53	113.4, 118.97
			132.54, 133.79, 142.67	137.67, 141.15, 145.30
Carbonyl (CO)	153.79	153.78	153.76	153.47, 167.12
R moiety	Phenyl C[a]	*p*-Chlorophenyl C[a]	129.64 C_{vinyl}	52.3, OCH_3
			145.40 C_{vinyl}	126.47, 147.26, CH

[a] Overlapped signals.

5d as yellow oil (587.0 mg, 81.5%). For ^{1}H NMR (200 MHz, $CDCl_3$) and ^{13}C NMR (50 MHz, $CDCl_3$) assignments, see Tables 1 and 2; MS (IC with NH_3) *m/z*: 398.8 $[M + 1]^+$ for $C_{17}H_{23}N_2O_7P$.

4.4. Plant material

Madagascar periwinkle (*C. roseus* [L.] G. Don) cell suspensions (line C20) were subcultured every week (dilution rate 1:10) in the Gamborg et al. (1968) B5 medium with 58 mM sucrose and 4.5 μM 2, 4-dichlorophenoxyacetic acid (Mérillon et al., 1993). They were maintained in 250 ml Erlenmeyer flasks containing 50 ml culture medium on a rotary shaker (100 rpm) at 24 °C, in darkness.

For experimental purpose, *C. roseus* cells were subcultured in an alkaloid-inducing medium, i.e. a 2,4-D-free B5 medium to which 5 μM zeatin were added on the third day after subculture (Ouelhazi et al., 1993). Treatments were performed at the third day: the compounds to be tested were dissolved in MeOH–distilled water. Then, the solutions were filter-sterilized (MILLEX GV 0.22 μM, Millipore) before addition to the culture 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.

4.5. Alkaloid determination

Alkaloids were extracted from 25 mg freeze-dried cells with methanol, then separated by TLC (Mérillon et al., 1993). Ajmalicine, the chosen marker of alkaloid accumulation, was quantified by spectrofluorodensitometry (TLC Scanner III, Camag λ_{ex}: 365 nm, $\lambda_{em} > 400$ nm).

Acknowledgements

We express our appreciation to the Le STUDIUM (Agence Régionale de Recherche et d'Accueil International de Chercheurs Associés – Orléans, France) which supported two years a research associate position for Z. Mincheva. This work was also supported by the "Conseil Régional" (Région Centre, France).

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