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Multidrug resistance reversal agent from *Jatropha elliptica*

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Abstract

As part of an ongoing project to identify plant natural products as resistance-modifying agents, bioassay-guided fractionation of an extract of *Jatropha elliptica* (Pohl) Muell Arg. led to the isolation of a penta-substituted pyridine, namely 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester (**8**). The structure was established by spectroscopic methods. This known compound was assayed for in vitro antibacterial and resistance-modifying activities against strains of *Staphylococcus aureus* possessing the MsrA and NorA resistance efflux mechanisms. Antibiotic efflux studies indicated that (**8**) acts as an inhibitor of the NorA efflux pump and restores the level of intracellular drug concentration.

Keywords: *Jatropha elliptica*; *Staphylococcus aureus*; Fluoroquinolone; Resistance; Efflux pump inhibitor

1. Introduction

Active efflux of toxic compounds out of cells is a general mechanism that bacteria were able to develop as a protective mechanism against antimicrobial agents. It has became apparent that efflux-related multidrug resistance (MDR) is a significant complicating factor in the chemotherapy of bacterial infections (van Bambeke et al., 2000, 2003). The efflux pumps are capable of extruding out of the cells a large variety of antibacterial compounds structurally unrelated and having different modes of action. Most genes encoding these MDR pumps are normal constituents of chromosomes, and confer an efflux that is not apparent in wild-type cells. But such genes can be over-expressed under antibiotic pressure. Furthermore, the genes coding for specific systems may be associated with mobile genetic elements which can be transmitted between bacteria (Butaye et al., 2003). The discovery and use of bacterial Efflux Pump Inhibitors (EPIs) could be an attractive approach to restore the activity of antibiotics against bacteria expressing efflux proteins (Lewis, 2001; Renau and Lemoine, 2001; Lomovskaya et al., 2001).

Although widely used for therapeutic purposes, plants are practically not used as antimicrobials (Cowan, 1999; Lewis, 2001). In parallel with the screening of synthetic chemical librairies (Markham et al., 1999; Lomovskaya et al., 2001), seek for natural compounds isolated from plants has proved to be a successful way to identify such EPIs (Stermitz et al., 2001, 2003; Gibbons et al., 2003a; Oluwatuyi et al., 2004). The porphyrin pheophorbide A and the flavonoligan 5′-methoxyhydnocarpin, isolated from *Berberis* plants (Stermitz et al., 2000a,b; Guz et al., 2001; Musumeci et al., 2003), have been identified as inhibitors of the

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Staphylococcus aureus NorA efflux pump. These compounds are devoid of antibacterial activity but they potentiate the activity of berberine, an alkaloid produced by the same *Berberis* plants against the NorA pump. This illustrates that plants producing antimicrobials may have developed other secondary metabolites, which are inhibitors and act synergistically with their antibacterial compounds (Stermitz et al., 2000a,b; Tegos et al., 2002). Recently, a chalcone isolated from *Dalea versicolor* was shown to improve the antibiotic activity of berberine and other antibiotics against *S. aureus* (probably by acting on the NorA pump) and *Bacillus cereus* (Belofsky et al., 2004).

Having access to some of the Brazilian biodiversity, we performed a screening work looking for antibacterial activities in various extracts from more than 25 species of plants used in the Brazilian folk medicine (Ferreira de Lima et al., unpublished results). Some extracts showed an interesting activity, and our attention was attracted by crude rhizome extracts of *Jatropha elliptica*, which, besides antibacterial activity, seemed to potentiate the activity of some antibiotics.

J. elliptica is a plant of the Euphorbiaceæ family. It is a shrub annual herb distributed throughout the North and the West of Brazil, it has been reported to possess several medicinal properties, and is used in the folk medicine for the treatment of several diseases (Corrëa and Penna, 1984; Duke, 1985; Calixto and Sant'Ana, 1987, 1990). The compounds already isolated from the rhizome of this plant are the diterpenoids jatrophone (**1**) and jatropholones A and B (**2** and **3**), the ester pentatriacontanyl ferulate (**4**), the coumarine fraxetine (**5**), the coumarin lignoid propacine (**6**), the triterpenoid 3-*O*-acetyl aleuritolic acid (**7**) and a mixture of steroids, jatrophone being the most abundant component (Fig. 1). The Euphorbiaceæ family is rich in active compounds as illustrated by antibiotic activity reported from *Jatropha podagrica* (Odebiyi, 1985), and by the molluscicidal activity found in various extracts (Zani et al., 1993; Schmeda-Hirschmann et al., 1996; Dos Santos and Sant'Ana, 1999; Goulard et al., 1993). Interestingly, compounds isolated from *Euphorbia* species have been found to be inhibitors of the efflux-pump activity mediated by the MDR transporter P-glycoprotein of tumor cells (Hohmann et al., 2002, 2003; Correa et al., 2004).

We assayed extracts from *J. elliptica*, for a screening of EPI activity on *S. aureus* harbouring efflux pumps. We chose to work with *S. aureus*, since this Gram-positive species, devoid of an outer-membrane, has a greater cell-wall permeability than the Gram-negative organisms (Tegos et al., 2002). Furthermore, it may present resistance mechanisms such as those caused by efflux pumps. The isolation and identification of several fractions from *J. elliptica* were already described by some of us (Goulard et al., 1993). We report here the results of a bioguided fractionation of ethanolic extracts of the rhizomes of *J. elliptica* based on inhibition of the MDR efflux pump NorA (Kaatz et al., 1993; Neyfakh et al., 1993), and of the ATP-binding cassette and macrolide-selective pump represented by the MsrA protein (Ross et al., 1990; Reynolds et al., 2003). A thorough study of the more active component was then carried out.

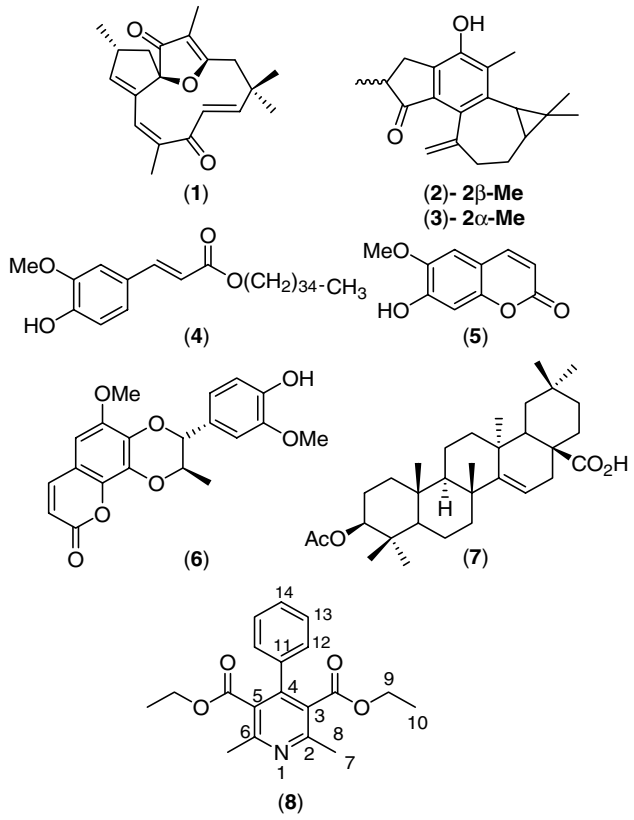

Fig. 1. Compounds isolated from the Euphorbiaceae family.

2. Results and discussion

Antibacterial and inhibitory activities of the extracts and fractions from the rhizomes of *J. elliptica*, tested at 100 mg/l against the three *S. aureus* strains used in this study are shown in Table 1 (no activity was observed for the extracts against *Escherichia coli*, data not shown). These strains will be designated in the text as susceptible *S. aureus* for the susceptible reference strain ATCC 25923; NorA *S. aureus* for the resistant SA-1199B strain harbouring the NorA pump; MsrA *S. aureus* for the resistant strain harbouring the MsrA pump. Ampicillin at 16 mg/l was used as a positive control (its MIC is 8 mg/l). Ciprofloxacin (CIP), has a MIC of 0.37 mg/l against susceptible *S. aureus* and of 16 mg/l against NorA *S. aureus*. Erythromycin (ERY) has a MICs of 0.5 mg/l against the susceptible *S. aureus*, and of 128 mg/l against the resistant MsrA *S. aureus*.

Table 1
Antibacterial activities induced by extracts from *J. elliptica* rhizome against *S. aureus* strains (100 mg/l) with or without antibiotic

Entry		*S. aureus* ATCC 25923	SA-1199B	SA-1199B + CIP[a]	*S. aureus* MsrA + ERY[b]
1	Crude ethanol	+	+	++	+
2	Jatropholone (**2/3**)	−	−	−	−
3	Propacine (**6**)	−	−	+	−
4	3-*O*-Acetyl aleuritolic acid (**7**)	−	−	−	−
5	F-(1–7)	++[c]	++	++	++
6	F-(16–21)(**8**)	−	−	++	−
7	CIP 2 mg/ml	++	−	ND	ND
8	ERY 16 mg/ml	++	++	ND	ND
9	Ampicillin 16 mg/ml	++	++	ND	ND

A fraction was considered as very active (indicated as ++ in the table) if there is no bacterial growth, as active (+ in the table) if bacterial growth is less than 10% of the negative control, and as inactive (− in the table) if bacterial growth is more than 10% of the control without antibiotic.
ND: not done.
[a] Concentration of ciprofloxacin was equal to 2 mg/l.
[b] Concentration of erythromycin was equal to 16 mg/l.
[c] MIC = 100 mg/l.

On entry 1, the crude ethanol extract of rhizome shows a fair activity against both the susceptible and the NorA *S. aureus* strains when assayed alone (columns 1 and 2). When CIP was added at sub-inhibitory concentration (MIC/8, i.e. 2 mg/l), and the mixture assayed against NorA *S. aureus*, the activity of the extract was enhanced, as shown in column 3. This suggests the presence of an inhibitor of the pump which could restore the activity of CIP. No such effect was observed with MsrA *S. aureus* upon addition of ERY at sub-inhibitory concentration (MIC/8, i.e. 16 mg/l) (column 4). Fractionation of the crude extract was then undertaken, and the resulting fractions were assayed. No activity was found for compounds (**2**, **3** and **7**). On entry 3, compound (**6**), devoid of any antibacterial activity, shows a moderate restoration of the activity of CIP on NorA *S. aureus*. On entry 5, F-(1-7) is active against susceptible and resistant strains with a MIC of 100 mg/l. This activity is probably responsible for the antibacterial activity of the crude extract; but this fraction was not further studied. On entry 6, fraction F-(16-21), without any antibacterial activity alone, was shown to abolish the growth of NorA *S. aureus* when tested with sub-inhibitory CIP concentration, thus able to inhibit the NorA pump, and to restore the activity of CIP. No effect was observed against *S. aureus* MsrA, in the presence of ERY at 16 mg/l (MIC/8).

Compound **6** has already been identified as proparcine (Fig. 1). The fair inhibitory action of the coumarin lignoid proparcine might be due its lignan part as flavonoligans have been demonstrated by Guz et al. (2001) to inhibit the NorA efflux pump of *S. aureus*. Characterization of fraction F-(16-21) showed that it was a single compound, and analysis of the spectroscopic data prompted us to describe it as 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester, shown as (**8**). In fact a survey of the literature confirmed this proposal since this penta-substituted pyridine has been synthetically obtained by Nakamichi et al. (2002).

As compound **8**, in association with ciprofloxacin, showed a strong effect against NorA *S. aureus*, we carried out its characterization as a potential NorA inhibitor. MICs against NorA *S. aureus* of several antibacterial agents, all substrates of the pump, were determined in combination with several concentrations of **8** (Table 2). Pefloxacin, an hydrophobic quinolone known as a poor substrate of the pump (Yoshida et al., 1990; Zeller et al., 1997), was used as a negative control. Entry 1 shows the MICs of the assayed molecules alone against NorA *S. aureus*. The addition of **8** at 100 mg/l resulted in reduction of the MICs of the substrates of the pump (ciprofloxacin, norfloxacin, ethidium bromide (EtBr) and acriflavine), by factors ranging from 5.5 to 17-fold. The inhibitory activity of **8** was still good at 50 mg/l for ciprofloxacin, acriflavine and EtBr (3-, 4-, 4-fold, respectively), and similar to that of reserpine at 20 mg/l, but only fair, 2-fold, for norfloxacin, As expected, even with high concentration of **8**, no effect was observed for pefloxacin, which is a poor substrate of the pump. Similar experiments carried out on the susceptible strain SA-1199 showed that **8** and reserpine have no significant effect on the MICs of the two fluoroquinolones substrates of the NorA pump.

To further comfort the possible efflux inhibitor effect of **8** against the NorA pump, efflux of EtBr and uptake of CIP by NorA *S. aureus* were monitored fluorometrically.

Fig. 2 illustrates the effect of **8** at different concentrations, ranging from 100 to 25 mg/l, and of the reference inhibitors reserpine and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), (20 mg/l) on the efflux of EtBr. In the presence of CCCP and reserpine, a decrease in the efflux of about 85% and 72%, respectively, was observed. A reduction of the efflux of EtBr similar to that obtained with reserpine was obtained in the presence of **8** at 100 mg/l (72%), 75 and 50 mg/l (60%), the effect being weaker at 25 mg/l (37%).

Table 2
Effect of the association of compound **8** on the MICs of several antibacterial agents, against the NorA resistant *S. aureus* strain, SA-1199B, and its parental susceptible strain, SA-1199

Entry		MICs (mg/l)					
		(**8**)	CIP	NOR	PEF	ACR	EtBr
	SA-1199B						
1	No association	>500	16	64	8	48	48
2	+ reserpine[b]		4 (4)[a]	24 (3)	8 (1)	24 (2)	12 (4)
3	+ (8) (100 mg/l)		3 (5.5)	12 (5.5)	6 (1.5)	4 (8.5)	3 (17)
4	+ (8) (75 mg/l)		4 (4)	16 (4)	6 (1.5)	6 (4)	6 (8.5)
5	+ (8) (50 mg/l)		6 (3)	32 (2)	8 (1)	12 (4)	12 (4)
6	+ (8) (25 mg/l)		8 (2)	48 (1.5)	8 (1)	48 (1)	24 (2)
	SA-1199						
7	No association		0.37	1			
8	+ reserpine[b]		0.37	0.75			
9	+ (8) (100 mg/l)		0.25	0.5			

CIP: ciprofloxacin; NOR: norfloxacin; PEF: pefloxacin; ACR: acriflavine; EtBr: ethidium bromide.
[a] Values in parentheses are fold-reduction in MICs.
[b] Concentration of reserpine was equal to 20 mg/l.

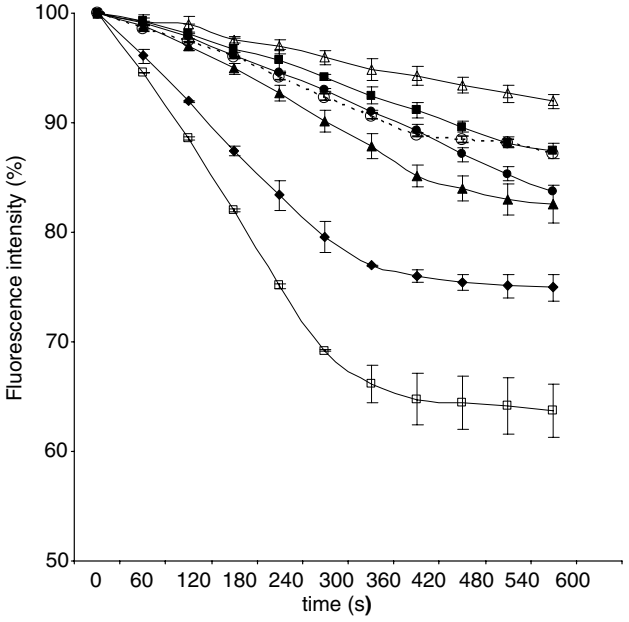

Fig. 2. Effect of inhibitors on EtBr (10 mg/l) efflux by SA-1199B. □ SA-1199B without inhibitor; △ CCCP 20 mg/l; –○– Reserpine 20 mg/l; ■ (**8**) 100 mg/l; ● (**8**) 75 mg/l; ▲ (**8**) 50 mg/l; ◆ (**8**) 25 mg/l. Fluorescence is expressed as the percentage of that at the first time point (100%). Error bars show standard deviation.

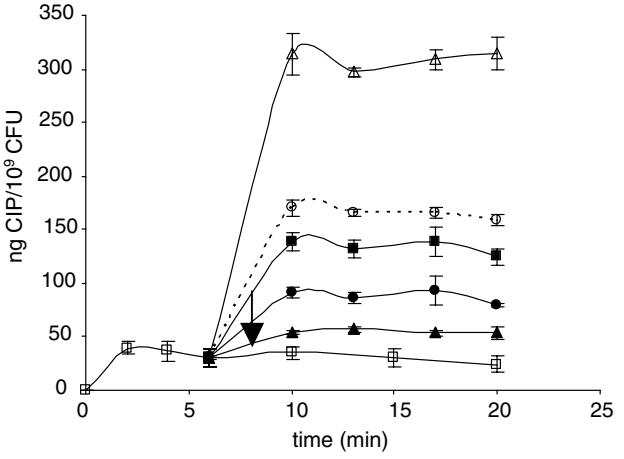

Fig. 3. Effect of inhibitors on the ciprofloxacin (10 mg/l) uptake by SA-1199B. □ SA-1199B without inhibitor; △ CCCP 20 mg/l; –○– Reserpine 20 mg/l; ■ (**8**) 100 mg/l; ● (**8**) 75 mg/l; ▲ (**8**) 50 mg/l. Inhibitors are added at 6 min, as indicated by the arrow. The amount of CIP inside the cells is expressed as ng of CIP per 10^9 CFU. Error bars show standard deviation.

Fig. 3 shows the effect of the inhibitors on the uptake of CIP by NorA *S. aureus*. CIP accumulated rapidly (2 min), and its intracellular amount reached a plateau around 40 ng CIP/10^9 Colonies Forming Units, CFU and, in the absence of any inhibitor, this level was stable. The addition of CCCP (20 mg/l) at 6 min dramatically increased ciprofloxacin uptake from 40 to 320 ng CIP/10^9 CFU. In the presence of reserpine (20 mg/l), the amount of intracellular ciprofloxacin also increased (40–170 ng CIP/10^9 CFU) but twice less than with CCCP. A similar enhancement of the accumulation of ciprofloxacin was observed in the presence of **8** at 100 mg/l. At 75 and 50 mg/l the effect was lower; at 25 mg/l, it was not significant (data not shown).

Therefore, compound **8** satisfies the criteria proposed by Lomovskaya et al. (2001) to be an EPI. We found that this compound, devoid of a proper antibacterial activity, potentiated ciprofloxacin activity against a resistant *S. aureus* strain over-expressing NorA. MICs of several NorA substrates were significantly reduced in presence of **8** at 50, 75 and 100 mg/l, whereas no effect was observed on the quinolone pefloxacin, not substrate of the pump, nor on the parental susceptible strain SA-1199. A concentration-dependent effect of **8** on uptake and efflux of two substrates of the NorA pump was also observed. At 100 mg/l, its action on the

inhibition of EtBr efflux and on the enhancement of ciprofloxacin uptake was similar to that caused by reserpine at 20 mg/l.

A clinical relevant EPI needs to be active at an achievable serum concentration, and have a minimal toxicity. The plant alkaloid reserpine, a known inhibitor (Neyfakh et al., 1991) does not fulfill such criteria, and there is still a need for other inhibitors. It is important to note that the NorA *S. Aureus*, SA-1199B, a ciprofloxacin-resistant derivative (MIC = 16 mg/l) of the susceptible SA-1199, harbours two mutations, an increased transcription of the NorA gene, plus a mutation into the gene of the topoisomerase IV. The MIC of CIP conferred by the latter mutation alone being 2 mg/l (Kaatz et al., 1991; Ferrero et al., 1998), this MIC is the minimum that can be obtained through inhibition of the pump. Using **8**, we reached a MIC of 3 mg/l for CIP, which is close to this value.

Compound **8** belongs to the class of substituted pyridinedicarboxylate esters which biological activities are connected with calcium channel antagonistic activity like in the case of the related dihydropyridine class (Scholz et al., 1997; Muck and Bode, 1994; Kumar et al., 2002; van Rhee et al., 1996). It is noteworthy that some compounds enhancing the activities of anticancer agents against mammalian cells overexpressing the MDR pump P-glycoprotein are closely related to compound **8** and have also emerged as leads for the study of multidrug resistance in cancer chemotherapy (Asoh et al., 1989; Kawase et al., 2002; Avendano and Menéndez, 2002). Bacterial and eukaryotic pumps have been shown to have similar pharmacological characteristics, and some common inhibitors (van Veen et al., 1998; Gibbons et al., 2003b). This indicates that inhibitors of bacterial pumps might be used as tools to study eukaryotic efflux pumps.

3. Conclusion

In this study, we described the characterization of a compound from the rhizome of *J. elliptica*, a plant which has shown many biological activities, and its activity as inhibitor of the NorA transporter. Compound **8**, 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester, was isolated from the ethanol extract and was identified through comparison of its analytical data with published ones. Further studies demonstrated that **8**, a penta-substituted pyridine, is an EPI against the NorA efflux pump of *S. aureus*, according to the criteria proposed by Lomovskaya et al. (2001). It enhances the activities of ciprofloxacin, norfloxacin and other substrates of NorA such as acriflavine and EtBr; it does not potentiate the activity of pefloxacin, which is not a substrate; it has no significant effect on a susceptible strain; it increases intracellular level of antibiotics, which is an additional confirmation that the mode of action of **8** is that of an EPI. The investigation of a series of analogues of compound **8** to acceed to compounds with higher efficiency seems reasonable, since, as pointed by others (Lomovskaya et al., 2001; Gibbons et al., 2003b; Daporta et al., 2004), the combination of a broad-spectrum MDR pump inhibitor with antibiotics known as substrates of the pump could reduce the morbidity and mortality that might result from serious infections caused by Gram-positive organisms.

4. Experimental

4.1. General experimental procedures

Melting points were measured on a Kofler micro hot stage melting point apparatus and are uncorrected. IR spectra were measured with a Perkin-Elmer 783 spectrometre. The EIMS were obtained with a Nermag R 10 mass spectrometre at 70 eV. NMR spectra were recorded on a Bruker AM-400 spectrometre in $CDCl_3$; δ values are in ppm and relative to internal solvent pic and J values are in Hz. Fluorescence of EtBr intercalated into DNA or of ciprofloxacin was measured with a spectrofluorimetre Hitachi F-2000 [($\lambda_{exc} = 530$ nm, $\lambda_{em} = 600$ nm) or ($\lambda_{exc} = 282$ nm, $\lambda_{em} = 447$ nm), respectively]. Absorbances were measured on a Kontron Uvikon 810 spectrophotometre.

4.2. Plant material

J. elliptica Muel. Arg. was collected in the state of Goias, Brazil, in 1994 and identified by Professor José Elias de Paula. A voucher [JEP 1863 (UB)] is deposited in the herbarium of the Universidade de Brasilia.

4.3. Extraction and isolation

The preparation of extracts was already described (Dos Santos and Sant'Ana, 1999; Goulard et al., 1993). For the obtention and identification of compound **8**, the rhizomes of *J. elliptica* (2.5 kg) were collected, dried and powdered and extracted by percolation with 90% ethanol. The solvent was removed by distillation under reduced pressure, giving 313.1 g of crude ethanol extract. A sample of 183.4 g of crude extract was incorporated into silica gel (300 g) and extracted successively with hexane (3 l), chloroform (3 l), ethyl acetate (3 l), ethanol (3 l) and ethanol + acetic acid (1%) (3 l) giving respectively the hexane (52.9 g), chloroform (33.7 g), ethyl acetate (18.8 g), ethanol (50.2 g) and the ethanol + acetic acid 1% (9.30 g) fractions after removal of the solvents. The ethyl acetate fraction (18.8 g) was submitted to chromatography on

a silica gel column (380 g). Fractions F-(16-21) eluted with ethyl acetate showed the same characteristics when compared by TLCand were put together (2.50 g). Recrystallisation from hot hexane furnished (**8**) as a white homogeneous solid (1.5 g). The isolation and identification of some other fractions were already described by us (Goulard et al., 1993). In order to perform the microbiological assays, a 10 mg/l DMSO solution of each compound or extract was prepared, and stored at −20 °C until required.

4.3.1. 2,6-Dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester (*8*)

White powder (hexane) Mp: 60–61 °C. IR (KBr) υ_{max} cm^{-1}: 2980, 1718, 1554, 1496, 1460, 1370, 1291, 1226, 1095, 1038, 857, 752, 702; ^1H NMR (400 MHz, $CDCl_3$): δ 7.2–7.4 (5H, *m*, H-12, H-13, H-14), 4.00 (4H, *q*, $J = 7.3$ Hz, H-9), 2.60 (6H, *s*, H-7), 0.90 (6H, *t*, $J = 7.3$ Hz, H-10); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.7 (*C* OOEt), 155.3 (C, C-2 and C-6), 146.0 (C, C-4), 136.4 (C, C-11), 128.3 (CH, C-14), 128.0 (CH, C-12), 127.9 (CH, C-13), 126.8 (C, C-3 and C-5), 61.2 (CH_2, C-9), 22.8 (CH_3, C-7), 13.4 (CH_3, C-10); EIMS *m*/*z*: 327 $[M]^+$ (100), 282 (69), 254 (48), 236 (89), 209 (41), 139 (81), 115 (45), 77 (27); Found: C 69.90; H 6.69; N 4.52. $C_{19}H_{21}NO_4$ requires: C 69.70; H 6.47; N 4.28%.

4.4. Bacterial strains

The following bacterial strains were used: *E. coli* (ATCC 25922, Gram-negative susceptible strain), *S. aureus* (ATCC 25923, Gram-positive susceptible strain), *S. aureus* SA-1199B (harbouring resistance to fluoroquinolones through over-expression of the NorA efflux pump) and its susceptible parental strain *S. aureus* SA-1199 (Kaatz and Seo, 1995), *S. aureus* MsrA (resistant to 14- and 15-membered macrolides, harboring the multicopies plasmid pUL 5054) (Ross et al., 1990). Strains were grown for a 24 h period in Mueller-Hinton broth (MH, Bio Rad), or Luria-Bertani broth (LB, Difco) at 37 °C.

4.5. Determination of antibacterial susceptibilities

The antibacterial activity of the assayed compounds was carried out into liquid medium by using a Biomek 2000 robot (Beckman) and micro titration plates. The final volume in each well was 200 μl. Two μl of the DMSO solution of compound or extracts were added to each well of the plate containing MH medium and 10^6 CFU/ml (Colonies Forming Units) of the bacteria. Final concentration of extract was thus 100 mg/l. The plates were incubated at 37 °C, and bacterial growth was monitored at 650 nm after 1, 2, 8 and 24 h of growth. Ampicillin (16 mg/l) was used as a positive control and 2 μl of DMSO as a negative control. The extract was considered as very active (indicated as ++ in the tables) if there was no bacterial growth after 24 hours incubation, as active (+ in the tables) if bacterial growth was less than 10%, and as inactive (−) if bacterial growth was more than 10% of the negative control. The Minimal Inhibitory Concentrations (MICs)were determined by using 2-fold dilution technique in Mueller-Hinton broth, to which 10^6 CFU of bacteria per ml was added. Cultures were incubated for 18 h at 37 °C. The MIC is defined as the lowest concentration at which there was no visible growth at 37 °C for 18 h. Experiments were carried out in triplicate.

4.6. Inhibition of resistance mechanisms

Experiments were carried out as above, but using the resistant strains, and the effect of combining the assayed compound and the antibiotic to which the strains were resistant was looked at. For that purpose, ciprofloxacin (CIP) (*S. aureus* NorA) or erythromycin (*S. aureus* MsrA) were added to each well at a concentration of MIC/8, i.e. 2 and 16 mg/l, respectively. The extract, added at a concentration of 100 mg/l was considered as a good (++), fair (+) or not (−) inhibitor of the pump if bacterial growth was totally, at 90% or at less than 90% inhibited, respectively. Reserpine, a plant alkaloid inhibiting several MDR efflux pumps (Neyfakh et al., 1991) was used at 20 mg/l as a positive control for SA-1199B.

4.7. Ethidium bromide efflux

Efflux of EtBr from cells was performed essentially as described previously (Neyfakh et al., 1991). LB medium was inoculated with an overnight culture of NorA *S. aureus* and cultured at 37 °C with agitation to an A_{660} of 0.7–0.8. The cells were first loaded with EtBr (10 mg/l) by incubation in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, CCCP (20 mg/l) during 10 min at 37 °C. Cells were then centrifuged and resuspended in 1 ml of LB medium without EtBr at 37 °C, the inhibitors CCCP or reserpine (20 mg/l) or compound **8** being eventually added at this moment, and the fluorescence was continuously measured for 10 minutes ($\lambda_{exc} = 530$ nm, $\lambda_{em} = 600$ nm). All experiments were performed at least three times, results are expressed as percentage of the initial fluorescence.

4.8. Ciprofloxacin accumulation

Ciprofloxacin uptake was performed as previously described (Denis and Moreau, 1993) using the silicone oil method. Briefly, cells were grown in LB medium to an OD_{660} of 0.7–0.8, then washed twice and

concentrated 20-fold in 100 mM phosphate buffer pH 7.2 containing 1 mM $MgSO_4$ and 0.4% glucose. Following 5 min pre-incubation at 37 °C, ciprofloxacin was added at 10 mg/l to the cell suspension. Efflux pump inhibitors were added 6 min after addition of the fluoroquinolone. At various time intervals, 0.5 ml samples were removed, placed on 0.5 ml aliquots of silicone oil (density 1.03, Fluka), into small plastic tubes, centrifugated for 2 min at 10,000*g* and immediately frozen. The tubes were cut in the middle of the silicone layer and inverted to eliminate the residual oil. The cell pellets were resuspended in 1 ml of lysis buffer (0.1 M glycine–HCl, pH 3.0) and lysed by incubation at 100 °C for 7 min. After centrifugation (2 min, 10,000*g*, fluorescence of supernatants was measured and compared with a ciprofloxacin standard curve ($\lambda_{exc} = 282$ nm, $\lambda_{em} = 447$ nm). Ciprofloxacin accumulation was then expressed as nanograms of product per 10^9 CFU.

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