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N-Caffeoylphenalkylamide derivatives as bacterial efflux pump inhibitors

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Abstract—As part of an ongoing project to identify plant natural products as efflux pump inhibitors (EPIs), bioassay-guided fractionation of the methanolic extract of *Mirabilis jalapa* Linn. (Nyctaginaceae) led to the isolation of an active polyphenolic amide: *N-trans*-feruloyl 4′-*O*-methyldopamine. This compound showed moderate activity as an EPI against multidrug-resistant (MDR) *Staphylococcus aureus* overexpressing the multidrug efflux transporter NorA, causing an 8-fold reduction of norfloxacin MIC at 292 μM (100 μg/mL). This prompted us to synthesize derivatives in order to provide structure–activity relationships and to access more potent inhibitors. Among the synthetic compounds, some were more active than the natural compound and *N-trans*-3,4-*O*-dimethylcaffeoyl tryptamine showed potentiation of norfloxacin in MDR *S. aureus* comparable to that of the standard reserpine.

In recent years bacterial resistance to antibiotics has become a serious problem of public health that concerns almost all antibacterial agents. Among the mechanisms involved (target modification, enzymatic inactivation or reduction of accumulation within the cell), active efflux has received particular attention since it has been recognised as one of the most important causes of intrinsic antibiotic resistance in bacteria.[1]

One species is particularly resistant to treatment, notably *Staphylococcus aureus*. This pathogen has evolved from the MRSA phenotype (methicillin-resistant *S. aureus*) to the VRSA phenotype (vancomycin-resistant *S. aureus*), whereas vancomycin was the drug of last-resort for treatment of MRSA.[2] Resistance has also been reported for newer agents such as linezolid and daptomycin.[3,4]

Of particular concern is the presence of multidrug resistance (MDR) efflux pumps that extrude a wide range of structurally unrelated compounds. The NorA protein of *S. aureus* is a drug/proton antiporter belonging to the major facilitator family (MFS) of transporters and is responsible for the efflux of substances such as norfloxacin, ciprofloxacin, ethidium bromide and acriflavin.[5,6] It has been recognised as one of the major efflux pumps protecting *S. aureus* from antibiotics.[7]

One of the strategies employed to overcome bacterial resistance is the use of EPIs that could restore antibiotic activity in resistant strains. This approach is promising as it would be a way to improve the efficacy and/or extend the clinical utility of existing antibiotics.[8] The combination of a resistance inhibitor with an antibiotic has already proven its efficacy with the clavulanic acid/amoxicillin association. Furthermore, this combination can reduce the in vitro frequency of emergence of resistant mutant strains.[9] At present this combination therapy is taken into account by a number of pharmaceutical companies and several strategies are being developed by different groups.[10]

Keywords: EPI; *Staphylococcus aureus*; *N*-Hydroxycinnamic acid derivatives; *Mirabilis jalapa*.
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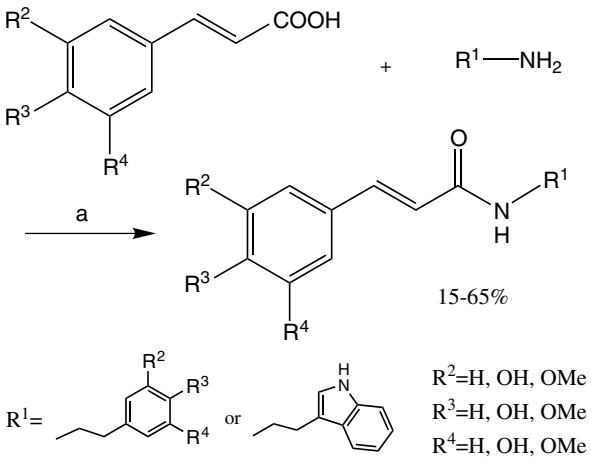
Plants use antimicrobials to protect themselves from environmental stresses, but unlike fungi or bacteria activities are often weak and with a narrow spectrum. This poses the interesting question of how plants react towards increasing antimicrobial resistance. Some authors postulate that plants use an anti-MDR strategy to potentiate their antimicrobials.[11] This is the case for the alkaloid berberine which has a weak antimicrobial activity that is significantly enhanced by 5′-methoxyhydnocarpin (5′-MHC), a flavonolignan produced by the same *Berberis* species.[12] 5′-MHC does not have any antibacterial activity on its own but potentiates the activity of berberine by inhibiting its efflux. A number of inhibitors produced by plants as secondary metabolites and belonging to many chemical classes have been identified but few are potent and have a broad spectrum of action.[13] This includes the alkaloid reserpine which is used as a standard but cannot be used in therapy because of its toxicity. Searching for inhibitors from natural sources is therefore an attractive strategy to access a greater range of active compounds.

In this context, previous screening of ornamental and exotic plants showed activity associated with extracts of *Mirabilis jalapa* Linn. (Nyctaginaceae) in reversing fluoroquinolone resistance in *S. aureus* overexpressing the NorA MDR efflux pump (SA-1199B strain).[14] This plant is used in folk medicine as an anti-infective.[15,16] Bioassay-guided fractionation of the methanolic extract from leaves and stems of *M. jalapa* led to the isolation of an active phenolic compound, namely *N*-*trans*-feruloyl 4′-*O*-methyldopamine, which has already been reported in the plant kingdom.[17] This compound caused an 8-fold reduction of norfloxacin MIC when tested at 100 μg/mL (292 μM). Synthesis of derivatives was then undertaken as this small molecule was a good starting point for structure–activity relationships (SARs), and could be a good candidate for combination therapy.

We chose couplings between cinnamic acid derivatives and amines that occur in nature, as our project was to identify active compounds from natural sources. Several structure–activity relationship criteria were examined: substitution on the aromatic ring on each part, methoxy or hydroxyl substitution influence, double bond influence, aromatic ring nature on the amine part (we chose here to test tryptamine combinations as indolic compounds are known to be good inhibitors).[18] We chose *N*-*trans*-3,4-*O*-dimethylcaffeoyl dopamine as lead compound as it showed the same activity as the natural compound, and its asymetrical substitution allowed us to have a better understanding of the influence of each substituant (i.e., hydroxyl or methoxy) on each part of the molecule. Each time, one part of the lead compound was conserved and the other part was varied in order to provide structure–activity relationships that could lead to further optimisation. Compounds were obtained by coupling of a cinnamic acid (or dihydrocinnamic acid in the case of compound **8**) derivative with the corresponding amine in DMF in the presence of triethylamine and BOP (benzotriazol-1-yloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate) as a coupling agent (Scheme 1).[19] Nine compounds were synthesized and their related activities are presented in Table 1.[20] MICs of the modulators were determined when good potentiation was observed; a good resistance reverser should actually not show any antibacterial effect at the tested concentrations.



Scheme 1. Reagents and condition: (a) BOP, Et_3N, DMF, 20 h. Compound **8** was prepared with 3,4-dimethoxy-dihydrocinnamic acid.

Interpretation of the results led us to draw several conclusions:

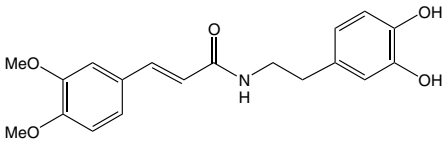
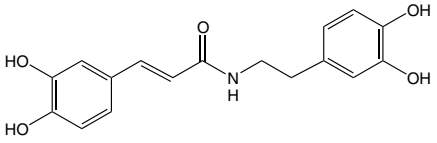
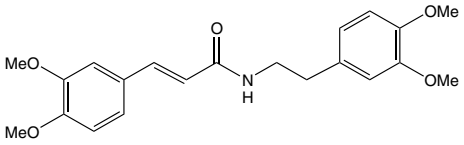
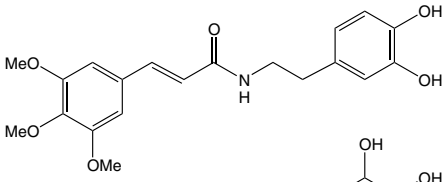
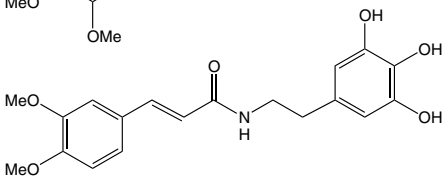
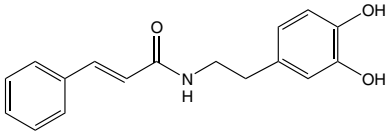
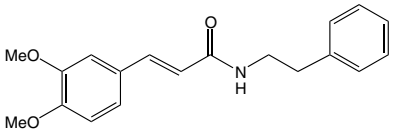
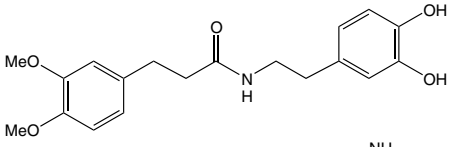
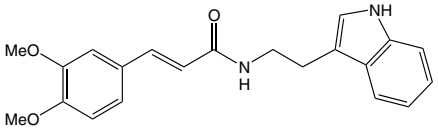
- For the cinnamic part: an hydroxyl substitution on the aromaticring appears to be better than methoxy or unsubstituted (compound **2** versus compounds **1** and **6**); the double bond is essential for activity.
- For the amine part: trisubstitution on the aromatic ring led to a decrease in activity but antibacterial activity was observed (compound **5**); methoxy substitution gave better results than hydroxyl substitution (compound **3** vs compound **1**) which, in turn, were better than no substitution (compound **1** vs compound **7**); tryptamine combinations showed the best results (compound **9**).

Among the 9 compounds tested, compound **9** showed potentiation of norfloxacin comparable to that of the reference alkaloid reserpine. In order to confirm the inhibitory mechanism, we performed an efflux inhibition assay using ethidium bromide (EtBr), a good substrate of NorA, whose efflux can be measured by loss of fluorescence within the cell (Fig. 1).[21,22]

Based on estimation of IC_{50}, compound **9** was approximately 2-fold less active than reserpine at inhibiting EtBr efflux (Fig. 1). However, this difference disappeared at a concentration of 30 μM for each compound.

Structure–activity relationships (SARs) showed that better activities were obtained when the phenyl ring on the cinnamic portion was substituted with 2 hydroxyls. According to this result, we would expect *N*-*trans*-caffeoyl tryptamine (dihydroxylated equivalent of compound **9**) to be more active than compound **9**. It

Table 1. Potentiation of norfloxacin MIC on SA-1199B strain cells by synthetic compounds

Compound	Formula	[Concentration]/fold reduction of norfloxacin MIC
1 (lead)		[292 μM]/**8** [146 μM]/**4**
2		[317 μM]/**8** [63 μM]/**4** MIC ⩾ 406 μM
3		[162 μM]/**8** [54 μM]/**2**
4		[268 μM]/**4**
5		[56 μM]/**0** MIC = 356 μM
6		[353 μM]/**16** [70 μM]/**4** MIC > 452 μM
7		[322 μM]/**4**
8		[290 μM]/**0**
9		[286 μM]/**16** [57 μM]/**8** [29 μM]/**4** MIC > 366 μM

would also be interesting to evaluate whether this type of compound would be a good EPI in Gram-negative bacteria or in other models.

In summary, we have demonstrated that *N*-cinnamoylphenalkylamides are a class of EPIs that could be used to potentiate the bactericidal effect of antibiotics such as norfloxacin in multidrug-resistant pathogenic bacteria such as *S. aureus*. The ease of synthesis and the small size of these compounds make them potential candidates for SARs as EPIs in multidrug efflux systems.

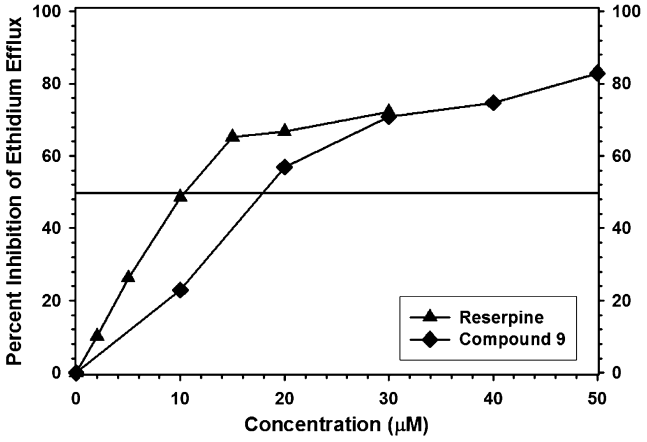


Figure 1. Ethidium efflux inhibition assay from SA-1199B strain cells: (▲) reserpine; (♦) compound **9**.

Acknowledgments

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19. Analytical data for new compounds: for Compound **1**: ^{1}H NMR (400 MHz, CD_3OD): δ 7.46 (d, 1H, J = 15.6 Hz), δ 7.16 (d, 1H, J = 2.0 Hz), δ 7.13 (dd, 1H, J_1 = 8.4 Hz; J_2 = 2.0 Hz), δ 6.97 (d, 1H, J = 8.4 Hz), δ 6.70 (d, 1H, J = 8.0 Hz), δ 6.69 (d, 1H, J = 2.0 Hz), δ 6.57 (dd, 1H, J_1 = 8.0 Hz; J_2 = 2.0 Hz), δ 6.46 (d, 1H, J = 15.6 Hz), 3.87 (s, 3H); 3.87 (s, 3H), δ 3.48 (t, 2H, J = 7.6 Hz), δ 2.68 (t, 2H, J = 7.6 Hz), RMN ^{13}C (100 MHz, CD_3OD): δ 167.6; δ 150.8; δ 149.3; δ 144.9; δ 143.4; δ 140.2; δ 130.7; δ 128.0; δ 121.8; δ 119.6; δ 118.3; δ 115.5; δ 115.0; δ 111.3; δ 109.9; δ 55.0; δ 55.0; δ 41.2; δ 34.6. EIMS: m/z = 343 [M^+]. For Compound **4**: RMN ^{1}H (400 MHz, CD_3OD): δ 7.45 (d, 1H, J = 15.6 Hz), δ 6.87 (s, 2H); δ 6.71 (d, 1H, J = 8.0 Hz), δ 6.69 (d, 1H, J = 2.0 Hz), δ 6.57 (dd, 1H, J_1 = 8.0 Hz; J_2 = 2.0 Hz), δ 6.52 (d, 1H, J = 15.6 Hz), δ 3.87 (s, 3H), δ 3.87 (s, 3H), δ 3.80 (s, 3H), δ 3.48 (t, 2H, J = 6.8 Hz), δ 2.62 (t, 2H, J = 6.8 Hz); RMN ^{13}C (100 MHz, CD_3OD): δ 167.2; δ 155.4; δ 144.6; δ 143.5; δ 142.3; δ 140.2; δ 130.9; δ 130.8; δ 120.0; δ 119.6; δ 117.6; δ 115.5; δ 115.0; δ 114.9; δ 114.6; δ 104.9; δ 59.8; δ 55.3; δ 55.3; δ 41.1; δ 34.6. EIMS: m/z = 373 [M^+]. For Compound **5**: RMN ^{1}H (400 MHz, CD_3OD): δ 7.39 (d, 1H, J = 15.6 Hz), δ 7.08 (d, 1H, J = 2.0 Hz), δ 7.05 (dd, 1H, J_1 = 8.0 Hz; J_2 = 2.0 Hz), δ 6.89 (d, 1 H, J = 8.4 Hz), δ 6.39 (d, 1H, J = 15.6 Hz), δ 6.62 (s, 2H), δ 3.80 (s, 3H), 3.79 (s, 3H), δ 3.39 (t, 2H, J = 7.2 Hz), δ 2.57 (t, 2H, J = 7.2 Hz); RMN ^{13}C (100 MHz, CD_3OD): δ 167.6; δ 150.8; δ 149.4; δ 145.7; δ 140.2; δ 131.3; δ 130.1; δ 128.1; δ 121.8; δ 118.4; δ 118.4; δ 111.4; δ 110.1; δ 107.3; δ 107.3; δ 55.1; δ 55.1; δ 41.0; δ 34.9. EIMS: m/z = 359 [M^+]. For Compound **8**: RMN ^{1}H (400 MHz, $CDCl_3$): δ 6.70 (d, 1H, J = 8.4 Hz), δ 6.68 (d, 1H, J = 8.0 Hz), δ 6.60 (d, 1H, J = 1.6 Hz), δ 6.59 (dd, 1H, J_1 = nd; J_2 = 1,6 Hz), δ 6.54 (d, 1H, J = 2.0 Hz), δ 6.38 (dd, 1H, J_1 = 8.0 Hz; J_2 = 2.0 Hz), δ 5.71 (t, 1H, J = 5.6 Hz), δ 3.75 (s, 3H), δ 3.72 (s, 3H), δ 3.31 (m, 2H), δ 2.78 (t, 2H, J = 7.2 Hz), δ 2.49 (t, 2H, J = 7.2 Hz), δ 2.35 (t, 2H, J = 7.6 Hz); RMN ^{13}C (100 MHz, $CDCl_3$): δ 173.5; δ 148.9; δ 147.5; δ 144.3; δ 143.1; δ 133.1; δ 130.6; δ 120.5; δ 120.4; δ 115.6; δ 115.4; δ 111.8; δ 111.5; δ 56.0; δ 55.9; δ 41.01; δ 38.7; δ 34.8; δ 31.3. EIMS: m/z = 345 [M^+]. For Compound **9**: RMN ^{1}H (400 MHz, $CDCl_3$): δ 7.97 (s, 1H), δ 7.60 (d, 1H, J = 8.0 Hz), δ 7.54 (d, 1H, J = 15.6 Hz), δ 7.37 (d, 1H, J = 8.0 Hz), δ 7.17 (td, 1H, J_1 = 8.0 Hz; J_2 = 7.2 Hz; J_3 = 1.2 Hz), δ 7.10 (td, 1H, J_1 = 8.0 Hz; J_2 = 7.2 Hz; J_3 = 1.2 Hz), δ 7.03 (s, 1H), δ 7.01 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), δ 6.96 (d, 1H, J = 1.6 Hz), δ 6.79 (d, 1H, J = 8.4 Hz), δ 6.24 (d, 1H, J = 15.6 Hz), δ 6.12 (t, 1H, J = 6.0 Hz), δ 3.87 (s, 3H), δ 3.84 (s, 3H), δ 3.72 (m, 2H), δ 3.02 (t, 2H, J = 6.8 Hz); RMN ^{13}C (100 MHz, $CDCl_3$): δ 167.6; δ 150.8; δ 149.4; δ 140.2; δ 136.6; δ 128.0; δ 127.3; δ 122.0; δ 121.8; δ 120.9; δ 118.4; δ 118.2; δ 117.9; δ 111.9; δ 111.3; δ 110.8; δ 110.0; δ 55.0; δ 55.0; δ 40.2; δ 25.0. EIMS: m/z = 350 [M^+].
20. Modulation assays were conducted according to National Committee for Clinical Laboratory Standards, 1999. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Norfloxacin MIC on SA-1199B cell is 100 μM (32 μg/mL), and the reference alkaloid reserpine causes an 8-fold reduction of norfloxacin MIC at 33 μM (20 μg/mL).
21. *Ethidium bromide assay*: EtBr is a substrate for many MDR pumps, including NorA. The efficiency of efflux pumps for which EtBr is a substrate can be assessed fluorometrically by the loss of fluorescence over time from cells loaded with EtBr. SA-1199B was loaded with EtBr as described previously and the effects of varying concentrations of compound **9** and reserpine were determined to generate dose–response profiles. The total time course for the efflux assay was 5 min. Assays were performed in duplicate and mean results were expressed as the percentage reduction of total efflux observed for test strains in the absence of inhibitors.
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