

Biocatalyzed synthesis of vanillamides and evaluation of their antimicrobial activity

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23 **Abstract**

24 A series of vanillamides were easily synthesized exploiting an acyltransferase from *Mycobacterium*
25 *smegmatis* (MsAcT). After their evaluation as antimicrobial agents against a panel of Gram-positive and
26 Gram-negative bacteria, three compounds demonstrated to be 9-fold more effective towards
27 *Pseudomonas aeruginosa* than the vanillic acid precursor. Taking into consideration the scarce permeability
28 of the Gram-negative bacteria cell envelope when compared to Gram-positive strains or yeasts, these
29 molecules can be considered the basis for the generation of new nature-inspired antimicrobials. To
30 increase the process productivity and avoid any problem related to the water solubility of the starting
31 material, a tailored flow biocatalyzed strategy in pure toluene was set up. While a robust immobilization
32 protocol exploiting glyoxyl-agarose was employed to increase the stability of MsAcT, in-line work up
33 procedures were added downstream the process to enhance the system automation and reduce the overall
34 costs.

35 **Keywords**

36 Vanillamides, antimicrobial agents, MsAcT, biocatalyzed reactions, enzyme immobilization, flow chemistry.

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45 **Introduction**

46 Microorganisms are frequently associated with a variety of diseases in a wide range of infections. During
47 the last years, a significant increase of antimicrobial resistance, prolonged treatment times, therapy toxicity
48 and related costs was observed.¹ Well-known multidrug-resistant strains, such as the methicillin-resistant
49 *Staphylococcus epidermidis* (MRSE), *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*
50 *faecalis* (VRE), among the Gram positive bacteria, and *Escherichia coli*, *Klebsiella pneumoniae*,
51 *Pseudomonas aeruginosa*, considering the Gram-negative ones, have appeared to be the main source of
52 community and hospital-associated severe infections.² Among the causes, the abuse of antimicrobials and
53 interrupted therapies in humans or animals seem to play a key role, thus making antimicrobial resistance a
54 global threat worldwide.^{3–5} Natural products typically produced by living organisms (*e.g.*, plants, bacteria,
55 fungi, sponges and animals) against pathogens or stress factors have been successfully used for treating
56 several illnesses and represent a great source of inspiration for the development of novel bioactive
57 molecules.^{6,7} The chemical diversity of natural compounds as well as the wide range of activities
58 (anticancer, antimicrobial, immunomodulatory, antioxidant and anti-inflammatory among others) have
59 attracted the attention of many researchers during the years. In this context, identifying natural
60 antimicrobial agents, understanding their mechanism of action and optimizing their structure-activity
61 relationship is an important task. Many phenolic compounds derived from plants exhibit antimicrobial
62 properties and therefore have the potential to be applied as new varieties of antimicrobial agents.^{8–10} For
63 example benzoic acid derivatives (*i.e.*, gentisic, vanillic and *p*-hydroxybenzoic acid) demonstrated to have
64 antibacterial and antifungal properties.¹¹ Among them, vanillic acid together with vanillin, a widely used
65 dietary flavoring agent, have been employed as natural food preservative due to their antioxidant and
66 antimicrobial characteristics.¹² Aiming at improving the chemo-physical properties and the stability profile
67 of vanillic acid, a small collection of differently *N*-substituted vanillamides was prepared to be tested as
68 antimicrobial agents.^{2,11} In tune with the increasing emphasis placed today on sustainable processing in
69 chemistry, a biocatalytic approach exploiting the efficient and selective acyl transferase from
70 *Mycobacterium smegmatis* (MsAcT)^{13,14} was set up and optimized. While batch strategies^{15–17} have been

employed to generate a sufficient amount of compounds to assess their antibacterial activities, a tailored flow intensified process was developed for the best hits.^{18,19} We recently exploited the immobilized MsAcT (imm-MsAcT) for the flow synthesis of esters and amides both in water and organic solvents, starting from primary alcohols or amines and short-chain esters.^{20–23} The first immobilization attempts involved the use of carbon nanotubes to exploit the MsAcT perhydrolase properties.^{24,25} A better catalyst activity and stability for the condensation reactions (*i.e.*, esterification and amidation reactions) has been observed using hydrophilic supports, among them glyoxyl-agarose showed the best results (*i.e.*, recovered activity: 73%, enzymatic loading: 1 mg_{MsAcT}/g_{matrix}, reusability: 100 cycles).²² In the present work, the catalytic power of MsAcT was demonstrated using vinyl vanillate (*i.e.*, vinyl 4-hydroxy-3-methoxybenzoate **2**, Fig.1) and several primary amines in pure toluene, thus guaranteeing a good solubility of the hydrophobic substrates while achieving highly productive protocols. It is worth noting that conventional chemical methods for the preparation of vanillamides usually require energy-consuming procedures, harsh reaction conditions, use of unstable coupling agents and anhydrous environment, generating a significant amount of waste.²⁶ In this scenario, biocatalytic approaches can be considered an alternative to classical chemistry and their combination with flow facilities represents a powerful tool to enhance productivity while reducing the process related costs.

Materials and Methods

Materials. All reagents and solvents were purchased from commercial suppliers and used without any further purification. Toluene (ACS reagent, 99.5%) from Merck was employed as solvent for flow biotransformations. R2C/R4 flow reactor, commercially available from Vapourtec®, was equipped with Omnifit® glass columns (6.6 mm i.d. 100 mm length). A Zaiput liquid-liquid separator was employed for in-line extraction, while a peristaltic pump has been connected for the in-let of HCl. ¹H NMR and ¹³C NMR spectra were recorded with a Bruker AV600 (¹H, 600 MHz; ¹³C 150 MHz) and a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. TLC analyses were performed using commercial silica gel 60 F254 aluminum sheets. HPLC analyses were performed using

96 a Waters 1525 Binary HPLC Pump, equipped with a Waters 2489 UV–vis detector (Waters, Milford, MA) and
97 an Ascentis C18 column (250 mm x 4.6 mm, 5 μ m particle size).

98 **Synthesis of vinyl vanillate (2).** To a solution of vanillic acid (2.5 g, 14.9 mmol) in dry THF (15 mL), vinyl
99 acetate (22 mL, 237.9 mmol) and palladium(II) acetate (0.334 g, 1.49 mmol) were added under nitrogen
100 atmosphere. After stirring for 30 min at room temperature, 10 % w/w of sulfuric acid in THF (500 μ L) was
101 added and the temperature increased at 60 °C overnight. After cooling to room temperature, the reaction
102 mixture was filtered through celite and the solvent evaporated. The crude mixture was purified by column
103 chromatography (cyclohexane/EtOAc 7:3) giving the desired product as a white solid (70% yield). ^1H NMR
104 (600 MHz, CDCl_3): δ 7.75 (dd, J = 1.8, 8.2 Hz, 1H), 7.59 (d, J = 1.8 Hz, 1H), 7.50 (dd, J = 6.2, 14 Hz, 1H), 6.99 (d,
105 J = 8.2 Hz, 1H), 6.09 (s, 1H), 5.05 (dd, J = 1.6, 14 Hz, 1H), 4.65 (dd, J = 1.6, 6.2 Hz, 1H), 3.95 (s, 3H); ^{13}C NMR
106 (600 MHz, CDCl_3): δ 163.4, 150.7, 146.3, 141.5, 124.9, 120.9, 114.2, 112.0, 97.8, 56.1.

107 **Enzyme preparation and immobilization.** MsAct was expressed and purified as previously reported.¹⁶ The
108 enzyme was immobilized onto glyoxyl-agarose²² and its activity assayed as previously described.^{22,27}

109 **General procedure for biocatalyzed batch synthesis of vanillyl amides (4a-j) using free MsAct.** In 10 mL
110 screw cap tubes a solution 0.25 M (1 mmol, 194.2 mg) of vinyl vanillate (2) in phosphate buffer (0.1 M, pH
111 8.0) and 10% v/v of DMSO was prepared. 0.5 M of the required amine (3a-j) (2 mmol) were then added,
112 together with free MsAct (1 mg/mL). The obtained reaction mixture (final volume: 4 mL) was gently stirred
113 at 28 °C for 24 hours. After this time, dichloromethane (10 mL) and HCl 0.5 M (6 mL) were added. The
114 aqueous phase was extracted three times with dichloromethane. The organic phases were washed with
115 brine, dried over Na_2SO_4 and filtered, while the solvent was evaporated under reduced pressure. The crude
116 product was purified by flash chromatography (cyclohexane/EtOAc 7:3 to 1:1) giving the desired products.

117 **4-Hydroxy-N-isobutyl-3-methoxybenzamide (4a):** colorless sticky solid, 25% yield. ^1H NMR (600 MHz, CDCl_3):
118 δ 7.47 (s, 1H), 7.17 (d, J = 8.1 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.16-6.04 (m, 1H), 5.90 (bs, 1H), 3.9 (s, 3H),
119 3.30-3.25 (m, 2H), 1.93-1.85 (m, J = 6.7 Hz, 1H), 0.98 (d, J = 6.7, 6H); Spectroscopic data matched with those
120 reported in the literature.¹¹

121 *N-Benzyl-4-hydroxy-3-methoxybenzamide (4b)*: yellow oil, 15% yield. ¹H NMR (600 MHz, CDCl₃): δ 7.49 (d, *J*=
122 1.8 Hz, 1H), 7.38-7.34 (m, 3H), 7.32-7.28 (m, 2H), 7.20 (dd, *J*= 1.8, 8.2 Hz, 1H), 6.90 (d, *J*= 8.2 Hz, 1H), 6.41-
123 6.30 (m, 1H), 5.90 (bs, 1H), 4.63 (d, *J*= 5.4 Hz, 2H), 3.9 (s, 3H); Spectroscopic data matched with those
124 reported in the literature.¹¹

125 *N-Cyclohexyl-4-hydroxy-3-methoxybenzamide (4c)*: white solid, 18% yield. ¹H NMR (600 MHz, CDCl₃): δ 7.44
126 (s, 1H), 7.16 (d, *J*= 8.3 Hz, 1H), 6.90 (d, *J*= 8.3 Hz, 1H), 5.98-5.88 (m, 1H), 3.90 (s, 3H), 2.02 (d, *J*= 11.3 Hz, 2H),
127 1.74 (d, *J*= 13.2 Hz, 2H), 1.64 (d, *J*= 13.2, 1H), 1.47-1.37 (m, 2H), 1.28-1.15 (m, 4H); Spectroscopic data
128 matched with those reported in the literature.¹¹

129 *4-Hydroxy-3-methoxy-N-(4-methoxybenzyl)benzamide (4d)*: yellow wax, 27% yield. ¹H NMR (600 MHz,
130 CDCl₃): δ 7.49 (d, *J*= 1.9 Hz, 1H), 7.30-7.27 (m, 1H), 7.19 (dd, *J*= 1.9, 8.2 Hz, 1H), 6.90-6.87 (m, 3H); 6.29-6.22
131 (m, 1H), 5.90 (bs, 1H), 4.56 (d, *J*= 5.4 Hz, 2H), 3.94 (s, 3H), 3.80 (s, 3H); Spectroscopic data matched with
132 those reported in the literature.¹¹

133 *N-(4-Fluorobenzyl)-4-hydroxy-3-methoxybenzamide (4e)*: orange solid, 40% yield. ¹H NMR (600 MHz, CDCl₃):
134 δ 7.49 (d, *J*= 1.8 Hz, 1H), 7.34-7.30 (m, 2H), 7.20 (dd, *J*= 1.8, 8.2 Hz, 1H), 7.06-7.01 (m, 2H), 6.90 (d, *J*= 8.2 Hz,
135 1H), 6.38-6.31 (m, 1H), 5.90 (bs, 1H), 4.60 (d, *J*= 5.7 Hz, 2H), 3.94 (s, 3H); Spectroscopic data matched with
136 those reported in the literature.¹¹

137 *N-(3,4-Dimethoxybenzyl)-4-hydroxy-3-methoxybenzamide (4f)*: orange solid, 30% yield. ¹H NMR (600 MHz,
138 CDCl₃): δ 7.49 (d, *J*= 1.8 Hz, 1H), 7.19 (dd, *J*= 1.8, 8.2 Hz, 1H), 6.92-6.88 (m, 3H), 6.84 (d, *J*= 8.2 Hz, 1H), 6.30-
139 6.25 (m, 1H), 5.90 (bs, 1H), 4.57 (d, *J*= 5.5 Hz, 2H), 3.95 (s, 3H), 3.88 (s, 3H); Spectroscopic data matched
140 with those reported in the literature.¹¹

141 *N-(Benzo[d][1,3]dioxol-5-ylmethyl)-4-hydroxy-3-methoxybenzamide (4g)*: yellow sticky solid, 27% yield. ¹H
142 NMR (600 MHz, CDCl₃): δ 7.48 (d, *J*= 1.7 Hz, 1H), 7.19 (dd, *J*= 1.7, 8.2 Hz, 1H), 6.89 (d, *J*= 8.2 Hz, 1H), 6.84 (s,
143 1H), 6.80 (d, *J*= 7.9 Hz, 1H), 6.76 (d, *J*= 7.9 Hz, 1H), 6.43-6.30 (m, 1H), 5.94 (s, 2H), 4.52 (d, *J*= 5.6 Hz, 2H),
144 3.93 (s, 3H); Spectroscopic data matched with those reported in the literature.¹¹

145 *N*-(2-(1*H*-indol-2-yl)ethyl)-4-hydroxy-3-methoxybenzamide (**4h**): white spongy solid, 40% yield. ¹H NMR (600
146 MHz, CDCl₃): δ 8.09 (s, 1H), 7.65 (d, *J* = 7.8 Hz, 1H), 7.46-7.36 (m, 2H), 7.22 (t, *J* = 7.8 Hz, 1H), 7.13 (t, *J* = 7.8
147 Hz, 1H), 7.07 (s, 1H), 7.04 (dd, *J* = 8.2, 1.8 Hz, 1H), 6.48 (d, *J* = 8.2 Hz, 1H), 6.23-6.10 (m, 1H), 6.10 (bs, 1H),
148 3.89 (s, 3H), 3.78 (q, *J* = 6.1 Hz, 2H), 3.09 (t, *J* = 6.6 Hz, 2H); ¹³C NMR (600 MHz, CDCl₃): δ 167.2, 148.8, 146.8,
149 136.7, 127.6, 127.1, 122.5, 122.3, 119.8, 119.8, 119, 114.0, 113.4, 111.5, 110.5, 56.3, 40.6, 25.5.

150 *N*-Hexyl-4-hydroxy-3-methoxybenzamide (**4i**): white solid, 50% yield. ¹H NMR (600 MHz, CDCl₃): δ 7.46 (d, *J* =
151 1.0 Hz, 1H), 7.17 (dd, *J* = 1.0, 8.3 Hz), 6.90 (d, *J* = 8.3 Hz, 1H), 6.16-6.04 (m, 1H), 5.98 (bs, 1H), 3.93 (s, 1H),
152 3.43 (q, *J* = 6.7 Hz, 2H), 1.60 (q, *J* = 7.2 Hz, 2H), 1.43-1.28 (m, 6H), 0.89 (t, *J* = 6.7 Hz, 3H); ¹³C NMR (600 MHz,
153 CDCl₃): δ 167.3, 148.8, 146.9, 127.3, 119.6, 114.0, 110.7, 56.3, 40.4, 31.7, 29.9, 26.9, 22.8, 14.2.

154 4-Hydroxy-3-methoxy-*N*-(4-methoxyphenyl)benzamide (**4j**): white solid, 5% yield. ¹H NMR (300 MHz, CDCl₃):
155 δ 7.72 (s, 1H), 7.55-7.49 (m, 3H), 7.32 (dt, *J* = 8.2, 1.9 Hz, 1H), 6.99-6.89 (m, 3H), 3.96 (s, 3H), 3.82 (s, 3H).
156 Spectroscopic data matched with those reported in the literature.¹¹

157 **Bacterial Strains and Culture Conditions.** *Escherichia coli* ATCC 25922 (*Ec*), *Salmonella enterica* Enteritidis
158 ISM 8324 (*Se*), *Pseudomonas aeruginosa* IMV 1 (*Pa*) and *Staphylococcus aureus* ATCC 6538 (*Sa*) were plated
159 on Tryptic Soy Agar + 5% sheep blood (Microbiol, Italy) and incubated aerobically at 37 °C for 24 h.

160 **Determination of the Minimum Inhibitory Concentration.** The minimum inhibitory concentration (MIC)
161 was determined using the microdilution assay, according to the Clinical and Laboratory Standards Institute
162 (CLSI) guidelines.²⁸ All the strains were grown on Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) and 3 or 4
163 isolated colonies were suspended in fresh sterile saline solution to reach an initial concentration of 1.5 x
164 10⁸ CFU/mL (equivalent to 0.5 MacFarland standard).

165 One hundred microliters of the 1:100 diluted cell suspensions were dispensed into each well of a 96-well
166 microtiter plate. The strains were exposed to 2-fold dilution series of each vanillamides. After incubation
167 for 24 h at 37 °C, the MICs were determined as the lowest dilution of molecules able to inhibit visible
168 bacterial growth.

169 **Biocatalyzed batch synthesis of vanillyl amides (4b, 4c, 4h) with imm-MsAcT.** In 10 mL screw cap tubes
170 0.25 M of vinyl vanillate (**2**) (48.5 mg, 0.25 mmol) were dissolved in toluene. 0.5 M of amine **3b**, **3c** or **3h**
171 and immobilized MsAcT (50 mg, 1 mg/g_{agarose}) were added, and the reaction mixture was gently stirred
172 overnight at 28 °C. After this time, the reaction output was evaluated by HPLC (see below).

173 **HPLC analysis.** Mobile phase: (A) H₂O+0.05% TFA; (B) ACN+0.05% TFA; gradient conditions: 0–4 min 80% (A)
174 / 20% (B), 4–8 min 65% (A) / 35% (B); 8-12 min 50% (A) / 50% (B); 12-16 min 35% (A) / 65% (B); 16-20 min
175 20% (A) / 80% (B); 20-24 min 20% (A) / 80% (B); flow rate: 1.0 mL/min; λ : 254 nm. Injection volume: 10 μ L.
176 Reaction samples (150 μ L) were diluted with a solution 1:50 H₂O/ACN + 0.05% TFA (1.90 mL). Retention
177 times (t_R): vinyl vanillate (**2**) = 15.8 min; benzylamine (**3b**) = 2.3 min; *N*-benzyl-4-hydroxy-3-
178 methoxybenzamide (**4b**) = 21.2 min; *N*-cyclohexyl-4-hydroxy-3-methoxybenzamide (**4c**) = 20.9 min;
179 tryptamine (**3h**) = 2.2 min; *N*-(2-(1H-indol-2-yl)ethyl)-4-hydroxy-3-methoxybenzamide (**4h**) = 20.5 min.

180 **Intensified flow process.** An Omnifit® glass column (6.6 mm i.d.) was filled with 1.5 g of imm-MsAcT (1
181 mg/g_{agarose}). A 1 M amine solution (**3b**, **3c** or **3h**) and 0.5 M of vinyl vanillate (**2**) both in toluene were
182 prepared. The two solutions were mixed in a T-piece and the resulting flow stream (0.5 M amine and 0.25
183 M acyl donor) directed into the column packed with imm-MsAcT (packed bed reactor volume: 1.2 mL). The
184 flow rate was 0.04 mL/min for each pump. An in-line extraction was performed using a Zaiput liquid/liquid
185 separator and an inlet of HCl 1N (flow rate: 0.19 mL/min) which was mixed to the exiting reaction flow
186 stream using a T-junction. Both the organic and aqueous phase were analyzed by HPLC using the above
187 reported conditions. The desired amides were obtained with the following molar conversions: **4b**: 75%; **4c**:
188 68%; **4h**: 72%. For the gram-scale preparation of amide **4b**, the system was left operating for 10 h; the
189 organic phase was collected and the solvent evaporated under reduced pressure. Purification through
190 column chromatography (cyclohexane/EtOAc 7:3 to 1:1) was performed yielding 2.6 grams of desired
191 product (70% isolated yield).

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194 Results and discussion

195 **Batch biotransformations and vanillamides antimicrobial evaluation.** In our previous works, we already
196 demonstrated that vinyl esters are more efficient acyl donors than the ethyl ones for MsAct-mediated
197 transesterifications, so activated vinyl vanillate was prepared through a Pd(II)-catalyzed transvinylation
198 reaction starting from vanillic acid. (Fig. 1).^{16,22,23}

199 Using the newly formed ester and several primary amines, batch reactions were subsequently carried out
200 for the preparation of a variety of different vanillyl amides (Fig. 2). Following our previously optimized
201 reaction conditions,¹⁵⁻¹⁷ high substrate loading (0.25 M for the acyl donor and 0.5 M for the amines), free
202 MsAct (1 mg/mL) and phosphate buffer (0.1 M, pH 8.0) were employed. Unlike the biotransformations
203 described so far, where water-immiscible liquid acyl donors were employed creating a more favorable-to-
204 solubilization biphasic environment, vinyl vanillate is a solid compound slightly soluble in buffer media.
205 Adding co-solvents (*e.g.*, DMSO 10-20% v/v) or decreasing the substrate concentration (0.05 M and 0.1 M),
206 did not show any further improvement in the solubility.

207 Although the reactions were characterized by modest-to-good yields (15-40%) and prolonged reaction
208 times (24 h), mainly due to the poor solubility of **2**, the desired products **4a-j** were obtained in a sufficient
209 amount to assay their potential antimicrobial activity (Table 1).

210 According to the literature, vanillic acid has been reported to have antimicrobial activity against *Bacillus*
211 *cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi* with MICs in
212 the range of 2000-583 µg/mL.^{29,30} Therefore, compounds **4a-j** have been submitted to antibacterial
213 screening against a panel of Gram-positive and Gram-negative bacteria. Table 1 shows the results of MICs
214 against target microorganisms. Interestingly, all the amide derivatives exhibited better activity than the
215 vanillic acid precursor. It was reported that compounds such as geraniol, octanol, citronellol as well as
216 polyphenols showed antimicrobial activity through microbial membrane disruption.^{11,31-33} This suggests
217 that the increased lipophilicity of the generated molecules in combination with the polyphenolic moiety
218 may have strengthened their antimicrobial activity through their membrane-disrupting properties.

219 Compounds **4b**, **4c** and **4h** were the most potent being 9-fold more effective against *P. aeruginosa* than
220 vanillic acid precursor (64 µg/mL and 583 µg/mL, respectively). Taking into account the stronger cell
221 envelope of Gram-negative strains and their lower sensitivity to polyphenolic compounds when compared
222 to Gram-positive bacteria or yeasts,³³ these molecules can be considered promising prototypes for the
223 development of antimicrobial agents active against Gram-negative bacteria. In order to maximize the
224 productivity of the best hits, a tailored flow process was developed.

225 **Flow intensified process for the synthesis of 4b, 4c and 4h.** To enhance the MsAcT stability, the enzyme
226 was immobilized onto glyoxyl-agarose, selected as best carrier after an in-depth study previously
227 performed by our group.²² Among the different hydrophilic supports (*i.e.*, agarose, cellulose, silica and
228 epoxy resins), aldehyde-agarose immobilization allowed not only for the highest recovered activity (*i.e.*,
229 73%) with low enzymatic loading (1 mg/g_{matrix}), but also the easy catalyst integration in flow chemistry
230 reactors (packed bed reactor, PBR), ensuring controlled fluid dynamics, acceptable residence times and
231 process efficiency.²⁷ In order to overcome the solubility limitations observed with water-media
232 biotransformations, pure toluene, a well-tolerated organic solvent from MsAcT,²³ was employed as reaction
233 medium. Batch reactions were firstly set up to check the stability of imm-MsAcT while monitoring the
234 reaction outcome by HPLC. Although improvements in the conversion were observed (**4b**: 30%, **4c**: 40%,
235 **4h**: 50%), prolonged reactions times (16 hours) were still necessary. A continuous process was then set up
236 (Fig. 3). Using a 1.2 mL bioreactor, different residence times were evaluated (*i.e.*, 15 min, 30 min and 60
237 min). As previously demonstrated for different MsAcT-mediated condensation reactions in flow
238 mode^{20,21,34,35} due to the high local concentration of the biocatalyst, the efficient mixing as well as the
239 increased catalyst stability improved yields and shorter reaction times were observed when compared with
240 batch transformations. In particular, using 0.25 M of **2** and 2 equivalents of the amines (**3b**, **3c** and **3h**) the
241 desired amides **4b**, **4c**, and **4h** were obtained with very good conversions (68-75%) in only 15 min of
242 residence time. In order to enhance the system automation and the overall sustainability, work-up
243 procedures were added downstream the process. An in-line extraction was performed by adding an inlet of

244 HCl and using a liquid-liquid separator. In this way, also the unreacted amines have been collected as salt
245 form. After basification and extraction in toluene they could be reused as fresh substrate into the system.

246 To evaluate the performance of our bioreactor over the time, the preparation of amide **4b** was selected
247 and 50 mL of corresponding solution were collected (10 h of continuous operations). 2.6 g of pure **4b** were
248 obtained after column chromatography (Table 2).

249 In summary, a series of vanillamides have been synthesized using a facile enzymatic strategy and tested for
250 their antimicrobial activity against a panel of Gram-positive and negative microorganisms. All the generated
251 molecules exhibited better antibacterial action than the vanillic acid precursor, attributable to the
252 increased lipophilicity as well as the enhanced membrane disrupting properties. The three best hits (*i.e.*,
253 **4b**, **4c** and **4h**) were efficiently synthesized through a flow biocatalytic protocol, intensifying their
254 preparation (gram scale), increasing the system automation (in-line work-up procedures), while reducing
255 the reaction times (15 min) and the process related costs. The recovery of the unreacted starting material
256 contributed to the generation of low environmental impact reactions. The use of MsAcT in pure toluene let
257 to overcome any solubilization problem of the acyl donor (**2**), giving rise to a more productive protocol (2.6
258 g of pure **4b** in 10 h) and demonstrating high stability and reusability of the enzyme. Due to the applicability
259 of the described system to larger bioreactors and the robustness of the imm-MsAcT, larger scale
260 continuous biotransformations seem to be feasible without any further optimization. In addition, due to
261 the difficult permeability of the barrier protecting Gram-negative bacteria compounds **4b**, **4c** and **4h**, (9-
262 fold more effective than the precursor against *P. aeruginosa*) represent promising prototypes, which could
263 serve as basis for further research in the field of antimicrobials.

264 **Supporting information statement**

265 SDS-page of the pure MsAcT (Figure S1); HPLC chromatography (Figure S2); ¹H NMR spectra of the obtained
266 molecules (**4a-g** and **4j**) (Figure S3-S10 and S13); ¹H NMR and ¹³C NMR of the newly generated compounds
267 (**4h** and **4i**) (Figure S11 and S12).

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372 Figure captions

373 **Figure 1.** Preparation of vinyl vanillate

374 **Figure 2.** Batch preparation of vanillamides in water medium.

375 **Figure 3.** Flow-based preparation of **4b**, **4c** and **4h**. **A.** Solution of vinyl vanillate **2** (0.5 M) in toluene. **B.** Solution
376 amines **3b**, **3c** or **3h** (1 M) in toluene.

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378

Tables

Table 1 Microbial evaluation of compounds **4a-j**

Compound	<i>Ec</i> ^a MIC (µg/mL)	<i>Se</i> ^b MIC (µg/mL)	<i>Pa</i> ^c MIC (µg/mL)	<i>Sa</i> ^d MIC (µg/mL)
4a	256	128	128	256
4b	256	128	64	256

4c	128	128	64	256
4d	128	128	128	256
4e	128	128	128	256
4f	128	128	128	256
4g	128	128	128	256
4h	256	128	64	128
4i	128	128	128	256
4j	128	128	128	256

^a *Ec* = *Escherichia coli*; ^b *Se* = *Salmonella enterica* Enteritidis; ^c *Pa* = *Pseudomonas aeruginosa*; ^d *Sa* =

Staphylococcus aureus

Table 2 Continuous preparation of **4b** in flow reactor. Reactor volume 1.2 mL, residence time 15 min

m. c. ^a (%)	Isolated yield (%) ^b	Productivity (g/h)	Catalyst Productivity (mmol/mg _{enzyme} h)
75	70	0.26 g	0.45

^a Determined by HPLC

^b After column chromatography

Figures

Figure 1

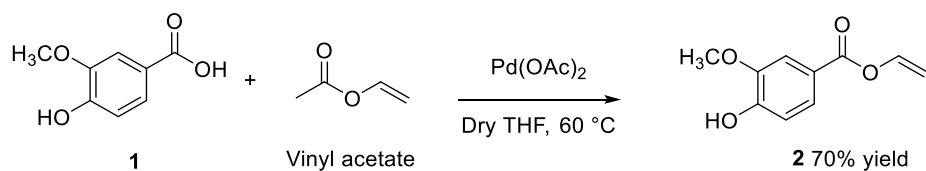


Figure 2

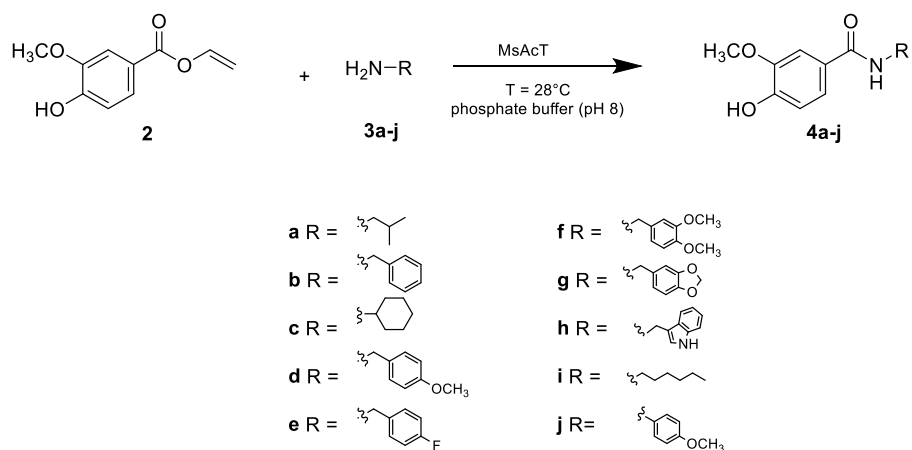
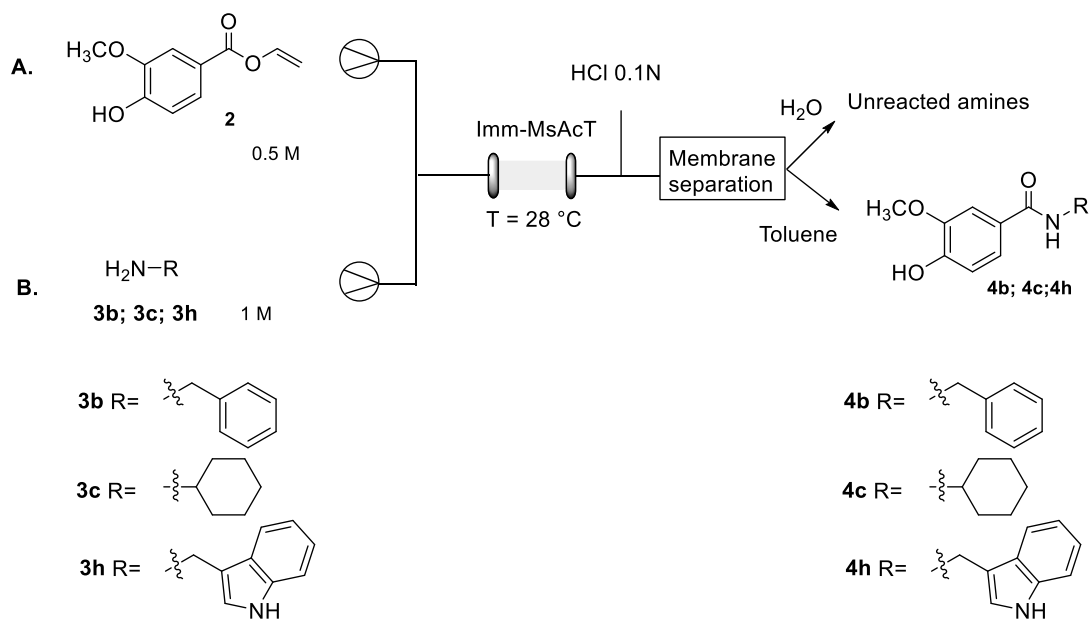


Figure 3



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