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1 **Antimicrobial and antioxidant activities of amines derived from vanillin as potential**
2 **preservatives: impact of length chain and polarity of substituent**

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14 Keywords: vanillin derivatives, antimicrobial, antioxidant, preservative agent, cosmetics,
15 structure-function

16 **Abstract**

17 The stability of cosmetic products in terms of microbial safety or oxidation is a major
18 challenge when faced with consumer's expectations for less controversial compounds used
19 in their formulation. In this context, we present new vanillin derivatives synthesized in a two
20 steps one-pot process. The synthesis was based on the reductive amination reaction
21 between vanillin and a selected panel of amines with different structural units such as alkyl
22 chains, halogens and aromatics. The different molecules were designed to bring a better
23 understanding of the structure-preservative activity relationships in vanillin-based
24 molecules. The antibacterial and antifungal properties were evaluated on several strains
25 known to be responsible for the contamination of cosmetic products. Most compounds
26 exhibited strong antioxidant activities in DPPH, ABTS and FRAP assays. Some of these vanillin
27 compounds derived with a *p*-ethylaniline (**2c**), with a *p*-butylaniline (**2d**), with a *p*-
28 hexylaniline (**2e**) and a *p*-iodoaniline (**3b**) showed a promising level of antibacterial activity
29 against *Bacillus subtilis* and *Micrococcus luteus*. In particular, **2d** was very active against most
30 of the tested bacterial and fungal strains and showed very promising antibacterial and

31 antifungal capabilities while displaying among the highest antioxidant capacity and low
32 cytotoxic effects on human fibroblasts. We investigated if a correlation between the
33 hydrophobicity and biological activities of the compounds could be established and we
34 highlighted that the presence of aliphatic chains grafted on the aromatic amine ring is
35 associated to the highest antimicrobial activities. The structure-activity relationships
36 highlighted in this study allow paving the way for future applications of these compounds in
37 cosmetics as alternative to controversial molecules.

38

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41 **1. Introduction**

42 The cosmetic industry is constantly adapting to meet the regulation requirements and to the
43 consumer demands. It must ensure, on one hand, a good preservation of the products, as
44 well as guaranteeing the protection of the consumer's health. A wide variety of synthetic
45 antimicrobial preservatives is authorized in Europe (Alvarez-Rivera et al., 2018). Among
46 them, esters of 4-hydroxybenzoic acid, also known as parabens, represent 80% of
47 preservatives used in cosmetics (Nowak et al., 2020). However, parabens are decried due to
48 potential health risks (Golden et al., 2005); and some of them, such as isopropylparaben,
49 isobutylparaben, phenylparaben, benzylparaben and pentylparaben, have been already
50 banned by the European Commission (Alvarez-Rivera et al., 2018). Butylated hydroxyanisole
51 (BHA) and butylated hydroxytoluene (BHT) are strong antioxidants widely used in cosmetic
52 creams and lipsticks (Alvarez-Rivera et al., 2018). However, they are suspected to be
53 carcinogenic by the International Agency for Research against Cancer and to be endocrine
54 disruptors by the European Commission (2019) (Jeong et al., 2005). There is a pressing issue
55 in addressing the need of products considered to be less harmful to the consumers by
56 looking for bio-based alternatives to these different preservatives (Alvarez-Rivera et al.,
57 2018). Plant extracts have long been associated with beneficial properties such as
58 antioxidant, antibacterial or antifungal activities (Al-Rifai et al., 2017; Kusuma et al., 2014).
59 Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the world's principal flavoring
60 compounds and has been shown to possess a wide panel of biological activities (Fitzgerald et
61 al., 2004; Kumar et al., 2004). Originally isolated from vanilla seedpods, this compound is
62 also now widely produced through biotechnological and chemical processes (Gallage and
63 Møller, 2015) to cope with the high demand for foods, beverages, cosmetics and drugs
64 industries. Optimization of vanillin bioproduction from lignin is currently under investigation
65 (Leriche-Grandchamp et al., 2020). Vanillin is generally recognized as safe (GRAS) and known
66 to have multifunctional effects such as antimutagenic (Keshava et al., 1997), antiangiogenic
67 (Lirdprapamongkol et al., 2009), anti-colitis (Wu et al., 2009), antioxidant (Burri et al., 1989),
68 antifungal effects, among the others (Fitzgerald et al., 2004, 2005). Regarding the
69 antimicrobial activities, vanillin showed a remarkable protective effect against both bacteria
70 and fungi in food products (Cerrutti and Alzamora, 1996; Fitzgerald et al., 2004; López-Malo
71 et al., 1995) although some studies have been stressing out its low antioxidant activity
72 (Santosh Kumar et al., 2002; Tai et al., 2011; Zhao et al., 2017). In order to improve its

73 efficiency, industries and researchers are now looking for developing synthetic derivatives of
74 vanillin that could cumulate antimicrobial and antioxidant activities.

75 Dalko described in 2012 new preservatives derived from ethylvanillin and alkyl ketones for
76 cosmetic applications (Dalko, 2012). These molecules demonstrated strong inhibitory effects
77 on the growth of different bacterial and fungal strains (*Escherichia coli*, *Pseudomonas*
78 *aeruginosa*, *Candida albicans* and *Aspergillus niger*). The effect on antimicrobial activity of
79 the phenyl ester substituent present in novel vanillin derived oxime esters was studied
80 (Harini et al., 2012). More recently, Scipioni et al. (2018) reported the strong antioxidant
81 properties of some new vanillin derivatives containing amine substituents. Aliphatic amines
82 derived from vanillin have been also synthesized and a relationship between their chemical
83 structure and their antimicrobial activities has been highlighted (Patterson et al., 2015).
84 Notably, it has been shown that the length of the aliphatic group could enhance the
85 antimicrobial activities. However, little is known about the parameters that govern the
86 structure-biological activity relationships of vanillin derivatives. A better understanding of
87 how enhancing the antioxidant and antimicrobial properties at the same time by modify the
88 chemical structure of vanillin is required for an efficient application of such derivatives in
89 cosmetics industries.

90 Herein with the purpose of combining both antimicrobial and antioxidant activities, we
91 studied the influence of structural units such as alkyl chains, halogens and aromatics found
92 in some antimicrobial compounds (Dalko, 2012; Odlaug, 1981; Zemek et al., 1987) on the
93 properties of new molecules possessing a vanillyl group. We investigated if a correlation
94 between the hydrophobicity and biological activities of the compounds could be established.

95

96 **2. Materials and methods**

97

98 2.1 Materials and instrumentation

99 Substrates were purchased from Sigma Aldrich and Acros. Solvents were purchased from
100 Acros and Fisher Scientific. All materials were used without further purification. Thin-Layer
101 Chromatography (TLC) was performed on silica gel 60 F254 plates (Merck). Compounds were
102 eluted with a mixture of ethyl acetate and cyclohexane with variable proportions and
103 revealed under UV light ($\lambda = 254$ nm) and with an ELSD (Evaporative Light Scattering
104 Detector). Purifications were performed using a flash chromatography system (GRACE,

105 Reverleris X2) and pre-packed columns (GRACE, Reverleris, Silica 40 μm , 12 g). ^1H and ^{13}C
106 NMR spectra were recorded on a 400 MHz Bruker UltraShield 400 MHz/54 mm Ultra-long
107 hold. Chemical shifts (δ) are quoted in ppm and are referenced to deuterated chloroform
108 CDCl_3 (Carlo-Erba) as an internal standard (7.26 ppm). Coupling constants (J) are quoted in
109 Hz. The following abbreviations were used to describe the spectra obtained, s: singlet, brs:
110 broad singlet, d: doublet, t: triplet, q: quartet, quin: quintet, sex: sextet, m: multiplet, dd:
111 doublet of doublet. The electrospray ionisation mass spectrum was recorded on an Agilent
112 QTOF 6538 (Wilmington, DE) coupled to an Agilent 1200 series fast resolution LC system. The
113 mobile phase consisted of 5 % Water – 95 % HPLC grade ACN. The samples were prepared in
114 the proper solvent and injected without HPLC column into the DUAL ESI at a flow rate of 400
115 $\mu\text{L}/\text{min}$. The mass spectrometer was operated in positive and negative ESI modes, in full scan
116 mode and with continuous infusion of a calibration solution. The capillary voltage was +3800
117 V and -3800 V. The ion source gas temperature was 300 $^\circ\text{C}$ with flow of 12 L/min of nitrogen.
118 Fragmentator was set at 120 V. The mass range for ESI experiments was from $m/z = 100$ to
119 $m/z = 1000$.

120

121 2.2 General procedure for synthesis of vanillin derivatives

122 All vanillin derivatives were obtained through the reductive amination in a one-pot reaction
123 following an adapted protocol previously reported (Patterson et al., 2015). A mixture of
124 vanillin and the corresponding amine were dissolved in ethanol followed by the addition of
125 NaBH_4 . The reaction mixture was stirred overnight under reflux.

126 An EtOH (10 mL) solution of the selected amine (1.13 mmol) was added dropwise to an EtOH
127 (10 mL) solution of vanillin (172 mg, 1.13 mmol) under stirring. The reaction mixture was
128 heated at reflux overnight. Upon cooling at 0 $^\circ\text{C}$, NaBH_4 (1.70 mmol) was added to the
129 solution and the mixture was allowed to proceed for 12h at room temperature. Once the
130 reaction was completed as shown by a TLC monitoring, the mixture was evaporated to
131 dryness using a rotary evaporator. The residue was dissolved in ethyl acetate then washed
132 by extraction with aqueous solution of saturated NaHCO_3 . The organic fraction was dried
133 using anhydrous sodium sulfate and filtered. The solvent was removed under vacuum to
134 yield the final products. The crude products were directly subjected to column
135 chromatography (AcOEt/Cyclo) to give pure products **1**, **2a-2e**, **3a-3b** and **4**. All compounds
136 synthesized were fully characterized by ^1H NMR, ^{13}C NMR, ESI-MS (electrospray ionization-

137 mass spectrometry) and the melting points of the solid compounds were experimentally
138 measured (see Supporting information).

139

140 2.3 Log $P_{o/w}$ determination

141 The octanol/water partition coefficient ($\log P_{o/w}$) of the tested compounds was provided by
142 calculation using Chemdraw Ultra 8.0 software.

143

144 2.4 Antioxidants studies

145 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-
146 picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
147 (ABTS), potassium persulfate (di-potassium peroxydisulfate), butylated hydroxytoluene (BHT),
148 butylated hydroxyl-anisole (BHA), vanillin were obtained from Merck. All other chemicals
149 were analytical grade and were obtained either from Merck.

150 A stock solution of each compound (**1** to **4** compounds as listed in Table 1, Trolox, BHA and
151 BHT) was prepared in absolute methanol by dissolving in order to get a concentration of 1
152 mM. From this stock solution, different concentrations of sample solution from 0 to 200 μ M
153 were prepared in methanol for the assays. Vanillin stock solution was prepared at 10 mM
154 and diluted in methanol from 0 to 2 mM. Trolox, BHA and BHT were used as the standard
155 antioxidants.

156

157 2.4.1 DPPH assay

158 Equivolume of DPPH methanolic solution (200 μ M) and sample solution in methanol or
159 methanol as the control (A_0) were mixed and well vortexed. The absorbance of the solutions
160 was measured at 515 nm with a spectrophotometer after 30 min at room temperature. The
161 blank solution was pure methanol. The absorbance of A_0 is adjusted to be about 1.

162

163 2.4.2 ABTS assay

164 Scavenging activities on ABTS radical of the vanillin derivatives were assessed as previously
165 described (Ksouda et al., 2018). $ABTS^{\bullet+}$ radical was prepared from a mixture of 7 mM of
166 ABTS and 0.75 mM potassium persulfate in distilled water. The reaction was performed in
167 the dark for 1 h at 40 °C. Prior use, the $ABTS^{\bullet+}$ solution was diluted in water. Equivolumes of

168 diluted radical and sample solution (A_s) or methanol for the control (A_0) were mixed and
169 were let to react during 30 minutes in protecting from light and room temperature. The
170 absorbance was measured at 734 nm with a spectrophotometer; the blank was made using
171 pure methanol. The absorbance of A_0 is adjusted to be about 1.

172

173 2.4.3 IC₅₀ calculation for DPPH and ABTS assay

174 For DPPH and ABTS assay, the radical scavenging capacity was calculated using the following
175 formula:

$$176 \text{ Radical scavenging activity (\%)} = [(A_{S0} - A_s) / A_{S0}] \times 100$$

177 where A_{S0} was the absorbance of the control and A_s was the absorbance of the samples or
178 the standard compounds. The antioxidant activity was expressed as an IC₅₀ value that is the
179 concentration in μM that scavenged the DPPH or ABTS radicals by 50 %, and was calculated
180 from the concentration-effect linear regression curve. The standard curves for DPPH and
181 ABTS assays were linear between 10 and 50 μM Trolox.

182

183 2.4.4 FRAP reducing activity assay

184 FRAP assay is based on the rapid reduction in ferric-tripyridyltriazine (Fe^{3+} -TPTZ) by
185 antioxidants present in the samples forming ferrous-tripyridyltriazine (Fe^{2+} -TPTZ), a blue-
186 coloured product (Benzie and Strain, 1996). The working FRAP reaction solution was
187 prepared by mixing 10 volumes of 300 mM acetate buffer at pH 3.6 with 1 volume of 20 mM
188 FeCl_3 solution and 1 volume of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl. 1 mL of
189 this reagent was mixed with 150 μL of sample solution. The mixtures were incubated 30
190 minutes in the dark at 37 °C in a water bath. The absorbance was measured at 595 nm, the
191 blank was made using S_0 where 150 μL of methanol was used instead of the sample solution.
192 All curves at absorbance vs concentration were drawn and fitted in order to get the linear
193 regression: $Y = a * X$. The FRAP values represents the ratio between the slope of the linear
194 plot for reducing Fe^{3+} -TPTZ reagent by the compounds compared to the slope of the this plot
195 for Trolox. The FRAP results were expressed in term of Trolox equivalent TE (μM Trolox/ μM
196 compound).

197

198 2.5 Antimicrobial activities measurements

199 2.5.1 Microorganisms

200 The tested strains were selected according to their documented occurrence in cosmetics
201 contamination or their recommendation by the Personal Care Products Council for validation
202 of preservative efficiency in cosmetics. Bacterial strains are *Escherichia coli* ATCC 25922™
203 (American Type Culture Collection, USA), *Micrococcus luteus* CRBIP 107660 (Institut Pasteur,
204 France) and *Bacillus subtilis* ATCC 6051 (American Type Culture Collection, USA). They were
205 maintained on Tryptic Soy Agar (TSA, CONDA, Madrid) at 30 °C under obscurity.

206 Concerning fungal strains, *Penicillium funiculosum* (MUCL 038968), *Aspergillus brasiliensis*
207 (MUCL 029039), and *Paecilomyces variotii* (MUCL 039890) were provided by the Agro-food
208 and Environmental Fungal Collection (BCCM/MUCL) of the Catholic University of Louvain.
209 *Cladosporium cladosporioides* and *Candida krusei* were previously isolated by the laboratory.
210 These fungi were grown on solid potato dextrose agar medium (Conda Laboratories, Madrid,
211 Spain) under 12/12 h photoperiod and replicated every week.

212

213

214 2.5.2 Determination of inhibition rates

215 Assays were carried out in 96-well clear flat-bottom plates (96 well Nunc™ edge 2.0 micro-
216 plates, ThermoFisher Scientific) in triplicates according to a method derived from CLSI broth
217 microdilution method (Clinical and Laboratory Standards Institute and Weinstein, 2018).
218 Concerning bacteria, the inoculum consisted in a 0.2 optical density (OD) calibrated
219 suspension in mineral medium (KCl 0.250 g/L, NaH₂PO₄ 6.464 g/L, Na₂HPO₄·2H₂O 10.408 g/L,
220 MgSO₄ 0.244 g/L, NO₃NH₄ 1 g/L, MgCl₂ 0.05 g/L; pH = 7, glucose 10 g/L). Concerning fungi,
221 inocula consisted in spores suspensions in mineral medium (KCl 0.250 g/L, NaH₂PO₄ 1.544
222 g/L, Na₂HPO₄·2H₂O 0.008 g/L, MgSO₄ 0.244 g/L, NO₃NH₄ 1 g/L, MgCl₂ 0.05 g/L; pH = 5.5, 5 g/L
223 of glucose) calibrated to 2x10⁴ spores/mL using a Malassez counting cell. In wells dedicated
224 to test cultures, 100 µL of the inoculum, 98 µL of mineral medium with glucose and 2 µL of
225 the 100 times concentrated mother solution of the tested compound in acetonitrile are
226 mixed. In controls to check the toxicity of the solvent, solutions of compounds were replaced
227 by acetonitrile without compound. In the so-called positive controls, 100 µL of inoculum and
228 100 µL of mineral medium with glucose were mixed to measure the normal growth of the
229 microorganism. The negative control was made of mineral medium with glucose. The
230 eventual evolution of absorbance of the tested compounds was also checked with wells
231 containing the compound with mineral medium without any microorganism. Fungal growth

232 was monitored by measuring absorbance at 600 nm every day over one week and bacterial
233 growth through kinetics of measurements every 15 min over 24 h. The growth inhibition of
234 the microorganisms in test wells was calculated as follow:

$$235 \quad \%_{Inhibition} = \left(1 - \frac{\Delta OD_{test}}{\Delta OD_{positive\ control}} \right) \times 100$$

236 To screen for the most active compounds, only a final concentration in the well of 50 mg/L
237 (mother solution in acetonitrile: 5 g/L) was tested. For minimum inhibitory concentration
238 (MIC) determination of the compound **2d**, final concentrations of 1.25, 5, 12.5, 25, 50, 75
239 and 100 mg/L were tested through variation of the mother solution concentration. MIC₅₀
240 corresponds to the lowest tested concentration of the compound enabling to reach 50 % of
241 inhibition in average of the considered microorganism growth, MIC₈₀ is the same for 80 % of
242 inhibition and MIC₁₀₀ for 100 %.

243

244 2.6 Cytotoxicity assay on human cells

245 Compound **2d** cytotoxicity was analysed by measuring the viability of skin fibroblasts
246 cultured with the compound. Human neonatal dermal fibroblasts (HDFn cat C-004-5C) were
247 purchased from Cascade Biologics and were grown on DMEM (Gibco) medium
248 supplemented with 10 % of fetal bovine serum (Hyclone), 100 U/mL pencillin and 100 µg/mL
249 streptomycin (both from Gibco) at 37 °C under humidified atmosphere (5 % CO₂, 95 % air).
250 HDFn cells were seeded on Falcon polystyrene-96 well plates (10⁴ cell/well) in 100 µL of
251 supplemented culture medium. 24 h post seeding, culture medium was replaced with 100 µL
252 of 1.25, 5, 12.5, 25, 50, 75 or 100 mg/L of compound 2d or methylparaben solutions
253 prepared in supplemented culture medium. Positive cytotoxic control (100 µL of extract
254 obtained by incubating for 24h pieces of sterile latex gloves under gentle agitation in
255 supplemented culture medium, according to the International Standard ISO 10993 (6
256 cm²/mL)) and negative control (100 µl of fresh supplemented culture medium) were
257 included during the assay. After additional 24 h of incubation of cells with the solutions to
258 be tested, 20 µL of reagent CellTiter 96® AQueous One Solution Cell Proliferation Assay from
259 Promega were added to each wells and incubated for 2 h at 37 °C. The absorbances were
260 read by a microplate reader at 490 nm and the cell viability compared to the negative
261 control was calculated as follow: (A_{solution}/A_{negative control}) x 100. The solutions are considered

262 toxic when the cell viability is less than 70 % of the negative control. The experiment was
263 performed in triplicate and the results are expressed as the mean.

264

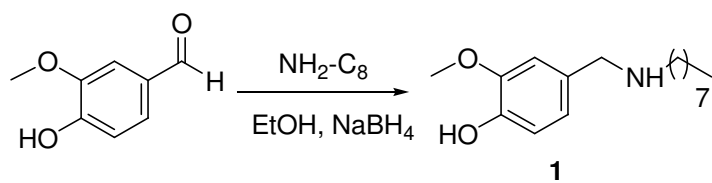
265 3. Results and discussion

266 3.1 Chemical synthesis

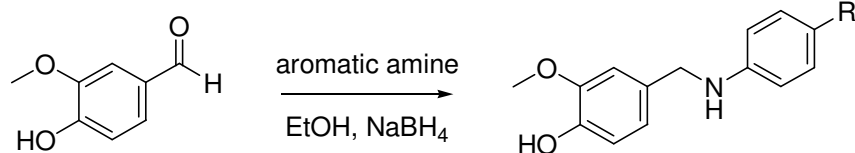
267 The synthesis of a series of vanillin derivatives (**1**, **2a-2e**, **3a-3b**, **4**) was achieved through
268 reductive amination (Figure 1) according to a procedure adapted from Patterson et al. (2015)
269 (see SI). Resulting amines were isolated with yields ranging from 15 to 84 %. The lowest
270 yields could be explained by the weaker nucleophilicity of some amines in particular for
271 iodoaniline and toluidine. All compounds were fully characterized by ¹H and ¹³C NMR (see
272 SI).

273 All the derivatives contain phenolic structures (Table 1) that are likely to trigger inhibition
274 against microorganisms by reacting with enzymes active sites through hydrogen bonds
275 (Papuc et al., 2017) and can quench reactive oxygen species by donating an electron (Lü et
276 al., 2010). The synthesized molecules contain different aliphatic chains or polar substituents
277 in the *para* position. Patterson found that lipophilic character of amine derivatives increased
278 antibacterial properties (Patterson et al., 2015). The compounds with aliphatic chains C₁, C₂,
279 C₄, C₆ (**2b**, **2c**, **2d**, **2e** respectively) and with no added chain (**2a**) will allow to compare the
280 influence of the substituted carbon chain length. Halogenated compounds such as chlorine
281 and bromine are known to have antibacterial activities (Odlaug, 1981). The influence of a
282 halogen (Br, I) substituent (compounds **3a** and **3b** respectively) will also be evaluated. The
283 compounds **2a** and **4** will allow to investigate the influence of aromatic methylene group
284 between the amine and the aromatic group and the compounds **1** and **2d** the impact of
285 supplementary aromatic ring.

A

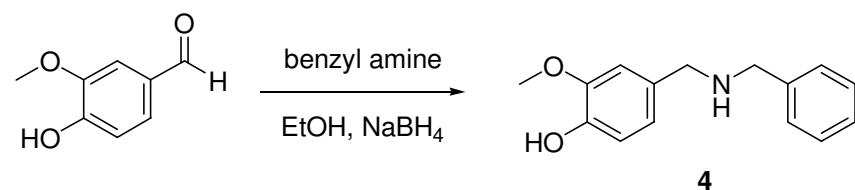


B



2a R= H
2b R= CH₃
2c R= C₂
2d R= C₄
2e R= C₆
3a R= Br
3b R=I

C



286

287 **Figure 1: Synthesis of vanillin derivatives 1, 2a-2e, 3a-3b, 4. Synthesis of aliphatic amine (A), synthesis of aromatic amines**

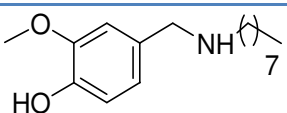
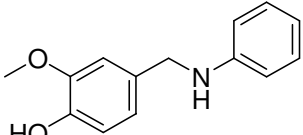
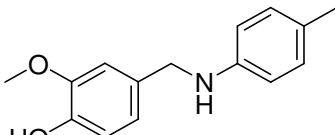
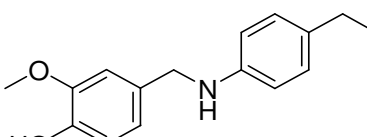
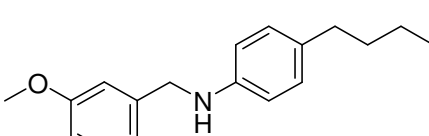
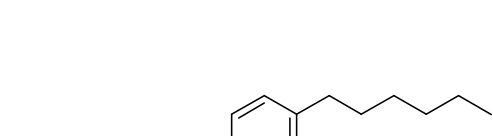
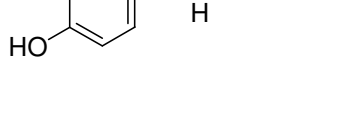
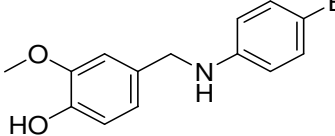
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(B), synthesis of benzyl amines (C).

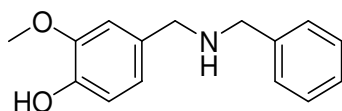
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290

Table 1: Chemical structures, yields and log $P_{o/w}$ of vanillin derivatives

Compounds	Structure	Yields	log $P_{o/w}$
1		81 %	3.99
2a		39 %	2.75
2b		25 %	3.23
2c		83 %	3.65
2d		76 %	4.49
2e		53 %	5.32
3a		84 %	3.58
3b		15 %	4.10

4



81 %

2.96

292

293 3.2 Antioxidant activities

294 Antioxidant activities of all synthesized compounds were assayed by the three methods
295 based on two different electron transfer principles: the methods based on free radical
296 scavenging (1,1-diphenyl-2-picrylhydrazyl) (DPPH) and 2,2'-azino-bis (3-ethylbenzo-
297 thiazoline-6-sulfonicacid) (ABTS)) and the ferric reducing antioxidant power (FRAP) assay
298 based on reduction of ferric ion (Huang et al., 2005). As the activities of some antioxidants
299 may vary depending of the radical sources, the reaction conditions and the mechanisms
300 involved, the use of multiple methods is recommended to get full information about the
301 antioxidative capacity of vanillin derivatives.

302 The antioxidant activities of the vanillin derivatives and reference compounds with half-
303 inhibitory scavenging concentration (IC_{50}) and Trolox equivalent (TE ($\mu M/\mu M$)) were shown
304 in Table 2. Trolox, BHA and BHT were used as the standard antioxidants. When measured
305 using DPPH assay, BHT showed about 3 times lower antioxidant capacity than Trolox while
306 they exhibited similar activities when evaluated by ABTS assay. The inversion in the
307 antioxidant capacity ranking between Trolox and BHT depending on the assay performed
308 was already reported (Singh et al., 2018; Topal, 2019).

309 Antioxidant activity depends on the hydroxyl group of the aromatic ring and the type of
310 substituent. High correlation was observed between the IC_{50} values of the tested vanillin
311 derivatives for DPPH and ABTS assays ($R^2 = 0.9618$). On the other hand, the correlation
312 coefficient R^2 between DPPH and FRAP methods is 0.6317 and for ABTS and FRAP methods
313 0.5217, which means a low correlation. Here, lower TE values obtained for vanillin and the
314 vanillin derivatives with FRAP assay can be explained by the shift of the redox potential of
315 phenolic derivatives under the acidic solvent conditions used in this assay as it was shown
316 that the high proton concentration inhibits the dissociation of phenolic hydroxyl (Singh et al.,
317 2018; Zhang et al., 2011).

318 It has been well reported that the antioxidant ability of vanillin is very low (Santosh Kumar et
319 al., 2002; Tai et al., 2011; Zhao et al., 2017). In this work, we obtained DPPH and FRAP

320 antioxidant values for vanillin in the same order of magnitude than the ones given in Scipioni
321 et al. (2018) for identical reaction times (30 min).

322 In DPPH and ABTS assays, the vanillin derivatives showed similar or stronger radical
323 scavenging activity than BHT, BHA and Trolox except for the compounds **1** and **4**. They all
324 exhibited a strong increase in activity when compared with vanillin between about 100 (for
325 compound **4**) to 300 times (for compound **2b**). In our conditions, the dominant mechanism
326 explaining the antioxidant activity of phenolic OH of the vanillin moiety is the hydrogen
327 transfer (HT) (Anouar et al., 2009; Galano et al., 2012; Mahal et al., 2001). The nature of the
328 group substitution in *para* position on phenol is on the first importance for the radical
329 scavenging activity of the phenolic OH (Galano et al., 2012). In vanillin, the electro-
330 withdrawing carbonyl group in *para* position causes a destabilization of the radical produced
331 by the HT reaction. This explains the very low antioxidant activity. Conversely, in all other
332 compounds (**1** to **4**), the presence of the electro-donor $-\text{CH}_2-\text{NH}-$ group leads to the
333 opposite effect, enhancing the scavenging capacity.

334 Among the vanillin derivatives, **1** and **4** have the lowest antioxidant capacity. The FRAP value
335 that we determined for the compound **4** are in accordance with the one reported in Scipioni
336 et al. (Scipioni et al., 2018). The evaluation in the antioxidant capacities of the vanillin
337 derivatives seems to be correlated to the basicity of the nitrogen, as the compounds **1** and **4**
338 have the more basic nitrogen. This observation is similar to the observations made by
339 Scipioni et al. (Scipioni et al., 2018), about importance of electron pair of nitrogen for
340 antioxidant activity but they have been compared with secondary imine, tertiary amine and
341 quaternary salts. In this study, we are comparing secondary amines. This result also suggests
342 that the presence of a NH-aromatic group has a significant effect on the antioxidant activity
343 as described before (Scipioni et al., 2018). The delocalisation of the lone pair of electrons on
344 the amino group towards the phenyl group could explain a better antioxidant activity
345 (Scipioni et al., 2018).

346 Compounds **2a** to **2e** exhibited similar activities. No correlation was found with the length of
347 chain on aromatic and the value of IC_{50} or TE values. These results are consistent with works
348 of Scipioni where no effect of the nature of alkyl chain on the antioxidant activity was found
349 (Scipioni et al., 2018). However, an increasing of the antioxidant capacity of rosmarinates
350 esters as the alkyl chain is lengthened was reported by Laguerre et al. (2010), with a
351 maximum after which further chain extension leads to a collapse in antioxidant capacity .

352

353 **Table 2: Antioxidant properties of vanillin derivatives 1,2a-2e, 3a-b, 4 and standard antioxidants Trolox, BHA, BHT by**
 354 **DPPH, ABTS and FRAP assays. The results of DPPH, ABTS were given as half-inhibitory scavenging concentration IC₅₀**
 355 **(μM) and FRAP values were expressed as Trolox equivalent (TE) (μM Trolox/ μM compound). The average and standard**
 356 **deviations were calculated from at least five independent experiments**

Compounds	DPPH (IC ₅₀ μM)	ABTS (IC ₅₀ μM)	FRAP (TE)
1	96.1 \pm 1.1	86.3 \pm 1.7	0.13 \pm 0.05
2a	42.4 \pm 3.0	37.5 \pm 4.4	0.20 \pm 0.08
2b	37.7 \pm 1.2	28.0 \pm 2.7	0.36 \pm 0.10
2c	40.5 \pm 2.2	29.5 \pm 3.1	0.31 \pm 0.07
2d	38.0 \pm 3.2	31.0 \pm 2.8	0.32 \pm 0.10
2e	41.0 \pm 3.2	33.9 \pm 3.4	0.26 \pm 0.07
3a	42.4 \pm 2.8	36.8 \pm 3.4	0.21 \pm 0.05
3b	49.0 \pm 4.0	41.7 \pm 3.4	0.19 \pm 0.04
4	59.1 \pm 4.3	60.7 \pm 1.8	0.18 \pm 0.05
Vanillin	9 187 \pm 25	8 550 \pm 35	0.12 \pm 0.03
BHT	125.0 \pm 10.1	32.4 \pm 2.2	1.17 \pm 0.21
BHA	50.1 \pm 5.1	39.8 \pm 2.6	1.44 \pm 0.18
Trolox	40.8 \pm 2.6	36.3 \pm 2.7	

357

358 3.3 Antimicrobial activities

359 The antibacterial activity of the nine synthesized compounds and vanillin was evaluated
 360 against different bacteria reported as contaminants in cosmetics, namely *Escherichia coli*
 361 (Adegoke and Arotupin, 2017; Brannan, 1997), *Micrococcus luteus* (Dao et al., 2017) and
 362 *Bacillus subtilis* (Adegoke and Arotupin, 2017; Brannan, 1997). Concerning the antifungal
 363 activities, our study focused on strains previously documented in cosmetic products such as
 364 *Paecilomyces variotii* (Geis, 2006), *Cladosporium cladosporioides* (Dao et al., 2017), or
 365 recommended by the Personal Care Products Council for validation of preservative in
 366 cosmetics, *i.e.* *Aspergillus brasiliensis* and *Penicillium funiculosum* (Brannan, 1997; Dadashi
 367 and Dehghanzadeh, 2016). The compounds were also tested on an emergent opportunistic
 368 pathogenic yeast named *Candida krusei* (Ashton Acton, 2012).

369 The results of inhibition of bacterial and fungal growth are given in Table 3. The inhibition
 370 rate calculation uses the comparison of the growths in presence of the tested compound
 371 versus inoculated control. The results can thus exceed 100 % when the antimicrobial agent
 372 decreases the initial OD due to the inoculum.

373 None of the tested molecules displayed an antimicrobial effect against the gram-negative
 374 fecal contamination indicator *E. coli*. This species is the sole gram negative bacteria used in
 375 this study. This result could thus be due either to a variation in the nature of the targeted

376 component within the bacterial cell or to an access limitation for the antimicrobial molecules
377 considering the difference in the cell wall structure. Indeed, *Escherichia coli*, as most
378 coliform bacteria, is known as being resistant to preservatives notably in relation to the
379 higher selectivity of its cell envelope (Halla et al., 2018).

380 The results obtained against the Gram-positive bacterium *Bacillus subtilis* showed a strong
381 inhibition, higher than 94 %, for the compounds **2b**, **2c**, **2d**, **2e**, **3a** and **3b**. The other
382 compounds, comprising vanillin, had no significant activities on the *Bacillus subtilis* growth.
383 Incidentally, all of these non-effective compounds on *B. subtilis* display a log $P_{0/w}$ below 3
384 except the compound **1**. A significant increase of the inhibition level was observed from the
385 compound **2a** without additional carbon chain to the compounds **2b** (Me), **2c** (C₂) and **2d**
386 (C₄). Replacing one hydrogen (**2a**) with bromine (**3a**) on the cycle increased the anti-bacterial
387 activity from a non-significant value to 96 %. The iodo compound **3b** showed the same high
388 inhibition rate as the bromo compound **3a**.

389 The inhibition rates of the Gram-positive bacterium *Micrococcus luteus* growth varied
390 between the compounds. Compounds **2c**, **2d**, **2e** and **3a** showed a strong inhibition
391 (between 87 and 99 %) while the compounds **2a** and **2b** led to a not significant inhibition.
392 Other compounds, including vanillin, had low inhibition rates. It seems to indicate that a
393 minimum chain length is necessary to obtain a significant inhibitory activity (chains C₂, C₄, C₆
394 have a better activity than CH₃ and no chain). It was previously described that the higher
395 lipophilicity of vanillin derivatives is able to inhibit the growth of Gram-positive bacteria by
396 causing a permeabilization of the Gram-positive cell envelope (Patterson et al., 2015). The
397 hydrophobic interaction between the alkyl chain and the lipid region in the membrane
398 promotes the lipid membrane destabilization. A balance between the hydrophilic and
399 hydrophobic parts of the molecule is required for antibacterial activity. On one hand, the
400 molecule has to diffuse into an aqueous environment in order to reach the cell surfaces and
401 on the other hand the molecule should insert into cell membranes. Interestingly in our
402 experiment, vanillin showed a low activity in comparison to its derivatives. Indeed, we used
403 the same mass concentrations for all the tests, but when we converted it to molar
404 concentration, we can noticed that the molar concentrations tested (0.16-0.19 mmol/L) for
405 active vanillin derivatives (**2c**, **2d**, **2e** and **3a**) are lower than vanillin concentration (0.33
406 mmol/L). The results revealed the determinant influence of the alkyl or bromo substituent
407 on the antimicrobial activity. The presence of aliphatic chains and free hydroxyl groups as

408 well as the delocalization of electrons within the structure are contributing factors to the
409 antimicrobial activity of aroma compounds (Arfa et al., 2006). As described by Sikkema et al.
410 (1994), aromatic compounds are able to penetrate into the lipid bilayer and disrupt the
411 cytoplasmic membrane. It was also shown that capsaicin which contains vanillyl group and
412 amine chain acted as bactericidal compound causing damage to cell membrane (Marini et
413 al., 2015). Additionally, the presence of an aromatic cycle in the substitutive group seems to
414 play a role in the inhibitory activity by comparison of the compounds **1** (C₈) and **2d** (C₄).
415 Contrary to *Bacillus subtilis*, the nature of the halogen is also crucial factor since the iodo
416 compound (**3b**) had no activity in contrast to the bromo analog (**3a**). This could be explained
417 by the higher electronegativity of bromine, which contributes to the delocalization of
418 electrons. Polarity or electronic effects could lead to a significant improvement of
419 antibacterial activities rather than the substituent size contrary to studies described by
420 Bahrin et al. (2016).

421 Some of the tested compounds presented also antifungal activities against filamentous
422 fungi, whereas the yeast *C. krusei* was more sensitive to the original vanillin than to the
423 vanillin derivatives. *P. variotii* appeared to be the most sensitive filamentous strain for the
424 tested compounds. The compounds **1**, **2d**, **2e** and **3a** showed the highest inhibitions (greater
425 than 93 %). Compound **1** without the aromatic part has an inhibition rate close to 99 %
426 whereas vanillin showed a low activity. This result indicates that the coupling of a
427 hydrocarbon chain to the vanillin aromatic ring could be suitable for the antifungal effect.
428 The mechanism of action could be hypothesized to occur through an interaction between
429 the vanillin part and the ergosterol aromatic structures present in the cell membrane
430 allowing a membrane disturbance through the hydrocarbon chain insertion in a similar way
431 than described for other fungicides such as amphotericin B (Brajtburg et al., 1990). Further
432 studies are required to investigate the possible interaction of the derivative **1** with fungal
433 membranes. *C. cladosporioides* was specifically inhibited by compounds **3a** and **3b**. The
434 inhibition activity observed against *A. brasiliensis* was restricted to the compound **2d**.
435 Compounds **2d** and **2e** showed strong inhibitions against *P. funiculosum*. Consequently, the
436 compound **2d** appeared to have the broader antifungal spectrum among the tested
437 compounds for the considered species.

438

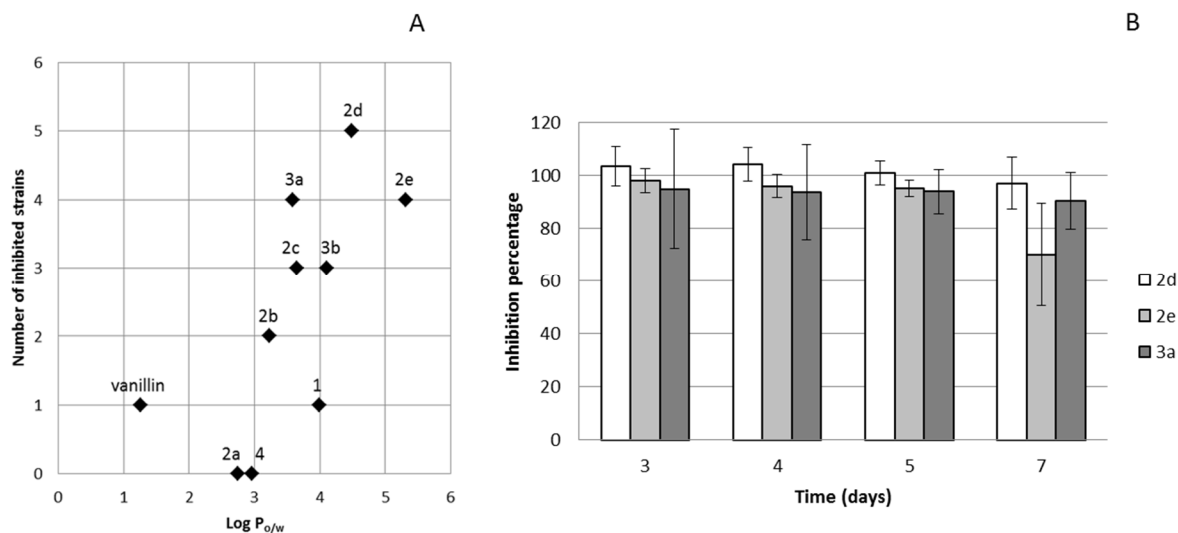
439
440

Table 3: Growth inhibition rates (%) of bacterial and fungal strains caused by vanillin derivatives (Average of three replicates, n.s. = not significant results in regard of the low inhibition result, i.e. < 70 %, and/or its high variability)

	Bacteria			Fungi				
	Gram +		Gram -	Yeast	Filamentous			
	Coccus	Bacillus	Bacillus		<i>C. krusei</i>	<i>P. variotii</i>	<i>P. funiculosum</i>	<i>C. cladosporioïdes</i>
	<i>M. luteus</i>	<i>B. subtilis</i>	<i>E. coli</i>					
Vanillin	n.s.	n.s.	n.s.	76.68 ±15.69	n.s.	n.s.	n.s.	n.s.
1	n.s.	n.s.	n.s.	n.s.	98.70 ±1.22	n.s.	n.s.	n.s.
2a	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2b	n.s.	94.83 ±6.88	n.s.	n.s.	77.54 ±9.51	n.s.	n.s.	n.s.
2c	95.29 ±6.21	100.00 ±14.51	n.s.	n.s.	70.95 ±14.90	n.s.	n.s.	n.s.
2d	87.28 ±9.73	109.91 ±12.55	n.s.	n.s.	100.76 ±4.55	99.06 ±4.43	n.s.	81.80 ±14.28
2e	87.79 ±8.30	98.49 ±6.41	n.s.	n.s.	94.92 ±3.20	95.66 ±3.45	n.s.	n.s.
3a	99.11 ±12.33	95.69 ±18.04	n.s.	n.s.	93.74 ±8.24	n.s.	89.04 ±11.82	n.s.
3b	n.s.	95.47 ±23.08	n.s.	n.s.	70.63 ±20.44	n.s.	87.62 ±24.20	n.s.
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

441

442 In our study, compounds **2d**, **2e**, **2c**, **3a** and **3b**, which presented the broader spectrum of
 443 antimicrobial activities, had the higher log $P_{o/w}$ as shown in Figure 2A. Correlations between
 444 the antimicrobial activities of phenolic compounds and log $P_{o/w}$ values have been already
 445 described (Ultee et al., 2002). A greater hydrophobicity seems to be important to increase
 446 the antimicrobial activity. According to Weber et al. (1996), compounds with a high log $P_{o/w}$
 447 will partition further into the cell membrane, which will cause a more significant membrane
 448 damage and will increase the passive proton flux across the membrane. This is in accordance
 449 with our study since vanillin and derivatives **2a** and **4** with a log $P_{o/w}$ lower than 3 presented
 450 low antimicrobial activities. However, compound **1** with a log P higher than 3 (3.99) did not
 451 inhibit the growth of most tested microorganisms in the studied conditions. This last result
 452 indicates than the limit of 3 could fluctuate according to other chemical factors as described
 453 by Ben Arfa (2006).



454

455 **Figure 2: Number of efficiently inhibited microbial strains according to the log P_{o/w} of the tested molecules (A) and**
 456 **evolution along time of the inhibitory effects of the broader-spectrum compounds against *P. variotii* (B)**

457

458 For the targeted applications, notably in the cosmetics domain, a broad spectrum of action
 459 on both bacteria and fungi is a major advantage (Halla et al., 2018). Consequently, the best
 460 candidates for this application field appear to be the molecules **2d**, **2e** and **3a**. In particular,
 461 the compound **2d**, bearing C₄ chain, led to remarkable results against a broad spectrum of
 462 both fungal and bacterial strains. Another required property in cosmetics formulation is the
 463 duration of action. In our experimental conditions, the evolution of the antimicrobial
 464 activities of the three best candidates against *P. variotii* was estimated over 1 week to have a
 465 first insight into the stability of the antifungal activities (Figure 2B). The antifungal activities
 466 appeared to be stable over the considered time in particular for the compounds **2d** and **3a**. It
 467 can be noted that the vanillin-derivatives **2d**, **2e** and **3a** are also among the best compounds
 468 tested regarding to their antioxidant activities equaling the activity level of the reference
 469 molecules (BHA, BHT and Trolox) and even potentially surpassing them for the compound
 470 **2d**. These biosourced molecules present the major potential to play the two antioxidants
 471 and antimicrobials functions in cosmetics formulation, allowing also to shorten the additive
 472 list.

473 These molecules required now further studies in order to evaluate their behavior when
 474 employed in cosmetic product and be compared to widely used preservatives. As a first step,
 475 minimum inhibitory concentrations of the compound **2d** against the 3 tested bacterial
 476 strains and the fungal strains recommended by the Personal Care Products Council for

477 validation of preservative in cosmetics were measured and compared to the cosmetic
 478 preservative methylparaben (Table 4). Methylparaben did not allow reaching 50 % of
 479 inhibition in the concentration range compatible with its solubility in our experimental
 480 conditions for all strains apart from *P. funiculosum* for which MIC₅₀ is reached with 75 mg.L⁻¹.
 481 In contrast, the compound **2d** reached MIC₈₀ for *A. brasiliensis* with 50 mg.L⁻¹ and MIC₁₀₀ for
 482 *M. luteus*, *B. subtilis* and *P. funiculosum* with 50, 75 and 50 mg.L⁻¹ respectively. Thus, the
 483 results confirmed the compound **2d** potential to be used in cosmetics as a broad-spectrum
 484 antimicrobial agent against both gram-positive bacteria and molds with lower MIC than the
 485 positive control methylparaben.

486

487 **Table 4: Comparison of the minimal concentrations of compound 2d and methylparaben required to inhibit 50 %, 80 % or**
 488 **100 % of the growth for the tested bacterial and fungal strains.**

	Concentrations (mg.L ⁻¹)	Bacterial strains			Fungal strains	
		<i>M. luteus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>P. funiculosum</i>	<i>A. brasiliensis</i>
Methylparaben	1.25	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	5	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	12.5	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	25	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	50	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	75	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	MIC₅₀	< MIC ₅₀
	100	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₈₀	< MIC ₅₀
Compound 2d	1.25	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	5	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀	< MIC ₅₀
	12.5	< MIC ₅₀	MIC₈₀	< MIC ₅₀	MIC₈₀	< MIC ₅₀
	25	< MIC ₅₀	< MIC ₁₀₀	< MIC ₅₀	< MIC ₁₀₀	< MIC ₅₀
	50	MIC₁₀₀	< MIC ₁₀₀	< MIC ₅₀	MIC₁₀₀	MIC₈₀
	75	100 %	MIC₁₀₀	< MIC ₅₀	100 %	< MIC ₁₀₀
	100	100 %	100 %	< MIC ₅₀	100 %	< MIC ₁₀₀

489

490 3.4 Cytotoxicity assay

491 To confirm the potential of compound **2d** for cosmetic applications, a cytotoxicity assay was
 492 realized on human skin fibroblasts treated with methylparaben or compound **2d** (Table 5).
 493 When the cells were cultured without the studied compounds, their viability was 100%
 494 (negative control). The overall cell viability remained high (values between 85% and 100%)
 495 when the cells were treated with methylparaben (a compound is considered as cytotoxic
 496 when the cell viability is below 70%), as expected (Spindola et al., 2018). For the same
 497 concentrations, we also observed no cytotoxic effect of compound **2d** on the human cells,

498 with cell viability always superior to 95%. These preliminary data underline the strong
 499 potential of compound **2d** as a safety preservative for cosmetic applications.

500

501 **Table 5: Human cell viability (%) after 24 hours of incubation with methylparaben or compound 2d**
 502

Controls		Methylparaben (mg.L ⁻¹)							Compound 2d (mg.L ⁻¹)						
Neg.	Pos.	1.25	5	12.5	25	50	75	100	1.25	5	12.5	25	50	75	100
100%	6 %	92%	90%	105%	93%	86%	99%	84%	98%	99%	97%	102%	97%	95%	95%

503 Neg. = negative control

504 Pos. = positive control (cells treated with cytotoxic latex extract)

505

506 4. Conclusion

507 This work enabled, for the first time, to synthesize bioderived amine derivatives with
 508 enhanced antioxidant and antimicrobial activities compared to the vanillin compound and to
 509 investigate the influence of the nature of the substituents on these activities. A simple
 510 synthesis of vanillin derivatives was achieved through a reductive amination in order to
 511 obtain various compounds bearing alkyl chain of different lengths or halogen groups. For all
 512 compounds, except for the compounds **1** and **4**, high antioxidant activities was determined
 513 using DPPH, ABTS and FRAP methods. The derivatives **2b** and **2d** presented the highest
 514 antioxidant capacities, even higher than for the standards BHA and BHT. The presence of
 515 phenyl group linked to NH seems to increase the antioxidant capability. The growth
 516 inhibition of bacteria and fungi was investigated as well. The most promising compounds as
 517 preservatives are the compounds **2d**, **2c**, **3a** and **2e**, then **2b**, **1**, **3b** to a lesser extent. We
 518 demonstrated that the presence of aliphatic chains or halogens enhances the *in vitro*
 519 antimicrobial properties of vanillin. A relation between the log $P_{o/w}$ of vanillin derivatives and
 520 antimicrobial activities was highlighted. Thus, the most hydrophobic compounds emerged as
 521 the best antimicrobial products with the largest broad spectrum of action. The compound
 522 **2d** with a butyl group and a log $P_{o/w}$ greater than 4 combines very good activities against
 523 almost all microbes, no cytotoxic effect on human cells and the highest antioxidant capacity
 524 among the evaluated molecules. We demonstrated that vanillin derivatives are good
 525 candidates for being used as biosourced preservatives in cosmetics. Their evaluation in such

526 applications as well as their safety assessment have now to be pursued. Notably, a challenge
527 test has to be carried out once these compounds will be included in a formulation.

528 **CRedit authorship contribution statement**

529 **Antoine Fayeulle** : methodology, validation, supervision, formal analysis, investigation,
530 writing original draft, writing - Review & Editing, **Elodie Trudel** : investigation, **Audrey**
531 **Damiens** : investigation, **Alexandra Josse** : investigation, **Nesrine Ben Hadj Youssef** :
532 investigation, methodology, **Pascale Vigneron** : investigation, methodology, formal analysis,
533 **Muriel Vayssade** : investigation, methodology, visualization, formal analysis, **Claire Rossi** :
534 methodology, investigation, validation, supervision, visualization, formal analysis, writing -
535 Review & Editing. **Claire Ceballos** : conceptualization, methodology, validation,
536 supervision, formal analysis, writing original draft, writing - Review & Editing. All authors
537 have read, edited and approved the final manuscript. All the authors of the manuscript are
538 agreed with the above mentioned credit author statement.

539

540 **Declaration of competing interest**

541 The authors declare that they have no known competing financial interests or personal
542 relationships that could have appeared to influence the work reported in this paper.

543

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551 **Appendix. Supplementary data**

552

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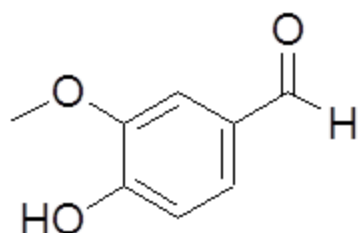
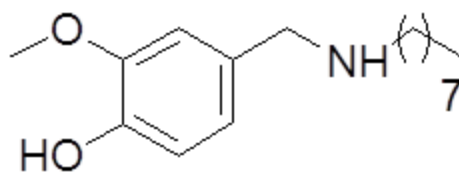
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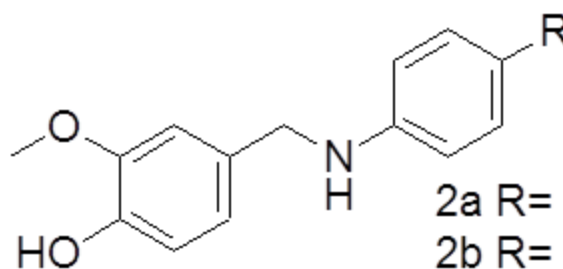
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One pot,
green synthesis

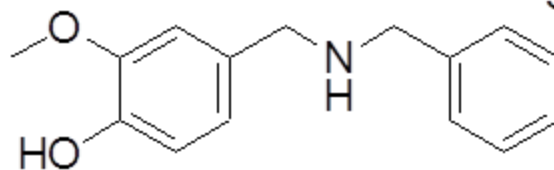


vanillin

Bio-sourced
compound



- 2a R= H
- 2b R= CH₃
- 2c R= C₂
- 2d R= C₄
- 2e R= C₆
- 3a R= Br
- 3b R= I



High antioxidant activities
High antimicrobial activities for
certain compounds
Best results for the butyl
derivative of vanillin **2d**

Potential preservatives
in cosmetics