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

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### Abstract

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

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

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## 1. Introduction

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

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

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

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

## **2. Materials and methods**

### **2.1 Materials and instrumentation**

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

Reverleris X2) and pre-packed columns (GRACE, Reverleris, Silica 40  $\mu$ m, 12 g).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a 400 MHz Bruker UltraShield 400 MHz/54 mm Ultra-long hold. Chemical shifts ( $\delta$ ) are quoted in ppm and are referenced to deuterated chloroform  $\text{CDCl}_3$  (Carlo-Erba) as an internal standard (7.26 ppm). Coupling constants (J) are quoted in Hz. The following abbreviations were used to describe the spectra obtained, s: singlet, brs: broad singlet, d: doublet, t: triplet, q: quartet, quin: quintet, sex: sextet, m: multiplet, dd: doublet of doublet. The electrospray ionisation mass spectrum was recorded on an Agilent QTOF 6538 (Wilmington, DE) coupled to an Agilent 1200 series fast resolution LC system. The mobile phase consisted of 5 % Water – 95 % HPLC grade ACN. The samples were prepared in the proper solvent and injected without HPLC column into the DUAL ESI at a flow rate of 400  $\mu\text{L}/\text{min}$ . The mass spectrometer was operated in positive and negative ESI modes, in full scan mode and with continuous infusion of a calibration solution. The capillary voltage was +3800 V and -3800 V. The ion source gas temperature was 300  $^\circ\text{C}$  with flow of 12 L/min of nitrogen. Fragmentator was set at 120 V. The mass range for ESI experiments was from  $m/z = 100$  to  $m/z = 1000$ .

## 2.2 General procedure for synthesis of vanillin derivatives

All vanillin derivatives were obtained through the reductive amination in a one-pot reaction following an adapted protocol previously reported (Patterson et al., 2015). A mixture of vanillin and the corresponding amine were dissolved in ethanol followed by the addition of  $\text{NaBH}_4$ . The reaction mixture was stirred overnight under reflux.

An EtOH (10 mL) solution of the selected amine (1.13 mmol) was added dropwise to an EtOH (10 mL) solution of vanillin (172 mg, 1.13 mmol) under stirring. The reaction mixture was heated at reflux overnight. Upon cooling at 0  $^\circ\text{C}$ ,  $\text{NaBH}_4$  (1.70 mmol) was added to the solution and the mixture was allowed to proceed for 12h at room temperature. Once the reaction was completed as shown by a TLC monitoring, the mixture was evaporated to dryness using a rotary evaporator. The residue was dissolved in ethyl acetate then washed by extraction with aqueous solution of saturated  $\text{NaHCO}_3$ . The organic fraction was dried using anhydrous sodium sulfate and filtered. The solvent was removed under vacuum to yield the final products. The crude products were directly subjected to column chromatography (AcOEt/Cyclo) to give pure products **1**, **2a-2e**, **3a-3b** and **4**. All compounds synthesized were fully characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, ESI-MS (electrospray ionization-

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

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

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

### 2.4 Antioxidants studies

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

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

#### 2.4.1 DPPH assay

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

#### 2.4.2 ABTS assay

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

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

#### 2.4.3 IC<sub>50</sub> calculation for DPPH and ABTS assay

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

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

where  $A_{S0}$  was the absorbance of the control and  $A_s$  was the absorbance of the samples or the standard compounds. The antioxidant activity was expressed as an IC<sub>50</sub> value that is the concentration in  $\mu\text{M}$  that scavenged the DPPH or ABTS radicals by 50 %, and was calculated from the concentration-effect linear regression curve. The standard curves for DPPH and ABTS assays were linear between 10 and 50  $\mu\text{M}$  Trolox.

#### 2.4.4 FRAP reducing activity assay

FRAP assay is based on the rapid reduction in ferric-tripyridyltriazine ( $\text{Fe}^{3+}$ -TPTZ) by antioxidants present in the samples forming ferrous-tripyridyltriazine ( $\text{Fe}^{2+}$ -TPTZ), a blue-coloured product (Benzie and Strain, 1996). The working FRAP reaction solution was prepared by mixing 10 volumes of 300 mM acetate buffer at pH 3.6 with 1 volume of 20 mM  $\text{FeCl}_3$  solution and 1 volume of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl. 1 mL of this reagent was mixed with 150  $\mu\text{L}$  of sample solution. The mixtures were incubated 30 minutes in the dark at 37 °C in a water bath. The absorbance was measured at 595 nm, the blank was made using  $S_0$  where 150  $\mu\text{L}$  of methanol was used instead of the sample solution. All curves at absorbance vs concentration were drawn and fitted in order to get the linear regression:  $Y = a * X$ . The FRAP values represents the ratio between the slope of the linear plot for reducing  $\text{Fe}^{3+}$ -TPTZ reagent by the compounds compared to the slope of the this plot for Trolox. The FRAP results were expressed in term of Trolox equivalent TE ( $\mu\text{M}$  Trolox/ $\mu\text{M}$  compound).

### 2.5 Antimicrobial activities measurements

#### 2.5.1 Microorganisms



The tested strains were selected according to their documented occurrence in cosmetics contamination or their recommendation by the Personal Care Products Council for validation of preservative efficiency in cosmetics. Bacterial strains are *Escherichia coli* ATCC 25922<sup>TM</sup> (American Type Culture Collection, USA), *Micrococcus luteus* CRBIP 107660 (Institut Pasteur, France) and *Bacillus subtilis* ATCC 6051 (American Type Culture Collection, USA). They were maintained on Tryptic Soy Agar (TSA, CONDA, Madrid) at 30 °C under obscurity. Concerning fungal strains, *Penicillium funiculosum* (MUCL 038968), *Aspergillus brasiliensis* (MUCL 029039), and *Paecilomyces variotii* (MUCL 039890) were provided by the Agro-food and Environmental Fungal Collection (BCCM/MUCL) of the Catholic University of Louvain. *Cladosporium cladosporioides* and *Candida krusei* were previously isolated by the laboratory. These fungi were grown on solid potato dextrose agar medium (Conda Laboratories, Madrid, Spain) under 12/12 h photoperiod and replicated every week.

#### 2.5.2 Determination of inhibition rates

Assays were carried out in 96-well clear flat-bottom plates (96 well Nunc<sup>TM</sup> edge 2.0 microplates, ThermoFisher Scientific) in triplicates according to a method derived from CLSI broth microdilution method (Clinical and Laboratory Standards Institute and Weinstein, 2018). Concerning bacteria, the inoculum consisted in a 0.2 optical density (OD) calibrated suspension in mineral medium (KCl 0.250 g/L, NaH<sub>2</sub>PO<sub>4</sub> 6.464 g/L, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O 10.408 g/L, MgSO<sub>4</sub> 0.244 g/L, NO<sub>3</sub>NH<sub>4</sub> 1 g/L, MgCl<sub>2</sub> 0.05 g/L; pH = 7, glucose 10 g/L). Concerning fungi, inocula consisted in spores suspensions in mineral medium (KCl 0.250 g/L, NaH<sub>2</sub>PO<sub>4</sub> 1.544 g/L, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O 0.008 g/L, MgSO<sub>4</sub> 0.244 g/L, NO<sub>3</sub>NH<sub>4</sub> 1 g/L, MgCl<sub>2</sub> 0.05 g/L; pH = 5.5, 5 g/L of glucose) calibrated to 2x10<sup>4</sup> spores/mL using a Malassez counting cell. In wells dedicated to test cultures, 100 µL of the inoculum, 98 µL of mineral medium with glucose and 2 µL of the 100 times concentrated mother solution of the tested compound in acetonitrile are mixed. In controls to check the toxicity of the solvent, solutions of compounds were replaced by acetonitrile without compound. In the so-called positive controls, 100 µL of inoculum and 100 µL of mineral medium with glucose were mixed to measure the normal growth of the microorganism. The negative control was made of mineral medium with glucose. The eventual evolution of absorbance of the tested compounds was also checked with wells containing the compound with mineral medium without any microorganism. Fungal growth

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

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

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

## 2.6 Cytotoxicity assay on human cells

Compound **2d** cytotoxicity was analysed by measuring the viability of skin fibroblasts cultured with the compound. Human neonatal dermal fibroblasts (HDFn cat C-004-5C) were purchased from Cascade Biologics and were grown on DMEM (Gibco) medium supplemented with 10 % of fetal bovine serum (Hyclone), 100 U/mL pencillin and 100 µg/mL streptomycin (both from Gibco) at 37 °C under humidified atmosphere (5 % CO<sub>2</sub>, 95 % air). HDFn cells were seeded on Falcon polystyrene-96 well plates (10<sup>4</sup> cell/well) in 100 µL of supplemented culture medium. 24 h post seeding, culture medium was replaced with 100 µL of 1.25, 5, 12.5, 25, 50, 75 or 100 mg/L of compound **2d** or methylparaben solutions prepared in supplemented culture medium. Positive cytotoxic control (100 µL of extract obtained by incubating for 24h pieces of sterile latex gloves under gentle agitation in supplemented culture medium, according to the International Standard ISO 10993 (6 cm<sup>2</sup>/mL)) and negative control (100 µl of fresh supplemented culture medium) were included during the assay. After additional 24 h of incubation of cells with the solutions to be tested, 20 µL of reagent CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega were added to each wells and incubated for 2 h at 37 °C. The absorbances were read by a microplate reader at 490 nm and the cell viability compared to the negative control was calculated as follow: (A<sub>solution</sub>/A<sub>negative control</sub>) x 100. The solutions are considered

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

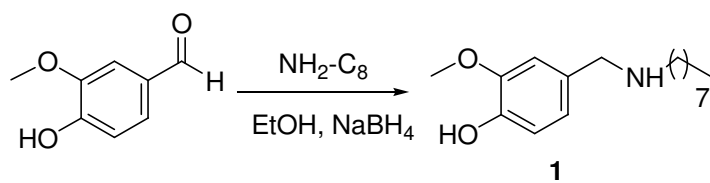
### 3. Results and discussion

#### 3.1 Chemical synthesis

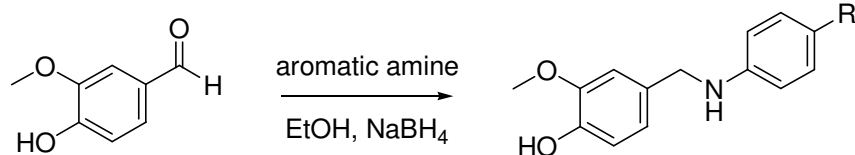
The synthesis of a series of vanillin derivatives (**1**, **2a-2e**, **3a-3b**, **4**) was achieved through reductive amination (Figure 1) according to a procedure adapted from Patterson et al. (2015) (see SI). Resulting amines were isolated with yields ranging from 15 to 84 %. The lowest yields could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

All the derivatives contain phenolic structures (Table 1) that are likely to trigger inhibition against microorganisms by reacting with enzymes active sites through hydrogen bonds (Papuc et al., 2017) and can quench reactive oxygen species by donating an electron (Lü et al., 2010). The synthesized molecules contain different aliphatic chains or polar substituents in the *para* position. Patterson found that lipophilic character of amine derivatives increased antibacterial properties (Patterson et al., 2015). The compounds with aliphatic chains C<sub>1</sub>, C<sub>2</sub>, C<sub>4</sub>, C<sub>6</sub> (**2b**, **2c**, **2d**, **2e** respectively) and with no added chain (**2a**) will allow to compare the influence of the substituted carbon chain length. Halogenated compounds such as chlorine and bromine are known to have antibacterial activities (Odlaug, 1981). The influence of a halogen (Br, I) substituent (compounds **3a** and **3b** respectively) will also be evaluated. The compounds **2a** and **4** will allow to investigate the influence of aromatic methylene group between the amine and the aromatic group and the compounds **1** and **2d** the impact of supplementary aromatic ring.

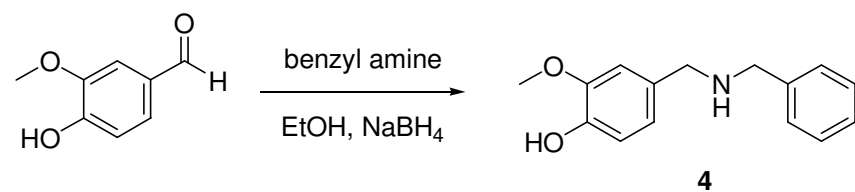
**A**



**B**



**C**

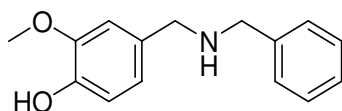


**Figure 1: Synthesis of vanillin derivatives 1, 2a-2e, 3a-3b, 4. Synthesis of aliphatic amine (A), synthesis of aromatic amines (B), synthesis of benzyl amines (C).**

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

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

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

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

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

**Table 2: Antioxidant properties of vanillin derivatives 1,2a-2e, 3a-b, 4 and standard antioxidants Trolox, BHA, BHT by DPPH, ABTS and FRAP assays. The results of DPPH, ABTS were given as half-inhibitory scavenging concentration IC<sub>50</sub> (μM) and FRAP values were expressed as Trolox equivalent (TE) (μM Trolox/μM compound). The average and standard deviations were calculated from at least five independent experiments**

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

### 3.3 Antimicrobial activities

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

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

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



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

The results obtained against the Gram-positive bacterium *Bacillus subtilis* showed a strong inhibition, higher than 94 %, for the compounds **2b**, **2c**, **2d**, **2e**, **3a** and **3b**. The other compounds, comprising vanillin, had no significant activities on the *Bacillus subtilis* growth. Incidentally, all of these non-effective compounds on *B. subtilis* display a log  $P_{o/w}$  below 3 except the compound **1**. A significant increase of the inhibition level was observed from the compound **2a** without additional carbon chain to the compounds **2b** (Me), **2c** (C<sub>2</sub>) and **2d** (C<sub>4</sub>). Replacing one hydrogen (**2a**) with bromine (**3a**) on the cycle increased the anti-bacterial activity from a non-significant value to 96 %. The iodo compound **3b** showed the same high inhibition rate as the bromo compound **3a**.

The inhibition rates of the Gram-positive bacterium *Micrococcus luteus* growth varied between the compounds. Compounds **2c**, **2d**, **2e** and **3a** showed a strong inhibition (between 87 and 99 %) while the compounds **2a** and **2b** led to a not significant inhibition. Other compounds, including vanillin, had low inhibition rates. It seems to indicate that a minimum chain length is necessary to obtain a significant inhibitory activity (chains C<sub>2</sub>, C<sub>4</sub>, C<sub>6</sub> have a better activity than CH<sub>3</sub> and no chain). It was previously described that the higher lipophilicity of vanillin derivatives is able to inhibit the growth of Gram-positive bacteria by causing a permeabilization of the Gram-positive cell envelope (Patterson et al., 2015). The hydrophobic interaction between the alkyl chain and the lipid region in the membrane promotes the lipid membrane destabilization. A balance between the hydrophilic and hydrophobic parts of the molecule is required for antibacterial activity. On one hand, the molecule has to diffuse into an aqueous environment in order to reach the cell surfaces and on the other hand the molecule should insert into cell membranes. Interestingly in our experiment, vanillin showed a low activity in comparison to its derivatives. Indeed, we used the same mass concentrations for all the tests, but when we converted it to molar concentration, we can noticed that the molar concentrations tested (0.16-0.19 mmol/L) for active vanillin derivatives (**2c**, **2d**, **2e** and **3a**) are lower than vanillin concentration (0.33 mmol/L). The results revealed the determinant influence of the alkyl or bromo substituent on the antimicrobial activity. The presence of aliphatic chains and free hydroxyl groups as

well as the delocalization of electrons within the structure are contributing factors to the antimicrobial activity of aroma compounds (Arfa et al., 2006). As described by Sikkema et al. (1994), aromatic compounds are able to penetrate into the lipid bilayer and disrupt the cytoplasmic membrane. It was also shown that capsaicin which contains vanillyl group and amine chain acted as bactericidal compound causing damage to cell membrane (Marini et al., 2015). Additionally, the presence of an aromatic cycle in the substitutive group seems to play a role in the inhibitory activity by comparison of the compounds **1** (C<sub>8</sub>) and **2d** (C<sub>4</sub>). Contrary to *Bacillus subtilis*, the nature of the halogen is also crucial factor since the iodo compound (**3b**) had no activity in contrast to the bromo analog (**3a**). This could be explained by the higher electronegativity of bromine, which contributes to the delocalization of electrons. Polarity or electronic effects could lead to a significant improvement of antibacterial activities rather than the substituent size contrary to studies described by Bahrin et al. (2016).

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

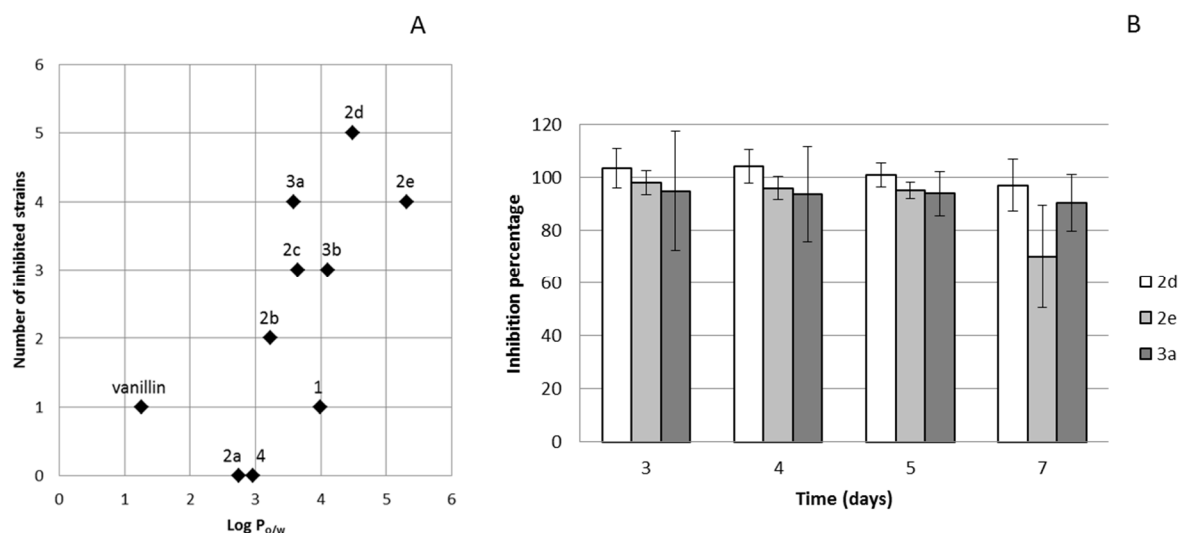
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**Table 3: Growth inhibition rates (%) of bacterial and fungal strains caused by vanillin derivatives (Average of three replicates, n.s. = not significant results in regard of the low inhibition result, i.e. < 70 %, and/or its high variability)**

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



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

For the targeted applications, notably in the cosmetics domain, a broad spectrum of action on both bacteria and fungi is a major advantage (Halla et al., 2018). Consequently, the best candidates for this application field appear to be the molecules **2d**, **2e** and **3a**. In particular, the compound **2d**, bearing  $C_4$  chain, led to remarkable results against a broad spectrum of both fungal and bacterial strains. Another required property in cosmetics formulation is the duration of action. In our experimental conditions, the evolution of the antimicrobial activities of the three best candidates against *P. variotii* was estimated over 1 week to have a first insight into the stability of the antifungal activities (Figure 2B). The antifungal activities appeared to be stable over the considered time in particular for the compounds **2d** and **3a**. It can be noted that the vanillin-derivatives **2d**, **2e** and **3a** are also among the best compounds tested regarding to their antioxidant activities equaling the activity level of the reference molecules (BHA, BHT and Trolox) and even potentially surpassing them for the compound **2d**. These biosourced molecules present the major potential to play the two antioxidants and antimicrobials functions in cosmetics formulation, allowing also to shorten the additive list.

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

validation of preservative in cosmetics were measured and compared to the cosmetic preservative methylparaben (Table 4). Methylparaben did not allow reaching 50 % of inhibition in the concentration range compatible with its solubility in our experimental conditions for all strains apart from *P. funiculosum* for which MIC<sub>50</sub> is reached with 75 mg.L<sup>-1</sup>. In contrast, the compound **2d** reached MIC<sub>80</sub> for *A. brasiliensis* with 50 mg.L<sup>-1</sup> and MIC<sub>100</sub> for *M. luteus*, *B. subtilis* and *P. funiculosum* with 50, 75 and 50 mg.L<sup>-1</sup> respectively. Thus, the results confirmed the compound **2d** potential to be used in cosmetics as a broad-spectrum antimicrobial agent against both gram-positive bacteria and molds with lower MIC than the positive control methylparaben.

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

	Concentrations (mg.L <sup>-1</sup> )	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 <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	5	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	12.5	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	25	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	50	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	75	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	<b>MIC<sub>50</sub></b>	< MIC <sub>50</sub>
	100	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>80</sub>	< MIC <sub>50</sub>
Compound <b>2d</b>	1.25	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	5	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>	< MIC <sub>50</sub>
	12.5	< MIC <sub>50</sub>	<b>MIC<sub>80</sub></b>	< MIC <sub>50</sub>	<b>MIC<sub>80</sub></b>	< MIC <sub>50</sub>
	25	< MIC <sub>50</sub>	< MIC <sub>100</sub>	< MIC <sub>50</sub>	< MIC <sub>100</sub>	< MIC <sub>50</sub>
	50	<b>MIC<sub>100</sub></b>	< MIC <sub>100</sub>	< MIC <sub>50</sub>	<b>MIC<sub>100</sub></b>	<b>MIC<sub>80</sub></b>
	75	100 %	<b>MIC<sub>100</sub></b>	< MIC <sub>50</sub>	100 %	< MIC <sub>100</sub>
	100	100 %	100 %	< MIC <sub>50</sub>	100 %	< MIC <sub>100</sub>

### 3.4 Cytotoxicity assay

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

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

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

Controls		Methylparaben (mg.L <sup>-1</sup> )							Compound <b>2d</b> (mg.L <sup>-1</sup> )						
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%

Neg. = negative control

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

#### 4. Conclusion

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

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

#### **CRedit authorship contribution statement**

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

#### **Declaration of competing interest**

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

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#### **Appendix. Supplementary data**

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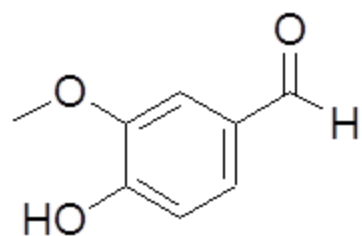
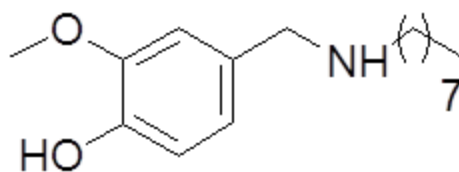


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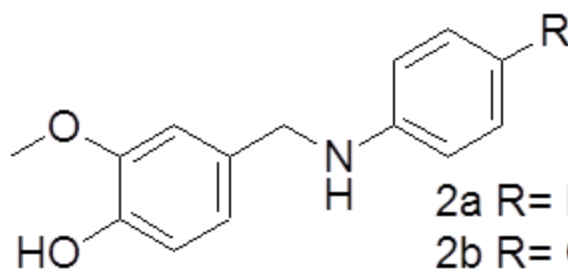
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One pot,  
green synthesis

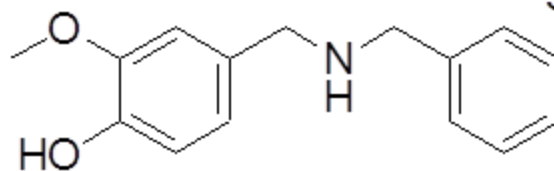


vanillin

Bio-sourced  
compound



- 2a R= H  
2b R= CH<sub>3</sub>  
2c R= C<sub>2</sub>  
2d R= C<sub>4</sub>  
2e R= C<sub>6</sub>  
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