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## Novel Vanillin Derivatives: Synthesis, anti-oxidant, DNA and Cellular protection properties

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### Abstract

Antioxidants have been the subject of intense research interest mainly due to their beneficial properties associated with human health and wellbeing. Phenolic molecules, such as naturally occurring Resveratrol and Vanillin, are well known for their anti-oxidant properties, providing a starting point for the development of new antioxidants. Here we report, for the first time, the synthesis of a number of new vanillin through the reductive amination reaction between vanillin and a selection of amines. All the compounds synthesised, exhibited strong antioxidant properties in DPPH, FRAP and ORAC assays, with compounds **1b** and **2c** being the most active. The latter also demonstrated the ability to protect plasmid DNA from oxidative damage in the presence of the radical initiator AAPH. At cellular level, neuroblastoma SH-SY5Y cells were protected from oxidative damage (H<sub>2</sub>O<sub>2</sub>, 400 μM) with both **1b** and **2c**. The presence of a tertiary amino group, along with the number of vanillin moieties in the molecule contribute for the antioxidant activity. Furthermore, the delocalization of the electron pair of the nitrogen and the presence of an electron donating substituent to enhance the antioxidant properties of this new class of compounds. In our opinion, vanillin derivatives **1b** and **2c** described in this work can provide a viable platform for the development of antioxidant based therapeutics.

Keywords: Vanillin derivatives, Synthetic antioxidants, Antioxidant assays, Oxidative stress, DNA and Cell protection.

## Introduction

In recent years, antioxidants have been the subject of intense research interest mainly due to their beneficial properties associated to human health and wellbeing [1]. Oxidative stress, the imbalance between reactive oxygen and nitrogen species (ROS and RNS) production and the antioxidant defence, is well known to play a fundamental role in different pathophysiological conditions [2]. In fact, a strong link between age related diseases such as cancer, cardiovascular, immune and neurodegenerative disorders with oxidative stress has been recently found [3-5]. With an aging world population, the occurrence of these conditions is on the increase and therefore there is intense interest in the search and study for novel antioxidant molecules for their potential use as therapeutic agents [6]. Furthermore, new synthetic antioxidants can help to decipher the mode of action of many diseases associated with oxidative stress. In the last decade, Choi *et al.* showed the beneficial effect of an antioxidant-based therapy on cognitive decline in patients suffering from Alzheimer disease (AD) [7]. In addition, Takahata *et al.* reported on the strong link between oxidative stress and the lipid peroxidation occurring in Parkinson's disease (PD), thus setting the basis for a possible antioxidant therapeutic approach [8]. Along with their potential therapeutic applications, the food industry uses popular synthetic antioxidants such as trolox, ethylvanillin, butylhydroxytoluene (BHT) and butylhydroxyaniline (BHA) to extend the shelf life of food products. Therefore, there is also high demand for new antioxidants in the food industry [9, 10].

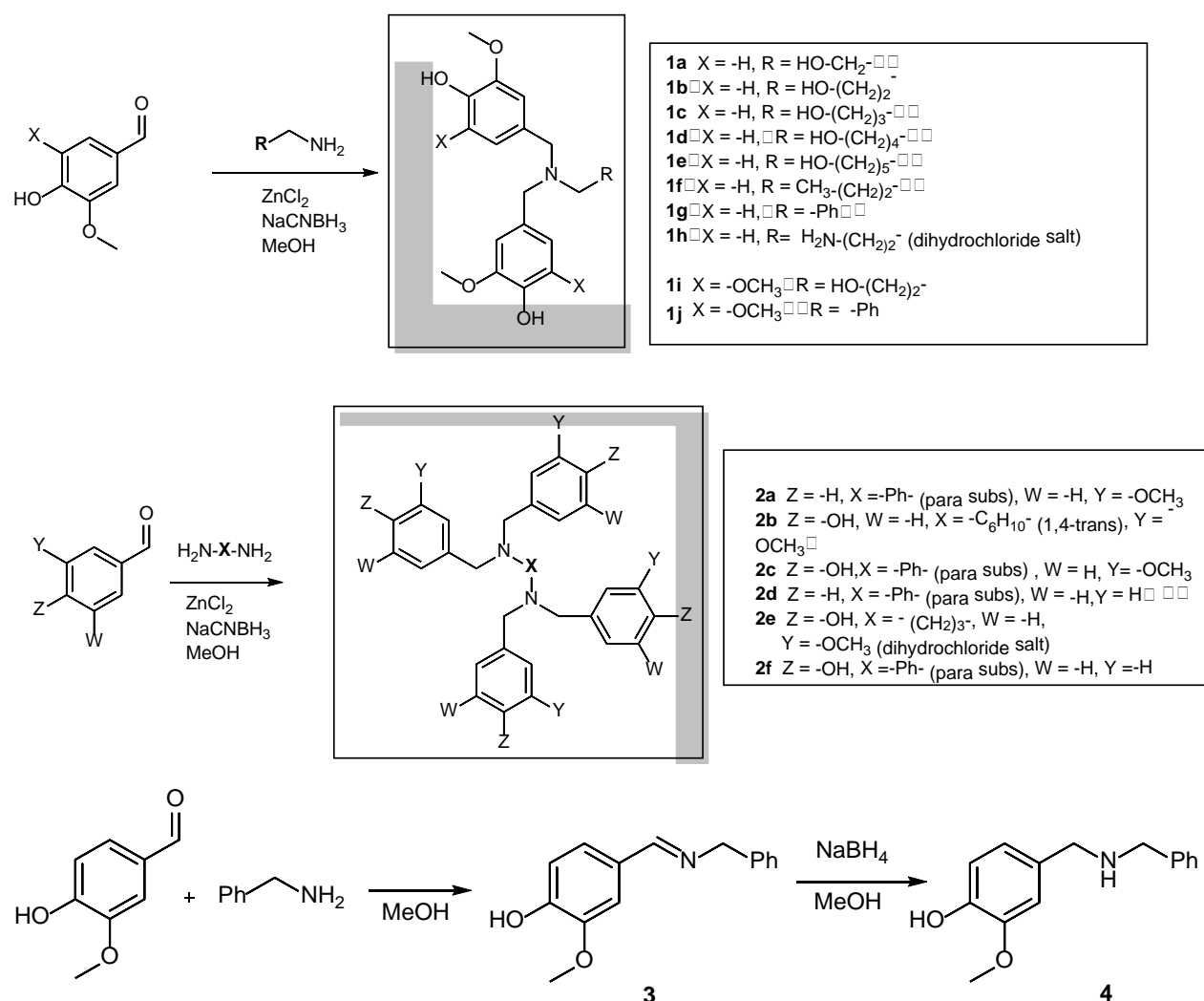
Although there have been many studies on the application of individual natural and synthetic polyphenolic compounds against cancer [11-13] and inflammatory conditions [14], there is limited information on the derivatives of vanillin. The latter is a phenolic compound widely available and applied in the food, drink and cosmetic industries [15, 16]. Furthermore it has been shown recently that vanillin exhibited neuroprotection ability in an experimental model of Huntington's disease and ischemia mainly due to its antioxidant, anti-inflammatory, and anti-apoptotic properties [17]. Recently, Lee *et al.* reported the synthesis of a number of vanillin based dendrimers with enhanced antioxidant properties. They also found those dendrimers to have protective effects on fatty acid (linoleic acid), DNA (pBR322), and lipoprotein (human LDL) against free radical damage. [18, 19]. In other reports diarylpyrazole derivatives bearing a vanillinic hydrazine moiety, had been designed and synthesised to demonstrate both *in vivo* hypoglycemic and *in vitro* antioxidant activities. It was also claimed that the presence of vanillinic group contributed to its radical scavenging properties [20, 21].

Here we report for the first time the synthesis and characterisation of a number of new vanillin derivatives (**Scheme 1**, **1a-1j**, **2a-2f**, **3** and **4**); the determination of their *in vitro* antioxidant properties together with their structure activity relationship (SAR) analysis and their ability to protect DNA from free radical damage. The protection of hydrogen peroxide stressed human neuroblastoma cells with the most active antioxidant derivatives (**1b**, **2c**) was also studied.

## Results and discussion

### Chemical Synthesis

The synthesis of a series of vanillin derivatives was achieved by reacting Vanillin with the corresponding amines under reductive amination conditions [22] ( $\text{NaCNBH}_3$ ,  $\text{ZnCl}_2$ ) in methanol to yield either the mono, di- or the tetra- vanillin derivatives in yields ranging from 12-90% (see scheme 1).



Scheme 1. Chemical strategy for the synthesis of Vanillin derivatives

Depending on the ratio of the reagents used in each reaction, either the di- or tetra- vanillin derivatives were obtained. For example, in the synthesis of divanillin derivatives **1a-1f** and **1i** an excess of amines was required. The excess amine was removed by extracting with  $\text{CHCl}_3$ /saturated  $\text{NaHCO}_3$  (the amines being water soluble together with  $\text{NaCNBH}_4$  and  $\text{ZnCl}_2$ , were efficiently removed by the bicarbonate solution wash) or by column chromatography ( $\text{CHCl}_3$ / MeOH 95:5). Interestingly, this reaction always yielded the di-*N*-alkylated product

(divanillin) rather than the mono-*N*-alkylated derivative. This finding will require further study and elaboration, but it was out with the scope of this work. For this reason, the synthesis of compound **4** was performed in a multistep reaction (see scheme 1); Compound **1** was obtained after the reaction of vanillin with an equimolar amount of amine to yield the corresponding imine which upon subsequent reduction, using sodium borohydride, afforded **4** in 68%. It is also of interest to note that only the *N,N*-divanillin derivative **1h** was obtained following the same above mentioned strategy and no *N<sup>l</sup>,N<sup>3</sup>*-disubstituted isomeric compound was obtained as a by-product.

Interestingly, some of the crude compounds when purified by solvent extraction gave poorer yields (12-23%). This is due to the fact that these products are sparingly soluble in the aqueous phase. In contrast using column chromatography for purification of the crudes afforded higher yields (65-82%). For the synthesis of **1g**, **1j**, **2a-2f** an excess of the aldehydic compound (2-3 equivalents) led to the production of the stoichiometrically desired compounds in good yields (38-90%) which precipitated out from the reaction solution except for compound **2b** (18%). All compounds were fully characterised by <sup>1</sup>H and <sup>13</sup>C NMR and high resolution mass spectroscopy (see experimental section and **Supplementary Information, SI**).

### ***Antioxidant activity***

All compounds synthesised were studied for their antioxidant properties using a range of assays with differing oxidative potential evaluating mechanisms. Furthermore, the ability of selected compounds to protect plasmid pBR322DNA from damage in the presence of the radical initiator AAPH was also studied. All the antioxidant results are listed in tables 1 and 2 below.

### **DPPH Assay**

Diphenyl-1-picrylhydrazyl (DPPH) is a stable organic nitrogen radical with an absorption maximum at 515nm. Its reduction in the presence of antioxidants was monitored spectrophotometrically [23]. The IC<sub>50</sub> value of each compound was determined (see table 1 and **Supplementary Information, SI**). All the Vanillin derivatives except **1h**, **2a** and **2e** showed strong radical scavenging activity with IC<sub>50</sub> ranging from 5.8 to 248 μM when compared with the starting compounds used in the synthesis i.e. vanillin and syringaldehyde (IC<sub>50</sub> 4050 and 1150 μM respectively). Interestingly the majority of the compounds synthesised, showed similar range of activity to known antioxidant Trolox (IC<sub>50</sub> 27.6 μM) [19].

Table 1. Antioxidant properties of vanillin derivatives **1a-1j**, **2a-2e**, **3** and **4**.

Compound	DPPH (IC <sub>50</sub> μM)	FRAP (TE)
<b>1a</b>	12.77 ± 0.11	0.62 ± 0.05
<b>1b</b>	13.65 ± 0.15	0.68 ± 0.02
<b>1c</b>	21 ± 0.40	0.62 ± 0.03
<b>1d</b>	24 ± 2.00	0.63 ± 0.03
<b>1e</b>	22.20 ± 0.60	0.64 ± 0.02
<b>1f</b>	21.90 ± 1.70	0.60 ± 0.01
<b>1g</b>	29.70 ± 1.30	0.55 ± 0.05
<b>1h</b>	INACTIVE*	0.28 ± 0.02
<b>1i</b>	12.30 ± 1.20	1.14 ± 0.04
<b>1j</b>	15.38 ± 1	1.11 ± 0.03
<b>2a</b>	INACTIVE*	2.54 ± 0.56
<b>2b</b>	137.50 ± 5.50	0.56 ± 0.05
<b>2c</b>	5.80 ± 0.10	5.29 ± 0.62
<b>2d</b>	144.30 ± 34.70	2.81 ± 0.45
<b>2e</b>	INACTIVE*	0.74 ± 0.13
<b>2f</b>	98 ± 5.51	2.78 ± 0.03
<b>3</b>	248.5 ± 24.50	0.06 ± 0.003
<b>4</b>	107 ± 1	0.16 ± 0.08
<b>Vanillin</b>	4050 ± 25	0.11 ± 0.07
<b>Syringaldehyde</b>	1150 ± 22	0.93 ± 0.03

Results from each assay are expressed as a mean ± SD of three independent experiments.\*Compounds were tested up to 250 μM.

Compounds **1a**, **1b**, **1c**, **1d**, **1e** and **1f** all exhibited similar activity (IC<sub>50</sub> ranging from 12.77 to 22.2 μM), confirming that the nature of the alkyl chain has no effect on the antioxidant activity. Furthermore, it is interesting to note that the terminal functional group does not have any impact on antioxidant activity. For example, **1c** has a terminal hydroxyl group whereas **1f** has

a methyl group, both compounds demonstrated similar range of antioxidant properties with  $IC_{50}$  values of 13.65 to 21.9  $\mu\text{M}$  respectively.

It can clearly be demonstrated that the structural feature contributing to the greatest impact on the radical scavenging property was the basicity of the nitrogen in the molecule. This is evident when comparing the activity of compounds **3**, **4** and **1g** ( $IC_{50}$  248.5, 107 and 29.7  $\mu\text{M}$  respectively) with **1g** bearing a tertiary amine, showed greater activity when compared to the secondary amine **4** and the imine **3**. Furthermore, quaternary salts **1h** and **2e** were devoid of any activity, suggesting the importance of the electron pair on the nitrogen atoms for radical scavenging property. In addition, the number of vanillin moieties present in each molecule had a significant effect on the antioxidant activity of each compound. For example **2c** has 4 vanillin moieties and is almost 2 times more active than **1b**, which has only two vanillin moieties ( $IC_{50}$  5.8 and 13.65  $\mu\text{M}$  respectively).

Compounds with a syringaldehyde moiety such as **1j** (an extra methoxy group when compared with vanillin) showed an increase in activity when compared with the corresponding vanillin derivative **1g** with  $IC_{50}$  values of 15.38 and 29.7  $\mu\text{M}$  respectively, thus highlighting the role of the extra methoxy group in the aromatic structure. At the same time, removal of the methoxy group caused a dramatic reduction of activity as shown in compounds **2c** and **2f** (5.8 and 98  $\mu\text{M}$  respectively). Finally, as expected the presence of a hydroxyl group in the vanillin moiety plays a fundamental role in antioxidant activity [24]. This is clearly demonstrated with compound **2a** which bears no hydroxyl group, exhibited no activity and this is compared with hydroxylated compound **2f** (98  $\mu\text{M}$ ). Interestingly, the substitution of the phenyl ring of **2c** with a cyclohexane ring (**2b**) also caused a dramatic loss of antioxidant activity (5.8 and 137.5  $\mu\text{M}$  respectively).

### **FRAP Assay**

The FRAP assay works at acidic pH and is based on the reduction of the ferric-tripyridyltriazine complex by antioxidants. The resulting formation of the ferrous-tripyridyltriazine complex leads to a measurable blue colour (593 nm) [25] and the results are expressed as Trolox Equivalent (TE) (see **SI**) by direct comparison with the standard trolox calibration curve. All the derivatives synthesised, showed significantly increase (up to 48 times) in activity in the FRAP assay (0.16- 5.29 TE) when compared with vanillin (0.11 TE) except for compound **3** (imine derivative, 0.06 TE). No statistical differences in activity were found for the derivatives



bearing different alkyl chain linked to the basic nitrogen (**1a-1g**) with values ranging from 0.55-0.68 TE.

As in the above DPPH assay the presence of the basic nitrogen in the molecule provides some impact on the antioxidant activity as evidenced by comparing compounds **3** (imine), **4** (2° amine) and **1g** (3° amine) showing a gradual increase in FRAP activity with 0.06, 0.16 and 0.55 TE respectively. Furthermore, the dihydrochloride salts **1h** and **2e** showed weak FRAP activity with 0.28 and 0.74 TE respectively. On the other hand, syringaldehyde derivatives showed improved activity (almost two times) when compared with the corresponding vanillin derivatives, as evidenced with **1j** with **1g**, 1.11 and 0.55 TE respectively and **1i** with **1b**, 1.14 and 0.68 TE respectively. However, the increase in activity of syringaldehyde derivatives does not follow the same trend of syringaldehyde itself when compared with vanillin (0.93 and 0.11 TE, respectively).

Compounds **2c**, **2d**, **2f** and **2a** showed the highest activities with 5.29, 2.81, 2.74 and 2.54 TE respectively, thus highlighting the importance of the number of vanillin moieties and the presence of both hydroxyl and methoxy groups in the molecule. Compounds bearing only hydroxy (**2f**), methoxy (**2a**) groups and no substituents on the aromatic ring (**2d**) showed similar activity (2.78, 2.54 and 2.81 TE, respectively). When both hydroxy and methoxy substituents are present at the same time on the aromatic ring (**2c**), this enhances the activity of the compound (5.29 TE). Interestingly, the substitution of the phenyl ring in **2c** with a cyclohexane ring **2b** resulted in a dramatic loss in antioxidant activity (5.29 and 0.74 TE respectively). For that reason, in contrast to the DPPH assay, the delocalization of the nitrogen's electrons onto the aromatic ring linking the vanillin /syringaldehyde moieties appears to be fundamental for enhanced antioxidant activity. It is of note that **2b** exhibited comparable FRAP activity to compounds **1a-1i**, all the latter compounds are not able to delocalize the tertiary amino electrons due to the absence any conjugated system linking vanillin /syringaldehyde moieties (see Figure 4).

### **ORAC Assay**

The ORAC assay is based on hydrogen atom transfer and measures the capacity of a compound to inhibit the peroxy free radical induced oxidation of fluorescein. The thermal decomposition of the oxidative stress inducer AAPH results in the formation of free radicals, emulating that

found commonly in the human body. Thus making this assay more relevant to biological systems [26].

Only a selection of compounds was put through the ORAC assay (see table 2). The selection (**1b**, **1g**, **1h**, **2c**, **2e**, **3**, **4**) was based on the number of vanillin moieties and the results from the previous antioxidant assays.

Table 2. Antioxidant properties of selected vanillin derivatives.

Compound	ORAC (TE)	DNA Protection (IC <sub>50</sub> μM)
<b>1b</b>	6.02 ± 0.56	3.60 ± 0.87
<b>1g</b>	5.34 ± 1.54	3.80 ± 1.40
<b>1h</b>	4.08 ± 0.82	61.50 ± 5.20
<b>2a</b>	INSOLUBLE	13.60 ± 1.20
<b>2c</b>	20.40 ± 1.30	0.60 ± 0.10
<b>2e</b>	7.54 ± 1.25	13.90 ± 4.20
<b>3</b>	1.85 ± 0.90	3.80 ± 1.20
<b>4</b>	3.16 ± 1.15	4.10 ± 1.90
<b>Vanillin</b>	2.19 ± 0.28	5.60 ± 0.60
<b>Syringaldehyde</b>	1.45 ± 0.12	9.50 ± 0.30

Results from each assay are expressed as a mean ± SD of three independent experiments.

All the derivatives showed increased activity with the ORAC assay compared with the starting compound vanillin (2.19 TE) except the imine derivative **3** (1.85 TE) (see table 2). The different types of alkyl chains has no effect on the activity in this assay. For example **1b** and **1g** showed similar activity (6.02 and 5.34 TE respectively); however, the number of vanillin moieties in the molecule appears to contribute significantly in the activity, since compound **1g** showed to be 2 fold more active than **4**. At the same time, compound **4** (a 2° amine) showed increased activity when compared with **3** (imine), confirming the importance of the nitrogen basicity as discussed above. In contrast to the DPPH and FRAP assays the hydrochloride salts **1h** and **2e**, turned out to be active in the ORAC assay with 4.08 and 7.54 TE respectively. This may be due to the weakly basic (pH 7.4) environment the experiment was carried out. Compound **2c** showed the highest activity (20.4 TE), highlighting the importance of the number of vanillin moieties in the molecule.

### DNA Damage Protection Assay

The same set of compounds used in the ORAC assay was applied to the DNA damage protection assay. As previously demonstrated in the ORAC assay above, AAPH has the ability to reduce fluorescence intensity of the used fluorescence probe, caused by radical generation.

AAPH is also able to cause oxidative DNA strand breakage in pBR322 plasmid DNA [27], from the supercoiled to both opened circular and linear form of the plasmid DNA. Previous work had demonstrated natural compounds such as phenolic extracts from *Sphallerocarpus gracilis* seeds are able to prevent plasmid DNA strand break. [28] Here all the derivatives tested, showed better activity in protecting oxidative DNA damage when compared with vanillin (IC<sub>50</sub> 5.6 μM, see table 2) except for derivatives **1h**, **2a** and **2e** which gave IC<sub>50</sub> values of 61.5, 13.6 and 13.9 μM respectively. Both of the hydrochloride salts showed lower activity (IC<sub>50</sub> values 61.5 and 13.9 μM respectively), confirming the importance of the electron pair on the nitrogen atoms for enhanced activity. For example in Figure 1, DNA protection can be observed with much higher vanillin concentration (34μM) while with **2c** only a concentration of 4μM was required to obtain maximum DNA protection (figure 1). Furthermore, while vanillin at 2.2μM concentration showed no protective effect, **2c** (at 0.11μM) on the other hand showed 14.7% DNA protection.

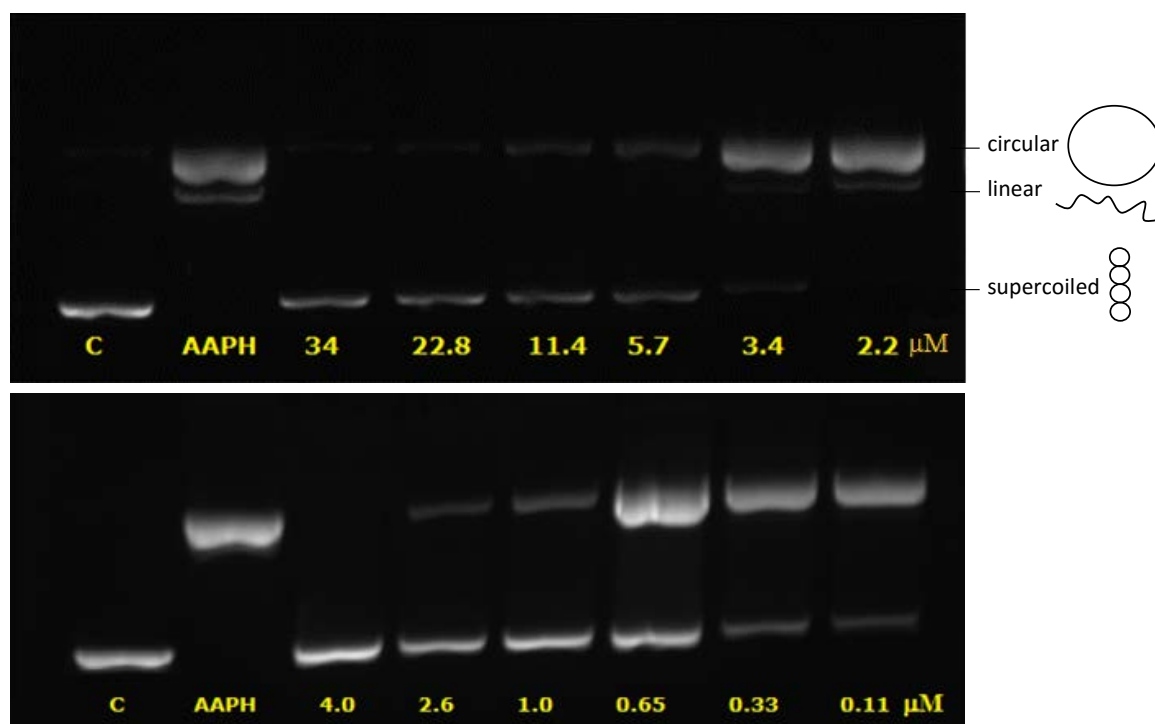


Figure 1. Gel image obtained after DNA protection assay for **vanillin** (above) and compound **2c** (below). The first band (c) at the base represents the DNA control; the band (AAPH) is the AAPH control, the bands from (34 – 2.2 μM) and from (4.0 – 0.11 μM) represent the DNA protection for different concentrations of the compounds. The intensity of each band was measured with image J software and the percentage of protection was measured for each concentration.

The substitution of the OH group with an H atom in the vanillin moieties caused a dramatic reduction of activity as shown in compounds **2a** (IC<sub>50</sub> 13.6 μM) and **2c** (IC<sub>50</sub> 0.6 μM). Although the type of alkyl chain (**1b**, IC<sub>50</sub> 3.6 and **1g**, IC<sub>50</sub> 3.8 μM) showed no effect on activity, the

number of vanillin moieties in the molecule however plays a crucial role on the activity as seen in derivative **2c** which bears four vanillin moieties ( $IC_{50}$  0.6  $\mu$ M). Interestingly in this assay no statistically difference in activity between imine **3** and its corresponding reduced form **4** was showed ( $IC_{50}$  3.8 and 4.1  $\mu$ M respectively).

### Cellular protection through MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is a colorimetric experiment to study cell viability. In this study, the protective potential of **1b** and **2c** was investigated using the neuroblastoma SH-SY5Y human derived cell line. In many age related diseases, cell death is often induced by the presence of Reactive Oxygen Species (ROS). Here  $H_2O_2$  (400  $\mu$ M) was used as a model neurotoxin since it gives rise to oxidative stress in SH-SY5Y cells followed by cell death. [29]

Compounds **1b** and **2c** were chosen in this assay due their strong antioxidant activities demonstrated in the different assays discussed above. Compounds **1b** and **2c** did not show cellular cytotoxicity up to 1 mM and 20  $\mu$ M respectively (see **SI**). Therefore the ability of **1b** to protect  $H_2O_2$  (400  $\mu$ M) treated cells, at concentrations ranging from 0.1 – 200  $\mu$ M and for **2c** from 0.01-10.00  $\mu$ M were studied. From Figure 2, it can clearly be seen that **1b** significantly protected (6%) stressed cells at concentration as low as 5  $\mu$ M ( $p < 0.05$ ). Maximum cellular protection (20%) was achieved with 200  $\mu$ M of **1b** ( $p < 0.001$ ).

Similarly from figure 2, compound **2c** significantly protected (8%)  $H_2O_2$  (400 $\mu$ M) stressed cells at a much lower concentration of 0.1  $\mu$ M ( $p < 0.01$ ) and the maximum cellular protection (30%) was achieved at 10  $\mu$ M ( $p < 0.001$ ) which is well below the cytotoxicity concentrations of **2c**. Therefore **2c** is 20 times better at protecting  $H_2O_2$  (400 $\mu$ M) stressed cells than **1b**. It is noteworthy to mention that vanillin itself up to 800  $\mu$ M concentration did not show any protection on  $H_2O_2$  (400  $\mu$ M) stressed cells (see **SI**).

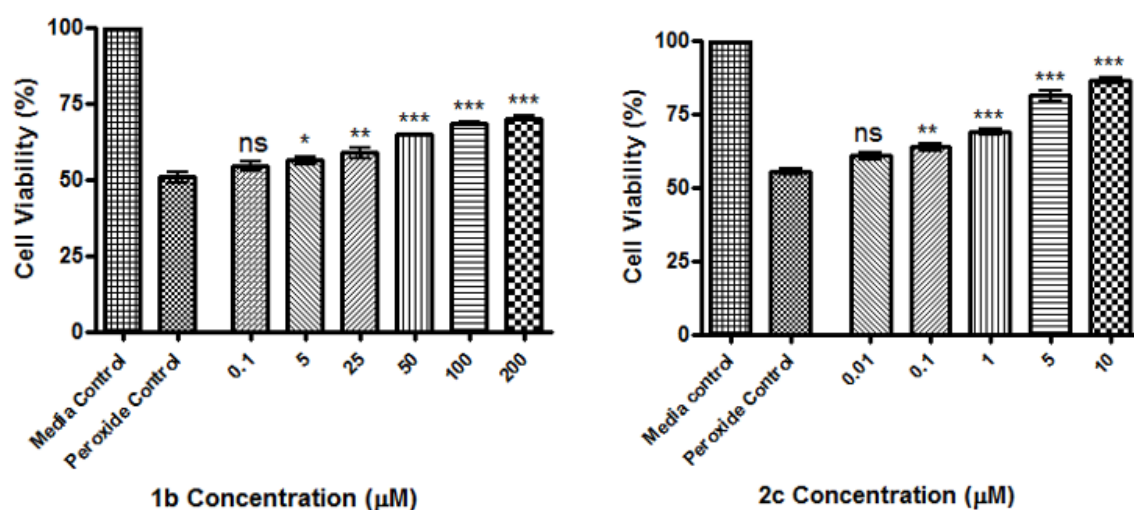


Figure 2. Protective effects of compound **1b** and **2c** in hydrogen peroxide ( $400\mu\text{M}$ )-stressed cells. Cells were incubated with **1b** ( $0.1\text{-}200\mu\text{M}$ ) or **2c** ( $0.01\text{-}10\mu\text{M}$ ) for 24 hours before the addition of hydrogen peroxide. After 24 hours the cell viability was measured using the MTT assay. Values are expressed as the percentage of the untreated control and represented as mean  $\pm$  SD of three independent experiments in each group. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns no significantly different compared to the control.

## SAR

Different antioxidant assays were performed on all the compounds synthesised to evaluate their antioxidant properties and to determine a structure-activity relationship (SAR). For enhanced antioxidant properties the presence of the following are important (see figure 3): (i) tertiary amine (ii) phenolic functionality (iii) the number of vanillin moiety (iv) electronic delocalisation.

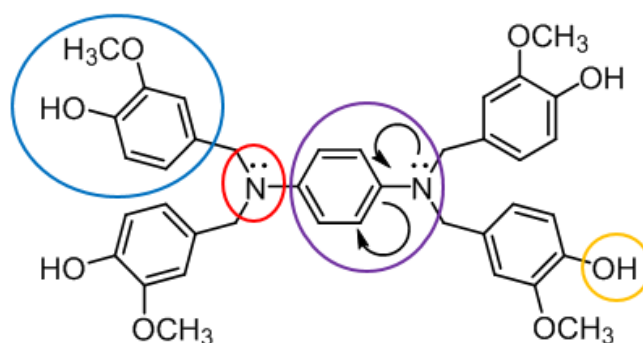


Figure 3. SAR study for new derivatives. The key features for the antioxidant activity are highlighted in different colours: vanillin moiety (blue), phenolic functionality (yellow), tertiary amine (red) and electronic delocalisation (purple).

The most interesting antioxidant compounds **1b** and **2c** showed their ability to protect plasmid DNA from radical damage with  $\text{IC}_{50}$  values  $3.6$  and  $0.6\ \mu\text{M}$ , respectively. Compound **2c**

exhibited total DNA protection up to concentration of 4  $\mu\text{M}$ , demonstrating improved activity compared with other synthetic compounds derived from natural occurring antioxidant resveratrol [30]. Interestingly a homologue compound of **2c** reported by Lee *et al.* [19] with a benzyl group linking the four vanillin moieties **A** (figure 4) instead of a phenyl group as in the case of **2c** (0.6  $\mu\text{M}$ ) showed much weaker DNA protection activity at concentration  $> 45 \mu\text{M}$ . Therefore the ability for the lone pair of electrons on the tertiary amino group to delocalise towards the phenyl group may be a contributory factor for increased antioxidant activity.

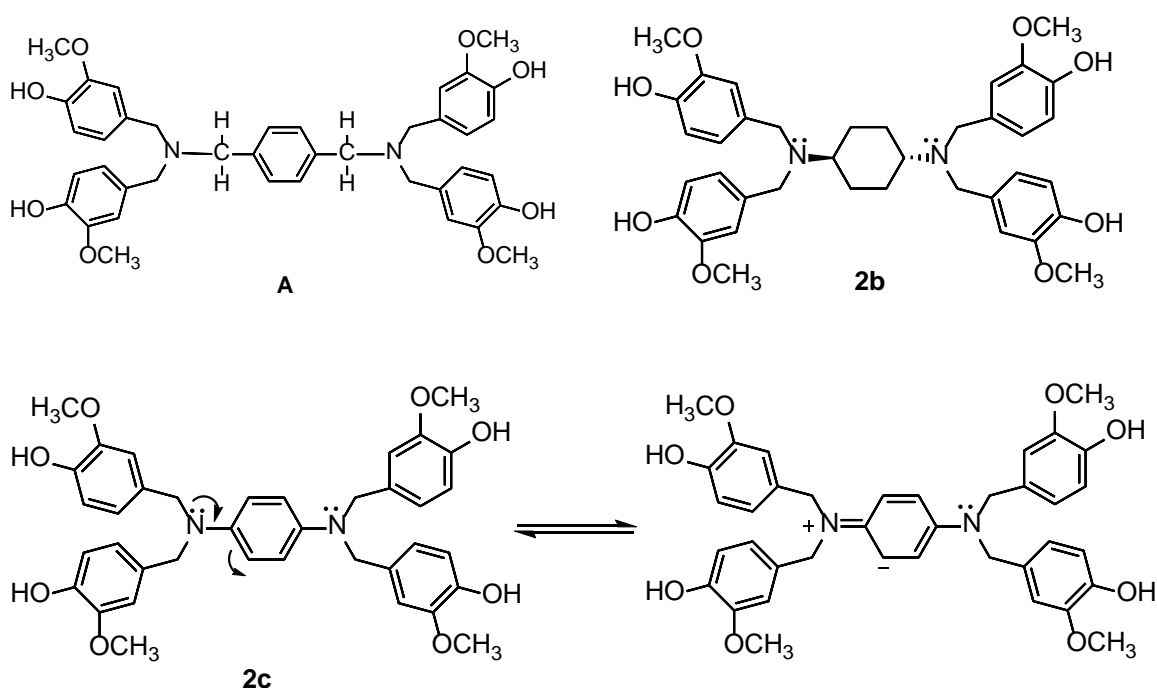


Figure 4. Benzyl Vanillin derivative, **A** [19] and Phenyl Vanillin derivatives, **2b** and **2c**. The nitrogen's electron pairs cannot be delocalized in the cyclic system nearby (compound **A** and **2b**). However, delocalization is possible for compound **2c**.

In a more complex cell based assay both **1b** and **2c** demonstrated cellular protection of  $\text{H}_2\text{O}_2$  treated neuroblastoma cells with **2c** exhibiting some protection at drug concentration as low as 0.01  $\mu\text{M}$ . Furthermore **1b** and **2c** showed an increase in the cell viability of almost 20% and 30% at concentrations of 200  $\mu\text{M}$  and 10  $\mu\text{M}$  respectively. Our results showed similar antioxidant effects when compared to huperzine A (10  $\mu\text{M}$ ), a naturally occurring sesquiterpene alkaloid compound. The latter is a cholinesterase inhibitor well-known for its protective effects on hydrogen peroxide-stressed cells and is currently undergoing phase I trial for Alzheimer disease treatment. [31-32]

## Conclusions

A structurally related series of vanillin derivatives were synthesized using reductive amination methodology. The yields of the reaction ranged from 12-90 %. Depending on the assay used, the majority of the compounds exhibited from good to very good antioxidant properties. Based on the results we have presented so far, the mechanism of DNA or/and cellular protection can either be *via* direct antioxidant activity or by upregulation of antioxidant defences. In the case of DNA protection assay, since this is an *in vitro* set up, most likely, the compounds (**2c** and **1b**) tested, acted as radical scavenging agent. However in the cellular assay, both mechanisms are possible. We are currently addressing these important questions at cellular levels and our findings will be reported elsewhere. Taken together the vanillin derivatives reported in this work can provide a viable platform in the development of antioxidant based therapeutics. [33]

## Experimental Section

All the reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific unless otherwise stated. All  $^1\text{H}$  and  $^{13}\text{C}$  spectra were collected using a Magnet Ultrashield Bruker 400 MHz. High-resolution Mass spectrometry was performed at EPSRC National Mass Spectrometry Service Centre at Swansea University, Swansea using Thermo Scientific LTQ Orbitrap XL or Waters Xevo G2-S spectrometers. The progress of each reaction was monitored by thin layer chromatography (TLC Aluminium foil silica gel 60 with fluorescence indicator 254 nm, Sigma-Aldrich) viewed under UV light (254-265 nm). Column chromatography was carried using silica gel (Alfa Aesar 70-230 mesh) as stationary phase and chloroform/methanol as mobile phase. The chemical drawing and nomenclature of the compounds were applied according to ChemBioDraw Ultra version 13.0 (CambridgeSoft). Neuroblastoma SH-SY5Y cells were from the European Collection of Authenticated Cell Cultures (ECACC). Cells were maintained in DMEM medium (containing GlutaMAX-1 with 25 mM HEPES), supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (10,000  $\mu\text{g}/\text{mL}$ ). Cells were grown at 37°C (5%  $\text{CO}_2$ ). GelRed™ Nucleic Acid Gel Stains 10000x in water (Biotium) was from VWR.



## General Method

All the Vanillin derivatives (**1a-1j**, **2a-2e**) were obtained through the reductive amination in a one pot reaction as previously reported (with modifications). [34] In general a mixture of Vanillin, Syringaldehyde or Benzaldehyde and the corresponding amine or diamine were dissolved in methanol followed by the addition of NaCNBH<sub>3</sub> and ZnCl<sub>2</sub>. The reaction mixture was stirred overnight at room temperature. After the reaction was completed (monitored by tlc), it was evaporated to dryness using a rotary evaporator, then the residue was dissolved in chloroform followed by extraction with saturated NaHCO<sub>3</sub> (x 3). The organic fraction was dried with anhydrous sodium sulphate, filtered and the solvent removed under *vacuo* to yield the final products. In some cases as indicated in the method section, the crude products were directly subjected to column chromatography (CHCl<sub>3</sub>/ MeOH 95:5) to give pure products. In other reactions, when the reaction was complete, the product precipitated out of solution. The resulting precipitate was collected by filtration, washed thoroughly and dried under *vacuo*.

### *Synthesis of 4,4'-(((2-hydroxyethyl)azanediyl)bis(methylene))bis(2-methoxyphenol)- 1a*

Vanillin (1g, 6.6 mmol) and 2-aminoethanol (0.30 ml, 5 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1a** in 12 % yield as a thick orange oil after extraction with CHCl<sub>3</sub> and saturated NaHCO<sub>3</sub>. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.21), 6.76-6.67 (m, Ar-H, 6H), 3.75 (s, -OCH<sub>3</sub>, 6H), 3.50 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH, *J* = 5.6 Hz, 2H), 3.45 (s, Ar-CH<sub>2</sub>-N, 4H), 2.57 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 5.6 Hz, 2H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.4-76.8), 146.8-111.2, 58.5, 57.9, 56.0, 54.6. HRMS calcd for C<sub>18</sub>H<sub>24</sub>NO<sub>5</sub> [M+H]<sup>+</sup> 334.1649, *m/z* found 334.1648.

### *Synthesis of 4,4'-(((3-hydroxypropyl)azanediyl)bis(methylene))bis(2-methoxyphenol)- 1b*

Vanillin (1 g, 6.6 mmol) and 3-amino-1-propanol (0.326 ml, 5 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1b** in 70% yield as a thick orange oil after column chromatography of the crude. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.21), 6.82-6.72 (m, Ar-H, 6H), 3.83 (s, -OCH<sub>3</sub>, 6H), 3.63 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH, *J* = 5.2 Hz, 2H), 3.45 (s, Ar-CH<sub>2</sub>-N, 4H), 2.60 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 5.6 Hz, 2H), 1.72 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.6-74.8), 147.0-110.7, 64.3, 58.4, 55.9, 53.3, 27.4. HRMS calcd for C<sub>19</sub>H<sub>26</sub>NO<sub>5</sub> [M+H]<sup>+</sup> 348.1805, *m/z* found 348.1801.

### *Synthesis of 4,4'-(((4-hydroxybutyl)azanediyl)bis(methylene))bis(2-methoxyphenol)- 1c*

Vanillin (1 g, 6.6 mmol) and 4-amino-1-butanol (0.461 ml, 5 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1c** in 14 % yield as a thick orange oil after extraction with CHCl<sub>3</sub> and saturated NaHCO<sub>3</sub>. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.21), 6.83-6.70 (m, Ar-H, 6H), 3.80 (s, -OCH<sub>3</sub>, 6H), 3.65 (s, Ar-CH<sub>2</sub>-N, 4H), 3.51 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH, *J* = 5.6 Hz, 2H), 2.64 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 5.6 Hz, 2H), 2.38 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.60 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H). <sup>13</sup>CNMR: (DMSO solvent peak δ: 40.6-39.3), 147.8-112.8, 60.5, 56.1, 46.8, 29.9, 23.3. HRMS calcd for C<sub>20</sub>H<sub>28</sub>NO<sub>5</sub> [M+H]<sup>+</sup>. 362.1962, *m/z* found 362.1962.

*Synthesis of 4,4'-(((5-hydroxypentyl)azanediyl)bis(methylene))bis(2-methoxyphenol)- 1d*

Vanillin (1 g, 6.6 mmol) and 5-amino-1-pentanol (0.326 ml, 5 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1d** in 82% yield as thick orange oil after column chromatography. <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ:2.52-2.50), (H<sub>2</sub>O 3.38), 6.90-6.78 (m, Ar-H, 6H), 3.99 (s, Ar-CH<sub>2</sub>-N, 4H), 3.75 (s, -OCH<sub>3</sub>, 6H), 2.81 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH, *J* = 6.4 Hz, 2H), 2.50 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 6.4 Hz, 2H), 1.63 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.41 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.35 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H). <sup>13</sup>CNMR: 147.7-113.8, 61.2, 57.2, 55.9, 50.7, 46.5, 30.6, 25.2, 22.2. HRMS calcd for C<sub>21</sub>H<sub>23</sub>O<sub>5</sub> [M+H]<sup>+</sup> 376.2118, *m/z* found 376.2108.

*Synthesis of 4,4'-(((6-hydroxyhexyl)azanediyl)bis(methylene))bis(2-methoxyphenol)- 1e*

Vanillin (1 g, 6.6 mmol) and 6-amino-1-hexanol (0.59g, 5 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1e** in 17 % yield as thick orange oil after extraction with CHCl<sub>3</sub> and saturated NaHCO<sub>3</sub>. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.22), 6.83-6.62 (m, Ar-H, 6H), 3.76 (s, -OCH<sub>3</sub>, 6H), 3.48 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH, *J* = 6.8 Hz, 2H), 3.38 (s, Ar-CH<sub>2</sub>-N, 4H), 2.68 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 6.8 Hz, 2H), 1.62 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.56 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.32 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.28 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.5-76.9), 147.2-110.1, 67.8, 61.4, 58.2, 55.2, 33.0-27.3. HRMS calcd for C<sub>22</sub>H<sub>32</sub>NO<sub>5</sub> [M+H]<sup>+</sup> 390.2275, *m/z* found 390.2272.

*Synthesis of 4,4'-((butylazanediyl)bis(methylene))bis(2-methoxyphenol)- 1f*

Vanillin (1 g, 6.6 mmol) and n-butylamine (0.5ml, 5mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded **1f** in 23 % yield as a thick orange oil after extraction with CHCl<sub>3</sub> and saturated NaHCO<sub>3</sub>. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.20), 6.81-6.63 (m, Ar-H, 6H), 3.73 (s, Ar-CH<sub>2</sub>-N, 4H), 3.64 (s, -OCH<sub>3</sub>, 6H), 2.57 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 7.2 Hz, 2H), 1.42 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H), 1.25 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>, 2H), 0.94 (t, CH<sub>2</sub>-CH<sub>3</sub>, *J* = 6.8

Hz, 3H).  $^{13}\text{CNMR}$ : ( $\text{CDCl}_3$  solvent peak  $\delta$ : 77.8-76.7), 146.6-110.8, 55.8, 49.2, 32.2-20.5. HRMS calcd for  $\text{C}_{20}\text{H}_{27}\text{NO}_4$   $[\text{M}+\text{H}]^+$  346.2013,  $m/z$  found 346.2013.

*Synthesis of 4,4'-((benzylazanediy)bis(methylene))bis(2-methoxyphenol)- 1g*

Vanillin (1.5 g, 10mmol) and benzylamine (0.55 ml, 5 mmol) in presence of  $\text{NaCNBH}_3$  (1.2 equiv.) and  $\text{ZnCl}_2$  (1.2 equiv.) in methanol gave a precipitate which after filtration, washing with deionized water and methanol, afforded **1g** in 90 % yield.  $^1\text{HNMR}$ : ( $(\text{CD}_3)_2\text{SO}$  solvent peak  $\delta$ :2.42-2.40), ( $\text{H}_2\text{O}$ , 3.26), 7.30-6.59 (m, Ar-H, 11H), 3.67 (s, -OCH<sub>3</sub>, 6H), 3.56 (s, Ar-CH<sub>2</sub>-N, 2H), 3.47 (s, Ar-CH<sub>2</sub>-N, 4H),  $^{13}\text{CNMR}$ : ( $(\text{CD}_3)_2\text{SO}$  solvent peak  $\delta$ : 40.6-39.3), 141.34-115.5, 55.9, 52.5, 45.6. HRMS calcd for  $\text{C}_{23}\text{H}_{26}\text{NO}_4$   $[\text{M}+\text{H}]^+$  380.1856,  $m/z$  found 380.1849.

*Synthesis of  $N^1,N^1$ -bis(4-hydroxy-3-methoxybenzyl)propane-1,3-diaminium chloride- 1h*

Vanillin (1 g, 6.6 mmol) and 1,3 diaminopropane (0.84 ml, 10 mmol) in presence of  $\text{NaCNBH}_3$  (1.2 equiv.) and  $\text{ZnCl}_2$  (1.2 equiv.) in methanol afforded a precipitate which was filtered and washed with deionized water and methanol. Suspension of the precipitate in methanol (5 mL) followed by the addition of concentrated hydrochloric acid, gave a solid. All the solvent was removed using a rotary evaporator and the resulting solid washed with diethyl ether and dried under *vacuo* to yield **1h** (67%) as a white solid.  $^1\text{HNMR}$ : ( $(\text{CD}_3)_2\text{SO}$  solvent peak  $\delta$ :2.51) ( $\text{H}_2\text{O}$ , 3.64), 7.12-6.79 (Ar-H, 6H), 4.00 (s, Ar-CH<sub>2</sub>-N, 4H), 3.78 (s, -OCH<sub>3</sub>, 6H), 2.95 (t, N-CH<sub>2</sub>-CH<sub>2</sub>,  $J = 7.6$  Hz, 2H), 2.87 (t, CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>3</sub><sup>+</sup>  $J = 7.6$  Hz, 2H), 1.92 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H).  $^{13}\text{CNMR}$  147.6-113.8, 55.9, 51.0, 43.6, 36.5, 23.7. HRMS calcd for  $\text{C}_{19}\text{H}_{26}\text{ClN}_2\text{O}_4$  347.1965  $[\text{M}-\text{H}_2\text{Cl}]^+$ ,  $m/z$  found 347.1965.

*Synthesis of 4,4'-(((3-hydroxypropyl)azanediy)bis(methylene))bis(2,6-dimethoxyphenol)- 1i*

Syringaldehyde (0.5 g, 2.7 mmol) and 3-amino-1-propanol (0.1 ml, 1.7 mmol) in presence of  $\text{NaCNBH}_3$  (1.2 equiv.) and  $\text{ZnCl}_2$  (1.2 equiv.) in methanol afforded **1b** in 65% yield as thick colourless oil after column chromatography.  $^1\text{HNMR}$ : (MeOD solvent peak  $\delta$ :3.34), 6.81 (s, Ar-H, 4H), 4.17 (s, Ar-CH<sub>2</sub>-N, 4H), 3.84 (s, -OCH<sub>3</sub>, 12H), 3.73 (t, CH<sub>2</sub>-CH<sub>2</sub>-OH,  $J = 5.6$  Hz, 2H), 3.23 (t, N-CH<sub>2</sub>-CH<sub>2</sub>,  $J = 5.6$  Hz, 2H), 1.97 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H),  $^{13}\text{CNMR}$ : (MeOD solvent peak  $\delta$ : 48.2), 149.0-106.8, 59.4, 55.7, 51.6, 45.6, 28.2. HRMS calcd for  $\text{C}_{21}\text{H}_{30}\text{NO}_7$   $[\text{M}+\text{H}]^+$  408.2017,  $m/z$  found 408.2012.

*Synthesis of 4,4'-((benzylazanediy)bis(methylene))bis(2,6-dimethoxyphenol)- 1j*

Syringaldehyde (1 g, 5.4 mmol) and benzylamine (0.2 ml, 1.8 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol, gave a precipitate which was filtered and washed with deionized water and methanol and dried to afford **1j** in 46 % yield. <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ:2.40), (H<sub>2</sub>O, 3.25), 7.28-6.48 (m, Ar-H, 9H), 3.64 (s, -OCH<sub>3</sub>, 12H), 3.56 (s, Ar-CH<sub>2</sub>-N, 4H), 3.46 (s, Ar-CH<sub>2</sub>-N, 2H). <sup>13</sup>CNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ: 40.6-39.3), 140.3-127.0, 56.3, 45.4. HRMS calcd for C<sub>25</sub>H<sub>30</sub>NO<sub>6</sub> [M+H]<sup>+</sup> 440.2058, m/z found 440.2058.

*Synthesis of N<sup>1</sup>,N<sup>1</sup>,N<sup>4</sup>,N<sup>4</sup>-tetrakis(3-methoxybenzyl)benzene-1,4-diamine – 2a*

m-Anisaldehyde (0.82 g, 6 mmol) and p-Phenylendiamine (0.1 g, 1 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded a precipitate which upon filtration, washed with deionized water and methanol and drying gave **2a** in 54 % yield. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ: 6.81), 7.38-7.33 (m, Ar-H, 4H), 7.01-6.89 (m, Ar-H, 16H), 4.62 (s, Ar-CH<sub>2</sub>-N, 8H) 3.87 (s, -OCH<sub>3</sub>, 12H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.6-77.0), 160.0-112.2, 55.5, 55.2. HRMS calcd for C<sub>38</sub>H<sub>41</sub>N<sub>2</sub>O<sub>4</sub> [M+H]<sup>+</sup> 589.3061, m/z found 589.3047.

*Synthesis of 4,4',4'',4'''-(((1r,4r)-cyclohexane-1,4-diyl)bis(azanetriyl))tetrakis(methylene))tetrakis(2-methoxyphenol) – 2b*

Vanillin (1.82 g, 12 mmol) and trans-1,4-diaminocyclohexane (0.2 g, 2 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded a precipitate which after filtration and washing with methanol to give a solid. The latter was dissolved in water, filtered and extracted with chloroform. The water phase was subjected to freeze drying to yield **2b** (18 %). <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ:2.40), (H<sub>2</sub>O, 3.26), 7.10-6.67 (m, Ar-H, 12H), 3.88 (s, Ar-CH<sub>2</sub>-N, 8H), 3.68 (s, -OCH<sub>3</sub>, 12H), 2.79 (m, N-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>2</sub>, 2H), 2.10 (m, CH-CH<sub>2</sub>-CH<sub>2</sub>, 4H), 1.30 (m, CH-CH<sub>2</sub>-CH<sub>2</sub>, 4H). <sup>13</sup>CNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ: 39.9-39.0), 148.0-114.4, 56.2, 55.0, 48.2, 27.3. HRMS calcd for C<sub>38</sub>H<sub>47</sub>N<sub>2</sub>O<sub>8</sub> [M+H]<sup>+</sup> 659.3327, m/z found 659.3327.

*Synthesis of 4,4',4'',4'''-((1,4-phenylenebis(azanetriyl))tetrakis(methylene))tetrakis(2-methoxyphenol) – 2c*

Vanillin (1 g, 6.6 mmol) and p-Phenylenediamine (0.1 g, 1 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded a precipitate which was filtered and washed with deionized water and methanol. The solid was then dissolved in chloroform and extracted with saturated NaHCO<sub>3</sub>. The organic phase was dried and evaporated to dryness to give **2c** in 74 % yield. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ: 7.30), 6.88-6.76 (m, Ar-H, 16H), 4.39

(s, Ar-CH<sub>2</sub>-N, 8H), 3.83 (s, -OCH<sub>3</sub>, 12H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.4-76.7), 164.5-109.9, 55.9, 55.1. HRMS calcd for C<sub>38</sub>H<sub>41</sub>N<sub>2</sub>O<sub>8</sub> [M+H]<sup>+</sup> 653.2857, *m/z* found 653.2851.

*Synthesis of N<sup>1</sup>,N<sup>1</sup>,N<sup>4</sup>,N<sup>4</sup>-tetrabenzylbenzene-1,4-diamine – 2d*

Benzaldehyde (1.27 g, 12 mmol) and p-Phenylenediamine (0.2 g, 2 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded a precipitate which was filtered and washed with deionized water and methanol. The solid was then dissolved in chloroform and extracted with saturated NaHCO<sub>3</sub>. The organic phase was dried and evaporated to dryness to afford **2d** in 80 % yield. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ: 7.25), 7.40-7.25 (m, Ar-H, 20H), 6.68 (s, Ar-H, 4H), 4.55 (s, Ar-CH<sub>2</sub>-N, 8H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.4-76.7), 141.7-114.77, 55.1. HRMS calcd for C<sub>34</sub>H<sub>32</sub>N<sub>2</sub> [M]<sup>+</sup> 468.2560, *m/z* found 468.2550.

*Synthesis of N<sup>1</sup>,N<sup>1</sup>,N<sup>3</sup>,N<sup>3</sup>-tetrakis(4-hydroxy-3-methoxybenzyl)propane-1,3-diaminium chloride – 2e*

Vanillin (1.8 g, 12 mmol) and 1,3 daminopropane (0.17 ml, 2 mmol) in presence of NaCNBH<sub>3</sub> (1.2 equiv.) and ZnCl<sub>2</sub> (1.2 equiv.) in methanol afforded a precipitate. The latter was washed with deionized water and methanol. The resulting solid was suspended in methanol (5 mL), followed by the addition concentrated hydrochloric acid. All the solvent was removed by rotary evaporator and the solid obtained, was washed with diethyl ether to give **2e** in 38% yield. <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO +D<sub>2</sub>O solvent peak δ:2.51), (H<sub>2</sub>O, 4.03), 7.05-6.78 (m, Ar-H, 12H), 3.99 (s, Ar-CH<sub>2</sub>-N, 8H), 3.76 (s, -OCH<sub>3</sub>, 12H), 2.96 (t, N-CH<sub>2</sub>-CH<sub>2</sub>, *J* = 2.4 Hz, 4H), 1.98 (m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>, 2H). <sup>13</sup>CNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ: 39.8-38.6), 149.0-114.2, 56.1, 50.7, 43.8, 22.5. HRMS calcd for C<sub>35</sub>H<sub>44</sub>ClN<sub>2</sub>O<sub>8</sub> [M-H<sub>2</sub>Cl]<sup>+</sup> 619.3014, *m/z* found 619.3002 [M-H<sub>2</sub>Cl]<sup>+</sup>.

*Synthesis of 4,4',4'',4'''-((1,4-phenylenebis(azanetriyl))tetrakis(methylene))tetraphenol – 2f*

The reaction between 4 hydroxybenzaldehyde (1.5 g, 12 mmol) and p-Phenylenediamine (0.2 g, 2 mmol) in methanol was evaporated to dryness. The resulting solid was washed thoroughly with water, chloroform and after drying, gave **2f** in 66%. <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ:2.52-2.50), (H<sub>2</sub>O, 3.37), 9.23 (s, Ar-OH, 4H), 7.02-6.55 (m, Ar-H, 20 H), 4.27 (s, Ar-CH<sub>2</sub>-N, 8H). <sup>13</sup>CNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ: 40.6-39.3), 156.5-115.5, 54.8. HRMS calcd for C<sub>34</sub>H<sub>33</sub>N<sub>2</sub>O<sub>4</sub> [M+H]<sup>+</sup> 533.2435, *m/z* found 533.2427.

*Synthesis of 4-((benzylimino)methyl)-2-methoxyphenol - 3*

Vanillin (1g, 6.6 mmol) was reacted with benzylamine (1.07 g, 10 mmol) in methanol overnight at room temperature. Once the reaction was completed, it was dissolved in chloroform followed by extraction with saturated NaHCO<sub>3</sub> solution. The organic phase was collected, dried with anhydrous sodium sulphate and evaporated to dryness to afford **3** in 63% yield. <sup>1</sup>HNMR: (CDCl<sub>3</sub> solvent peak δ:7.38), 8.32 (s, Ar-CH=N, 1H), 7.54-6.95 (m, Ar-H, 8H), 4.85 (s, Ar-CH<sub>2</sub>-N, 2H), 3.93 (s, -OCH<sub>3</sub>, 3H). <sup>13</sup>CNMR: (CDCl<sub>3</sub> solvent peak δ: 77.4-76.7), 161.8, 148.4-108.3, 64.9, 56.1. HRMS calcd for C<sub>15</sub>H<sub>16</sub>NO<sub>2</sub> [M+H]<sup>+</sup> 241.1176, *m/z* found 242.1170.

#### *Synthesis of 4-((benzylamino)methyl)-2-methoxyphenol – 4*

Compound **3** (0.7 g, 3 mmol) and sodium borohydride (0.11 g, 3 mmol) in methanol were stirred for 2 hours. Evaporation of the solvent yielded a solid which was washed thoroughly with deionized water and dried under *vacuo* to afford **4** in 68% yield as a pale yellow solid. <sup>1</sup>HNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ:2.42-2.40), (H<sub>2</sub>O, 3.25), 7.33-6.62 (m, Ar-H, 8H), 3.86 (s, Ar-CH<sub>2</sub>-N, 2H), 3.65 (s, -OCH<sub>3</sub>, 3H), 3.55 (s, Ar-CH<sub>2</sub>-N, 2H). <sup>13</sup>CNMR: ((CD<sub>3</sub>)<sub>2</sub>SO solvent peak δ: 40.6-39.4), 147.9-112.9, 56.0, 52.2. HRMS calcd for C<sub>15</sub>H<sub>18</sub>NO<sub>2</sub> [M+H]<sup>+</sup> 244.1332, *m/z* found 244.1333.

### **Antioxidant activity**

#### *DPPH Assay*

The ability of the new compounds to scavenge DPPH free radical was determined by following Payet *et al.* [23] procedure with minor modifications on a 96-well platform. A dilution series of antioxidant ranging from 1.5 to 1000 μM was made in eppendorf tubes (1.5 mL). Those solutions (50 μl) were transferred to the corresponding well while methanol (50 μl) was used as negative control wells. Thereafter DPPH solution (100 μl, 0.1 mM) were added to each well and the 96-well plate was kept in the dark for 30 minutes after which the absorbance was measured at 517 nm using a Bio-Rad iMark microplate reader.

#### *FRAP Assay*

The reducing capacity of the new compounds was measured using FRAP assay following the method described by Firuzi *et al.* [25], with minor modifications on a 96-well plate. A dilution series of antioxidant and Trolox ranging from 500 to 5000 μM was made in eppendorf tubes, then each solution (10 μl) was pipetted in the corresponding well in a 96 well-plate. FRAP reagent (190 μl), prepared by mixing 2.5 ml of 10 mM TPTZ solution (in 40 mM HCl), 2.5 ml

of 20 mM FeCl<sub>3</sub> (in deionized water) and 25 ml of 300 mM sodium acetate buffer (pH 3.6) was added in each well. The plate was stored in the dark for 30 minutes before the absorbance was measured at 593 nm using a Bio-Rad iMark microplate reader.

#### *ORAC Assay*

The ability of the new compounds to prevent the oxidative degradation of the fluorescent molecule fluorescein was measured using ORAC assay following the methods described by Roy *et al.* [35] and Huang *et al.* [36] with minor changes on a black-walled 96-well plate. A dilution series of antioxidant and Trolox was made in eppendorf tubes. 25 µl of solution from each tubes were pipetted in the corresponding well in the plate whereas phosphate buffer (25 µl, 75 mM, pH 7.4) were transferred in the control wells. Fluorescein solution (150 µl, 25 nM) were added in each well and the plate was incubated in the plate reader at 37 °C for 30 minutes. Subsequently AAPH solution (25 µl, 0.15 M) was added in the positive control and in the antioxidant wells whereas phosphate buffer (25 µl) was pipetted in the negative control and the fluorescence was measured every 2 minutes over a period of 2 hours (485/20 nm excitation, 525/20 nm emission) using a BioTek Synergy HT microplate reader.

#### *DNA Damage Protection Assay*

The ability of the novel derivatives to prevent oxidative stress-mediated strand breakage in supercoiled DNA plasmid was measured following the method previously described by us [37] and others [38] with minor changes. 0.7% agarose gel (50 mL, TAE buffer). Gel red dye (5 µl) was used as DNA stain. A dilution series of antioxidant was made in 6 different eppendorf tubes depending on their protective effect. Plasmid pbr322 DNA was used (0.5 µg/µl, PBS). 8 different solutions were prepared in eppendorf tubes; the negative control (1 µl of DNA plasmid solution and 22 µl PBS); the positive control (1 µl of DNA plasmid solution, 14 µl of PBS and 8 µl of 10 mM AAPH solution) and the working solutions (1 µl of DNA plasmid solution, 6 µl of PBS, 8 µl of 10 mM AAPH solution and 8 µl of antioxidant solution at different concentrations (refer to **SI** for concentrations used for each compound). The tubes were incubated in the dark at 37°C for 1 hour. 2 µl of loading dye (0.5% bromophenol blue, 50% glycerol in deionized water) were added in each tube and mixed thoroughly. Finally, 10 µl of each solution was loaded in each well of the agarose gel (0.7%) and it was electrophoresed for 70 minutes at 80 volts. The gels were analysed with a Fusion FX7 system for chemiluminescence and fluorescence and a picture was taken with a high resolution camera system.

### *Cell viability in the presence of H<sub>2</sub>O<sub>2</sub> stressor using the MTT Cell Assay*

SH-SY5Y cells were seeded ( $7 \times 10^3$  cells/well) in a 96-well plate. After attachment (24 hr), cells were treated with different concentrations (0.1-200.0  $\mu$ M for **1b** and 0.001-10.0  $\mu$ M for **2c**) of drug and incubated for 24 hours. Subsequently H<sub>2</sub>O<sub>2</sub> solution (50  $\mu$ l, 1.6 mM) were then added in the positive control and in the drug wells (to give a final concentration of 400  $\mu$ M) followed by incubation for another 24 hours. Then, all the solutions were pipetted off from all the wells and 100  $\mu$ l of MTT solution (0.5 mg/ml) were added in each well. The plate was covered with aluminium foil and incubated for 4 hours at 37 °C. The solutions in each well were removed and 100  $\mu$ l of DMSO were added in each well in order to dissolve the formazan crystals. After 20 minutes of gentle shaking, absorbance was measured at 490 nm with a Bio-Rad iMark microplate reader. Cell viability was expressed as a percentage of the absorbance from control cells.

### **Statistical analysis**

Data are shown as mean  $\pm$  standard deviation (SD) and all the experiments were run at least in triplicate. Statistical analysis was performed using both Microsoft Excel 2013 and GRAPH PAD prism 4 using ONE WAY ANOVA and Bonferroni's multiple comparison test. Significant differences are labelled accordingly (ns- not significant,  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ).

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### **Abbreviations used**

AAPH- 2,2'-azobis (2-amidinopropane) dihydrochloride, AUC- area under the curve, BHA- butylhydroxyaniline, BHT- butylhydroxytoluene, DMSO- dimethyl sulfoxide, DPPH- Diphenyl-1-picrylhydrazyl, FRAP- Ferric reducing ability of plasma, MTT- Methylthiazolyldiphenyl-tetrazolium bromide, ORAC- Oxygen radical absorbance capacity,



PBS- phosphate buffer saline, ROS- Reactive oxygen species, TE- Trolox equivalent, TPTZ- 2,4,6-tripyridyl-s-triazine.

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