Pergamon

Tetrahedron 57 (2001) 1277–1282

TETRAHEDRON

Synthesis of polyhydroxylated aromatic mandelic acid amides and their antioxidative potential

Jakob P. Ley* and Heinz-Jürgen Bertram

Haarmann & Reimer GmbH, Flavor Division, Research and Development Flavors, Synthesis New Flavor Ingredients, P.O. Box 1253, D-37601 Holzminden, Germany

Received 15 November 2000; accepted 4 December 2000

Abstract—Six hydroxymandelic acid amides of phenolic amines were synthesized by condensation of 3,4-dihydroxymandelic or 4-hydroxy-3-methoxymandelic acid *N*-succinimidyl esters and several phenolic benzylamino and phenethylamino hydrochlorides in moderate to good yield. The radical scavenging activities determined by 2,2-diphenyl-1-picrylhydrazyl assay and superoxide trapping assay were superior compared to standards L-ascorbic acid, α-tocopherol, and butylated hydroxytoluene (BHT). The antioxidative activities tested by accelerated autoxidation of bulk lipids were 2–3.5 times more potent compared to standards α-tocopherol and BHT. 3,4-Dihydroxymandelic acid dopamide (**4b**) and 4-hydroxy-3-methoxymandelic acid dopamide (**4a**) showed the best overall performance as antioxidants.

1. Introduction

Antioxidants play a crucial role in organisms or tissues like human skin as well as in non-living systems like foodstuffs. In man, photoinduced oxidative stress is suspected as the main cause of skin ageing.[1–3] In addition, it is assumed that many severe diseases like arteriosclerosis, cataract formation and cancer are caused or accelerated by oxidative stress, which can be generated by natural and artificial sources.[4–6]

A complex antioxidant defense system has evolved in organisms, which protects against reactive oxygen species (ROS). However, a high level of ROS can tilt the balance toward a prooxidant state. The resulting oxidative stress is responsible for the damage of cellular molecules, especially DNA, proteins and unsaturated lipids, e.g. human sebum lipid squalene is oxidized to its corresponding hydroperoxide. This situation leads to the degeneration of cellular and interstitial structures and, therefore, to apoptosis or necrosis of cells or tissue. Finally, the organs develop pathological conditions such as the sagging or wrinkling of skin, lesions of blood vessels, clouding of eye lens and even cancer.

In cells, ROS are formed by different pathways. For example, mediated by photosensitizers (e.g. porphyrins), UVA radiation generates singlet oxygen or superoxide (SO) radical anions from triplet oxygen.[7] Additionally, SO radical anions are formed as intermediates in the respiratory chain. The SO can be accidentally liberated from mitochondria.

Under physiological conditions, the SO is converted mainly by SO dismutase into hydrogen peroxide, which is degraded by catalase or by glutathione peroxidase. Alternatively, hydrogen peroxide can be converted to the highly aggressive hydroxyl radical by heavy metal ions, e.g. Fe^{2+} (Fenton reaction). Hydroxyl radicals are especially responsible for oxidative damage of the lipids in cell membranes or intercellular lipids. The hydroperoxides of the lipids are able to propagate the radical chain mechanism of lipid autoxidation.

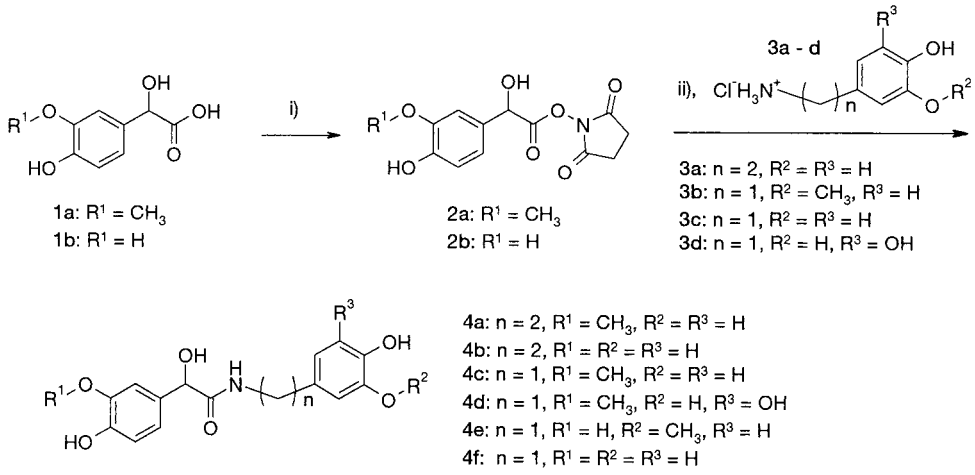
The most important molecular antioxidants in mammals are α-tocopherol, L-ascorbic acid, ubiquinone, lipoic acid and glutathione, which are able to scavenge the ROS directly. The highly reactive hydroxyl radical is also trapped by many other organic cell compounds, e.g. glucose and proteins.

If cells are exposed to strong oxidative stress, the high amount of ROS depletes the endogenous molecular radicals that accelerate the development of cell damage.[8] It is assumed that the administration of antioxidants or radical scavengers to tissues like human skin can shift the antioxidative balance back towards protection against oxidative stress. In fact, recent clinical studies showed that topically applied antioxidants were able to prevent skin lipid peroxidation and photoageing.[9,10] For arteriosclerosis, some facts suggest that dietary antioxidants like tocopherols and flavonoids prevent or retard the development of typical sclerotic lesions of blood vessels.[6]

Keywords: antioxidants; radical scavengers; hydroxymandelic acid amides; polyphenols.

* Corresponding author. Fax: +49-5531-903883; e-mail: jakob.ley.jl@hr-gmbh.de

0040–4020/01/$ - see front matter
PII: S0040-4020(00)01136-4



Scheme 1. Synthesis of hydroxymandelic acid amides **4a**–**4f**. (i) 1,4-Dioxan, NHOSu (1 equiv.), DCC (1 equiv.), rt, 16 h; (ii) H_2O, $NaHCO_3$ (1 equiv.), 1 h, 50°C, then HCl (5% w/w).

Antioxidants are added to food mainly to inhibit autoxidation of lipids or proteins. In particular, the autoxidation of lipids generates typical off-flavors in many foodstuffs. The long chain polyunsaturated fatty acids are degraded to aliphatic C-9 or C-6 aldehydes like (*E*)-2-nonenal, (2*E*,4*E*)-2,4-nonadienal and different hexenals like *n*-hexanal, which cause the characteristic rancid flavor of oxidized fats and oils.[11]

In our efforts to develop new antioxidants for cosmetic or food use we have synthesized and investigated new phenolic acid amides of different phenolic amines as potent antioxidants.[12,13] As most of these amides showed a limited water solubility (<0.1%), we decided to prepare the hydroxymandelic acid amides of phenolic amines as more hydrophilic derivatives.

2. Results and discussion

2.1. Synthesis

In the literature, 3,4-dihydroxymandelic or 4-hydroxy-3-methoxymandelic acid amides of phenolic amines have not been described so far. In our earlier studies regarding phenolic acid amides, we used protected active esters for synthesis.[12] The classical coupling of acid chlorides with amines in basic mixtures was not applicable because self-condensation of the phenolic acids occurred. The phenolic acids, e.g. caffeic acid, were protected at the phenolic hydroxyl moieties by methoxycarbonyl groups and esterified with *N*-hydroxysuccinimide (NHOSu) by *N*,*N*′-dicyclohexylcarbodiimide (DCC) methodology. The active esters were isolated, purified, and subsequently condensed with phenolic amines at pH 8–9.

For the synthesis of the new hydroxymandelic acid amides **4a**–**4f**, we have used a variation of this method (cf. Scheme 1). The active succinimidyl esters **2a** and **2b** were prepared directly from the non-protected hydroxymandelic acids **1a** and **1b** by the NHOSu/DCC method in 1,4-dioxan as solvent. The reaction mixtures were only filtered to remove the precipitated *N*,*N*′-dicyclohexylurea (DCU) and the filtrates were treated immediately with the appropriate phenolic amino hydrochlorides **3a**–**3d** in aqueous solution.

3,4,5-Trihydroxybenzylamino hydrochloride (**3d**), which is not available commercially, was obtained from 3,4,5-trihydroxybenzaldehyde via the benzaldoxime and subsequent Pd–C-catalysed reduction by hydrogen in hydrochloric acid containing ethanol.[12]

The isolated yield of hydroxymandelic acid amides **4a**–**4f** varied widely between 25 and 77%. In particular, the yield of the amide of 3,4,5-trihydroxybenzylamine (**4d**) was only moderate. In order to remove all the remaining DCU, the crude products have to be chromatographed in two steps: the first eluent used was ethyl acetate; in a second step, the solvent system chloroform/methanol was used for further purification. Especially in solution, the products are sensitive toward oxidation. Therefore, during reaction and isolation, precautions against oxygen have to be taken, such as degassing of solvents and inert gas.

For the chromatographed products, the purity detected by HPLC was higher than 95% (UV detection by a diode array detector). The amount of remaining DCU was determined by ^{1}H NMR spectroscopy because the urea showed no characteristic absorption in the UV detection system. The chromatographic purification steps yielded products with a DCU amount lower than the detection limit.

The hydroxymandelic acid amides **4a**–**4f** were identified by NMR, LC–MS and HRMS. For recording the ^{1}H NMR spectra in CD_3OD, a suppressing sequence in the range 4.9–5.0 ppm for the CD_3OH signal was used. Because the α-H signal of the mandelic acid moiety occurs in the same region (4.95 ppm), it was at least partially suppressed by the sequence and the integral was much too low. All other signals and their integrals in ^{1}H NMR spectra corresponded to the proposed structures. The characteristic ^{13}C NMR signal of mandelic acid α-C could be detected for each product from 74 to 75 ppm. The identification of HPLC peaks was performed by MS coupling measuring in atmospheric pressure chemical ionization (APCI) negative mode. The $[M-H]^-$ ions were found for all compounds. With the

Table 1. Results of evaluation of radical scavenging and antioxidative potential for hydroxymandelic acid amides **4a**–**4f**. Conditions for Rancimat assay: A, soybean oil, 100°C, 0.05%; B, evening primrose oil, 100°C, 0.05%; C, squalene, 80°C, 0.005%

	DPPH assay		SO assay, IC_{50}/nM	Rancimat assay AOI			Solubility (%), 5% DMSO in H_2O
	EC_{50}/mmol/mmol	s.s.[a]/h		A	B	C	
4a	0.13±0.01	24	150	13±1	7.7±0.5	28±1	5–10
4b	0.10±0.01	5	80	17±1	6.4±0.8	7.1±0.8	>10
4c	0.20±0.01	7	110	9.8±1.3	8.0±0.4	15±3	>10
4d	0.20±0.01	7	800	11±1	9.3±1.4	7.5±1.4	0.5–1
4e	0.20±0.01	7	140	12±1	4.4±0.6	13±1	0.05–0.1
4f	0.07±0.01	7	70	17±2	7.4±1.0	6.4±0.6	0.1–0.5
ascorbic acid	0.27±0.02	0.2	700	–	–	–	>10
α-tocopherol	0.25±0.02	0.3	–	5.1±0.1	4.6±1.3	39±4	<0.05
BHT	0.24±0.01	7	–	4.6±0.7	–	19±1	<0.05

[a] s.s.: steady state.

exception of **4c** and **4e**, the $[2M-H]^-$ ions could be detected, too. For identification, satisfactory HRMS spectra could be recorded. The difference between the calculated and observed molecular weights was below 1.3×10^{-3} mass units.

2.2. Antioxidative activity

The free radical scavenging action of the amides **4a**–**4f** was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay[14] and an SO radical trapping assay.[15] For performing the DPPH assay, a solution of the purple coloured DPPH radical was mixed with the test compound and the decrease of the absorption was determined photometrically until a steady state was reached. The results were calculated as the effective concentration EC_{50} of the test compound in relation to starting DPPH concentration, at which 50% of the DPPH radicals remained. The SO radical was generated in a hydrogen peroxide/horseradish peroxidase system. The SO radical was detected by recording chemiluminescence amplified by luminol. The SO scavenging activity of lipophilic test compounds such as α-tocopherol could not be measured correctly in the SO assay because of their poor solubility in the required aqueous solvent. Results were expressed as the inhibitor concentration IC_{50}, at which 50% of SO radicals had been trapped. L-Ascorbic acid was used as standard for both assays.

For the determination of the antioxidative potential for lipid systems, the accelerated autoxidation of bulk lipids by air was used.[12,16] The oxidation was carried out with or without test substance in the Rancimat apparatus with stripped soybean oil and stripped evening primrose oil at 100°C and squalene at 80°C. α-Tocopherol and butylated hydroxytoluene (BHT) were used as standards. Squalene was chosen as an example of a typical skin lipid component, soybean oil as an important nutritional fat, and evening primrose oil as a valuable cosmetic lipid, which is very sensitive towards oxidation. Commercial squalene was used after purification over alumina type N and stabilization with 1 ppm α-tocopherol. Oxidation of the unsaturated lipids proceeds only very slowly for the induction period (IP) and then suddenly increases when autoxidation accelerates. The IP of the stripped soybean oil at 100°C was between 2 and 3 h. Squalene stabilized with 1 ppm α-tocopherol showed IPs between 0.5 and 1 h at 80°C. The antioxidative indices (AOIs) were calculated by dividing the $IP_{test\ compound}$ by $IP_{control}$.[12] In soybean and evening primrose oil, we have used test concentrations of 0.05% (500 μg g^{-1}) and 0.005% squalene (50 μg g^{-1}).

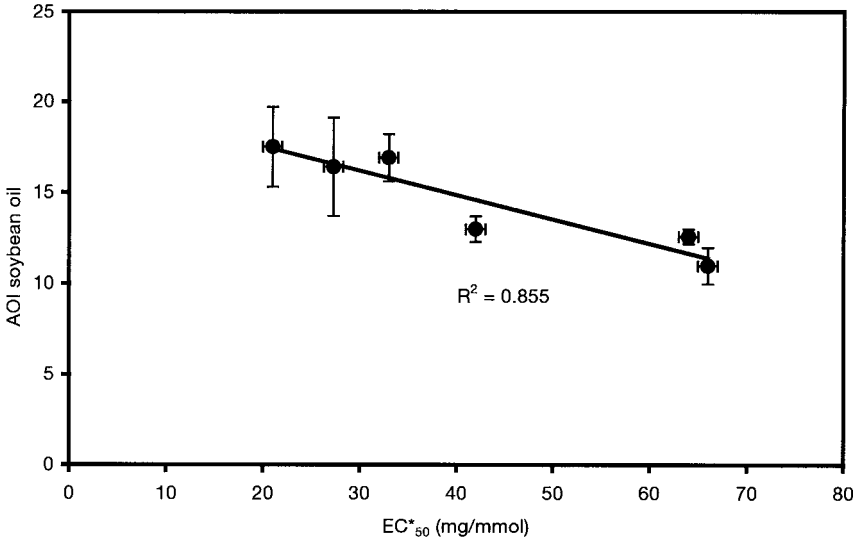


Figure 1. Correlation between radical scavenging activity determined by DPPH assay and antioxidative potential determined in soybean oil for amides **4a**–**4f**. EC^*_{50} is the mass related EC_{50} calculated by $EC^*=EC\times MW$ (mg $mmol^{-1}$).

Solubilities of the test compounds were determined in water containing 5% DMSO. This aqueous system was chosen because it would be tolerated by cell cultures, which will be performed in the future. The results of all tests are listed in Table 1.

The amides **4a**–**4f** are superior radical scavengers in respect to L-ascorbic acid, α-tocopherol and BHT. One equivalent of **4c**–**4e** is able to scavenge 5 equiv. of DPPH radicals, and for **4a**, **4b**, and **4f** the ratio is still 1:10. In the SO assay, only the 4-hydroxy-3-methoxymandelic acid amide **4d** shows a lower radical trapping activity. All other amides are 4–10 times more potent than L-ascorbic acid. The AOIs of hydroxymandelic acid amides in soybean oil are in the range of 10–17 and are 2–3.5 times higher compared to α-tocopherol and BHT, respectively. In evening primrose oil, the AOI of **4e** is comparable to tocopherol, but the other amides are superior to the standards. In squalene, only **4a** has an antioxidative potential of the same order compared to α-tocopherol. **4c** and **4e** show good action similar to BHT, but the remaining amides **4b**, **4d**, and **4f** are only weak to moderate antioxidants in squalene.

In Fig. 1, a plot of mass related EC^*_{50} (calculated by $EC^*=EC\times MW$) against AOI of soybean oil is shown. There is a fairly good linear correlation with a Pearson coefficient of 0.86. In contrast, we could not detect any correlations between EC^*_{50} and AOI for squalene or evening primrose oil, and between EC_{50} of DPPH assay and IC_{50} of SO assay. The used test systems are not redundant and therefore they show different aspects of antioxidative activity.

In addition, we found some structure–activity relations for hydroxymandelic acid amides **4a**–**4f**. In soybean oil and evening primrose oil, the methoxy groups of **4a**, **4c**, **4d**, and **4e** show a strong suppressing effect on the AOI. Additionally, the number of catechol moieties has a negative influence on AOI in squalene and evening primrose oil. In contrast, the catechol number correlates positively with AOI in soybean oil and the effectiveness as DPPH radical scavengers. A trihydroxy group in the molecule decreases the SO trapping ability as well as the potential to protect squalene.

The solubilities of compounds **4a**–**4f** in water/DMSO were much better compared to the phenolic acid amides synthesized earlier. Most of the hydroxymandelic acid amides show solubilities greater than 1% in the mixture. As expected, the α-hydroxy group of the mandelic acid moiety has a positive effect upon the solubility. When the results of the assays were scored, 3,4-dihydroxymandelic acid dopamide (**4b**) shows the best performance as antioxidant and radical scavenger, followed by the 4-hydroxy-3-methoxymandelic acid dopamide (**4a**).

3. Conclusions

The new hydroxymandelic acid amides of phenolic amines **4a**–**4f** are efficient radical scavengers and antioxidants and more effective as radical scavengers when compared with the standards α-tocopherol, BHT and L-ascorbic acid. The investigated amides are equal or more potent antioxidants for soybean and evening primrose oil with respect to tocopherol. They are able to protect squalene against oxidation fairly well, but are inferior to classic antioxidants like α-tocopherol or BHT.

4. Experimental

4.1. General

Squalene was obtained from E. Merck (Darmstadt, Germany) and evening primrose oil and soybean oil from Henry Lamotte (Bremen, Germany). All other chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) or Lancaster Synthesis (Mülheim, Germany). Flash chromatography was performed using Biotage Flash 40 equipment (LCTech, Dorfen, Germany) with disposable pre-packed columns. NMR spectra were recorded using Varian VXR400S (^{1}H: 400 MHz) or Gemini 2000 (^{1}H: 200 MHz) spectrometers (Varian, Darmstadt, Germany) at 25°C using tetramethylsilane as internal standard. LC–MS spectra were recorded using the LCQ HPLC system Finnigan MAT HP1100 (Finnigan MAT, Egelsbach, Germany; APCI). High resolution mass spectra were recorded using a Finnigan MAT 8200 by flash evaporation of samples after dissolving in methanol (resolution >8000). The oxidative stability of bulk lipids was measured with a Rancimat apparatus (Deutsche Metrohm GmbH, Filderstadt, Germany). Chemiluminescence was determined using a Berthold LB96P (Perkin–Elmer GmbH, Überlingen, Germany).

4.1.1. *N*-(3,4-Dihydroxyphenethyl)-2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)acetamide (4a). 2-(4-Hydroxy-3-methoxyphenyl)-2-hydroxyacetic acid (**1b**) (400 mg, 2.02 mmol) and NHOSu (232 mg, 2.02 mmol) were dissolved in 1,4-dioxan (20 ml) under nitrogen. *N*,*N*′-Dicyclohexylcarbodiimide (416 mg, 2.02 mmol) was added to the mixture at room temperature, and the mixture was stirred at that temperature for 48 h. The by-product that precipitated out was filtered off, and the filtrate was added to a solution of 2-(3,4-dihydroxyphenyl)-ethylamino hydrochloride (**3a**) (383 mg, 2.02 mmol) in a mixture of water and 1,4-dioxan (2:1, 20 ml) under nitrogen, followed by sodium hydrogen carbonate (187 mg, 2.22 mmol). The mixture was stirred at 50°C for 4 h, then rendered acidic using hydrochloric acid (c=10%), and the reaction solution was extracted 3 times with ethyl acetate (a total of 90 ml). The organic phase was washed with saturated NaCl solution, dried over Na_2SO_4, filtered, and the filtrate was evaporated under reduced pressure. Purification was carried out by flash chromatography on silica gel using the eluent ethyl acetate. The yield was 170 mg of an amorphous, colourless solid (25% of theory based on the acid used). HPLC >99%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.94 (1H, d, 1.9 Hz), 6.79 (1H, ddd, 8, 2, 0.5 Hz), 6.74 (1H, dd, 8.1 Hz), 6.654 (1H, d, 7.9 Hz), 6.647 (1H, d, 2 Hz), 6.48 (1H, dd, 8.1, 1.9 Hz), 4.87 (partially suppressed, s), 3.82 (3H, s), 3.46–3.36 (2H, m), 2.68–2.63 (2H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ=175.7 (C), 149.0 (C), 133.1 (C), 131.8 (C), 121.0 (2×CH), 116.8 (CH), 116.4 (CH), 116.1 (CH), 111.6 (CH), 75.5 (CH), 56.4 (CH_3),

41.8 (CH_2), 35.9 (CH_2) ppm; MS (APCI-): *m/e*=332.1 (100%, $[M-H]^-$), 664.6 (17%, $[2M-H]^-$); HRMS: MW_{found}=333.1216; MW_{calcd} for $C_{17}H_{19}NO_6$=333.1213.

4.1.2. *N*-(3,4-Dihydroxyphenethyl)-2-(3,4-dihydroxyphenyl)-2-hydroxyacetamide (4b). 2-(3,4-Dihydroxyphenyl)-2-hydroxyacetic acid (**1b**) (300 mg, 1.63 mmol) and NHOSu (188 mg, 1.63 mmol) were dissolved in 1,4-dioxan (20 ml) under nitrogen, and *N*,*N*′-dicyclohexylcarbodiimide (336 mg, 1.63 mmol) was added to the mixture at room temperature, which was stirred at that temperature for 16 h. The by-product which precipitated out was filtered off, and the filtrate was added to a solution of 2-(3,4-dihydroxyphenyl)ethylamino hydrochloride (**3a**) (309 mg, 1.63 mmol) in water (20 ml). Sodium hydrogen carbonate (151 mg, 1.8 mmol) was added, and the reaction mixture was stirred under nitrogen at 50°C for 1.5 h. The mixture was rendered acidic using hydrochloric acid (*c*=5%) and extracted 3 times with ethyl acetate (a total of 90 ml). The organic phase was washed with saturated NaCl solution, dried over Na_2SO_4, filtered, and the filtrate was evaporated under reduced pressure. The product was purified by flash chromatography on silica gel using the eluent ethyl acetate. The yield was 266 mg of a colourless solid (51% of theory based on the acid used). HPLC >96%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.83 (1H, d, 2 Hz), 6.72 (1H, d, 8 Hz), 6.674 (1H, ddd, 8, 2, 0.5 Hz), 6.672 (1H, d, 8 Hz), 6.65 (1H, d, 2 Hz), 6.50 (1H, dd, 8, 2.1 Hz), 3.43–3.35 (2H, m), 2.65 (t, 7.6 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ=175.8 (C), 146.5 (C), 146.3 (C), 146.26 (C), 144.9 (C), 133.3 (C), 131.9 (C), 121.2 (CH), 119.9 (CH), 116.9 (CH), 116.5 (CH), 116.2 (CH), 115.4 (CH), 75.4 (CH), 41.9 (CH_2), 36.0 (CH_2) ppm; MS (APCI-): *m/e*=318.8 (100%, $[M-H]^-$), 636.4 (23%, $[2M-H]^-$); HRMS: MW_{found}=319.1043; MW_{calcd}=319.1056 for $C_{16}H_{17}NO_6$.

4.1.3. *N*-(3,4-Dihydroxybenzyl)-2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)acetamide (4c). This compound was obtained analogous to **4a** from 2-(4-hydroxy-3-methoxyphenyl)-2-hydroxyacetic acid (**1a**) (500 mg, 2.53 mmol) and 3,4-dihydroxybenzylamino hydrobromide (**3c**) (667 mg, 3.03 mmol) as a colourless amorphous solid. The product was purified by flash chromatography on silica gel using the eluent ethyl acetate. Yield: 618 mg (77% of theory based on the acid used). HPLC >98%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.94 (1H, d, 2.1 Hz), 6.87 (1H, ddd, 8.1, 2.0, 0.5 Hz), 6.75 (1H, d, 8.1 Hz), 6.73 (1H, d, 2.0 Hz), 6.68 (1H, d, 8.0 Hz), 6.60 (1H, ddd, 8.0, 2.1, 0.6 Hz), 4.94 (partially suppressed, s), 4.32 (1H, d, 14.5 Hz), 4.21 (1H, d, 14.5 Hz), 3.78 (3H, s) ppm; ^{13}C NMR (100 MHz, CD_3OD): (δ=175.5 (C), 149.0 (C), 147.7 (C), 146.4 (C), 145.7 (C), 133.3 (C), 131.6 (C), 121.3 (CH), 120.3 (CH), 116.3 (CH), 116.1 (CH), 115.9 (CH), 111.4 (CH), 75.5 (CH), 56.4 (CH_3), 43.5 (CH_2) ppm; MS (APCI-): *m/e*=318.1 (100%, $[M-H]^-$); HRMS: MW_{found}=319.1063; MW_{calcd}=319.1056 for $C_{16}H_{17}NO_6$.

4.1.4. 2-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-*N*-(3,4,5-trihydroxybenzyl)acetamide (4d). This compound was obtained analogous to **4a** from 2-(4-hydroxy-3-methoxyphenyl)-2-hydroxyacetic acid (**1a**) (300 mg, 1.52 mmol) and 3,4,5-trihydroxybenzylamino hydrochloride[12] (**3d**) (290 mg, 1.52 mmol) as a colourless amorphous solid. The product was purified by flash chromatography on silica gel two times using subsequently the eluent ethyl acetate and a gradient chloroform/methanol 10:1 to 2:1. Yield: 200 mg (39% of theory based on the acid used). HPLC >98%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.93 (1H, d, 2.1 Hz), 6.86 (1H, ddd, 8.1 Hz, 2.1 Hz, 0.5 Hz), 6.75 (1H, d, 8.1 Hz), 6.31 (2H, t, 0.6 Hz), 4.93 (partially suppressed, s), 4.27 (1H, d, 14.3 Hz), 4.14 (1H, d, 14.3 Hz), 3.79 (3H, s) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ=175.5 (C), 149.0 (C), 147.7 (C), 147.1 (2×C), 133.3 (C), 130.9 (C), 121.3 (CH), 116.0 (CH), 111.4 (CH), 107.9 (2×CH), 75.5(CH), 56.4 (CH_3), 43.7 (CH_2) ppm; MS (APCI-): *m/e*=334.0 (100%, $[M-H]^-$), 668.7 (5%, $[2M-H]^-$); HRMS: MW_{found}=335.1010; MW_{calcd}=335.1005 for $C_{16}H_{17}NO_7$.

4.1.5. 2-(3,4-Dihydroxyphenyl)-2-hydroxy-*N*-(4-hydroxy-3-methoxybenzyl)acetamide (4e). This compound was obtained analogous to **4b** from 2-(3,4-dihydroxyphenyl)-2-hydroxyacetic acid (**1b**) (300 mg, 1.63 mmol) and 4-hydroxy-3-methoxybenzylamino hydrochloride (**3b**) (309 mg, 1.62 mmol) as a colourless amorphous solid. The product was purified by flash chromatography on silica gel using the eluent ethyl acetate. Yield: 296 mg (57% of theory based on the acid used). HPLC >98%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.90 (1H, dt, 2.1, 0.4 Hz), 6.78 (1H, ddd, 8.2, 2.1, 0.6 Hz), 6.74 (1H, d, 1.6 Hz), 6.72 (1H, d, 8.2 Hz), 6.71 (1H, dd, 8.0, 0.4 Hz), 6.68 (1H, dd, 8.0, 1.8 Hz), 4.90 (partially suppressed, s), 4.36 (1H, d, 14.7 Hz), 4.28 (1H, d, 14.7 Hz), 3.73 (3H, d, 0.4 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ=175.8 (C), 149.1 (C), 146.7 (C), 146.5 (C), 146.4 (C), 133.4 (C), 131.5 (C), 121.1 (CH), 119.7 (CH), 116.1 (CH), 116.0 (CH), 115.1 (CH), 112.0 (CH), 75.4 (CH), 56.3 (CH_3), 43.4 (CH_2) ppm; MS (CI-): *m/e*=318.0 (100%, $[M-H]^-$), 301.7 (22%), 300.5 (34%); HRMS: MW_{found}=319.1054; MW_{calcd}=319.1056 for $C_{16}H_{17}NO_6$.

4.1.6. *N*-(3,4-Dihydroxybenzyl)-2-(3,4-dihydroxyphenyl)-2-hydroxyacetamide (4f). This compound was obtained analogous to **4b** from 2-(3,4-dihydroxyphenyl)-2-hydroxyacetic acid (**1b**) (1.00 g, 5.4 mmol) and 3,4-dihydroxybenzylamino hydrochloride (**3c**) (1.15 g, 6.6 mmol) as a colourless amorphous solid. The product was purified by flash chromatography on silica gel using the eluent ethyl acetate. Yield: 640 mg (39% of theory based on the acid used). HPLC >99%; ^{1}H NMR (400 MHz, CD_3OD with CD_3OH suppression): δ=6.87 (1H, dt, 1.9, 0.5 Hz), 6.75 (1H, ddd, 8.1, 1.9, 0.5 Hz), 6.72 (1H, dd, 8.1, 0.5 Hz), 6.72 (1H, dm, 2.1, 0.3 Hz), 6.69 (1H, d, 8.0 Hz), 6.59 (1H, ddt, 8.0, 2.1, 0.6 Hz), 4.88 (partially suppressed, s), 4.25 (2H, s) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ=175.6 (C), 146.6 (C), 146.5 (C), 146.4 (C), 145.8 (C), 133.2 (C), 131.3 (C), 120.2 (CH), 119.8 (CH), 116.3 (CH), 116.1 (CH), 116.0 (CH), 115.3 (CH), 75.4 (CH), 43.6 (CH_2) ppm; MS (APCI-): *m/e*=304.1 (10%, $[M-H]^-$), 608.7 (100%, $[2M-H]^-$); HRMS: MW_{found}=305.0894; MW_{calcd}= 305.0899 for $C_{15}H_{15}NO_6$.

4.2. DPPH assay

In disposable polystyrol cuvettes, 2.5 ml of a methanolic

DPPH solution (100 μmol l^{-1}) was mixed with 500 μl of test solutions of different concentrations (methanol as control) and the decrease of the purple coloured radical was determined photometrically at 514 nm until the decrease was less than 2% per hour against negative control (steady state). The concentration dependent activity of the test compounds as free radical scavengers was calculated using the following equation:

$$\text{activity}_{\text{conc}}(\%) = 100 - (\text{remaining DPPH}) \quad (1)$$

The remaining DPPH was calculated from absorptions by

$$\text{remaining DPPH}\ (\%) = \text{absorption}_{(\text{test at steady state})}/\text{absorption}_{(\text{control at } t_0)} \quad (2)$$

The activity (%) in a series of dilutions of one test compound was used to calculate the effective relative concentration EC_{50} (based on the starting concentration of DPPH, $EC = c_{\text{test}}/c_{(\text{DPPH at } t_0)}$), at which 50% of DPPH has been removed. The assay was performed in triplicate and the absorptions were averaged before calculation. α-Tocopherol, L-ascorbic acid and BHT were used as standards.

4.3. SO assay

The test compounds were diluted using a dimethyl sulfoxide stock solution (10 mM) with phosphate buffered saline (PBS). Each well of a 96-well plate of white polystyrene was subsequently charged with 50 μl luminol (200 μM in PBS), 50 μl horseradish peroxidase (HRPO, 1 U ml^{-1} in PBS), and 50 μl of the test solutions of different concentrations (e.g. 40 μM). The mixtures were incubated at 37°C for 5 min. Finally, 50 μl aliquots of H_2O_2 (40 μM) were added and the chemiluminescence was recorded immediately for 10 s. The activity was calculated as inhibitor concentration IC_{50}, at which 50% of SO generated was depleted. L-Ascorbic acid was used as standard.

4.4. Rancimat assay

Prior to the assays, the lipids were purified as follows: soybean oil, evening primrose oil and squalene were dissolved in *n*-heptane. The solutions were passed through a glass column charged with activated alumina type N by pressurized nitrogen. The eluent was collected under nitrogen. The solvents of the purified solutions were distilled off under reduced pressure. In the case of squalene, a solution of α-tocopherol in *n*-heptane was added to give a final concentration of 1 ppm tocopherol prior to evaporation.

The test compounds were dissolved in methanol or acetone (15 mg ml^{-1} for soybean and evening primrose oil and 1.5 mg ml^{-1} for squalene, respectively) and 100 ml of these solutions were added to a pre-prepared 3 g lipid sample in the reaction vessels of the Rancimat apparatus. Pure solvent was added to control samples. A constant dry air stream (20 l h^{-1}) was blown through the sample while heating to 100°C for soybean oil and evening primrose oil and 80°C for squalene, respectively. The volatile oxidation products were collected in the measuring vessels containing water and the conductivity of the aqueous solution was measured continuously and recorded. The IPs were calculated automatically by the Rancimat. All tests were run in duplicate and averaged before calculation. The following equation was used to calculate the AOI:

$$\text{AOI} = \text{IP}_{\text{test}}/\text{IP}_{\text{control}} \quad (3)$$

α-Tocopherol and BHT were used as standards.

4.5. Solubility

Of the test compound, 5 mg was weighed into a glass vessel, mixed with a defined amount of 5% DMSO in water (final masses: 50 mg, 100 mg, 500 mg, 1 g, 5 g, 10 g), and treated with ultrasound at room temperature for 5 min. This procedure was repeated until the compound had completely dissolved. At a solubility of $<0.05\%$ by weight, the experiment was terminated.

Acknowledgements

I am very grateful to Mr Strempel and Mrs Schlichter for their excellent technical assistance and to Dr Werkhoff, Dr Krammer, Dr Schmidt and Dr Sommer for the analysis of the compounds. Special thanks are dedicated to Professor Dr Müller-Peddinghaus and Dr Grützmann (both Bayer AG) for conducting the SO assay.

References

1. Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I. *Crit. Rev. Food Sci. Nutr.* **1995**, *35* (1/2), 7–20.
2. Tyrrell, R. M. *Biochem. Soc. Symp.* **1995**, *61*, 47–53.
3. Scharffetter-Kochanek, K. Photoaging of the connective tissue of skin: its prevention and therapy. In *Antoxidants in Disease Mechanisms and Therapy*; Sies, H., Ed.; Academic: San Diego, 1997; Vol. 38, pp 639–655.
4. Ames, B. N.; Shigenaga, M. K.; Hagan, T. M. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7915–7922.
5. Cheeseman, K. H. *Toxicol. Ind. Health* **1993**, *9* (1/2), 39–51.
6. Gordon, M. H. *Nat. Prod. Rep.* **1996**, 265–273.
7. Carbonare, M. D.; Pathak, M. A. *J. Photochem. Photobiol. B* **1992**, *14*, 105–124.
8. Podda, M.; Traber, M. G.; Weber, C.; Yan, L.-J.; Packer, L. *Free Radical Biol. Med.* **1998**, *24* (1), 55–65 (publ. 1997).
9. Pelle, E.; Muizzuddin, N.; Mammone, T.; Marenus, K.; Maes, D. *Photodermatol. Photoimmunol. Photomed.* **1999**, *15* (3/4), 115–119.
10. Stäb, F.; Lanzendörfer, G.; Schönrock, U.; Wenck, H. *SÖFW J.* **1998**, *124* (10), 604 (see also pages 606, 608–610, 612, 613).
11. Fielder, S.; Rowan, D. D. *J. Labelled Compd. Radiopharm.* **1999**, *42* (1), 83–92.
12. Ley, J. P. *Int. J. Cosm. Sci.* **2001**, *23* (1) (in press).
13. Ley, J. P. *Haarmann and Reimer GmbH* EP 0,900,781, 1998; *Chem. Abstr.* **1999**, *130* (16), 209510.
14. Brand-Williams, W.; Cuvelier, M.-E.; Berset, C. *Lebensm.-Wiss.u.-Technol.* **1995**, *28*, 25–30.
15. Müller-Peddinghaus, R.; Grützmann, R. Personal communication.
16. Hadorn, H.; Zürcher, K. *Deutsche Lebensmittel-Rundschau* **1974**, *70* (2), 57–65.