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New compounds obtained by enzymatic oxidation of phloridzin

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Abstract—Oxidation of phloridzin was studied in model system in the presence of apple polyphenol oxidase. In addition to 3-hydroxy phloridzin, two major oxidation products were purified by reversed phase HPLC at the semi-preparative scale. Their structures were elucidated by UV, ESI-MS^n and NMR spectroscopies. The first compound was a colourless product, which novel structure strongly differs from its precursor showing a biphenyl moiety and a propionic acid chain. The second product was an oxidised form of the first one and corresponded to a stable yellow pigment with two isomeric forms. A mechanism of formation of these products, which implied successive oxidation and nucleophilic addition steps was proposed.

Among polyphenols in commonly consumed fruit and vegetables, dihydrochalcones are specific to apple and apple-derived products. Phloridzin **1** is one of the most ubiquitous constituent of this class[1–3] and its structure has been largely studied by ^{13}C and ^{1}H NMR spectroscopy.[2,4,5] During apple juice and cider making, oxidation of polyphenols catalysed by polyphenol oxidase (PPO) takes place leading to new polyphenolic compounds. These oxidation products contribute to the colour of ciders and juices[6–8] and may influence the sensory properties of these beverages. Since phloridzin oxidation has already been studied,[4,9–11] no full structural characterisation of the oxidised products has been reported yet. Moreover, some of these compounds are yellow pigments, which could contribute to the colour of the apple-derived products.[6,8] This work describes the detection, the purification and the identification of two new phenolic compounds **3**, **4** (Fig. 1) formed from the oxidation of phloridzin catalysed by apple PPO in an apple juice like malate buffer. Their structures have been investigated by UV–vis, ESI-ion trap MS^n and NMR spectroscopy.

The enzymatic oxidation of phloridzin (1 mM) was achieved in a malate buffer (28 mM, pH 3.8) using an insoluble apple PPO extract prepared from apple pulp as already described.[9] The incubation was carried out for 6 h at 30 °C under air agitation. The medium was filtered, stabilised by adding sodium fluoride, a strong PPO inhibitor, and analysed by reversed phase HPLC equipped with a UV–vis diode array detector and using a gradient of acetonitrile and diluted acetic acid. Two compounds (**3** and **4**) appeared as well-separated major products on chromatograms at 280 nm. Incubation was also performed by adding an excess of ascorbic acid as a quinone reducing agent: those reducing conditions permitted to accumulate 3-hydroxy-phloridzin (**2**) as an intermediate in the course of phloridzin oxidation. These three products were purified for structural analyses: incubations were performed at a larger scale and compounds were separated by reversed phase HPLC at the semi-preparative scale. Each product was collected at the outflow of the column. Acetonitrile was evaporated under vacuum and fractions were freeze-dried. Thus, products were available in pure form for analysis. UV–vis spectra were obtained by the diode array detection of the HPLC system and mass spectroscopy was performed in the negative mode on an ion trap spectrometer equipped with an electrospray source. NMR spectroscopy of compounds **2**–**4** was performed in DMSO-d_6-TFA (9-1, v/v) using a 500 MHz spectrometer. Complete structure elucidation of these compounds was achieved by both 1D and 2D homonuclear ^{1}H and heteronuclear ^{1}H ^{13}C experiments, which allowed ^{1}H and ^{13}C chemical shift assignments (Table 1).

Keywords: Phloridzin; Dihydrochalcone; Apple; Polyphenol oxidase; Oxidation products; Browning.
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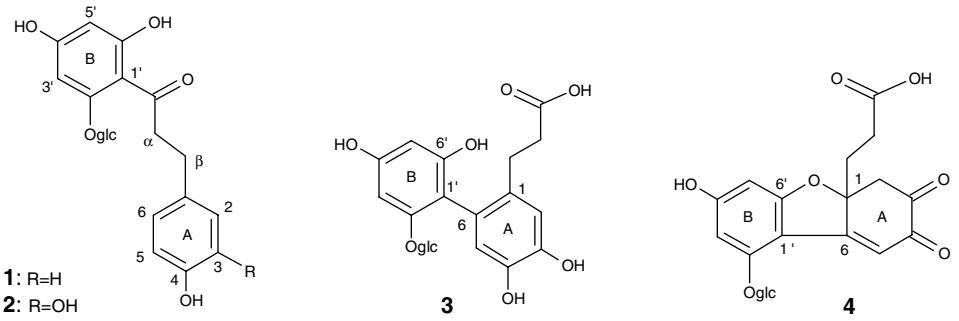

Figure 1.

Table 1. ^{1}H and ^{13}C NMR assignments of compounds **2**–**4** in DMSO-d_6-TFA

Position	2		3		4	
	δ ^{1}H (ppm); m, J (Hz)	δ ^{13}C	δ ^{1}H (ppm); m, J (Hz)	δ ^{13}C	δ ^{1}H (ppm); m, J (Hz)	δ ^{13}C
1A		133.0		131.4		89.2
2A	6.62; d, J=1.9	116.0	6.56; s	115.5	3.39; 3.32; m	49.9
3A	—	145.4	—	142.3*	—	192.4
4A	—	143.6	—	144.0*	—	178.9
5A	6.59; d, J=8	115.7	6.36; s	119.3	6.81; s	114.7
6A	6.48; dd, J=8, 1.9	119.3	—	124.0	—	165.8
1′B	—	105.2	—	109.2	—	104.1
2′B	—	161.5	—	156.5	—	157.7
3′B	6.13; d, J=2.1	94.5	6.05; d, J=2.2	96.5	6.30; m	97.1
4′B	—	165.1	—	157.1	—	167.9
5′B	5.93; d, J=2.1	97.1	6.07; d, J=2.2	94.0	6.11; m	91.9
6′B	—	166.1	—	155.7	—	167.2
α_1, α_2	3.31; 3.37; m	45.2	2.25; m	34.8	1.84; 2,08; m	28.5
β_1, β_2	2.72; t, J=7	29.4	2.39; 2.46; m	27.9	2.10; 2.16; m	39.3
CO	—	205.3	—	174.7	—	173.4
1″	4.93; m	101.1	4.69; d, J=7.9	100.4	4.99; d, J=7.0	100.0
2″	3.30; m	73.5	2.90; m	73.3	3.25; m	69.7
3″	3.30; m	77.1	3.18; m	76.9	3.34; m	76.9
4″	3.22; m	69.8	3.10; m	69.6	3.35; m	73.4
5″	3.33; m	77.5	3.17; m	76.9	3.38; m	77.4
6″a, b	3.53; dd, J=12, 1.5 3.72; dd, J=12, 5.4	60.5	3.67; 3.49; m	60.8	3.52; 3.71; m	60.8

The UV–vis of phloridzin and three oxidation products are given in Figure 2. The first oxidation compound **2** was the 3-OH phloridzin (m/z=451), which resulted from the cresolase activity of PPO on phloridzin as already reported.[4,11] Its UV–vis spectrum, similar to that of phloridzin, showed one maximum absorption at 283 nm. Asymmetry of the band absorption is related to conjugated system between ketonic function and phloroglucinol (B ring). The 3-OH-phloridzin was rapidly transformed in a colourless product **3**. Its UV spectrum revealed that the ketonic conjugated system was lost. Furthermore, its mass spectrum showed a molecular ion peak at m/z=467 indicating an additional hydroxyl group with respect to 3-OH phloridzin. Compound **3** was then converted to a yellow compound **4** with m/z value of 465 and maximum absorption at 417 nm. UV–vis spectra and reversed phase chromatographic behaviours of products **3** and **4** were highly similar to phloridzin oxidation products previously observed in comparable studies.[4,8]

Chemical shifts obtained for compound **2** were in agreement with those already reported for 3-OH-phloridzin.[4] In the ^{1}H NMR spectrum of compound **3**, two sets of signals were observed indicating the presence of two isomers in the ratio 60:40. Besides, ^{1}H spectrum integration showed the existence of 15 protons, that is one proton less than 3-OH phloridzin. In fact, only two aromatic singlets arising from A ring at 6.36 and 6.56 ppm were observed, suggesting an additional substitution of this ring. Moreover, the lack of coupling between these two protons implied that they are *para* related. This allowed to attribute these two signals to H2 and H5, C6 position being substituted. Two aromatic mutually coupled doublets (J=2 Hz) at 6.05 and 6.07 ppm were indicative of an asymmetrically substituted B ring as observed in phloridzin and 3-OH-phloridzin. α and β methylene proton signals were located in the region of 2–2.5 ppm. Glucoside protons were identified with the help of 2D ^{1}H COSY experiment. The high coupling constant (J=7.9 Hz) indicated the anomeric sugar conformation to be β. Short-range and long-range ^{1}H ^{13}C correlations obtained from HSQC and HMBC spectra, respectively, allowed to assign all ^{1}H and ^{13}C resonances (Fig. 3). The A ring proton at 6.56 ppm was attributed to H2 since it showed correlation with one methylene

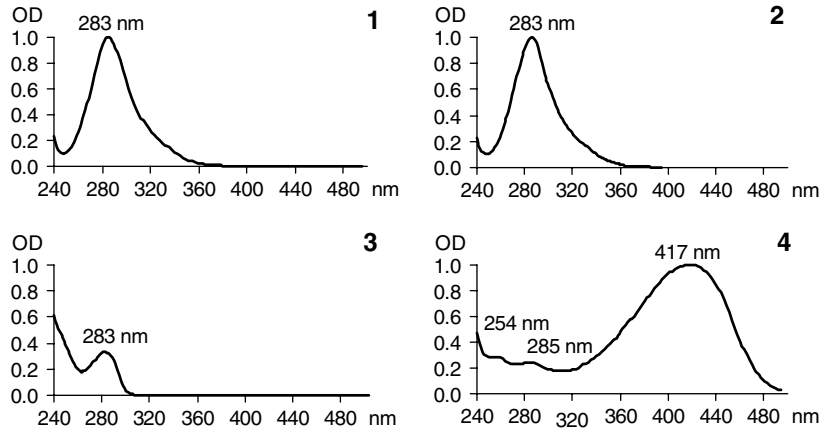

Figure 2. UV–vis spectra of compounds **1**–**4** (in diluted acetic acid–acetonitrile mixture).

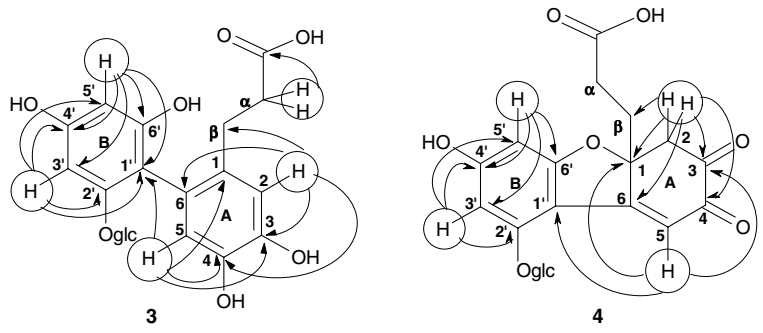

Figure 3. Main HMBC correlations observed in compounds **3** and **4**.

group carbon at 27.9 ppm, which was assigned to β CH2. The other methylene group was then attributed to the α CH2. Consequently, the other A ring singlet at 6.36 ppm was assigned to H5. Long-range correlations arising from H2 and H5 allowed to assign all quaternary carbons of A ring. Identification of quaternary carbons of the B ring was deduced on the basis of long-range correlations implying H3′ and H5′. Linkage of sugar moiety position was confirmed by a correlation between the glucose anomeric proton and carbon C2′ of the B ring. The structure of the compound **3** was definitely determined by the presence of a correlation between H5 and C1′, which can only occurred if the two aromatic rings are linked through a bond implying C6 and C1′ positions. This is in agreement with the chemical shift of C6 (δ 124.0), which is characteristic of a nonhydroxylated aromatic quaternary carbon. Furthermore, ^{13}C chemical shift of C=O group was upfield shifted in comparison with compound **2** of about 25 ppm. This ^{13}C chemical shift at 174.7 ppm was too shielded to be a ketonic group but was characteristic of a carboxylic one.

The presence of a carboxylic group in the structure of compound **3** was supported by MS^n experiments. The MS^2 experiment showed a loss of 162 mass units corresponding the glucose moiety. Then, the MS^3 experiment on the 305 aglycone ion showed a main fragment ($m/z = 261$) that corresponded to the loss of the carboxyl group (44 mass units). The presence of the carboxylic group was also confirmed by esterification of the compound in methanol/HCl 0.2 N. The conversion into the corresponding methylester was showed by observing the molecular ion peak at $m/z = 481$ corresponding to an increase of 14 mass units and a loss of 74 in MS^3 experiment, which were in good agreement with a β cleavage of the methylester moiety. The esterification of the carboxylic group was unambiguously confirmed by the presence of a long-range correlation between the methyl protons and the carbonyl function in HMBC spectrum.

All of the results allowed to propose the structure of compound **3** as shown in Figure 1. Rotation around the C1′C6 bond should be indeed restricted due to steric effects of the propionic acid group and glucoside moiety favouring two conformations of lower energy. As a consequence, the two isomers likely corresponded to two atropisomers as proposed in Figure 4.

In the case of compound **4**, two NMR signal sets were also observed indicating the presence of two isomers in a ratio 65:35. This compound possessed 15 protons as compound **3** but several differences were observed. First, the A ring presented only one ethylenic proton signal, which appeared as a singlet at 6.81 ppm. On the other hand, HSQC edited spectrum showed an additional methylene group. Chemical shift of protons and corresponding carbons was obtained from the HSQC spectrum. Quaternary carbons identification and structure

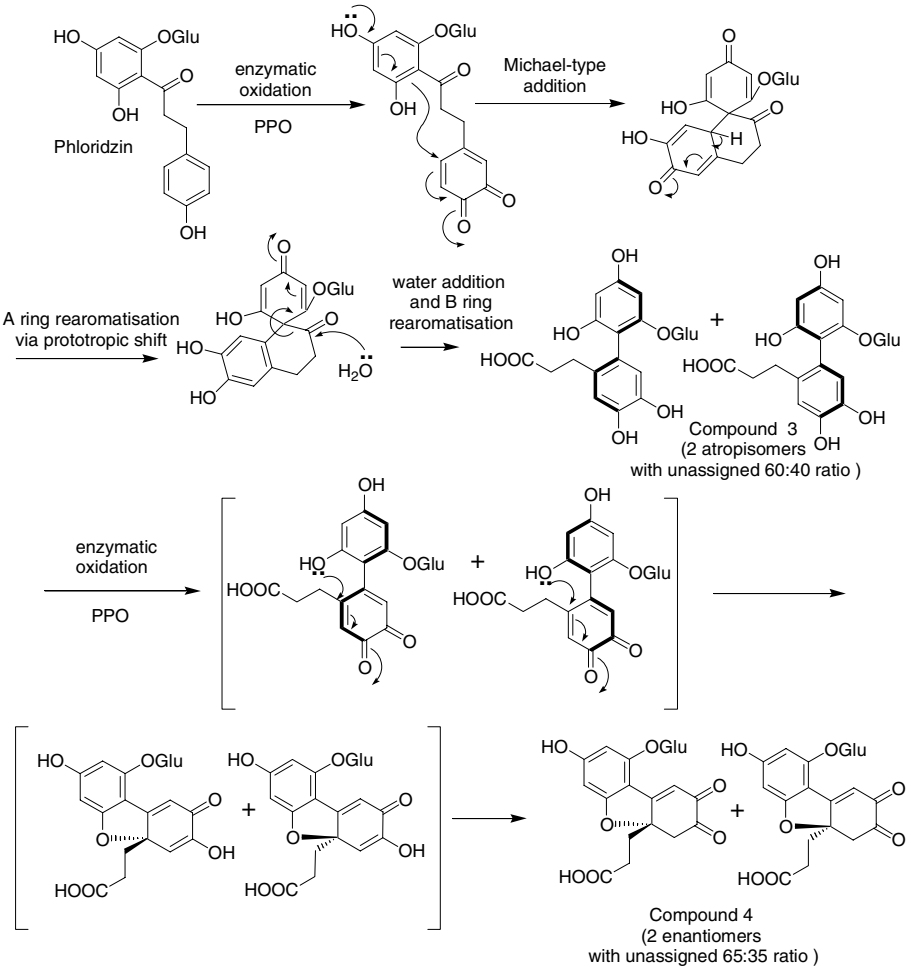

Figure 4. Proposed mechanism for the formation of compounds **3** and **4**.

determination were then realised with the help of long-range correlation HMBC (Fig. 3). Compared with compound **3**, only small differences in chemical shifts were observed concerning the B ring, the propionic acid group and the glucoside moiety. All ^{1}H and ^{13}C of these moieties were thus easily attributed as described for compound **3**. Moreover, the presence of the carboxylic group was confirmed by both MS^{n} and NMR experiments as described above for compound **3**.

The additional methylene protons at 3.32 and 3.39 ppm were attributed to A ring H2 since these protons had correlations with both quaternary carbons of A ring and β methylene carbons. The aromatic proton at 6.81 ppm was thus attributed to H5. All quaternary carbons of the A ring were identified on the basis of their correlations with H5 and H2 protons. The upfield chemical shifts of both C3 and C4 at 192.4 and 178.9 ppm, respectively, were characteristic of a di-ketonic group. The downfield shift of C1 at 89.2 ppm indicated a non aromatic quaternary carbon and confirmed the disappearance of A ring aromaticity. Moreover, the presence of several carbons having chemical shifts above 160 ppm was indicative of conjugated ketonic systems between the A and B rings. All of these results allowed to propose the structure of compound **4**, which was in accordance with UV–vis and mass spectroscopy data. The two isomers were probably stereo isomers since the C1 was an asymmetric carbon.

A mechanism for the formation of phloridzin oxidation products is proposed (Fig. 4). The first steps in the formation of compound **3** would be the enzymatic hydroxylation of phloridzin into 3-OH phloridzin immediately followed by oxidation of the later into the corresponding *o*-quinone as already observed in previous studies.[10–12] In fact, we observed that the addition of ascorbic acid in the oxidation medium permitted to accumulate 3-OH phloridzin by reducing the *o*-quinone back to the hydroquinone form as it was already showed in previous works.[9] In the absence of such a reducing agent, the 3-OH phloridzin *o*-quinone led to the formation of the compound **3** that may correspond to the highly polar and colourless product. The formation of **3** may be explained by a nucleophilic attack of the C1′ carbon of B ring on the C6 of the *o*-quinone A ring in an intramolecular Michael type addition (Fig. 4). The following hydration permitted to form the stable biphenyl structure with simultaneous formation of a propionic acid group. Two atropisomers were favoured because of the hindered rotation around the C1′–C6 linkage. The formation of compound **4** first implied an oxidation of

the compound **3** leading to the corresponding *o*-quinone, which further reacted according to an intramolecular nucleophilic Michael addition as described in Figure 4. As a consequence of the presence of two atropisomers of compound **3**, the stereoselective addition led to the formation of two enantiomers of compound **4** as shown in Figure 4.

In most previous works about the phloridzin oxidation products, authors suggested that major compounds corresponded to coupling dimers with an analogous biphenyl structure.[4,10,11] Although the formation of phloridzin dimers is not excluded, our results showed for the first time the complete structural elucidation of the main phloridzin oxidation products, which were monomers resulting from successive oxidation reactions and nucleophilic intramolecular additions. Compound **4** is a yellow pigment that may contribute to the colour of apple juices, ciders and other apple-derived products.

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References and notes

1. Durkee, A. B.; Poapst, P. A. *J. Agric. Food Chem.* **1965**, *13*, 137–139.
2. Dick, A. J.; Redden, P. R.; Demarco, A. C.; Lidster, P. D.; Grindley, T. B. *J. Agric. Food Chem.* **1987**, *35*, 529–531.
3. Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J. F. *J. Agric. Food Chem.* **1999**, *47*, 4847–4853.
4. Goodenough, P. W.; Kessell, S.; Lea, A. G. H.; Loeffler, T. *Phytochemistry* **1983**, *22*, 359–363.
5. Lommen, A.; Godejohann, M.; Venema, D. P.; Hollman, P. C. H. L.; Spraulm, M. *Anal. Chem.* **2000**, *72*, 1793–1797.
6. Lea, A. G. H. *Flüssiges Obst.* **1984**, *8*, 356–361.
7. Oleszek, W.; Lee, C. Y.; Jaworski, A.; Price, K. R. *J. Agric. Food Chem.* **1988**, *29*, 430–432.
8. Oszmianski, J.; Lee, C. Y. *J. Agric. Food Chem.* **1991**, *39*, 1050–1052.
9. Le Bourvellec, C.; Le Quéré, J. M.; Sanoner, P.; Drilleau, J. F.; Guyot, S. *J. Agric. Food Chem.* **2004**, *52*, 122–130.
10. Raa, J.; Overeem, J. C. *Phytochemistry* **1968**, *7*, 721–731.
11. Sarapuu, L.; Kheinaru, E. *Biokhimiya* **1971**, *36*, 8–17.
12. Sarapuu, L. P. *Biokhimiya* **1971**, *36*, 343–353.