Glucosides from MBOA and BOA Detoxification by *Zea mays* and *Portulaca oleracea*

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Incubation of *Zea mays* cv. Nicco seedlings with 6-methoxybenzoxazolin-2(3*H*)-one (MBOA) led to a minor detoxification product hitherto only found in Poaceae. This new compound was identified as 1-(2-hydroxy-4-methoxyphenylamino)-1-deoxy-*β*-glucoside 1,2-carbamate (**1**) (methoxy glucoside carbamate) and represents an analogue to the previously described 1-(2-hydroxyphenylamino)-1-deoxy-*β*-glucoside 1,2-carbamate (glucoside carbamate) from benzoxazolin-2(3*H*)-one (BOA). In *Portulaca oleracea* var. *sativa* cv. Gelber treatment with BOA resulted in further unknown detoxification products, which were not synthesized in detectable amounts after BOA absorption in all other species tested. Compound **1** easily undergoes decay into BOA-5-*O*-glucoside (**2**). *Z. mays* seedlings, known to produce BOA-6-*O*-Glc on incubation with BOA, are able to transform BOA-5-OH into BOA-5-*O*-glucoside (**2**). Besides the known compounds, maize contained a formerly unseen product that accumulated during late stages of the detoxification process. It was isolated and identified as 1-(2-hydroxyphenylamino)-6-*O*-malonyl-1-deoxy-*β*-glucoside 1,2-carbamate (**3**) (malonyl glucoside carbamate).

Phytotoxic benzoxazolin-2(3*H*)-one (BOA) results from a two-step degradation of the acetal glucoside (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one. These compounds are secondary compounds in several species of the Acanthaceae, Lamiaceae, Poaceae, Ranunculaceae, and Scrophulariaceae families.[1−5] Once released to the environment, BOA acts as an allelochemical, causing dose-dependent growth inhibitions in dicotyledoneous and, to a smaller extent, in monocotyledoneous species. Therefore, the compound was considered by agronomists as a natural herbicide. However, about 30 tested plant species are able to detoxify the compound and, in general, monocots with a higher efficiency than dicots.[6] Four detoxification products have been characterized: 1-(2-hydroxyphenylamino)-1-deoxy-*β*-glucoside 1,2-carbamate (glucoside carbamate), gentiobioside carbamate, BOA-6-OH (6-hydroxybenzoxazolin-2(3*H*)-one), and the corresponding 6-*β*-*O*-glucoside.[7,8] Glucosylation of BOA-6-OH is possible in all species tested, e.g., in *Arabidopsis thaliana*, where a corresponding glucosyltransferase activity was upregulated during BOA incubation. BOA-6-*O*-glucoside is the major product in dicotyledoneous species, whereas nontoxic glucoside carbamate is the major one in Poaceae. Gentiobioside carbamate production was restricted to maize. This compound accumulates during the late state of the detoxification process together with a new, hitherto unseen, product very similar to glucoside carbamate.

Portulaca oleracea (Portulacaceae) was the only dicotyledoneous species that accumulates new detoxification products resembling the known BOA-6-OH compounds after BOA application.

Incubations with 6-methoxybenzoxazolin-2(3*H*)-one (MBOA) also result in accumulation of BOA-6-*O*-glucoside.[9] In *Z. mays*, but also in *Hordeum vulgare* and *Avena sativa* roots, a second product was found.

Here, three new glucosides from the detoxification of MBOA and BOA by *Z. mays* and *P. oleracea* are described. The known detoxification products together with the new ones demonstrate the metabolic flexibility and variability of higher plants in detoxification of the allelochemically active compounds BOA and MBOA.

Results and Discussion

When maize seedlings were incubated with MBOA for 24 h, large amounts of BOA-6-*O*-glucoside were found in the MeOH extracts of the roots but only traces of a second product assigned as 1-(2-hydroxy-4-methoxyphenylamino)-1-deoxy-*β*-glucoside 1,2-carbamate (**1**) (methoxy glucoside carbamate). This compound accumulated in higher concentrations when the incubation time was extended to 48 h. It appeared only after MBOA incubations with the tested cereals *A. sativa*, *H. vulgare*, and *Z. mays*, but not in *A. thaliana*, *Brassica oleracea*, and other dicots tested.[9] Compound **1** was isolated, purified, and subjected to structural analysis.

For solubility reasons, NMR data for glucoside **1** have been recorded in CD_3OD. The ^1H NMR spectrum shows signals for a 1,2,4-trisubstituted phenyl ring and well-resolved signals in the sugar region. In the ^{13}C NMR spectrum the C-1′ resonance appears at 84.6 ppm. The spectra are very similar to a carbamate derivative described earlier.[7] The complete assignment was made by use of COSY, HMQC, and HMBC. Cross-peaks in the HMBC from H-1′ (5.25 ppm) to 70.1 (C-2′), 122.7 (C-1), and 154.7 ppm (NCOO) caused by couplings via two and three bonds show the connection with formation of the carbamate structure.

The CD_3OD solution was directly introduced into the ESI-FT-ICR-MS instrument. In the positive mode, an intensive peak at 351.09248 Da was observed, resulting from the monomer of compound **1** containing one deuterium atom in the form of a sodium adduct (exact theoretical mass 351.09146 Da) as well as a minor peak at 352.09583 Da for the corresponding analogue with two deuterium atoms. Mass analytical calculations gave rise to the conclusion that the formula of the nondeuterated sodium-free compound **1** is $C_{14}H_{17}NO_8$.

The slower detoxification of MBOA to methoxy glucoside carbamate (**1**) is in agreement with the known higher toxicity of MBOA to both dicots and monocots, in comparison to BOA. Whereas almost all of the dicotyledoneous weeds and crops tested were able to perform BOA-N-glucosylation, at least in traces, MBOA was only slowly converted in the Poaceae tested. The slower conversion together with the accumulation of high amounts of still toxic BOA-6-*O*-glucoside is assumed to be an important reason for the higher toxicity of the methoxylated benzoxazolinone, also for grasses.

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Extracts from BOA-incubated roots of *P. oleracea* contained besides BOA-6-*O*-glucoside the new compound **2** and small amounts of an additional, related detoxification product with a more hydrophobic character. This compound was isolated from the extract in microgram amounts only, allowing for an HRMS measurement and some 600 MHz NMR data, but it was rather unstable and the decay resulted in compound **2**. A complete set of HRMS and NMR spectra could be obtained, which allowed a structural assignation as 5-β-D-glucopyranosyloxybenzoxazolin-2(3*H*)-one for **2** (BOA-5-*O*-glucoside) as follows.

NMR investigations of a low-concentration sample of **2** have been carried out in DMSO-d_6 to identify also the OH protons of the sugar moiety. The structural assignment was possible especially by means of two-dimensional methods (COSY, HMQC, HMBC). In the aromatic region of the proton spectrum three signals at 7.17 (d, $^3J = 8.7$ Hz), 6.78 (d, $^4J = 2.4$ Hz), and 6.75 ppm (dd, $^3J = 8.7$ Hz, $^4J = 2.4$ Hz) could be observed, showing the coupling pattern of a 1,2,4-trisubstituted benzene derivative. The anomeric proton of the sugar at 4.76 ppm gives a doublet with a coupling constant of 7.6 Hz, which is typical for the axial position (β-configuration). In the HMBC spectrum additionally a cross-peak from the anomeric proton to a quaternary carbon at 155.1 ppm could be found, which is caused by a three-bond coupling from the glucose to the aromatic part of the heterocycle.

The negative mode ESIMS of **2** with ammonia as buffer gave a main peak at 312.07176 Da (exact theoretical mass 312.07249 Da). In addition, a dimer signal at 625.15246 Da (exact theoretical mass 625.15226 Da) was found, and the mass analysis yields the formula $C_{13}H_{14}NO_8$ for the monomer peak, which leads to $C_{13}H_{15}NO_8$ for compound **2**.

The structure of the unstable derivative of **2** mentioned above may be a monoacetylated BOA-5-*O*-glucoside; however detailed information on the position of acetylation is not possible. The identity of compound **2** was further ascertained by assaying with β-glucosidase from almonds. The enzymatic hydrolysis led to an aglycone identical with 5-hydroxybenzoxazolin-2(3*H*)-one (BOA-5-OH), which was synthesized as a reference and substrate in three steps from 2-nitrophenol, as reported below.

Incubations of maize seedlings and *P. oleracea* plants with BOA-5-OH were performed to complete the study. Both species absorbed the compound, and in the root extracts BOA-5-*O*-glucoside was detected. In maize, however, no further metabolite could be found. As a final check, the product **2** was isolated from maize incubated with BOA-5-OH and used for a full structural analysis by means of NMR and HRMS. Thus, *P. oleracea* when incubated with BOA produces via BOA-5-OH the BOA-5-*O*-glucoside **2**, which is further modified.

Maize is able to synthesize BOA-5-*O*-glucoside **2**, also, but the detoxification pathway via BOA-5-OH is not realized in vivo. Instead, BOA-6-*O*-glucoside and glucoside carbamate production is favored, with the latter accumulating in micromole amounts when the plants are incubated in the presence of 500 μmol of BOA for 24 h. The use of an analytical Nucleodur 100-5 C18 HPLC column, however, revealed a further detoxification product, 1-(2-hydroxyphenylamino)-6-*O*-malonyl-1-deoxy-β-glucoside 1,2-carbamate (**3**) (malonyl glucoside carbamate), which is present in traces after 24 h but became a major product after at least 48 h of incubation. Compound **3** has a UV spectrum similar to that of BOA and glucoside carbamate and eluted only 1.5 min earlier than BOA. For these reasons it had been overlooked in former studies where HPLC runs with extracts from roots incubated for 24 h were performed only with an analytical ultrasphere ODS RP 18 column, where the new compound **3** coeluted with BOA. In subsequent studies compound **3** was found in the highest concentration in the 100.000 g supernatant prepared according to Matsushima et al.[10] It was isolated from the supernatant and subjected to structural analysis.

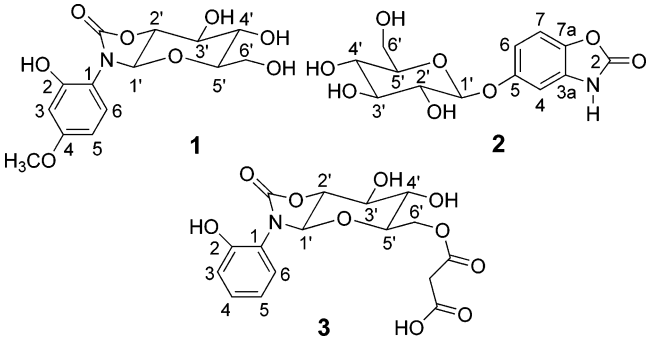

Figure 1. Detoxification products **1**−**3**.

The NMR spectra of **3** in CD_3OD showed signals that led to the structural assignment shown in Figure 1. In the ^1H spectrum of **3** a singlet at 3.35 ppm representing the CH_2 group of the malonyl unit was found close to the solvent signal. However, we could not detect the corresponding carbon correlation cross-peak in the 2D spectra. An explanation for this peculiar effect came from the NMR investigation of a 6-*O*-malonyl-D-glucose sample, prepared as a reference compound according to Kasai et al., which showed an analogous behavior.[11] Again, the methylene singlet appears at 3.35 ppm, whereas in the carbon spectrum only a very broad signal at around 41.94 ppm could be observed. In both cases, this behavior is caused by a slow H−D exchange of the acidic malonyl-methylene unit with the deuterated solvent causing line broadening: The ^{13}C NMR spectra of **3** showed three carbonyl absorptions (170.19, 168.45 (typical for malonates), and 155.29). The HMBC spectrum of **3** shows cross-peaks from H-1′ (d, 5.31 ppm) to the carbamate CO at 155.29 ppm and from the diastereotopic 6′-OCH_2 group (4.35 and 4.56 ppm) to the malonyl ester carbonyl group at 168.45 ppm. Therefore, the carbonyl absorption at 170.19 ppm probably belongs to the malonyl carboxy group.

HRESIMS spectra of glucoside **3** have been measured in CH_3OH solution after NMR spectra have been recorded from the same sample in CD_3OD solution, which led to H−D exchange. Therefore, these mass spectra showed several single peaks as well as peak groups. No intensive peak resulting from a potential metabolite was found in the low molecular range that corresponded to a dimer signal. However, by using different buffers (MeOH−H_2O−NH_3, MeOH−Et_3N, MeOH−H_2O−Et_3N) one peak group (starting from 766 to 772 Da) in the molecular weight range of dimers was persistent and increased with increasing base strength. By using an NH_3 buffer solution a second peak group starting from 787 to 791 Da was also found, due to the corresponding sodium adduct.

The mass analysis yields $C_{32}H_{31}D_2N_2O_{20}$ and $C_{32}H_{30}D_2NaN_2O_{20}$ for the two most intensive dimer peaks (767.17561 Da, exact mass 767.17577; 789.15811 Da, exact mass 789.15771 Da) of the partially deuterated ions. Therefore, we postulate the formula $C_{16}H_{17}NO_{10}$ for the monomeric form of the metabolite. This is in accordance with the structural proposal derived for **3** from the NMR spectra. For the validation of the intrinsic nondeuterated pseudomolecular ion the solution was evaporated a second time and redissolved in MeOH. The most intensive peaks are now 765.16262 Da $(M - H)^-$ (calcd 765.16322 Da) and 787.14655 Da $(M + Na - 2H)^-$ (calcd 787.14516 Da).

Ensuing from the structural determination of **3** we believe that the reported 6-*O*-oxalyl glucoside has instead to be regarded as a malonyl derivative, also.[12,13] In view of our findings, the reported oxalyl derivative torachrysone 8-*O*-β-D-(6-oxalyl)glucopyranoside is a malonyl derivative. Similarly to compound **3**, two downfield signals for carbonyl carbons (168.9 and 170.5 ppm in DMSO-d_6) were reported; however, no carbon for the CH_2 unit was mentioned. We assume that the same effect as with compound **3** has happened, which "hides" this special carbon by proton exchange as a dynamic effect. It is well known that oxalic acid monoester carbons have to

be expected at about 160 ppm, whereas the carbonyl shifts for malonic acid monoesters are expected distinctively downfield at about 170 ppm.

Malonylation is a common step in higher plant detoxification strategies. More than 175 6-*O*-malonylated glucosides have been reported as natural products. For instance, 2,4-dichlorophenoxyacetic acid or dichloroaniline accumulates as a malonyl glucoside.[14,15] An elusive indigo precursor in *Isatis tinctoria* has been shown to be a malonyl glycoside of 3-hydroxyindole.[16] Recently, an acyltransferase from tobacco cells has been characterized that catalyzes malonic acid transfer to exogenous flavonoid- and naphthol-glycosides.[17] The modification facilitates or manages the transport of already glucosylated secondary compounds or xenobiotics by ATP-binding cassette transporters to target compartments, such as the vacuole.[18] Malonylation of glucoside carbamate may facilitate the transport into the vacuole. As maize roots exude at least a part of the detoxification products and no extractable detoxification product is detectable in the plants a few days after exposure to BOA,[7] it is also possible that malonylation is involved in the transfer into the apoplastic space.

Experimental Section

General Experimental Procedures. Caryopses of *Z. mays* L. were aeroponically grown on cheesecloth for 5 days. Seeds of *P. oleracea* were grown under field conditions for 6 weeks. The plants were carefully harvested, cleaned from soil particles, and washed until material adhering to the roots was completely removed. Maize seedlings and *Portulaca* plants were incubated with 250 mL of medium containing either 500 μM BOA, 250 μM MBOA, or 100 μM BOH-5-OH for 48 h. The medium was prepared as described by Schulz and Wieland.[6] After incubation the roots were extracted with 50% MeOH. The extracts were centrifuged for 15 min at 10.000*g* and the supernatants used for HPLC analysis. HPLC was performed with a Beckman Coulter model 126 chromatograph equipped with a diode array detector and an analytical ultrasphere ODS RP 18 column (Beckman Coulter). Elution of the compounds was done with a gradient described in Wieland et al.[19] The new detoxification products (300−400 μg) with retention times of 19 min (for **2**) and 27 min were collected during 30−40 HPLC runs. The corresponding fractions were combined and evaporated to dryness, and the residues were dissolved in MeOH to check the purity by HPLC. The remaining solutions were dried by vacuum centrifugation at 4 °C. The residues were used for structural analyses. β-Glucosidase from almonds (Sigma) was used for enzymatic hydrolysis of the glucose moiety from BOA-5-*O*-glucoside **2**. Enzyme activity was assayed with acetate buffer pH 5.5 at 30 °C for 15 min. The assays were stopped by boiling for 5 min, centrifuged, and checked for the product BOA-5-OH by HPLC. Compound **3** was isolated from a 100.000*g* supernatant obtained by the procedure described by Matsushima et al.[10] Maize roots (4 g) harvested from BOA incubated plants (500 μM BOA, 48 h) were chopped with a razor blade in chopping buffer (50 mM Hepes pH 7.5, 5 mM EDTA, 0.4 M sucrose, 100 μL of protease inhibitor cocktail (Sigma)). The chopped material was filtered though Miracloth (Calbiochem) and centrifuged twice at 1.000*g* and then at 8.000*g* prior to the final centrifugation at 100.000*g* for 1 h at 4 °C. The resulting supernatant was extracted with EtOAc, the aqueous phase was evaporated in a vacuum centrifuge to dryness, and the residue was dissolved in 50% MeOH. The procedure was repeated four times. From the combined MeOH solutions compound **3** was isolated by 50 HPLC runs using an analytical Nucleodur 100-5 C18 column (Macherey & Nagel) and the gradient described above. Fractions containing the compound were combined and evaporated to dryness, yielding about 0.5 mg of compound **3**.

NMR spectra for the detoxification products **1** and **2** were recorded on a Bruker DRX-600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz). NMR spectra for **3** were measured on a Bruker DRX-700 Avance spectrometer (^{1}H: 700 MHz, ^{13}C: 175 MHz). Chemical shifts are reported in ppm; the signals of CD_3OD (^{1}H: $\delta = 3.31$ ppm; ^{13}C: $\delta = 49.00$ ppm) and DMSO-d_6 (^{1}H: $\delta = 2.50$ ppm; ^{13}C: $\delta = 39.52$ ppm) were used as internal references. HRESIMS spectra were recorded with syringe infusion with a Bruker FT-ICR mass spectrometer APEX II, equipped with a 7 T magnet.

Synthesis of 5-Hydroxybenzoxazolin-2(3*H*)-one. 5-Hydroxybenzoxazolin-2(3*H*)-one was synthesized starting from 2-nitrophenol, which was oxidized with alkaline ammonium persulfate to form 2-nitrohydroquinone in 35% yield according to the literature.[20] Hydrogenation of this compound in diluted HCl under normal pressure over Pd−C followed by filtration and complete removal of the solvent by lyophilization led to a pale greenish powder of 2-aminohydroquinone hydrochloride (mp 202−205 °C). This salt was heated together with urea at 145 °C for 3 h following the method reported[21] to yield black crystals of crude BOA-5-OH (mp 205−211 °C). Both recrystallization and column chromatography did not result in BOA-5-OH pure enough for the incubation studies. Eventually, sublimation of 250 mg of crude product at 3 mbar in a silicon oil bath at 205 °C afforded a pure 100 mg sample of colorless BOA-5-OH (mp 218−218.5 °C, lit. Zinner et al. 209−210 °C): ^{1}H NMR (DMSO-d_6) δ 11.29 (s, 1H, OH), 9.41 (s 1H, NH), 7.07 (d, 1H, $J_{H-7,H-6} = 8,7$ Hz, H-7), 6.50 (d, 1H, $J_{H-6,H-4} = 2.1$ Hz, H-4), 6.45 (dd, 1H, H-6); ^{13}C NMR (DMSO-d_6) δ 155.1 (C-2), 154.2. (C-5), 136.4 (C-7a), 131.0 (C-3a), 109.9 (C-4), 108.0 (C-7), 97.4 (C-6). These spectra have been measured with a Gemini 300BB spectrometer.

1-(2-Hydroxy-4-methoxyphenylamino)-1-deoxy-β-D-glucoside 1,2-carbamate (1): ^{1}H NMR (CD_3OD, H,H-COSY) δ 7.24 (d, $^3J = 8.7$ Hz, 1H, H-6), 6.94 (d, $^4J = 2.4$ Hz, 1H, H-3), 6.79 (dd, $^3J = 8.7$ Hz, $^4J = 2.4$ Hz, 1H, H-5), 5.25 (d, $^3J = 9.3$ Hz, 1H, H-1′), 4.00 (dd, $^3J = 9.3$ Hz, $^3J = 9.0$ Hz, 1H, H-2′), 3.90 (dd, $^2J = 12.2$ Hz, $^3J = 1.9$ Hz, 1H, H-6′a), 3.80 (s, 3H, OCH_3), 3.71 (dd, $^2J = 12.2$ Hz, $^3J = 5.7$ Hz, 1H, H-6′b), 3.53 (dd, $^3J = 9.3$ Hz, $^3J = 9.0$ Hz, 1H, H-3′), 3.51 (m, 1H, H-5′), 3.47 (dd, $^3J = 9.3$ Hz, $^3J = 9.1$ Hz, 1H, H-4′); ^{13}C NMR (CD_3OD, HMQC, HMBC) δ 157.0 (C-4), 154.7 (NCOO), 143.8 (C-2), 122.7 (C-1), 111.8 (C-6), 109.6 (C-5), 97.1 (C-3), 84.6 (C-1′), 80.1 (C-5′), 77.7 (C-3′), 70.3 (C-4′), 70.1(C-2′), 61.7 (C-6′) 55.4 (OCH_3).

5-β-D-Glucopyranosyloxybenzoxazolin-2(3*H*)-one (2): ^{1}H NMR (DMSO-d_6, H,H-COSY) δ 7.17 (d, $^3J = 8.7$ Hz, 1H, H-7), 6.78 (d, $^4J = 2.4$ Hz, 1H, H-4), 6.75 (dd, $^3J = 8.7$ Hz, $^4J = 2.4$ Hz, 1H, H-6), 6.63 (brs), 1H, NH), 5.28 (d, $^3J = 4.9$ Hz, 1H, 2′-OH), 5.05 (d, $^3J = 4.7$ Hz, 1H, 3′-OH), 4.99 (d, $^3J = 5.3$ Hz, 1H, 4′-OH), 4.76 (d, $^3J = 7.6$ Hz, 1H, H-1′), 4.57 (t, $^3J = 5.7$ Hz, 1H, 6′-OH), 3.69 (1H, H-6′a), 3.46 (1H, H-6′b), 3.29 (m, 1H, H-5′), 3.25 (m, 1H, H-3′), 3.21 (m, 1H, H-2′), 3.14 (m, 1H, H-4′); ^{13}C NMR (DMSO-d_6, HMQC, HMBC) δ 155.4 (HNCOO), 155.1 (C-5), 139.2 (C-7a), 131,8 (C-3a), 110.7 (C-6), 110.5 (C-7), 102.6 (C-1′), 100.1 (C-4), 77.9 (C-5′), 77.3 (C-3′), 74.1 (C-2′), 70.6 (C-4′), 61.6 (C-6′).

1-(2-Hydroxyphenylamino)-6-*O*-malonyl-1-deoxy-β-D-glucoside 1,2-carbamate (3): ^{1}H NMR (CD_3OD, H,H-COSY) δ 7.34 (d, $^3J = 7.3$ Hz, 1H, H-6), 7.26 (d, $^3J = 7.8$ Hz, 1H, H-3), 7.23 (dd, $^3J = 7.3$ Hz, $^4J = 1.0$ Hz, 1H, H-5), 7.18 (dd, $^3J = 7.8$ Hz, $^4J = 1.0$ Hz, 1H, H-4), 5.31 (d, $^3J = 9.3$ Hz, 1H, H-1′), 4.56 (dd, $^2J = 12.1$ Hz, $^3J = 2.1$ Hz, 1H, H-6′a), 4.35 (dd, $^2J = 12.1$ Hz, $^3J = 5.2$ Hz, 1H, H-6′b), 4.00 (dd, $^3J = 9.3$ Hz, $^3J = 9.0$ Hz, 1H, H-2′), 3.75 (m, 1H, H-5′), 3.56 (m, 1H, H-3′), 3.56 (m, 1H, H-4′), 3.35 (s, 2H, CH_2); ^{13}C NMR (CD_3OD, HMQC, HMBC) δ 170.10 (COOH), 168.45 (*C*$OOCH_2$), 155.29 (NCOO), 144.08 (C-2), 130.18 (C-1), 125.23 (C-5), 124.06 (C-4), 112.61 (C-6), 110.85 (C-3), 85.50 (C-1′), 78.25 (C-3′), 78.09 (C-5′), 71.01 (C-4′), 70.89 (C-2′), 65.06 (C-6′), 40.42 broad, weak (HOOC−*C*H_2−CO−).

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