Identification of Novel Low-Weight Sulfhydryl Conjugates of Oxidized 5-O- and 6-O-Substituted Betanidin Pigments

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ABSTRACT: The formation of conjugates of oxidized betacyanin pigments with selected low-weight sulphydryl scavengers was studied. Short-lived quinonoids, quinone methides, and aminochromes derived from oxidized betacyanins are able to form adducts with different efficiencies. In this report, mass spectrometric and NMR identifications of CS-linked conjugates of cysteine, cysteamine, N-acetylcysteine, and N,N-dithiobisethanol with quinonoid forms generated through oxidation of betanidin, betanin, and gomphrenin is presented. An adduct that formed between cysteine and quinonoid generated from betanin by its oxidation and decarboxylation (2-decarboxy-xanbetanin) was detected and reported for the first time. The most stable gomphrenin CS-conjugate, N-acetylcysteinylated gomphrenin, was isolated by semipreparative chromatography and its structure was established by NMR analysis. This enabled to confirm the conjugation position at carbon C-4 and to indicate the presence of a dopachromic intermediate during oxidation of gomphrenin. Conjugation of betacyanins with thiol-bearing moieties may generate new molecules with modified chemical and biological properties. Obtained results confirm that gomphrenin is capable of forming CS-conjugates with higher efficiency than betanin.

INTRODUCTION

Medicinal plants are regarded as a major source of therapeutic agents capable of curing human diseases, which may also be utilized as functional plant-based foods. Natural compounds including betalains gained much attention due to the increasing social awareness of the positive impact of natural products on human health. In addition, natural dyes obtained from plants are renewable and sustainable bioresource products with minimum environmental impact. In contrast to synthetic colorants, they do not cause allergic, toxic, and carcinogenic responses, which is why they have gained importance for the food industry. Betalains are unique, water-soluble, and indole-derived natural pigments, which occur in most families of the Caryophyllales order. They are present at high concentrations in red and yellow beetroot (Beta vulgaris L.), colored Swiss chard (B. vulgaris L. ssp. cicla), leafy or grainy amaranth (Amaranthus sp.), cactus fruit (Opuntia sp. and Hylocereus sp.), and Basella alba L. fruits and leaves. Betanin (5-O-β-glucoside of betanidin) is the most abundant and the most frequently studied betacyanin occurring in red beetroot (Beta vulgaris L.), while gomphrenin (6-O-β-glucoside of betanidin), which is an isomeric pigment to betanin, is identified at high concentrations in fruits of B. alba L. as well as in leaves of its variety B. alba var. Rubra.

Betalain pigments are not only considered as safe food additives with prominent coloring attributes but also as bioactive compounds with therapeutic and nutraceutical potential, which may inhibit formation of reactive oxygen species (ROS) or free radicals responsible for chronic inflammation in the human body, delay the aging process, and prevent from chronic diseases such as hypertension, dyslipidemia, and even cancer. It has been shown that some of the betalain pigments exhibit even higher antioxidant activity in comparison to common natural antioxidants such as ascorbic acid, rutin, and catechin. Furthermore, studies with different cell lines have demonstrated that some extracts rich in betalains proved to be effective growth inhibitors and apoptosis inducers in cell lines of immortalized ovarian, cervical, bladder, and melanoma cancer cells. Due to their high biological activity, they deserve particular attention and detailed investigation.

So far, very little progress has been made in establishing pharmacokinetics and pharmacodynamics of betalains, with aspects concerning their metabolism, biological activity, and...
bioaccumulation in living organisms being also unstudied.\textsuperscript{14,15} Some studies indicate that natural antioxidants from dietary sources (especially their oxidation semiproducts) can also act as pro-oxidants, which produce free radicals and cause DNA damage and mutagenesis, depending on their concentration and cellular microenvironment.\textsuperscript{16,17} In spite of being effective antioxidants, quinones generated from betacyanins may also be toxic under certain conditions.\textsuperscript{18} Anthocyanins, carotenoids, and flavonoids are examples of substances with such dual in vitro behavior, but there is no information about betalains.\textsuperscript{16,17}
The initial studies on the enzymatic and nonenzymatic oxidation of betanin-type betacyanins and betanin as well as the research on the final or intermediate reaction products were performed. According to our previous reports,19 in the course of betacyanin oxidation, α-quinone or other possible quinonoid structures, aminochromes/quinone methides can be formed depending on the 5-O- and 6-O-substitution pattern in betanin.19 Such derivatives may undergo further oxidation stages, thus reducing next portions of radicals being scavenged.

An important approach of direct detection of the short-lived, reactive intermediates is to utilize trapping agents that form stable adducts with the reactive forms that can be detected and characterized using MS/MS techniques.23 Research on adduct formation between thiols and quinones generated from betalains is a fundamental step toward better understanding of their function in biological systems. The conjugation reactions of different nucleophilic thiols with various compounds have been reported.24–29 Sulphydryl-containing compounds such as cysteine (Cys), cysteamine (CH), N-acetylcysteine (NAC) as well as dithiothreitol (DTT), which are electron-rich nucleophilic thiol molecules may form covalent bonds via 1,4-Michael addition reaction with electrophiles such as quinones generated during oxidation of polyphenolic compounds.30 They scavenge both one- and two-electron oxidants and play a vital role in redox-mediated or oxidant-induced processes in the cell.21 Furthermore, cysteine as well as N-acetylcysteine conjugates are excreted in urine, which is why their quantitative and qualitative analysis may provide information about the amount of conjugates produced.23 Therefore, such adducts may be regarded as quantitative biomarkers of oxidative stress.32

Oxidation of protein side chains leads to the formation of protein radicals, which subsequently cross-link with other side chains, forming dityrosine or disulfide bonds.33–36 The presence of polyphenolic compounds in foods not only prevents the thiol moiety (–SH) from oxidation but also leads to polyphenol oxidation to quinones, which may react with thiol groups of protein and form thiol–quinone adducts.30 CS-conjugates play a significant role in blocking the thiol groups in food proteins contributing to improved food quality.37

CS-conjugates are also responsible for aroma generation of many fermented foods and wines.38–40 Most probably, the formation of thiols is also involved in the fresh beer flavor.40 In addition, certain CS-linked compounds are strong in-mouth flavor precursors. For instance, conjugation between i-cysteine and reducing sugars in the Maillard reaction is important in the formation of sulfur-containing odorants.41 The reaction of i-cysteine with pentoses (ribose, xylose, or arabinose) also contributes to the aroma of cooked meat and heated food.42

According to our previous research,43 short-lived quinones derived from oxidized betanin and gomphrenin pigments can form stable adducts with glutathione at certain stages of their oxidation and can be detected and characterized using MS/MS and NMR techniques. It was also reported that glutathione reacts with quinones at carbon C-4 of the betanin ring. In the case of betanin, however, its predicted quinonoid intermediate (quinone methide) conjugate with glutathione was not observed, presumably because of a steric hindrance at carbon C-7 of betanin (predicted conjugation position), which reacts with quinones at carbon C-4 of the betanidin ring. In the case of betanin; however, its predicted quinonoid intermediate (quinone methide) conjugate with glutathione was not observed, presumably because of a steric hindrance at carbon C-7 of betanin (predicted conjugation position), which indirectly confirms that the quinonoid form generated during betanin oxidation is actually a quinone methide.22 For that reason, the experimental approach was changed, and smaller

### Table 1. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed CS-Conjugates (Cys, CSH, NAC, and DTT) with Oxidized Betacyanins (Bd, Bt, and Gp) and Their Diastereomers

| no. | compound abbreviation | R [min] | λ<sub>max</sub> [nm] | m/z | m/z from MS/MS of [M + H]⁺ |
|-----|------------------------|---------|---------------------|-----|-----------------------------|
| A1  | betanin-Cys Bd-Cys      | 8.8     | 541                 | 508 | 421; 375; 331               |
| A2  | betanin-CHS Bd-CHS      | 9.0     | 541                 | 464 | 420; 376; 331               |
| A2' | isobetanin-CHS Bd-CHS   | 9.4     | 541                 | 464 | 420; 376; 331               |
| A1' | isobetanin-Cys Bd-Cys   | 9.7     | 541                 | 508 | 421; 375; 331               |
| A3  | betanin Bd              | 10.2    | 541                 | 389 | 345                         |
| A3' | isobetanin Bd           | 11.1    | 541                 | 389 | 345                         |
| A4  | betanin-NAC Bd-NAC      | 12.4    | 541                 | 550 | 421; 375; 331               |
| A5  | betanin-DTT Bd-DTT      | 13.5    | 541                 | 541 | 495; 421; 375               |
| A4' | isobetanin-NAC Bd-NAC   | 13.8    | 544                 | 550 | 421; 375; 331               |
| A5' | isobetanin-DTT Bd-DTT   | 14.3    | 541                 | 541 | 495; 421; 375               |
| B6  | gomphrenin-Cys Gp-Cys   | 8.6     | 544                 | 670 | 626; 583; 508               |
| B7  | gomphrenin-CHS Gp-CHS   | 8.8     | 545                 | 626 | 582; 538; 464               |
| B6' | isogomphrenin-Cys Igp-Cys | 9.4     | 544                 | 670 | 626; 583; 508               |
| B7' | isogomphrenin-CHS Igp-CHS | 9.5    | 545                 | 626 | 582; 538; 464               |
| B8  | gomphrenin Gp           | 9.9     | 540                 | 551 | 389; 345                    |
| B8' | isogomphrenin Igp       | 11.1    | 540                 | 551 | 389; 345                    |
| B9  | gomphrenin-NAC Gp-NAC   | 12.0    | 545                 | 712 | 583; 550; 421               |
| B9' | isogomphrenin-NAC Igp-NAC | 12.6   | 545                 | 712 | 583; 550; 421               |
| B10 | gomphrenin-DTT Gp-DTT   | 12.6    | 540                 | 703 | 569; 541; 497               |
| B10' | isogomphrenin-DTT Igp-DTT | 13.3   | 540                 | 703 | 569; 541; 497               |
| C11 | betanin Bt              | 6.9     | 535                 | 551 | 389; 345                    |
| C12 | 2-decarboxy-xanbetanin-Cys 2-dXbt-Cys | 8.0 | 520 | 624 | 493,331 |
| C11' | isobetanin Ib              | 8.6     | 535                 | 551 | 389; 345                    |
| C12' | 2-decarboxy-xanisobetanin-Cys 2-dXibt-Cys | 9.0 | 620 | 624 | 493,331 |
| C13 | 2-decarboxy-xanobetanin 2-dXbt | 12.4 | 465 | 505 | 343,297,251 |
| C13' | 2-decarboxy-xanisobetanin 2-dXibt | 13.0 | 475 | 505 | 343,297,251 |
molecules of sulphydryl reagents, which would be presumably able to form conjugates also at carbon C-7, were applied. Additionally, in order to definitely confirm the conjugation position at carbon C-4 in the case of 6-O-glucoylated betacyanin, CS-conjugates of gomphrenin with smaller thiol molecules were generated with higher efficiencies for structural determination by NMR. A previously obtained GSH conjugate of gomphrenin CS-conjugates as it is discussed in the following section based on current NMR results.22

For this aim, we examined Cys, CSH, NAC, and DTT CS-conjugates with quinones derived from betacyanins (Bd, Bt, and Gp) by LC-DAD-ESI-MS/MS and LC-IT-TOF. The most stable CS-conjugate of N-acetylcyesteine with oxidized gomphrenin (Gp-NAC) was isolated and its structure was established by NMR techniques.

**RESULTS AND DISCUSSION**

Due to the fact that short-lived intermediates derived from the oxidized betacyanins are characterized by high reactivity and fast rearrangement to more stable products, their detection in reaction mixtures is problematic. Therefore, the utilization of a trapping agent capable of freezing the quinonoid forms enables detection and characterization of such unstable entities. Previously,22 stable adducts of oxidized betanin and gomphrenin with glutathione were obtained; however, presumably due to a steric hindrance at carbon C-7 of betanin, its glutathione conjugate was not observed. For that reason, four low-molecular weight thiol reagents, Cys, CSH, NAC, and DTT, were tested toward their ability to conjugate with oxidized betanin but also with oxidized betanidin and gomphrenin to enhance the possibilities of a new conjugate formation.

Formation of CS-Conjugates between Oxidized Betanin and Sulphydryl Reagents. The LC–MS chromatograms of selected ions of CS-conjugates of betanin with Cys, CSH, NAC, and DTT are presented in Figure 1. The discussed adducts were formed by the sulphydrylation of betanin/isobetanin quinonoids with Cys, CSH, NAC, and DTT generated during betanin/isobetanin A3/A3′ oxidation by ABTS cation radicals. In the case of Bd-NAC A4/A4′ and Bd-DTT A5/A5′, resulting CS-conjugates were eluted after the pigment substrates, which indicates that they are less polar than their corresponding precursors A3/A3′ (Table 1). These results are in contrast to Bd-Cys A1/A1′ and Bd-CSH A2/A2′ as well as to glutathionylated betanin (Bd-GSH) reported in our previous study,22 which were eluted before the starting pigment. Bd-Cys and Bd-NAC and their isoforms were formed with the highest efficiencies, whereas Bd-DTT and Bd-CSH exhibited lower signal intensity.

Initially, the fragmentation spectra of conjugates were obtained by low-resolution triple quadrupole mass spectrometry (Table 1). The molecular masses as well as the fragmentation patterns of obtained CS conjugates were confirmed by multistage fragmentation of high-resolution IT-TOF mass spectrometry (Table 2). Based on previous NMR results,22 the proposed pathway (Oxidation A) of betanin CS-conjugate formation is depicted in Figure 2. This scheme is in accordance with the pathway leading to the formation of gomphrenin CS-conjugates as it is discussed in the following section based on current NMR results.

Interpretation of LC-DAD-ESI-MS/MS and LC-IT-TOF spectra revealed that the adducts A1/A1′, A2/A2′, A4/A4′, and A5/A5′ exhibited the absorption maxima at λmax 541 nm and characteristic protonated molecular ions in the positive scan mode at m/z 508, 464, 550, and 541, respectively. Said

**Table 2. High-Resolution Mass Spectrometric Data Obtained by IT–TOF for Bd-Cys A1, Bd-CSH A2, Bd-NAC A4, Bd-DTT A5, Gp-Cys B6, Gp-CSH B7, Gp-NAC B9, Gp-DTT B10, and 2-dXBt-Cys C12 Conjugates and for Their Fragmentation Ions**

| no. | fragmentation ions | molecular formula | [M + H]+ observed | [M + H]+ predicted | error [mDa] | error [ppm] | MS+ ions |
|-----|-------------------|--------------------|-------------------|-------------------|------------|------------|----------|
| Betanin Conjugates |
| A1 | Bd-Cys | C21H22N3O10S | 508.1010 | 508.1020 | −1.0 | −1.97 | 421; 377; 375; 329 |
| A2 | Bd-CSH | C21H22N3O10S | 464.1106 | 464.1122 | −1.6 | −3.45 | 420; 403; 376; 375; 359; 331; 315; 287 |
| A4 | Bd-NAC | C21H22N3O10S | 550.1138 | 550.1126 | 1.2 | 2.18 | 421; 377; 375; 329 |
| A5 | Bd-DTT | C21H22N3O10S | 541.0932 | 541.0945 | −1.3 | −2.40 | 497; 495; 453; 449; 421; 375; 331; 329 |
| Gomphrenin Conjugates |
| B6 | Gp-Cys | C21H22N3O10S | 670.1522 | 670.1549 | −2.7 | −4.03 | 626; 583; 508; 464; 421; 377 |
| B7 | Gp-CSH | C21H22N3O10S | 626.1669 | 626.1650 | 1.0 | 3.03 | 464; 377 |
| B9 | Gp-NAC | C21H22N3O10S | 712.1639 | 712.1654 | −1.5 | −2.11 | 583; 550; 421 |
| B10 | Gp-DTT | C21H22N3O10S | 703.1497 | 703.1473 | 0.8 | 1.37 | 421; 375; 331; 292; 287 |
| Betatin Conjugate |
| C12 | 2-dXBt-Cys | C21H22N3O10S | 624.1486 | 624.1494 | −0.8 | −1.28 | 580; 493; 462; 418; 331; 287 |

nl: Neutral losses from the conjugates.

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CS-adducts were generated between previously formed quinonoid intermediates of betanidin diastereomers A3/A3′ (m/z 387) and Cys (508−387 = 121), CSH (464−387 = 77), NAC (550−387 = 163), and DTT (541−387 = 154), respectively.

Bd-Cys A1 and Bd-NAC A4 conjugates yielding ions at m/z 508.1010 and m/z 550.1138 with the confirmed molecular formulas C_{21}H_{22}N_{3}O_{10}S (calculated m/z: 508.1020) and C_{23}H_{24}N_{3}O_{11}S (calculated m/z: 550.1126), respectively, share a common pattern in CID experiments in positive ionization mode. Scission of 2-aminopropanoic acid (alanine) within Cys as well as within NAC moieties produces fragment ions [M + H]+ at m/z 421, corresponding to thiol of betanidin (Bd-SH). Furthermore, fragmentation of the latter ions results in the generation of ions at m/z 377 derived from a neutral loss of CO_{2} (−44 Da). Other characteristic daughter ions detected at m/z 375 (375 = 421−46) and 329 (329 = 421−92) were assigned to the loss of one and two formic acid molecules, respectively, from Bd-SH ions (m/z 421).

Further experiments with another small molecule reagent, CSH, confirmed its betanidin conjugation possibility. The HRMS determination of the molecular formula of C_{20}H_{22}N_{3}O_{8}S for the conjugate protonated molecular ions at m/z 464 supported the presence of Bd-CSH A2 (determined m/z 464.1106; calculated m/z 464.1122). The fragmentation pattern afforded signals at m/z 420 (420 = 464−44) and 376.
(376 = 420−44), indicating the formation of mono- and bi-decarboxylated conjugates, respectively. In addition, a neutral loss of NH₃ within the cysteamine moiety, which afforded ions at m/z (403 = 420−17) and 359 (359 = 376−17), respectively was observed. Further decarboxylation of the latter ion (315 = 359−44) as well as a loss of ethene within the rest of the cysteamine moiety in ions at m/z 403, 359, and 315 resulted in a generation of ions at m/z 375 (375 = 403−28),
Gomphrenin and Sulfhydryl Reagents. Based on our previous research, due to the presence of the phenolic group protonated molecular ions at (Cys, CSH, NAC, and DTT) reacted with the dopachromic obtained by the derivatization reaction in which the thiols (Cys, CSH, NAC, and DTT) reacted with the dopachromic intermediate. The fact that gomphrenin may exhibit different oxidation pathways compared to betanin should be considered during the determination of the overall betacyanin oxidation mechanism.

Analogously to adducts of A3/A3′, gomphrenin CS-conjugates B6/B6′, B7/B7′, B9/B9′, and B10/B10′ were obtained by the derivatization reaction in which the thiols (Cys, CSH, NAC, and DTT) reacted with the dopachromic intermediate (m/z 549). Said adducts exhibit characteristic protonated molecular ions at m/z 670, 626, 712, and 703 in the positive scan mode, respectively. All of the mentioned parent ions were subjected to MS/MS fragmentation experiments.

The representative MS spectra for the Cys, CSH, NAC, and DTT conjugates formed with the dopachromic intermediate resulting from gomphrenin B8/B8′ oxidation are shown in Figure 3. Similar to CS-adducts of Bd/IBd A3/A3′ as well as glutathionylated gomphrenin reported previously, Gp-NAC B9/B9′ and Gp-DTT B10/B10′ are less polar than starting pigments B8/B8′, while Gp-Cys B6/B6′ and Gp-CSH B7/B7′ are characterized by greater polarity with respect to B8/B8′.

The fragmentation pattern of Gp-Cys B6 (m/z 670) (Table 2) was based on the neutral loss of glucosyl (m/z 508 = 670−162) as well as mono-decarboxylation (m/z 626 = 670−44), resulting in a formation of the fragment ions at m/z 508 and 626, respectively. Subsequent decarboxylation (m/z 464 = 508−44) and deglucosylation (m/z 464 = 626−162) of the generated ions led to the formation of fragment ions at m/z 464. Based on the research of Dunkelblum et al., primary decarboxylation at position C-17 is rather preferred than C-15. Another fragmentation pathway led through scission of alanine producing fragment ions at m/z 583 (583 = 670−87), corresponding to Gp-SH ions as well as their subsequent deglucosylation to thiol of betanin m/z 421 (421 = 583−162) and decarboxylation (m/z 377 = 421−44).

Similar to A1 and B6, the initial step of Gp-CSH B7 fragmentation (yielding protonated molecular ion at m/z 626) is based on mono- (−44 Da) and bi- (−88 Da) decarboxylation as well as the neutral loss of the glucosyl moiety (−162 Da) and leads to a formation of fragment ions at m/z 582, 538, and 464, respectively (Table 2). Other fragment ions in the spectrum (m/z 420, 376, and 374) were derived from deglucosylated Gp-CSH (m/z 464) by mono- (m/z 420 = 464−44) and bi- (m/z 376 = 420−44) decarboxylation as well as a loss of HCOOH (m/z 374 = 420−46).

In addition, a neutral loss of NH3 within the cysteamine moiety which afforded ions at m/z 403 (403 = 420−17) and...
359 (359 = 376–17), respectively, with further decarboxylation (315 = 359–44) was observed. Subsequent loss of ethene within the rest of the cyssteanine moiety in the latter ions resulted in a generation of dehydrogenated (presumably at carbons C-2,3) fragment ions at m/z 375 (375 = 403–28), m/z 331 (331 = 359–28), and m/z 287 (287 = 315–28), respectively.

Daughter ions detected at m/z 377, 333, and 331 (thiols of gomprenin decarboxylated derivatives) indicated additionally a detachment of aminothene from the CSH moiety in ions at m/z 420 (m/z 377 = 420–43), 376 (m/z 333 = 376–43), and 374 (m/z 331 = 374–43), respectively.

For Gp-NAC B9, the molecular formula C_{29}H_{35}N_{2}O_{15}S_{2} was confirmed by IT-TOF analyses (observed m/z 712.1639; predicted m/z 712.1654), and the fragmentation pattern afforded the characteristic fragment ions [M + H]^+ of m/z 583 and m/z 550, corresponding to the scission of N-acetylanine and glucose moieties, respectively (Table 2, Figure 4). Furthermore, subsequent collision-induced fragmentation experiments performed on the latter ions led to cleavages of glucose and N-acetylanine, respectively, leading to fragment ions at m/z 421, corresponding to Bd-SH (Figure 4). A neutral loss of HCOOH (−46 Da) from the Bd-SH ion at position C-2 resulted in a formation of a fragment ion at m/z 375. Further CO_{2}, 2CO_{2}, and HCOOH cleavages in the latter ion released daughter ions at m/z 331, 287, and 329, respectively.

Additional NMR results obtained for Gp-NAC B9 (next section) confirmed the conjugation position at carbon C-4 and the proposed pathway (Oxidation A) of the CS-conjugate formation (Figure 2) which is the same as the pathway leading to a formation of glutathionylated betanidin identified previously\textsuperscript{22} and presumably the betanidin-based adducts studied in this report.

The LC-IT-TOF analysis of Gp-DTT B10 in the positive mode yielding high-resolution m/z 703.1497 (C_{28}H_{35}N_{2}O_{15}S_{2}, calculated mass: 703.1473) supported their identification. In accordance with Gp-CSH B7 experiments, mass spectrometric fragmentation of the protonated ion [M + H]^+ at m/z 703 of conjugate Gp-DTT B10 displayed the characteristic fragment ion derived from its deglucosylation (m/z 541 = 703–162) (Table 2). Further fragmentation of the ion at m/z 541 displayed other daughter ions at m/z 497 resulting from mono-decarboxylation (m/z 497 = 541–44) and m/z 421, indicating detachment of 1-sulfanyl-butane-2,3-diol from the DTT moiety (m/z 421 = 541–120) and leading to the formation of Bd-SH ions.

Additionally, the product ion scan showed the ions at m/z 375 (decarboxy-Bd-SH) resulting from the loss of HCOOH (375 = 421–46). Subsequent decarboxylation (331 = 375–44) of the formed ions as well as a loss of HCOOH (329 = 375–46) yielded fragment ions at m/z of 331 and 329, respectively.

**NMR Structural Analysis of N-Acetyl-Cysteinylated Gomprenin**

The characteristic NMR signals of the chromophoric moiety confirmed the presence of substituted gomprenin.\textsuperscript{20,22–28} Three individually coupled \textsuperscript{1}H-spin systems of the aglycone (H-2 and H3a/b, indicating the lack of decarboxylation at C-2; H-11 and H-12 distinguished by the low- and high-field positions; H-14a/b and H-15) were readily assigned in the COSY spectra, and the H-18 proton was indicated by its position and width (Table 3).\textsuperscript{20,26} The HSQC correlations were established for H-2, H3a/b, and H-7 in the dihydroindolic system. The following cross-peaks in HMBC (D_{2}O) were determined for the dihydroindolic system, H-7 to C-4,5,6,7,9,11, H-3a/b to C-8,9,10, and H-11 to C-2,8, as well as for the dihydropyridinic system, H-11 to C-13, H-12 to C-14,18, H-14a/b to C-13,15,18,19, H-15 to C-13,14,17,19, and H-18 to C-12,14 (Figure 4, Table 3).\textsuperscript{27,28} An HMBC correlation between H-11 and C-12, confirming the presence of the vicinal system was also observed.

**Table 3. NMR Data for N-Acetyl-Cysteinyl-Gomprenin B9 (Important HMBC and NOESY NMR Correlations for B9 Are Depicted in Figure 5)**

| no. | \textsuperscript{1}H NMR\textsuperscript{a} | \textsuperscript{13}C NMR\textsuperscript{b} |
|-----|--------------------------------|----------------|
| 2   | 4.97, bdd | 66.8 |
| 3a/b| 3.56 (overlap); 3.30 (overlap) | 36.6 |
| 4   |                     | 119.4 |
| 5   |                     | 148.3 |
| 6   |                     | 149.8 |
| 7   | 7.26, s | 104.4 |
| 8   |                     | 136.6 |
| 9   |                     | 133.8 |
| 10  |                     | 177.1 |
| 11  | 8.17, d, 13.7 | 147.0 |
| 12  | 5.89, d, 13.6 | 110.4 |
| 13  |                     | 165.3 |
| 14  | 3.26 (overlap); 3.19 (overlap) | 29.5 |
| 15  | 4.53, bt | 55.7 |
| 16  |                     | 157.5 |
| 17  | 6.24, bs | 108.4 |
| 18  |                     | 177.0 |
| 19  |                     | 167.9 |
| 20  |                     | 105.4 |
| 1′  | 4.93, d, 7.2 | 95.9 |
| 2′  | 3.59 (overlap) | 78.4 |
| 3′  | 3.58 (overlap) | 72.5 |
| 4′  | 3.47 (overlap) | 79.6 |
| 5′  | 3.64 (overlap) | 16.7 |
| 6′a/b| 3.99, dd, 1.7; 12.0 77, 5.1; 10.0 | 63.7 |
| 2′a | 4.38, br | 56.4 |
| 4′a | 1.86, s | 24.6 |
| 5′a | 3.35 (overlap); 3.30 (overlap) | 36.0 |

\textsuperscript{a}H NMR δ [ppm], mult, J [Hz]. \textsuperscript{b}13C NMR δ [ppm]. \textsuperscript{c}13C Chemical shifts were derived from gHSQC and gHMBC.
correlations, and the carbons C-1″ and 4″ were assigned by HMBC.25

The attachment position of the N-acetyl-cysteinylo moiety was primarily indicated by a strong long-range correlation of H-6α/b″ protons to quaternary carbon C-4. The lack of the H-4 signal in the 1H spectrum unambiguously confirmed this position (Figure 5). Additionally, the downfield chemical shift of the H-6α/b″ proton signal in comparison to the signal obtained for free N-acetyl-cysteine (data not shown) supported the CS-conjugation of gomphrenin.26

Furthermore, the NOESY correlation between H-7 and H-11,14 as well as between H-7 and glucosidic protons H-1′,5′ enabled the confirmation of the presence of proton H-7 in the structure, thus excluding the attachment position to C-7. The S-conjugation position at carbon C-4 confirms a formation of the dopachromic intermediate as a result of gomphrenin oxidation.22 Therefore, the spectroscopic data were consistent with the structure of 4-((N-acetyl-l-cysteinyl)-gomphrenin.

Formation of CS-Conjugates between Oxidized Betanin and Cysteine (Cys). According to our previous studies,19,20 betanin oxidation proceeds solely through a quinone methide intermediate. During subsequent betanin dehydrogenation and decarboxylation at carbon C-2 accompanying the rearrangement of the quinone methide, 2-decarboxy-2,3-dehydro-betanin C13 (2-decarboxy-xanbetanin) is generated. Accordingly, except for betanin C11 (m/z 551), the presence of 2-decarboxy-xanbetanin C13 (m/z 505) as well as cysteinylated 2-decarboxy-xanbetanin C12 (m/z 624) accompanied by their C-15 isomers (C11′, C12′, and C13′) was indicated in the reaction mixture (Figures 6 and 7) by means of LC-DAD-ESI-MS/MS and obtained m/z signals of protonated molecular and fragmentation ions in the positive mode. The conjugate C12 exhibited an absorption maximum at $\lambda_{max}$ 520 nm and protonated molecular ion [M + H]$^+$ at m/z 624, which supported the presence of cysteinylated conjugate based on oxidized C13 (624 = 503 + 121).

The conjugate 2-decarboxy-xanbetanin-Cys C12 has never been reported in the literature and this is the first successful attempt of synthesis of a sulphydrylic conjugate of the betanin derivative. The chemical nature of the oxidized product (Figure 2) suggests that a previously searched cysteine conjugate (not detected during monitoring at m/z 670) with betanin quinonoid (670 = 549 + 121) may be rapidly oxidized (Oxidation E) after its formation (after Oxidation D) because a peculiar system of renewed quinone methide of the conjugate is generated in the second oxidation step and it freely rearranges to the 2,3-dehydrogenated derivative with accompanying decarboxylation (Figure 2). However, as mentioned above, the formation of the adduct C12 (after Oxidation C) is also possible directly from the generated 2-decarboxy-xanbetanin C13 after the oxidation of betanin (Oxidation B). This alternative pathway (Figure 2) is supported by the presence of C13 in the ABTS-treated betanin/cysteine solution.

The molecular formula and the fragmentation pathway of C12/C12′ were further evaluated by multistep ion-trap fragmentation experiments by the high-resolution IT-TOF technique (Table 2, Figure 7). Determination of the molecular formula (C26H30N3O13S) for the observed m/z value (624.1486) matching the predicted m/z value (624.1494) in combination with the fragmentation pattern of the precursor ion [M + H]$^+$ supported the detection of 2-dec-xanBt-Cys/xanBt-Cys C12/C12′.

The fragmentation experiments of C12 (m/z 624) (Table 2 and Figure 7) revealed two main pathways based on neutral losses of glucosyl (m/z 462 = 624−162) and CO$_2$ (m/z 580 = 624−44), leading to the formation of 2-decarboxy-xanbetanidin and 2,17-bidecarboxy-xanbetanin ions, respectively.

Figure 6. LC−MS chromatograms of CS-conjugates C12/C12′ obtained in the selected positive ion monitoring after 40 μM betanin C11/ C11′ oxidation by 120 μM ABTS cation radicals in 25 mM aqueous sodium acetate buffer (pH 5) to 2-decarboxy-xanbetanin/xanisobetanin C13/C13′ with further conjugation with 40 μM Cys.
Fragmentation of 2,17-bidecarboxy-xanbetanin ($m/z$ 580) resulted in the detection of ions at $m/z$ 493 and 331 which were assigned to a loss of the alanine moiety ($m/z$ 493 = 580−87) combined with further deglucosylation ($m/z$ 331 = 493−162). Another daughter ion detected at $m/z$ 287 was assigned to the thiol of tridecarboxylated-xanbetanidin as a result of further decarboxylation at carbon C-15 ($m/z$ 287 = 331−44).

In parallel, fragmentation of 2-decarboxy-xanbetanidin ($m/z$ 462) results in detachment of CO$_2$ at C-17 ($m/z$ 418 = 462−44) combined with the loss of the alanine moiety ($m/z$ 331 = 418−87) and further decarboxylation at C-15 ($m/z$ 287 = 331−44), leading to a formation of a fragment ion at $m/z$ 287, corresponding to the thiol of tridecarboxylated-xanbetanidin (Figure 7).

As in the case of the pilot study on glutathionylation of betanin, sulfhydrylation of oxidized betanin with other sulphydryl radical scavengers except cysteine (CSH, NAC, DTT,) did not result in any detection of CS-conjugates or their derivatives at pH 3−8 (data not shown). This may be a consequence not only of steric hindrance at carbon C-7 but also enhanced reactivity of the forming conjugates susceptible to subsequent oxidation, as in the case of C12, and generation of nonchromophoric degradation products.

**CONCLUSIONS**

This study provides first evidence for the formation of the CS-conjugates between selected thiols of low molecular weight and oxidized betanidin, gomphrenin, and betanin oxidation product, 2-dec-xanBt, which were monitored by LC−MS/MS techniques. This is the first report including structural elucidation of N-acetyl-cysteinylated gomphrenin as well as adducts formation between cysteinyl sulphydryl scavengers and oxidized betanin derivative. The findings suggest that formation of the quinone methide during betanin (5-O-glycoside of betanidin) oxidation and generation of dopachrome intermediate in the case of gomphrenin (6-O-glycoside of betanidin) oxidation is highly possible. The position of betanidin substitution with glucosyl at carbon C-5 or C-6 is significant in terms of betalain reactivity. Conjugation of betacyanins with thiol-bearing moieties such as cysteamine and cysteine may generate new molecules with modified chemical and biological properties. The sulphydryl part of the molecules may modulate their capacities to penetrate biological membranes as well as their absorption and metabolism. In food chemistry, a great number of attempts have been made to inhibit the oxidative protein cross-link formation affecting the quality, water content, and red color of meat products. In this respect, the CS-conjugates of betacyanins can play a significant role in blocking the thiol groups in food proteins, contributing to improved food quality. Hitherto, our results confirm that gomphrenin is capable of forming CS-conjugates with higher efficiencies than betanin. However, generation of stable conjugates with betanin dehydrogenated derivatives formed during oxidation of betanin or its intermediate CS-conjugates may also predestinate this pigment as a valuable food-protecting ingredient.

**EXPERIMENTAL SECTION**

Reagents. Formic acid, LC−MS grade methanol, and acetonitrile as well as cysteine, cysteamine, N-acetylcysteine, DL-dithiothreitol, the diammonium salt of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and almond β-glucosidase were obtained from Sigma Chemical Co. (St. Louis, MO). Water purified through a Milli-Q (Millipore) water system (ISO 9001) was used for all solution preparations and dilutions.

Preparation of Betanidin and 5-O- and 6-O-Glucosylated Betanidins. Gomphrenin and betanin diastereomers (2S,15S and 2S,15R) were isolated from fruits of *B. alba* and roots of *B. vulgaris*, respectively, in accordance to previously published reports.44,45 Betanin was obtained in the course of gomphrenin enzymatic deglucosylation, which was catalyzed
by almond β-glucosidase. According to previous protocols, all the pigments were fractionated and purified by semi-preparative liquid chromatography. At each stage of the pigment preparation, reaction mixtures were directly analyzed by the LC-DAD-ESI-MS technique.

**Conjugation Experiments of Oxidized Betacyanins with L-Cysteine, N-Acetylcysteine, Cysteamine, and D-L-Dithiothreitol.** Due to the fact that studied quinones are unstable, these compounds were coupled with various thiols by trapping the freshly formed quinones in situ with different sulfhydryl scavengers (Cys, CSH, NAC, and DTT). In order to trapping the freshly formed quinones in situ with different sulfhydryl scavengers (Cys, CSH, NAC, and DTT) in 25 mM phosphate buffer with 2.45 mM potassium persulfate in the presence of 0.002 M cation radicals were produced by reacting 7 mM ABTS salt and subjected to adduct formation with di-betacyanins were oxidized by 1.2 mM ABTS cation radicals, unstable, these compounds were coupled with various thiols by trapping the freshly formed quinones in situ with different sulfhydryl scavengers (Cys, CSH, NAC, and DTT). In order to trapping the freshly formed quinones in situ with different sulfhydryl scavengers (Cys, CSH, NAC, and DTT). In order to trapping the freshly formed quinones in situ with different sulfhydryl scavengers (Cys, CSH, NAC, and DTT).

**Isolation of Betalains and CS-Conjugates by Semi-preparative HPLC.** Pigment solutions extracted from *B. alba* fruits and *B. vulgaris* as well as their hydrolysis product betanidin were subjected to semipreparative HPLC fractionation using an HPLC system equipped with HPLC semipreparative column Luna C18(2) 250 × 25 mm i.d., 10 μm (Phenomenex) with a 20 mm × 25 mm i.d. guard column of the same material (Phenomenex). A gradient solvent system composed of 1% formic acid in water (solvent A) and acetonitrile (solvent B) for semisynthesized betanidin was used as follows: 0 min, 10% B; increasing to 10 min, 12% B; increasing to 20 min, 14% B; increasing to 30 min, 70% B. For betanin, it was used as follows: 0 min, 10% B; increasing to 10 min, 11% B; increasing to 20 min, 12% B; increasing to 30 min, 13% B; increasing to 40 min; 70% B, and for gomphrenin, it was used as follows: 0 min, 8% B; increasing to 10 min, 10% B; increasing to 20 min, 12% B; increasing to 30 min; 70% B at a flow rate of 3 mL/min at a column temperature of 22 °C. The injection volume of the pigment solution was 2 mL. Isolation of gomphrenin conjugates was performed under the following isocratic system composed of 1% aqueous formic acid (solvent A) and acetonitrile (solvent B) as follows: 0 min, 10%; B to 30 min. The injection volume of the pigment solution was 2 mL, and the flow rate was 3 mL/min. Detection was monitored at 538, 505, 480, and 440 nm. The eluates were pooled and concentrated under reduced pressure at 25 °C and finally freeze-dried.

**LCMS-IT-TOF Analyses.** Mass spectrometric analyses were carried out in the ESI positive ion mode using an LCMS-IT-TOF mass spectrometer coupled to a Prominence HPLC (Shimadzu). The gradient system used for chromatographic separation was the same as in the case of the LCMS-8030 mass spectrometer. High-resolution mass spectra (HRMS), including fragmentation mass spectra, which were recorded using a scan range of m/z 100–2000 Da and collision energy between 15 and 50%, dependent on the structure of the examined compound, were applied. The nebulizing gas flow was 1.5 L/min, the capillary voltage was 4.5 kV, and the curved desolvation line (CDL) and heat block temperature were set at 230 °C. The results of high-resolution mass spectrometric experiments (HRMS) were studied using the Formula Predictor within the LCMS Solution software. Only empirical formulas with a mass error below 5 ppm were taken into account.

**NMR Experiments.** The NMR spectra were recorded for 9 mg of the Gp-NAC B9 conjugate sample at 280 K on Bruker Avance III 700 (Bruker Corp., Billerica, MA, USA) and Agilent DD2 800 (Agilent Technologies, Santa Clara, CA, USA) spectrometers in non-acidiﬁed D2O.45 The 1H and 13C chemical shifts were determined relative to TMS (0.00 ppm for 1H, 0.0 ppm for 13C) as internal standard. All 1D (1H) and 2D (COSY, TOCSY, HSQC, HMBC and NOESY (gradient enhanced)) measurements were performed using standard pulse sequences.
Sustainable Gardening Australia who promoted the gardening project. We express our special gratitude to Mr. Malcolm Haines from Alberton Primary School (Adelaide), and many other gardeners from the Malabar spinach berries in Australia. Special thanks to Ms. Pauline Muir (Adelaide) for her astounding commitment to designing and coordinating the project to cultivate and collect betalains.

Notes

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