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# Purification of a Sinapine-Glucoraphanin Salt from Broccoli Seeds

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## ABSTRACT

*A sinapine* (*sinapoylcholine*)*-glucoraphanin salt has been isolated from broccoli seeds and characterized by NMR and mass spectrometry. This salt extraction method can be used to purify glucoraphanin free from contamination by glucoiberin.*

***Keywords*: *Broccoli, Brassica, Glucoraphanin, Sinapine, Glucosinolate, Salt***

## 1. Introduction

Broccoli seeds contain several glucosinolates, including glucoraphanin, glucoiberin, glucoerucin, sinigrin, 4-hydroxyglucobrassicin, glucobrassicin, neoglucobrassicin, and a number of other minor glucosinolates depending on the hybrid and cultivar [1,2]. Glucoraphanin has attracted attention due to studies linking its degradation products, including its isothiocyanate form sulphoraphane, to the prevention of cancer in humans [3,4]. Due to the complex mixture of the glucosinolates in the Brassica species, isolation of large quantities of pure glucosenolates for further study and biological characterization has been difficult, time consuming, and expensive [5-7].

Brassica species also contain significant quantities of substituted phenolic acids, including sinapine [8-13]. These phenolic acids have been shown to have antioxidant activity [14,15] and may also play a role in the health promoting activities of a diet high in crucifer vegetables. Two major UV absorbing compounds were isolated from broccoli seeds, which decreased during the course of germination and sprouting. Characterization of these compounds resulted in the identification of a unique salt formed from the combination of sinapine and glucoraphanin, while the second compound was shown to be the methyl ester of sinapic acid.

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## 2. Results

MeOH extracts prepared from broccoli seeds were separated and characterized with a general phenolic HPLC gradient system monitored at 285 nm for phenolic compounds. The resulting chromatograph showed two major peaks at 285 nm with retention times of 10 and 33 minutes. The two compounds (compounds 1 and 2) were purified from the MeOH extract using a combination of flash chromatography and preparative HPLC, yielding 115 mg of compound 1 and 133 mg of compound 2 from 1 kg of defatted broccoli seeds.

### 2.1. Spectral Analysis

Compound 1 appeared pure by HPLC DAD, and the $^{1}H$ and $^{13}C$ NMR spectra were obtained from five milligrams (**Table 1**). Initial NMR spectra showed that there were two components present in the purified compound 1 (in addition to acetate) that were not fixed in their stoichiometry. Different batches of the product always contained both components, but in slightly varying ratios.

The more abundant compound contained two coupled olefinic protons at 7.70 ppm (1H, d, $J$ = 15.9 Hz, H-7) and 6.47 ppm (1H, d, H-8) and an aromatic singlet at 6.96 ppm (2H, s, H-2/6), which suggested a symmetrically substituted ring system with an olefinic side chain. The geometry of the olefinic double bond was determined to be *trans* on the basis of the coupling constants. The NMR spectrum was consistent with a 2,4,6- or a 3,4,5-trioxy-substituted cinnamic acid fragments, including the carboxylic carbon at 166.4 ppm (C-9). Two

 

methoxy-resonances at 3.90 ppm (6H, s, H-3/5-OMe) showed correlations to the aromatic ring carbon at 148.1 ppm (C-3/5) in the HMBC NMR spectrum. Since the substitution pattern must be symmetric, the methoxy-substituents must be at the 3 and 5 positions, or alternately at the 2 and 6 positions, leaving position 4 as a phenolic moiety. Simulations conducted with ACDLabs CNMR predictor quickly eliminated the 2,4,6- substitution pattern and confirmed the 3,4,5- substitution pattern. Other signals included in this more abundant fragment included two strongly coupled methylene protons 4.68 ppm (2H, m, H-10) and 3.79 ppm (2H, m, H-11). The protons at 4.68 ppm (H-10) showed a weak correlation in the HMBC NMR spectrum to the carboxylic carbon C-9 while the protons at 3.79 ppm (H-11) showed a 1:1:1 splitting pattern, characteristic of coupling to a spin 1 quadrupolar nucleus such as nitrogen. This implied that the methylene at 4.68 ppm would have an ester linkage to the rest of the fragment, while there was probably a nitrogen atom attached to the methylene at 3.79 ppm. In addition, the singlet at 3.27 ppm (9H, s, N-Me) showed a correlation to the methylene carbon C-11 attached to the nitrogen atom in the HMBC NMR spectrum. This suggested a quaternary ammonium salt with three methyl substituents. The remaining position on the quaternary nitrogen was the $–CH_2$-$CH_2$- branch linked by an ester to the substituted cinnamic acid fragment. This compound was identified as sinapine. The NMR data for this compound compared well with those shown for (*E*)-sinapoylcholine 4-*O*-ß-glucopyranoside [16].

The minor component contained a hexose sugar. Inspection of the HSQC NMR spectrum indicated shifts typical of an anomeric proton 4.85 ppm (1H, H-1'), but in the $^{13}C$ dimension of the HSQC spectrum the C-1' carbon (82.2 ppm) did not have a shift typical of an anomeric carbon (normally 95-104 ppm). This suggested this fragment was not an *O*-glycoside and had some other linkage to the rest of the molecule. Overlap in the COSY NMR spectra made interpretation difficult, but the HSQC NMR spectrum showed the presence of a 4-carbon methylene chain (C-1,2,3,4), and an isolated methyl group (C-5). The carbon atom (C-4) at one end of this chain had a shift of 53.0 ppm, which is somewhat upfield for an oxygen-bearing carbon. It is more characteristic of a nitrogen- or sulfur-containing moiety attached to the carbon. Several simulations with ACDLab software suggested a sulfoxide would account for the $^{13}C$ NMR shifts for the methylene at C-4 and the isolated methyl at C-5. The protons at 2.75 ppm (2H, m, H-1) on the other end of this chain showed a weak correlation in the HMBC NMR spectrum to the carbon at 159.4 ppm (the oddly-named C-0 of glucosinolate nomenclature). This carbon at 159.4 ppm showed another HMBC NMR correlation from the "anomeric" proton H-1' of the sugar fragment. The NMR analysis, along with the mass spectrometry data, which supported the presence of a sulfate group, indicated this compound was glucoraphanin [16].

Full spectral assignments of the observed $^1H$ and $^{13}C$ NMR shifts indicated that purified compound 1 was a mixture of sinapine and glucoraphanin (**Figure 1**) .

**Table 1. NMR spectroscopic data ($CD_3OD$ , $^1H$ 500 MHz, $^{13}C$ 125 MHz) for compound 1.**

| Carbon # | NMR Spectroscopic data | | |
|---|---|---|---|
| | *$^1H$ Shift (ppm)* | *$^1H$ Splitting, coupling (Hz)* | *$^{13}C$ Shift (ppm)* |
| Sinapine | | | |
| 1 | - | | 125.0 |
| 2 | 6.96 | s | 105.8 |
| 3 | - | | 148.1 |
| 4 | - | | 138.7 |
| 5 | - | | 148.1 |
| 6 | 6.96 | s | 105.8 |
| 7 | 7.70 | d, 15.9 | 146.9 |
| 8 | 6.47 | d, 15.9 | 113.3 |
| 9 | - | | 166.4 |
| 10 | 4.68 | m | 57.4 |
| 11 | 3.79 | m | 64.9 |
| 3,5-OMe | 3.90 | s | 55.5 |
| N-Me | 3.27 | s | 53.1 |
| Glucoraphanin | | | |
| 0 | - | | 159.4 |
| 1 | 2.75 | m | 31.6 |
| 2 | 1.92 | * | 25.5 |
| 3 | 1.87 | m | 21.5 |
| 4 | 2.82, 2.91 | m; m | 53.0 |
| 5 | 2.64 | s | 36.6 |
| 1' | 4.85 | * | 82.2 |
| 2' | 3.26 | * | 72.8 |
| 3' | 3.41 | t, 8.8 | 78.2 |
| 4' | 3.29 | * | 69.9 |
| 5' | 3.36 | m | 81.0 |
| 6'a,b | 3.62, 3.86 | dd, 6.3, 12.0; m | 61.4 |
| acetate | 1.93 | s | 21.8; 177.4 |

*Multiplicity not determined due to spectral overlap with acetate and solvent peaks. **Chemical shifts (δ) are in ppm from TMS.

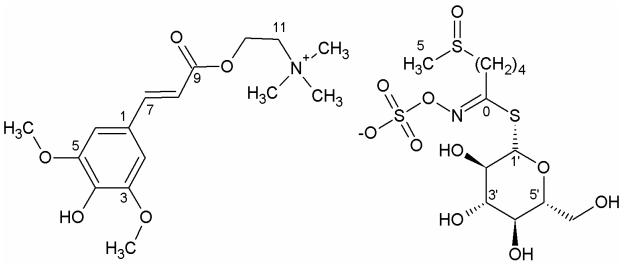

**Figure 1. Structure of compound 1: a sinapine-glucoraphanin salt, for glucosinolate numbering system see [16].**

 

The salt was contaminated with a small amount of acetate, likely due to the purification method, which contained acetic acid. This was probably acquired during the freeze-drying step to obtain the purified salt. The complete NMR spectra obtained for compound 1 agreed very closely with that published for a similar compound, Boreavan A purified and characterized from *Boreava orientalis*, which is a sinapine and sinigrin salt [17,18]. The NMR assignment for the sinapine fragment agreed closely with the similar (*E*)-sinapoylcholine 4-*O*-ß-glucopyranoside [19] and the NMR assignment for the glucoraphanin fragment agreed closely with the published spectra of the ammonium salt of glucoraphanin [16].

Compound 1 was infused under the conditions described in the materials and methods section into an ESI mass spectrometer in both the positive and negative modes. The positive ESI mass spectrum yielded a single major ion at *m/z* 310, which corresponds to the $[M]^+$ ion of sinapine. In the presence of formic acid, the spectrum showed *m/z* ions at 354, 663, and 973, corresponding to $[M]^+$, $[2M]^+$ and $[3M]^+$ adducts with formic acid, respectively. The negative ESI mass spectrum yielded two major ions at *m/z* 436 and 873, with a minor ion at *m/z* 895. These correspond to $[M]^-$, $[2M+H]^-$, and the $[2M+Na]^-$ ions of glucoraphanin. Electrospray mass spectrometry acquired in the negative mode of pure glucoraphanin resulting in similar spectra. From this data it was apparent that compound 1 was the salt of sinapine and glucoraphanin, contaminated with a small amount of acetate.

After assignment of the sinapine fragment of compound 1 it was obvious that compound 2 was a derivative of sinapic acid. Compound 2 was identified by NMR spectroscopy (**Table 2**) and mass spectrometry and found to be the methyl ester of sinapic acid (**Figure 2**). The $^1H$ and $^{13}C$ NMR spectrum compared closely with the published spectra of methyl-sinapate [20].

The spectral analysis of the sinapoylcholine-glucoraphanin salt was as follows—Isolated as light yellow crystals; mp 184-187℃; UV (MeOH) $\lambda_{max}$ (log e), 322 (3.60) nm; IR (KBr, disc) $\lambda_{max}$ ($\nu$ $cm^{-1}$): 3036, 3010, 2940, 2845, 1706, 1632, 1595, 1515, 1457, 1427, 1338, 1279 (sh), 1254, 1232 (sh), 1153, 1114, 1055; LREIMS positive mode *m/z* 310.2 $[M]^+$ and negative mode *m/z* 436.4 $[M]^-$; HREIMS positive mode *m/z* 310.1657 $[M]^+$ (calcd. for $C_{16}H_{24}NO_5$, 310.1654); negative mode *m/z* 436.0424 $[M]^-$ (calcd. for $C_{12}H_{22}NO_{10}S_3$, 436.0406). For $^1H$ and $^{13}C$ NMR spectroscopic data, see **Table 1**.

## 3. Discussion

Glucosinolates have previously been shown to be converted enzymatically or chemically to a number of degradation products, such as isothiocyanates, nitriles, and thiocyanates, once the plant tissue that they are contained in is damaged or consumed [21]. These degradation products have been shown to have a wide array of biological activities both *in vivo* and *in vitro* [1,3,4]. The isothiocyanate degradation product of glucoraphanin is sulforaphane which has been shown in extensive studies to have a number of interesting cancer chemo-preventive activities in both *in vivo* and *in vitro* studies [3,4]. Broccoli and related species have fairly high levels of glucoraphanin in their seeds and sprouts and are excellent sources of these compounds for purification and study. While conversion of glucoraphanin to sulforaphane is fairly simple, the purification of glucoraphanin is complicated by the presence of other glucosinolates in the seeds especially glucoiberin, which co-elutes in most chromatographic procedures.

A recently published method using counter current chromatography resulted in the purification of 61 grams of glucoraphanin from 500 grams of crude broccoli seed extract [22]. The authors did not state how many grams of broccoli seed were used to obtain the 500 grams of extract. The chromatographic isolation of the sinapine glucoraphanin salt is more straight-forward and provides

**Table 2. NMR spectroscopic data (CD3OD, 1H 500 MHz, 13C 125 MHz) for compound 2.**

| Carbon # | NMR Spectroscopic data | | |
|---|---|---|---|
| | $^1H$ *Shift* (*ppm*) | $^1H$ *Splitting, coupling* (*Hz*) | $^{13}C$ *Shift* (*ppm*) |
| 1 | - | | 125.2 |
| 2 | 6.91 | s | 105.6 |
| 3 | - | | 148.1 |
| 4 | - | | 138.3 |
| 5 | - | | 148.1 |
| 6 | 6.91 | s | 105.6 |
| 7 | 7.62 | d, 15.9 | 145.6 |
| 8 | 6.40 | d, 15.9 | 114.3 |
| 9 | - | | 168.2 |
| 10 | 3.78 | s | 50.6 |
| 3,5-OMe | 3.89 | s | 55.5 |

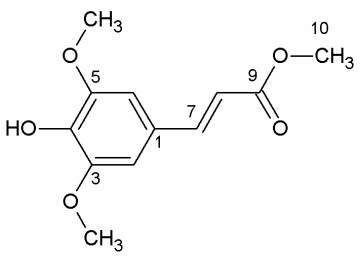


**Figure 2. Structure of compound 2: sinapic acid methyl ester.**

 

a good separation even with very crude chromatographic methods such as flash chromatography. The purified salt is free from contamination by glucoiberin. The purified sinapine-glucoraphanin salt can be passed through an anion exchange column to remove the sinapine and eluted with potassium sulfate if the potassium salt is needed. Further development and refinement of this purification technique promises to increase the yields and may provide an alternative method for the production of pure glucoraphanin than the conventional chromatographic isolation of the acid, or for the sodium or potassium salts. Sinapine also has biological activity [13-15], and the two compounds together may have interesting biological activities as an enhanced antioxidant and for the prevention of chronic diseases.

## 4. Experimental

### 4.1. Chemicals

All chemicals were of analytical reagent grade and purchased from national distribution venders. Solvents for chromatography were purchased from EMD-Merck (Gibbstown, New Jersey, USA). Water was purified using a ELGA Pure Lab Ultra system from Veolia Water Solutions and Technologies (Woodbridge, Illinois, USA).

### 4.2. Sample Preparation and Extraction

Broccoli seeds (*Brassica oleracea* L. var. *botrytis* cv. 'Liberty') were obtained from Sakata Seed America (Morgan Hill, CA). One kg of seeds were ground to a fine powder in a commercial coffee grinder and defatted overnight in four Soxhlet extractors (250 g each) with hexane for 24 hours. The hexane was then removed and the samples were dried in a fumehood for 24 hours. The defatted samples were then extracted in four Soxhlet extractors with MeOH for 72 hours. The pooled MeOH extracts from the original 1 kg of ground seed samples was evaporated to dryness in a rotary evaporator and resuspended in approximately 100 mL 50:50 MeOH:$H_2O$.

### 4.3. Isolation and Purification

A Büchi (Newcastle, DE) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord chromatography software was used. A Büchi C-670 Cartridger system to load 40 × 150 mm flash columns with approximately 90 grams of preparative C-18 reverse-phase bulk packing material (125Å, 55-105 µ, Waters Corp, Milford, MA). The columns were installed in the flash chromatography system and equilibrated with 20% MeOH and 0.5% HOAc in water for five minutes at a flow rate of 30 mL per minute. After samples (20 mL) were injected, the column was developed with a binary gradient to 50% MeOH over 30 minutes. The eluant was monitored at 237 nm and fractions based on absorbance were collected in the fraction collector by the software program. This was repeated 5 times to purify the entire extracted sample. Three major broad UV-absorbing peaks (fractions A, B, and C) were collected. The fractions were evaluated by analytical HPLC. Fraction A contained a single major UV absorbing peak with a retention time of 10 minutes (compound 1), and fraction C contained a single major UV absorbing peak at 33 minutes (compound 2). Fraction B contained a mixture of several peaks including those found in Fraction A and C. Fractions A and C were evaporated to dryness with nitrogen gas and resuspended in 30 mL of 1:1 mix of MeOH and water.

Fractions A and C were further purified using a Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, SCL 10Avp system controller all operating under the Shimadzu Class VP operating system. Ten mL sample aliquots in 1:1 MeOH: water were injected on a Phenomenex (Torrance, CA) Luna C-18(2) semi-preparative reversed-phase column (10 µ, 100Å, 250 × 50 mm). The column was pre-equilibrated with a solvent system consisting of 10% MeOH and 90% water (containing 1% HOAc) at a flow rate of 50 mL per minute and the eluant was monitored at 237 nm. The column was developed to 100% MeOH over 50 minutes. The major UV absorbing peak in each fraction was collected, pooled, allowed to evaporate for removal of the MeOH, then freeze-dried to remove the remaining water to afford approximately 115 mg of pure compound 1 and 133 mg of pure compound 2.

### 4.4. HPLC Analysis

General phenolic HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolution version 1.22 chromatography software, Columbia, MD, USA). The column used was an Inertsil ODS-3 reversed-phase C-18 column (5 µ, 250 × 4.6 mm, with a Metaguard column, from Varian). The initial conditions were 20% MeOH and 80% 0.01 M $H_3PO_4$ in water, at a flow rate of 1 mL per minute. The eluant was monitored at 285 nm on the PDA. After injection (typically 15 µL), the column was held at the initial conditions for 2 minutes, then developed to 100% MeOH in a linear gradient over 50 minutes.

For the glucosinolate analysis, a modification of a HPLC method developed by Betz and Fox was used [23] which gives excellent resolution of glucosinolates with-

 

out peak tailing, due to good ion pairing from the solvent. The extract was run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module) using the Shimadzu EZStart Version 7.3 software. The column was a C-18 Inertsil reversed-phase column (250 mm × 4.6 mm; RP C-18, ODS-3, 5 µ; with a Metaguard guard column; Varian, Torrance, CA). The glucosinolates were detected by monitoring at 237 nm. The initial mobile phase conditions were 12% MeOH/88% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL/minute. After injection of 15 µL of sample, the binary gradient was developed to 70% MeOH/30% aqueous 0.005 M TBS for 30 minute, and then to 50% MeOH/50% aqueous 0.005 M TBS over another 20 minutes.

### 4.5. HPLC-MS Analysis

To obtain the molecular weights of the compounds found in the seed extracts, aliquots were injected on a ion-trap LC-MS. Samples were run on a ThermoFinnigan LCQ DECA XP Plus LC-MS system with a Surveyor HPLC system (autoinjector, pump, degasser and PDA detector) and a nitrogen generator all running under the Xcaliber 1.3 software system. The MS was run with the ESI probe in either the positive or the negative mode. The column was a 3 mm × 150 mm Inertsil reversed-phase C-18, ODS-3, 3 µ, column (Varian, Torrance, CA) with a Metaguard guard column. Solvent A was 0.25% HOAc in $H_2O$ and Solvent B was 0.25% HOAc in MeOH. The source inlet temperature was set at 250℃, the sheath gas rate was set at 80 arbitrary units and the sweep (auxiliary) gas rate was set at 30 arbitrary units. The mass spectrometer was optimized for the detection of compounds in the extracts by using the autotune feature of the software while infusing a solution of the purified compounds in MeOH at a flow rate of 25 µL/minute with the eluant of the column (50:50 solvent A and B) at a flow rate of 100 µL/minute and tuning on the most prominent ion. Under these conditions the spray voltage was 4.0 kV and the capillary voltage was 40 V (positive mode) and -47 V (negative mode). An aliquot of the samples (typically 15 µL) was then run on the LC. The initial HPLC conditions were 12% MeOH/$H_2O$ (containing 0.25% HOAc) at a flow rate of 0.3 mL per minute, then the column was developed to 34% MeOH/$H_2O$ (containing 0.25% HOAc) over 20 minutes and then to 50% MeOH/$H_2O$ (containing 0.25% HOAc) over an additional 20 minutes. The eluant was also monitored at 237 and 285 nm on the PDA.

### 4.6. NMR Analysis

$^1$H, COSY, DEPT, and $^{13}$C NMR spectra were obtained on a Bruker (Billerica, MA, USA) Avance 500 NMR spectrometer equipped with a 5 mm inverse broadband Z-gradient probe ($^{13}$C NMR, 125 MHz; $^1$H, 500 MHz). NMR spectra were recorded in $CD_3OD$ and referenced to solvent resonances ($^{13}$C, 49.0 ppm, $^1$H, 3.30 ppm). The data was analyzed using the Advanced Chemistry Development, Inc., SpecManager 1D Processor and the HNMR and CNMR Predictor software suite version 12.01 (Toronto, ONT.)

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