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Identification and Analysis of a Mercapturic Acid Conjugate of Indole-3-methyl Isothiocyanate in the Urine of Humans who Consumed Cruciferous Vegetables

Pramod Upadhyaya^a, Adam T. Zarth^a, Naomi Fujioka^a, Vincent A. Fritz^b, and Stephen S. Hecht^{a,*}

^aMasonic Cancer Center, University of Minnesota, 2231 6th Street SE, Minneapolis, MN 55455, USA

^bSouthern Research and Outreach Center, University of Minnesota, 35838 120th Street, Waseca, MN 56093, USA

Abstract

Glucobrassicin, a quantitatively significant constituent of *Brassica* vegetables, gives rise to indole-3-carbinol (I3C) and its dimer di-indolylmethane (DIM) when the vegetables are chewed. I3C and DIM have been extensively studied with respect to their anti-carcinogenic properties. However, the presumed intermediate isothiocyanate in their formation, indole-3-methyl isothiocyanate (IMITC), has to our knowledge never been observed, despite the fact that isothiocyanates derived from cruciferous vegetables are known to have anti-carcinogenic properties. Therefore, we investigated the formation and presence in human urine of IMITC by analyzing for its *N*-acetylcysteine conjugate, IMITC-NAC, in order to gain a more complete understanding of the biochemical pathways leading to formation of I3C and DIM upon consumption of vegetables rich in glucobrassicin. Standard IMITC-NAC was synthesized and its structure confirmed by NMR and MS. IMITC-NAC was identified in extracts of Brussels sprouts chopped in the presence of *N*-acetylcysteine. An LC-ESI-MS/MS-SRM method for analysis of IMITC-NAC, with [¹³C, ¹⁵N]IMITC-NAC as internal standard, was developed and validated. Then, ten subjects (7 females) consumed a salad of Brussels sprouts and cabbage (containing 100 to 500 μmol glucobrassicin) once daily for 3 days. Urine was collected at intervals up to 24 h after vegetable consumption. Levels of IMITC-NAC in the urine of these 10 subjects ranged from 0.2 to 30.2 pmol/mL urine. These results provide the first evidence for the presumed intermediacy of IMITC in the formation of I3C and DIM in humans who consumed Brussels sprouts and cabbage as a source of glucobrassicin.

*To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, 2231 6th Street SE - 2-148 CCRB, Minneapolis, MN 55455, USA. phone: (612) 624-7604, fax: (612) 624-3869, hecht002@umn.edu.

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Keywords

Indole-3-carbinol; Cruciferous vegetables; Indole-3-methyl isothiocyanate; Glucobrassicin

Introduction

Extensive experimental data as well as the results of recent clinical trials indicate that consumption of cruciferous vegetables such as broccoli, cabbage, Brussels sprouts and watercress can ameliorate the properties of carcinogens, although overall evaluations of epidemiologic data on cruciferous vegetables and cancer prevention concluded that the evidence was limited or inadequate [1–8]. The bioactive compounds associated with the potential cancer-protective effects are isothiocyanates and their precursor glucosinolates (Figure 1), plant defense compounds which occur in substantial quantities in all cruciferous vegetables [1]. The glucosinolates are cellularly separated from the enzyme myrosinase. When the plants are damaged such as in chewing, myrosinase is released and catalyzes the conversion of the glucosinolates to pungent isothiocyanates, which can be toxic to predators. Well known examples of isothiocyanates with cancer protective properties include sulforaphane and phenethyl isothiocyanate (PEITC) (Figure 1), hydrolysis products of glucoraphanin and gluconasturtiin, respectively. One of the most common glucosinolates in cruciferous vegetables is glucobrassicin which occurs in relatively large amounts in Brussels sprouts, broccoli, cabbage, cauliflower, and kale among others [1]. Myrosinase-catalyzed hydrolysis of glucobrassicin produces indole-3-carbinol (I3C) which, along with its dimer di-indolyl methane (DIM), has been studied extensively in connection with its cancer preventive properties [9]. However, the presumed intermediate isothiocyanate on the pathway from glucobrassicin to I3C and DIM, indole-3-methyl isothiocyanate (IMITC, Figure 1), has to our knowledge never been reported as an intermediate in this process. IMITC, as a potentially reactive isothiocyanate, may have important biological properties related to the consumption of common cruciferous vegetables rich in glucobrassicin. Therefore, in the study described here, we investigated the formation of IMITC in chopped Brussels sprouts and the presence in human urine of IMITC-NAC (Figure 1), the *N*-acetylcysteine conjugate of IMITC, in order to gain a more complete understanding of the biochemical pathways leading to formation of I3C upon consumption of cabbage and Brussels sprouts, vegetables rich in glucobrassicin. IMITC-NAC is the product of glutathione conjugation of IMITC, followed by normal cellular processing of glutathione conjugates to mercapturic acids.

Materials and Methods

Study Design

The urine samples were from a previous study of cabbage and Brussels sprouts consumption and urinary DIM [10]. Briefly, 10 healthy nonsmoking non-vegetarian adults (7 females) ages 18 to 60 years were recruited. At enrollment, subjects were randomly assigned to consume 1 to 5 doses of glucobrassicin –100, 200, 300, 400 or 500 μmol - based on its concentration in specially grown cabbage and Brussels sprouts [10]. Subjects refrained from cruciferous vegetable consumption for a minimum of 5 days prior to the study. Salads were

prepared containing fixed proportions of raw cabbage and Brussels sprouts to attain the desired glucobrassicin dose. The amounts of salad consumed ranged from 173–293 g. All urine was collected for 24 h following each vegetable-eating session, divided into the following periods: 0–2, 2–6, 6–12, and 12–24h, as described [10]. This study was approved by the University of Minnesota Institutional Review Board.

Chemicals

[$^{13}\text{C}_3$, ^{15}N cysteine]*N*-Acetyl-L-cysteine was purchased from Cambridge Isotope Laboratories, Inc. MA, and indole-3-methylamine from ATK Pharm, Inc., Arlington Heights, IL. All other chemicals and reagents were obtained from Sigma-Aldrich (Milwaukee, WI).

Indole-3-Methylisothiocyanate (IMITC)

Warning: *thiophosgene is highly toxic and volatile, and must be handled in an efficient fume hood with great care.* Indole-3-methylamine (47.6mg, 0.33 mmol) was dissolved in 2 mL of *N,N*-dimethylacetamide, and triethylamine (200 μL) was added. The reaction mixture was cooled at -40°C for 10 min. Thiophosgene (37 μL , 0.33 mmol) in 1 mL of acetone was added dropwise and the mixture stirred for 15 min. The reaction mixture was quenched with 3 mL of H_2O , extracted with CH_2Cl_2 , and the extracts concentrated, dissolved in 100 μL CH_3CN , and 8 μL were injected immediately on an Agilent 1100 series capillary flow LC/MSD trap (Agilent Technologies, Palo Alto, CA) with a 150 mm $\times$ 0.5 mm Zorbax-C18 5 μm column eluted at 10 $\mu\text{L}/\text{min}$ with 2% CH_3OH in 15mM NH_4OAc to 75% CH_3OH over the course of 25 min. N_2 was used as the drying gas (200 $^\circ\text{C}$, 5 L/min). The system also contained an in line UV detector. The mass spectrometer was operated in the full scan mode (m/z 100–600) with target-ion abundance of 30,000, a maximum accumulation time of 300 ms, and a fragmentation amplitude of 0.9 V. Spectral properties of IMITC: MS, m/z 189 [$\text{M} + \text{H}]^+ \rightarrow m/z$ 130 ($\text{M} - \text{HNCS}$); UV (CH_3OH in 15mM NH_4OAc) λ_{max} 225, 275. IMITC was very unstable and decomposed to I3C and DIM during analysis.

S-(1H-indol-3-yl)methylcarbamoithioyl)-N-acetylcysteine (IMITC-NAC)

IMITC was immediately taken forward after addition of thiophosgene; *N*-acetylcysteine (159.4 mg, 0.978 mmol) in 200 μL pyridine was added and the mixture was stirred for 1h at -40°C and allowed to come to room temperature with stirring overnight. The reaction mixture was quenched with H_2O and extracted with CH_2Cl_2 . The organic layer was concentrated to dryness, dissolved in 5% $\text{CH}_3\text{CN}/95\%$ 15 mM NH_4OAc , and purified by HPLC. HPLC was carried out with a Waters (Milford, MA) system equipped with a model 1440 UV-visible detector set to 254 nm. A 300 $\times$ 3.9 mm, 10 μm C-18 Bondclone column (Phenomenex, Torrance, CA) was eluted at 0.7mL/min with 5–90% CH_3CN in 15mM NH_4OAc over the course of 40 min. The peak at 18.6 min was collected and concentrated to dryness yielding 2.9 mg IMITC-NAC (2.7%). UV (CH_3OH in 15 mM NH_4OAc) λ_{max} 210, 270; ^1H NMR (Bruker, 500 MHz, D_2O) δ ppm 1.79 (s, 3 H, $-\text{COCH}_3$), 3.47 (dd, $J = 14.34$, 7.32 Hz, 1 H, $\text{C}\beta\text{-H}$), 3.82 (dd, $J = 14.34$, 4.27 Hz, 1 H, $\text{C}\beta\text{-H}$), 4.43 (dd, $J = 7.17$, 4.43 Hz, 1 H, $\text{C}\alpha\text{-H}$), 4.99 (d, $J = 3.36$ Hz, 2 H, C3-CH_2), 7.07 – 7.12 (m, 1 H, C5-H), 7.19 (dd, $J = 8.15$, 7.20 Hz, 1 H, C6-H), 7.35 (s, 1 H, C2-H), 7.44 (d, $J = 8.24$ Hz, 1 H, C7-H), 7.63 (d, J

= 7.93 Hz, 1 H, C4-H); ^{13}C NMR (126 MHz, D_2O) δ ppm 21.81 (-COCH₃), 36.49 (C β), 42.72 (-C3-CH₂), 54.45 (C α), 109.59 (C3), 111.94 (C7), 118.54 (C4), 119.64 (C5), 122.13 (C6), 125.26 (C2), 126.06 (C3a), 136.16 (C7a), 173.64 (-COCH₃), 176.25 (-COO⁻), 196.81 (-NCS₂-); HRMS [LTQ Orbitrap Velos (Thermo Scientific, Carlsbad, CA)] calc'd 352.07841; found: 352.07883, MS-MS of m/z 352 $\rightarrow$ m/z 130 Calc'd for C₉H₈N 130.06622; found 130.06524

[$^{13}\text{C}_3$, ^{15}N]IMITC-NAC

Indole-3-methylamine (15.8 mg, 0.108 mmol) was dissolved in 1 mL of *N,N*-dimethylacetamide, triethylamine (100 μL) was added, and the reaction mixture cooled at -40°C for 10 min. Thiophosgene (12.3 μL , 0.108 mmol) in 0.5 mL of acetone was added dropwise and the mixture stirred for 15 min. [$^{13}\text{C}_3$, ^{15}N]N-acetylcysteine (53.2 mg, 0.326 mmol) in 200 μL pyridine was added and the mixture was stirred for 1 h at -40°C . The reaction mixture was allowed to come to room temperature, stirred overnight, and worked up as described above (0.8 mg, 2.2% yield). HRMS calc'd 356.0864; found 356.0863; MS/MS m/z 356 $\rightarrow$ m/z 130 Calc'd for C₉H₈N 130.0662; found 130.0652.

Formation of IMITC in Brussels sprouts

Brussels sprouts (64.5 g) were purchased from a local market and soaked in 100 mL 2% aqueous *N*-acetylcysteine, or H_2O alone, for 5 min. The Brussels sprouts, still submerged in the aqueous solution, were then cut into pieces, the entire mixture was transferred to a blender, and the Brussels sprouts were chopped for 5 min. The mixture was allowed to stand for 15 min, subjected to additional chopping for 5 min, then allowed to stand for 1 h. The resulting mixture was poured into 50 mL test tubes and centrifuged at 4000 rpm for 20 min. An aliquot of the supernatant (0.5 mL) was centrifuged at 8000 rpm for 20 min and the filtrate was adjusted to pH 9.0. One hundred μL of this mixture was mixed with 100 μL 15 mM NH_4OAc and analyzed by LC-ESI-MS/MS-SRM for IMITC-NAC, using the transition m/z 352.078 $\rightarrow$ m/z 130.065 and m/z 356.086 $\rightarrow$ m/z 130.065 for [$^{13}\text{C}_3$, ^{15}N]IMITC-NAC.

Analysis of Urine Samples

[$^{13}\text{C}_3$, ^{15}N]IMITC-NAC (17.1 pmol) was added to 1 mL urine and 1 mL H_2O , then loaded on a Strata-X solid phase extraction cartridge (200 mg/6 mL, Phenomenex) previously equilibrated using 6 mL MeOH followed by 6 mL H_2O . The cartridge was washed with 6 mL portions of H_2O , 10% aqueous MeOH, 50% aqueous MeOH, and 12 mL MeOH. The MeOH eluate was evaporated to dryness in a silanized vial on a Speedvac, dissolved in 400 μL of 1% CH_3CN in 15 mM NH_4OAc , and 4 μL were analyzed by LC-ESI-MS/MS-SRM on a TSQ Quantum Discovery Max instrument (Thermo Fisher Scientific) in the positive ion mode with N_2 as the nebulizing and drying gas. MS parameters were as follows: spray voltage 3.5 kV; sheath gas 40; capillary temperature 280°C ; collision energy 25 V for m/z 352; scan width 0.1 amu; Q2 gas pressure 1.0 mTorr; source CID 5V; tube lens offset 70V. MS data were acquired and processed by Xcalibur software version 1.4 (Thermo Fisher Scientific). Four μL of the sample were injected from an auto sampler using an Agilent 110 capillary LC system equipped with a 5 μm , 150 mm $\times$ 0.5 mm Zorbax SB-C18 column (Agilent Technologies). The flow rate was 10 $\mu\text{L}/\text{min}$ with a gradient from 5% CH_3CN in 15

mM NH₄OAc from 0 to 5 min, and from 5% CH₃CN to 30% CH₃CN in 25 min, then to 80% CH₃CN in 2 min. The mass transitions monitored were m/z 352 $\rightarrow$ m/z 130 for IMITC-NAC (typical retention time 19.7 min) and m/z 356 $\rightarrow$ m/z 130 for [¹³C₃,¹⁵N]IMITC-NAC. A calibration curve was prepared by plotting MS peak area ratios against concentration ratios using standard mixes containing constant levels of [¹³C₃,¹⁵N]IMITC-NAC and varying levels of IMITC-NAC. The peak area ratios were determined by LC-MS/MS analysis of each sample and quantitation was accomplished using the calibration curve and the known amount of [¹³C₃,¹⁵N]IMITC-NAC added to each sample.

Accuracy and precision of urinary IMITC-NAC assay

Accuracy was determined by adding IMITC-NAC (3.60 – 289 pmol) to 1.0 mL samples of a non-smokers' urine and performing the analysis as described above. Each determination was the mean of 3 analyses. Precision was determined by replicate analyses (N = 6) of a non-smokers' urine sample (1 mL) to which 72.3 pmol IMITC-NAC had been added.

Results and Discussion

The preparation of IMITC-NAC is summarized in Scheme 1. Indole-3-methylamine was allowed to react with thiophosgene at reduced temperature. The reaction mixture was analyzed immediately by LC-MS and UV. The MS showed peaks at m/z 189 corresponding to the protonated molecular ion of IMITC and m/z 130 (M – HNCS). The UV spectrum had maxima at λ 225 and 275. These data are fully consistent with the structure of IMITC, but it was quite unstable, decomposing during analysis to I3C and DIM. Therefore, IMITC was taken forward immediately for the preparation of IMITC-NAC by reaction with *N*-acetylcysteine (Scheme 1). IMITC-NAC was isolated by HPLC, in 2% overall yield from indole-3-methylamine. The proton and ¹³C NMR spectra were completely consistent with the structure and the assignments were confirmed by COSY, HMBC, and HSQC experiments. The HRMS showed a protonated molecular ion at m/z 352.07883 and a major 3-methyleneindolyl fragment ion at m/z 130.06622. The internal standard [¹³C₃,¹⁵N]IMITC-NAC was similarly prepared from [¹³C₃,¹⁵N]*N*-acetylcysteine. We determined that IMITC-NAC was stable for at least 24h in 10 mM phosphate buffer, pH 7.0, at 37 °C.

We began our investigation of IMITC formation by determining whether IMITC-NAC was detectable when Brussels sprouts were chopped in the presence of *N*-acetylcysteine. If IMITC were formed only transiently or were too unstable to be trapped by *N*-acetylcysteine under these favorable conditions, it would be very unlikely to detect it in humans who consumed cruciferous vegetables. As shown in Figure 2, we obtained clear LC-ESI-MS/MS-SRM evidence for the formation of IMITC-NAC in Brussels sprouts chopped in the presence of *N*-acetylcysteine. Quantitation was not performed at this point, as our major interest was the possible use of IMITC-NAC as a urinary biomarker of human cruciferous vegetable consumption.

For analysis of IMITC-NAC in human urine, the internal standard [¹³C₃,¹⁵N]IMITC-NAC was added to each urine sample, and they were subjected to solid phase extraction and analysis by LC-MS/MS using the analyte transitions m/z 352 [M + H]⁺ $\rightarrow$ m/z 130 [M + H

-NCS-NAC]⁺ and the internal standard transitions m/z 356 $\rightarrow$ m/z 130. Accuracy and precision of the method were tested by adding varying amounts of IMITC-NAC to urine samples and performing the analysis. The results which are summarized in Figure 3 demonstrate that the accuracy of the method was 95%. The precision was 1.2% (coefficient of variation, N = 6).

We analyzed urine samples from 10 subjects at various time points after consuming a salad consisting of cabbage and Brussels sprouts, and for whom we had previously determined levels of urinary DIM [10]. Typical LC-MS/MS chromatograms of urine samples before and after consumption of cabbage and Brussels sprouts are shown in Figure 4. IMITC-NAC and the internal standard are clearly evident in the urine samples after consumption of cabbage and Brussels sprouts.

We detected IMITC-NAC in the urine of all subjects and at most time points, with amounts generally reaching their maximum at the early time points, and ranging from 0.1 to 30.2 pmol/mL urine (Table 1). In a previous study, we found that urinary DIM represented about 0.01 – 0.03% of the amount of glucobrassicin in Brussels sprouts and cabbage. Levels of IMITC-NAC were 136 ± 310 (N = 34) times lower than those of DIM, indicating that IMITC-NAC represents an average of approximately 0.0002% of the dose of glucobrassicin [11].

These results provide the first evidence for the presumed intermediacy of IMITC in the formation of I3C and DIM in humans who consumed cabbage and Brussels sprouts as a source of glucobrassicin. The very low levels of IMITC-NAC detected in the urine of our subjects are consistent with the instability of IMITC which we observed. We hypothesize that IMITC is produced in the mouth when the cabbage and Brussels sprouts are chewed and that a portion of it reacts with glutathione in the oral mucosa to form a conjugate which is processed by the mercapturic acid pathway resulting ultimately in the excretion of IMITC-NAC in the urine.

Our discovery of IMITC as an intermediate in the processing of glucobrassicin could have profound implications with regard to the biological properties of the many cruciferous vegetables which contain this common glucosinolate. It has been assumed previously that these properties were due to indole-3-carbinol and its dimer DIM which is formed under the acidic conditions in the stomach along with other oligomers [1]. Indole-3-carbinol and DIM have diverse biological properties that align with their protective effects and those of the vegetables from which they are derived [12]. Our results however demonstrate that the protective properties of common cruciferous vegetables could also be due to IMITC. Since IMITC is readily trapped with *N*-acetylcysteine to form IMITC-NAC, it is reasonable to expect that it will also react easily with other cellular thiols. Important among these is the sulfhydryl-rich protein Keap1 which is readily modified by isothiocyanates such as sulforaphane, an interaction which allows for nuclear accumulation of Nrf2 resulting in enhanced transcription of a protective response. Further studies are required to examine the role of IMITC as an inducer of Nrf2, a pathway that could be particularly relevant in the oral cavity upon consumption of cruciferous vegetables containing glucobrassicin. Generation of IMITC *in situ* from IMITC-NAC could be one approach for such studies.

We have previously shown that DIM, formed in the stomach by dimerization of I3C, the hydrolysis product of IMITC, is an excellent biomarker of glucobrassicin consumption [11]. The results of this study also indicate that urinary DIM is preferable to urinary IMITC-NAC as a biomarker of glucobrassicin consumption in humans because of its substantially higher concentration. While not examined in our study, salivary IMITC-NAC may be a good biomarker of local generation of IMITC upon consumption of cruciferous vegetables. Further studies are required to explore the full potential of IMITC-NAC as a biomarker of cruciferous vegetable consumption and to explore the role of IMITC in the cancer preventive properties of glucobrassicin – containing vegetables.

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Highlights

Indole-3-carbinol (I3C) from cruciferous vegetables has cancer prevention properties

I3C is formed from glucobrassicin upon consumption of cruciferous vegetables

Indole-3-methyl isothiocyanate (IMITC) is the presumed bioactive intermediate

IMITC-NAC, the N-acetyl cysteine conjugate of IMITC, was synthesized

IMITC-NAC was identified for the first time in humans who ate cruciferous vegetables

Figure 1.
Formation of isothiocyanates from glucosinolates, catalyzed by plant myrosinase.

Figure 2. LC-ESI-MS/MS-SRM chromatograms obtained upon analysis of an extract of Brussels sprouts chopped in the presence of *N*-acetylcysteine. Top panel) m/z 352 $\rightarrow$ m/z 130 from IMITC-NAC; bottom panel) m/z 356 $\rightarrow$ m/z 130 from internal standard [$^{13}\text{C}_3, ^{15}\text{N}$]IMITC-NAC

Figure 3.
Relationship between added and measured amounts of IMITC-NAC added to non-smokers' urine samples as described in Materials and Methods.

Figure 4.

Representative LC-ESI-MS/MS-SRM chromatograms obtained upon analysis of A) a urine sample obtained prior to eating cabbage and Brussels sprouts, and B) 0–2h after eating cabbage and Brussels sprouts. Top panels, analyte, m/z 352 $\rightarrow$ m/z 130; bottom panels, internal standard, m/z 356 $\rightarrow$ m/z 130.

Scheme 1.
Preparation of IMITC and IMITC-NAC

Table 1

Levels of DIM and IMITC-NAC in the urine of subjects who consumed cabbage and Brussels sprouts. DIM data are from reference 10.

Subject	Glucobrassicin dose (μmol)	Time (h)	DIM (pmol/mL)	IMITC-NAC (pmol/mL)
1	100	0 to 2	14.79	0.8
		2 to 6	151	2.17
		6 to 12	8.41	0.35
		12 to 24	0.92	0.26
2	200	0 to 2	26.6	ND
		2 to 6	307	3.65
		6 to 12	3.29	0.13
		12 to 24	2.55	0.15
3	200	0 to 2	90.3	ND
		2 to 6	992	4.16
		6 to 12	52.3	0.86
		12 to 24	14.4	0.26
4	300	0 to 2	2.71	ND
		2 to 6	488	9.04
		6 to 12	25.2	1.23
		12 to 24	1.76	0.19
5	300	0 to 2	46.8	0.36
		2 to 6	387	0.21
		6 to 12	20.3	0.23
		12 to 24	2.35	0.1
6	400	0 to 2	239	11.3
		2 to 6	212	0.92
		6 to 12	255	1.3
		12 to 24	31.6	0.47
7	400	0 to 2	213	29.1
		2 to 6	359	2.01
		6 to 12	43.4	0.42
		12 to 24	9.84	0.23
8	500	0 to 2	1180	30.2
		2 to 6	970	13.4
		6 to 12	106	0.93
		12 to 24	11.7	0.16
9	500	0 to 2	145	ND
		2 to 6	344	ND

Subject	Glucobrassicin dose (μmol)	Time (h)	DIM (pmol/mL)	IMITC-NAC (pmol/mL)
10	500	6 to 12	434	2.53
		12 to 24	36.1	0.56
		0 to 2	179	2.6
		2 to 6	991	3.47
		6 to 12	95	0.76
		12 to 24	ND	0.24

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