IDENTIFICATION OF NEW DERIVATIVES OF SINIGRIN AND GLUCOTROPÆAOLIN PRODUCED BY THE HUMAN DIGESTIVE MICROFLORA USING $^1$H NMR SPECTROSCOPY ANALYSIS OF IN VITRO INCUBATIONS

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ABSTRACT:

One- and two-dimensional $^1$H NMR spectroscopy were used to study the biotransformation of two dietary glucosinolates, sinigrin (SIN), and glucotropaeolin (GTL) by the human digestive microflora in vitro. The molecular structures of the new metabolites issued from the aglycone moiety of the glucosinolate were identified, and the modulation of carbon metabolism was studied by quantifying bacterial metabolites issued from the xenobiotic incubation in the presence or absence of a source of free glucose. Unambiguously and for the first time, it was shown that SIN and GTL were transformed quantitatively into allylamine and benzylamine, respectively. The comparison of the kinetics of transformation of SIN and GTL with and without glucose clearly showed that the presence of glucose did not modify either the nature of the metabolites or the rate of transformation of the glucosinolates (complete degradation within 30 h). The main end products of the glucose moiety of glucosinolates were characteristic of anaerobic carbon metabolism in the digestive tract (acetate, lactate, ethanol, propionate, formate, and butyrate) and similar to those released from free glucose. This work represents the first application of $^1$H NMR spectroscopy to the study of xenobiotic metabolism by the human digestive microflora, demonstrating allyl- and benzylamine production from glucosinolates. Whether these amines are produced in vivo from dietary glucosinolates remains to be established. This would reduce the availability of other glucosinolate metabolites, notably cancer-protective isothiocyanates.

Glucosinolates are sulfur-containing phytochemicals present in edible cruciferous vegetables, such as Brussels sprouts, cabbage, radish, etc. Their common structure comprises a $\beta$-thioglucose group, a sulfonated oxime moiety, and a variable side chain derived from methionine, tryptophan, or phenylalanine. Upon disruption of plant tissues during food processing or ingestion, glucosinolates are hydrolyzed by the endogenous enzyme “myrosinase” (thioglucoside glucohydrolase EC 3.2.3.1.) to yield isothiocyanates, nitriles, and other minor products (Fenwick et al., 1983). Numerous experimental studies performed in animal and cellular models implicated isothiocyanates as the main bioactive agents responsible for the anti-cancer properties of cruciferous vegetables (Musk and Johnson, 1993; Nugon-Baudon and Rabot, 1994; Verhoeven et al., 1997). In comparison, the fate of glucosinolates following ingestion, in particular their breakdown site and rate, has received little attention. When plant myrosinase is active, glucosinolates are rapidly hydrolyzed in the food or in the proximal gut (De Vos and Blijleven, 1988; Campbell et al., 1995). Should the enzyme be deactivated by cooking (Jongen, 1996), glucosinolates reach the large bowel, where they are broken down by the resident microflora (Rabot et al., 1993; Michaelsen et al., 1994). A few recent experiments performed in humans and gnotobiotic rats associated with a humanecal flora have demonstrated that microbial breakdown of glucosinolates leads to the formation of isothiocyanates (Shapiro et al., 1998; Elfoul et al., 2001). The conversion is incomplete, however, suggesting that other metabolites are produced; at present, nothing is known of the identity of these latter products.

The aim of this study was to investigate the metabolism of glucosinolates by the human colonic microflora in vitro, using $^1$H NMR spectroscopy. Indeed, NMR signals are real fingerprints of molecules, and a wide range of metabolites can be measured simultaneously and without a priori hypothesis. In addition, despite a rather low sensitivity, this method is very convenient since it can be performed directly on biological samples without prior purification. Finally, quantitative data can be collected. $^1$H NMR spectroscopy has proved to be a powerful tool for analysis of the metabolite composition of biological fluids (Nicholson and Wilson, 1989; Fan, 1996; Kalic et al., 2000) and for the study of drug metabolism (Gartland et al., 1991; Holmes et al., 1995; Bollard et al., 1996; Foxall et al., 1996). More recently, this technique has been applied to the study of microbial metabolism (Matheron et al., 1998; Brecker and Ribbons, 2000; Weber and Brecker, 2000) and, in particular, microbial degradation of xenobiotics (Gaines et al., 1996; Besse et al., 1998; Combrouie et al., 1998a,b, 2000; Poupin et al., 1998; Delort and Combrouie, 2000).
In this work, we have used 1D and 2D 1H NMR spectroscopy to elucidate the microbial biotransformation of two structurally different glucosinolates, sinigrin and glucotropaeolin. Sinigrin (SIN; Fig. 1) is a simple aliphatic glucosinolate prevalent in a wide range of cruciferous vegetables, whereas glucotropaeolin (GTL; Fig. 1) is an aromatic glucosinolate specifically present in garden cress and papaya fruit. In comparative experiments, SIN and GTL were added to the culture media, either alone or concurrently with free glucosin. In this way, we sought to determine whether the availability of a simple source of energy and carbon, as is likely to be the case in the digestive tract, would hamper glucosinolate metabolism by digestive bacteria. In addition, data were collected from the NMR spectra to identify and quantify metabolites produced by the fermentation of the glucosinate sugar moiety.

Materials and Methods

Incubation Experiments. Freshly passed stools were collected from a healthy adult human subject who had not taken any antibiotics for at least 3 months preceding the study and who usually consumed a Western style diet. Feces were transferred into an anaerobic glove box where they were thoroughly mixed with the incubation buffer, using an Ultra-turrax blender (Janke and Kunkel GmbH, Staufen, Germany). Incubation buffer was 0.1 M sterile potassium phosphate, pH 7.0, with added yeast extract (Difco) 2 g · l⁻¹. The suspension was adjusted at 1 g · 100 ml⁻¹ by further addition of incubation buffer and divided into 20 ml fractions, transferred to amber glass vials, either alone (control without substrate) or together with SIN (Sigma-Aldrich, St. Louis, MO) and GTL (Merck, Darmstadt, Germany) (6 mM each) or SIN, GTL, and glucose (6 mM each). Sterile controls containing SIN and GTL were prepared to check the stability of the substrates in the absence of bacterial cells (controls without flora). SIN, GTL, and glucose were added as freshly prepared aqueous stock solutions sterilized by filtration (Milllex-GS 0.22 μm; Millipore Corporation, Bedford, MA). Vials were tightly closed with butyl-rubber stoppers and sealed with aluminum caps to maintain anaerobic conditions and to avoid loss of volatile isothiocyanates. They were incubated at 37°C in a shaking bath (50 rpm). All incubations were performed in duplicate.

Samples were collected by a puncture through the stoppers at 0, 3, 6, 18, and 20 h. They were centrifuged to remove bacteria (8000g, 10 min, 4°C), and supernatants were stored at −20°C until analysis.

HPLC Analysis. Supernatants were analyzed for residual intact SIN and GTL using HPLC analysis of desulfoglucosinolates, as recommended by the International Standardization Organization for glucosinolate analysis in Brassica (Anonymous, 1990), except that no methanol extraction was required before the desulfuration step.

NMR Spectroscopy. Preparation of the samples for NMR and quantification of the metabolites were performed as previously described (Combourieu et al., 1998a). No purification was performed before analysis, and pH was adjusted to 7 to avoid changes in chemical shifts. TSPd₄ (10% v/v of an 8 mM solution in D₂O) constituted a reference for chemical shifts (0 ppm) and quantification. All 1H NMR spectra were recorded on a Bruker Avance 300 spectrometer (Bruker, Newark, DE) at 300.13 MHz at 25°C with a 5-mm ¹H-/¹C-/¹N inverse probe equipped with z-gradients.

1D ¹H NMR experiments. Water was suppressed by presaturation or by the classical double-pulse field gradient echo sequence: WATERGATE (Price, 1999). In both cases, 64 scans were collected (relaxation delay, 5 s; acquisition time, 3.64 s; 32,000 data points). A 0.3-Hz line broadening was applied before Fourier transformation, and a baseline correction was performed on spectra before integration with Bruker software. The ¹H NMR spectra obtained were compared with those of the controls.

2D ¹H NMR experiments. Gradients-correlation spectroscopy was used to identify all the end products of carbon metabolism. The data were acquired as 2048 × 256 point files, accumulating eight transients per t₁ increment. Zero-filling in t₁ and unshifted sinusoidal window function in both time domains were employed before Fourier transformation. 2D phase-sensitive total correlation spectroscopy (TOCSY) experiments with water resonance suppression by a WATERGATE sequence (put at the end of the sequence) were used to assign all members of a coupled spin network. Spectral widths were adjusted in both dimensions to encompass all ¹H signals of interest. The “mixing period” (corresponding to several cycles of MLEV-17 spin-lock sequence) was 20 to 80 ms. The responses of eight scans for each of 512 t₂ increments were zero-filled. Acquisition in t₁ and sine window function in both dimensions were applied before 2D Fourier transformation.

GC-MS Analysis. Samples were extracted three times with CDCl₃, and the organic layers were dried over MgSO₄. The extracts were analyzed directly by GC-MS on an HP 5890 Series II Plus GC equipped with an HP 5989 B mass spectrometer (Hewlett Packard, Palo Alto, CA). Separation was achieved on a 30-m OPTIMA-5-MS capillary column (0.25-mm i.d. and 0.25-μm film) using the following temperature program: 55°C, 2-min hold, increased by 5°C · min⁻¹ to 80°C, 15-min hold. Helium was used as a carrier gas at a linear velocity of 41 cm · s⁻¹. The temperatures of injector, interface, and source were 250, 250, and 200°C, respectively. The ionization mode was electronic impact at 70 eV, and the detection mode chosen was single ion monitoring, which increases sensitivity by a factor of 10.

Results

Identification of Metabolites Issued from the Aglycone Moiety of SIN and GTL by 1D and 2D TOCSY ¹H NMR Spectroscopy. Incubations were performed with a mixture of SIN and GTL in the presence or the absence of glucose. In both cases, the same signals were present in the NMR spectra.

An example of kinetics monitored in the absence of glucose is presented in Fig. 2; these ¹H NMR spectra contained simultaneously the resonances of SIN, GTL, and their derivatives and also the resonances of characteristic glucose metabolites.

In the ¹H NMR spectrum obtained at 0 h (Fig. 2), many signals were visible: in particular, a singlet at 0 ppm that belongs to the methyl groups of TSPd₄ (used as a reference for chemical shifts and quantification) and an apparent singlet and a multiplet at 4.16 and 7.43 ppm corresponding to the side chain protons of GTL, methylene, and aromatics, respectively. Signals of glucose moieties, resonating between 3.20 and 3.95 ppm, cannot be distinguished. The two multiplets at 5.30 and 6.05 ppm correspond to the vinyl protons—CH₂ and CH, respectively—of SIN. The second methylene group of the SIN side chain cannot be assigned in a 1D spectrum.

At 18 h, the signals of GTL (4.16 and 7.43 ppm) decreased, whereas two new resonances at 4.20 and 7.49 ppm increased (named Y on Fig. 2). Also, close to the signals of SIN resonating at 5.30 and 6.05 ppm, two multiplets appeared at 5.40 and 5.95 ppm (named X on Fig. 2). At 20 h, a well resolved doublet of triplets was visible at 3.62 ppm. This signal was probably present at 18 h but overlapped with the glucose protons. At the end of the kinetics, the resonances of SIN and
GTL were no longer present, whereas the intensities of those of the new metabolites were at maximum.

To assign the different signals present in the 1D $^1$H NMR spectra, homonuclear 2D $^1$H TOCSY experiments were performed on the samples taken during the incubation of the fecal flora with SIN and GTL. Water, which generates strong samples taken during the incubation of the fecal flora with SIN and GTL were no longer present, whereas the intensities of those of the new metabolites were at maximum.

Apart from residual CHCl$_3$ and water, the $^1$H NMR spectrum exhibited only five well resolved signals [at a concentration of less than 100 ppm; Fukui, (1972)]. The following ions were detected in the mass spectrum: m/z 107 ([M]$^+$), 91 ([CH$_2$-C$_6$H$_5$])$^+$), 79, 51, 30.

Consequently, the hypothesis of an amine formation was checked. In the case of SIN, commercial allylamine (Fig. 1) was added to the samples, and its signals overlapped perfectly with those present in Fig. 2. To confirm this assignment definitively, liquid/liquid extraction of a 30-h supernatant was carried out with deuterated chloroform as a solvent. The residue was directly analyzed by $^1$H NMR and GC-MS. Apart from residual CHCl$_3$ and water, the $^1$H NMR spectrum exhibited only five well resolved signals [at a concentration of less than 100 μM, as estimated by comparison with satellite lines (1%) of residual non deuterated solvent (0.1%)] at different chemical shifts from those observed in the buffer (a typical solvent effect) but with the same multiplicity (data not shown). The following ions were detected in the mass spectrum: m/z 57 (M$^+$), 56, 42, 41, 30, 29, 27, and 15, which correspond to 2-propen-1-amine (allylamine), as shown in the spectrum of the commercial compound.

Regarding the transformation of GTL, the TOCSY (80-ms mixing time) experiment also indicated that the signal at 4.20 ppm was correlated (by J-coupling) to the signal at 7.49 ppm, suggesting that the corresponding protons belong to the same molecule (data not shown). These signals overlapped perfectly with those of commercial benzylamine (Fig. 1). The fragmentation obtained from GC-MS analyses of the 30-h CDCl$_3$ extract was identical to that of a commercial product: m/z 107 ([M]$^+$), 91 ([CH$_2$-C$_6$H$_5$]$^+$), 79, 51, 30.

Kinetics of Glucosinolate Degradation. Figure 4a shows the time course of GTL degradation and benzylamine formation in the absence and presence of glucose. The concentrations were calculated from the integrals of $^1$H NMR signals in 1D spectra of GTL and benzylamine [CH$_2$-C$_6$H$_5$] and TSP$^4$, as previously described (Combourieu et al., 1998a). First, it should be noted that the transformation of GTL into benzylamine was quantitative. Second, no differences were observed due to the presence of glucose; the nature of the metabolite and the kinetics of its appearance were similar. GTL was degraded within 30 h. The concentration of allylamine could not be estimated during the process since its signals overlapped with those of glucose and the vinyl protons of SIN; however, after 30 h, the methylene protons in the spectrum could be integrated. At that particular time, the concentrations of allylamine were 3.9 and 3.5 mM in the presence or the absence of glucose, respectively. In the first case, no residual saccha-
ride protons could be detected, whereas in the second case, a small amount (<0.5 mM) of glycone moiety was observed. As the integration of NMR signals was difficult in the case of SIN, HPLC was used. The time course of SIN concentration in the presence or the absence of glucose are presented in Fig. 4b. In both cases, SIN was degraded almost completely within 30 h; the presence of free glucose had no effect on the kinetics. Finally, to compare HPLC and NMR as methods for quantification, GTL degradation was also monitored by HPLC (Fig. 4c). The results obtained with HPLC are in very good agreement with those obtained with NMR (Fig. 4a). The very small differences in calculated concentrations could be due to the “excitation hole” produced by water signal suppression in the range of ±0.6 ppm from water resonance (4.7 ppm). In conclusion, these experiments showed that SIN and GTL were degraded in a similar fashion and that glucose had no effect on these biotransformations.

Identification of Metabolites Issued from the Glucose Moiety of SIN and GTL by 1D and 2D-gCOSY 1H NMR Spectroscopy. Some major signals in which the intensity increased with time were detected in 1D 1H NMR spectra (Fig. 2 and Fig. 5) in the incubation experiments without glucose. A characteristic singlet, resonating at 1.92 ppm, corresponds to CH₃ of acetate (Fan, 1996; Matheron et al., 1998). In the downfield region, a singlet at 8.46 ppm increased until 6 h and disappeared at the end of the kinetics; this typical signal was assigned to formate (Fan, 1996; Matheron et al., 1997).

Other major signals resonating at 0.90, 1.07, and 2.17 ppm (three triplets), at 1.56 ppm (hexuplet), and at 2.19 ppm (quadruplet) were identified unambiguously by 2D 1H NMR spectroscopy. An example of an NMR spectrum recorded with a correlation spectroscopy pulse sequence with gradients coherence pathways selection is presented in Fig. 5. It was obtained from a sample taken after 18 h of incubation with glucosinolates. A cross peak between triplet at 0.90 ppm and hexuplet at 1.56 ppm was visible. Another correlation between this latter signal and the triplet at 2.17 ppm was observed. These three signals were assigned, respectively, to CH₃(4), CH₂(3), and CH₂(2) of butyrate. The triplet at 1.07 ppm and the quadruplet at 2.19 ppm correspond to CH₃(3) and CH₂(2) of propionate, whereas the signals resonating at 1.17 and 3.66 ppm belong to CH₃(3) and CH₂(2) of ethanol. A doublet at 1.32 ppm and a quadruplet at 4.11 ppm were, respectively, assigned to CH₃ and CH of lactate. All these chemical shifts are in agreement (±0.05 ppm) with those described in the literature (Fan, 1996).

Kinetics of Glucose Moiety Degradation. By measuring 1H NMR integrals in 1D spectra of the different metabolites (CH₃ of acetate, CH₃ of propionate, CH₃ of lactate, CH of formate, and CH₃ of ethanol), it was possible to compare the kinetics of formation of these metabolites when the human fecal flora was incubated with SIN and GTL in the presence or absence of glucose. It should be noted that the butyrate concentration could not be measured since its signals partially overlapped with other unknown signals and thus could not be integrated properly.

These data were compared with a control incubation containing α-D-glucose (6 mM); under these conditions, the same metabolites as those produced in the presence of glucosinolates were obtained, and glucose was completely exhausted after 3 h of incubation (data not shown).

The time courses of the concentration of acetate, lactate, ethanol, propionate, and formate are reported in Fig. 6, a–e, respectively. First, it should be noted that the initial rates of formation of the different metabolites were similar when glucose was alone or in combination with glucosinolates. When glucosinolates were alone, the initial rate was lower except in the case of propionate. This could reflect a limitation due to the rate of glycone hydrolysis from glucosinolates. Second, end products (acetate, propionate, and ethanol) accumulated...
and intermediates (lactate and formate) disappeared differently according to the incubation conditions.

**Discussion and Conclusion**

By using 1D and 2D $^1$H NMR spectroscopy, we have shown unambiguously that SIN and GTL are transformed quantitatively by the human fecal flora into allylamine and benzylamine, respectively. These findings are in contrast with previous studies showing that human digestive bacteria produced isothiocyanates from glucosinolates (Shapiro et al., 1998; Getahun and Chung, 1999; Elfoul et al., 2001). However, it is worth noting that in these experiments low amounts of isothiocyanates were recovered accounting for 10 to 20% of the initial amounts of glucosinolates. As amines were not investigated, it is possible that up to 80 to 90% of the missing recovery was actually present in this form in the samples.

A question remains: do allylamine and benzylamine result from transformation of the corresponding isothiocyanates? Although isothiocyanates were not detected, they could be present at a concentration less than to 0.03 mM (limit of NMR detection). These intermediates do not accumulate because they are likely to be transformed very rapidly in allylamine and benzylamine. Indeed, Tang et al. (1972) demonstrated the conversion of benzyl isothiocyanate into benzylamine upon incubation with a suspension of *Enterobacter cloacae* isolated from papaya pulp. In order to check that such a transformation was possible under abiotic conditions, allyl isothiocyanate (Sigma-Aldrich) and benzyl isothiocyanate (Fluka, Buchs, Switzerland) were incubated in the buffer in the absence of cells for 48 h at 37°C under the same anoxic conditions as those employed with bacterial incubations. The supernatants were directly analyzed by 1D $^1$H NMR spectroscopy. Allylamine and benzylamine were detected in the medium (data not shown), indicating the high sensitivity of isothiocyanates to hydrolysis. One can imagine that this chemical reaction could take place in vivo; alternatively, this reaction could also be enzymatically catalyzed. The biological significance of a possible release of allylamine and benzylamine from SIN and GTL in vivo remains to be established. One can already conclude that the conversion of isothiocyanates to amines, whether of biotic or abiotic origin, would reduce the delivery of biologically active isothiocyanates to the tissues and thus decrease their cancer-protective potential.

The comparison of the kinetics of transformation of SIN and GTL with and without glucose clearly showed that the presence of glucose did not modify either the nature of the metabolites or the rate of transformation, suggesting that the availability of a simple source of energy is not an important factor in the degradation of these complex thioglucosides.

When glucosinolates are hydrolyzed, free glucose is released, which can be further metabolized anaerobically by the bacteria of the human flora. It was interesting to test whether this glucose moiety was metabolized in the same way as free glucose. Regardless of the experimental conditions, the main metabolites were similar, including acetate, lactate, ethanol, propionate, formate, and butyrate. These metabolites are characteristic of anaerobic carbon metabolism in the digestive tract (Wolin et al., 1998, 1999). Acetate, propionate, and ethanol, which are end products of bacterial fermentation, accumulated in the incubation medium, whereas lactate and formate, which are intermediates, decreased in the course of the incubations (Mac-Farlane and Gibson, 1996). Their kinetics of formation or degradation were different according to the experimental conditions; this results from the limitation of available free glucose and proves that metabolism of free glucose was quicker than hydrolysis of the glycone moiety of glucosinolates.

In this work, 1D and 2D TOCSY and gCOSY $^1$H NMR experiments were used both to elucidate the molecular structure of glucosinolate derivatives and to quantify the concentrations of metabolites. Different compounds of biochemical interest were analyzed simultaneously in NMR spectra, including SIN, GTL, allylamine, and benzylamine. Considering that the preparation of one sample takes 5 min and that recording a 1D $^1$H NMR spectrum and a 2D $^1$H NMR spectrum takes about 10 min and 60 min, respectively, NMR spectroscopy is a very powerful technique and can be used routinely. Another advantage is that this technique is without a priori hypothesis; consequently, unexpected metabolites, such as the amine derivatives, can be detected. Although $^1$H NMR is used increasingly in pharmacology and medicine to analyze biological fluids, this work represents the first application to the study of the degradation of naturally occurring xenobiotics from edible plants by the human digestive microflora. Wolin et al. (1998, 1999) applied $^{13}$C NMR for studying carbon metabolism by the human digestive microflora, using [3-$^{13}$C]glucose as a substrate. In regard to xenobiotics, no $^{13}$C-enriched molecules are commercially available, and thus, $^1$H NMR is the most suited current technique.

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**References**


