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Fe^{2+}-Catalyzed Formation of Nitriles and Thionamides from Intact Glucosinolates

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The ratio of isothiocyanates to nitriles formed upon the hydrolysis of glucosinolates is a key factor determining the physiological effect of glucosinolate-containing plants and materials. A micellar electrokinetic capillary chromatography (MECC) method was used to study the nonenzymatic Fe^{2+}-catalyzed transformation of glucosinolates. At room temperature, pH 5, and in the presence of only 2 molar excess of Fe^{2+} all glucosinolate was degraded in 24 h. At all molar excess Fe^{2+} tested, nitriles were the major compounds formed. Thionamides were also formed from glucosinolates that contained a side chain hydroxylated at C-2; in this case, trace amounts of oxazolidine-2-thione were also detected. The presence of Fe^{3+} had no effect. The nonenzymatic Fe^{2+}-catalyzed transformation of glucosinolates involves the binding of Fe^{2+} to the glucosinolate to form a complex.

Glucosinolates are alkyl-*N*-hydroximine sulfate esters with a β-D-thioglucopyranoside group attached to the hydroximine carbon in *Z*-configuration to the sulfate group.[1–3] Glucosinolates constitute a group of more than 140 structurally different compounds with well-defined chemotaxonomic occurrence in all plants of the order Capparales and in a few other plants.[4–6] Glucosinolates co-occur with myrosinase isoenzymes (thioglucohydrolases; EC 3.2.1.147),[7,8] which catalyze the hydrolysis of the β-D-thioglucopyranoside bond, giving rise to a variety of compounds with different structures, depending on the parent glucosinolate and the reaction conditions.[3,9,10] Isothiocyanates are often the quantitatively dominant compounds that are formed in the myrosinase-catalyzed hydrolysis of glucosinolates at neutral pH, and they have, therefore, been the most frequently studied glucosinolate hydrolysis products.[1,3,9,11,12] However, glucosinolate hydrolysis can also occur via a nonenzymatic mechanism catalyzed by Fe^{2+}, and in this case a nitrile, instead of an isothiocyanate, is yielded. In the case of glucosinolates hydroxylated at C-2 in the side chain, a thionamide is formed.[13–16]

Glucosinolate degradation products have been studied widely because of their biological activity, which includes antinutritional effects on monogastric farm animals,[17,18] beneficial effects on human health,[19,20] and fungicidal, herbicidal, and nematocidal properties.[21,22] Isothiocyanates are generally regarded as the most active compounds; however, they are often volatile, hydrophobic, and very reactive, which makes them very short-lived and limits the time span in which they have an effect.[23,24] Nitriles are more stable products; however their biological activity is generally lower than their equivalent isothiocyanates.[6,25,26]

Understanding the factors that influence the formation of nitriles or isothiocyanates upon hydrolysis of glucosinolates is of great importance in controlling and predicting the biological effects of glucosinolate-containing plants and materials. In particular, the study of the nonenzymatic formation of nitriles by Fe^{2+} is relevant, since iron is often present in machinery, agricultural soil, food, and feed,[27] and intact glucosinolates can persist because of natural (e.g., low pH in the stomach or soil) or artificial (e.g., food processing) inactivation of myrosinase.

We studied the nonenzymatic formation of nitriles using a recently developed micellar electrokinetic capillary chromatography (MECC) method that allowed us to follow the hydrolysis of glucosinolates and the product formation online.[28] Our aim was to determine the influence of different concentrations of Fe^{2+} on the quantity and types of products formed from various glucosinolates in the absence of myrosinase at pH 5. The effect of Fe^{3+} was also tested. In order to elucidate the mechanism responsible for the degradation of glucosinolates and the formation of transformation compounds, we studied the oxidation of Fe^{2+} during the reaction.

Results and Discussion

Glucosinolate Transformation Products Obtained by the Nonenzymatic Degradation of Glucosinolates Catalyzed by Fe^{2+}. Fe^{2+} catalyzed the nonenzymatic degradation of glucosibarin at pH 5, giving rise to both thionamide and nitrile. The degradation of glucosinolates in acidic solutions that contain Fe^{2+} has been reported for sinigrin,[16] epi-progoitrin,[13–15] progoitrin, and glucobarbarin.[14,29] The formation of thionamide has been documented only from glucosinolates hydroxylated at C-2 in the side chain.[13–15,29] The major product from the myrosinase-catalyzed hydrolysis of glucosibarin at pH 5 is generally (5*S*)-5-phenyloxazolidine-2-thione (OZT).[6,28] In our study, this compound was produced only in trace amounts, since it was only detected when a 50-fold injection volume was used (data not shown).

The MECC method used[28] proved to be valuable for following the hydrolysis of glucosibarin by Fe^{2+} in the absence of myrosinase. Figure 1 shows an electropherogram of the reaction in which glucosibarin (e) appears simultaneously with nitrile (b) and thionamide (c). The UV spectra of these compounds are available,[14,28] which permitted their immediate identification. In the course of the reaction, an additional peak (d) appeared between the degradation products and glucosibarin. According to its electrophoretic mobility, it was likely to be an uncharged compound of higher molecular weight than the thionamide or the nitrile. These characteristics, together with the fact that it was unstable (see below), indicated that it could be a Fe^{2+}-glucosinolate complex with one hydrated acidic Fe^{2+} and either one or two glucosinolates.[16] Furthermore, the compound had a UV spectrum similar to a glucosinolate with a maximum absorbance at 225–230 nm, which indicates that it had retained the characteristic glucosinolate core structure (C=N conjugated with S), although slightly changed with respect to the parent glucosinolate (e).

Glucosibarin degradation started immediately after adding 1 M excess Fe^{2+}, and the reaction proceeded during approximately 120 min (Figure 2), after which no significant changes in the concentration of glucosibarin, nitrile, and thionamide were observed. At this

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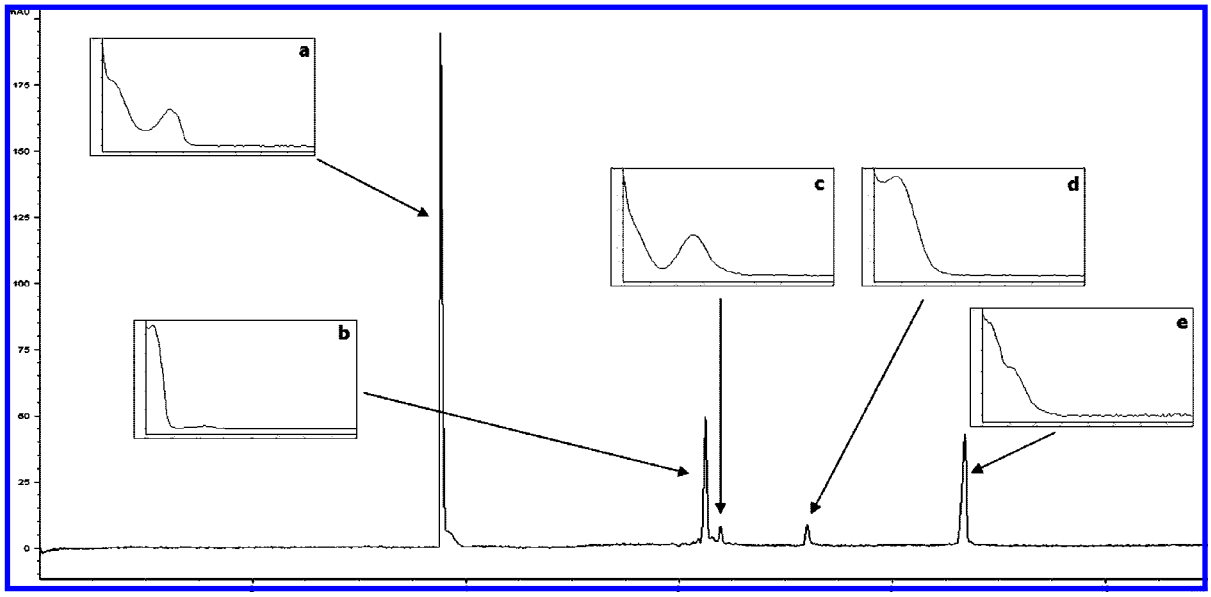

Figure 1. Electropherogram showing the simultaneous appearance of the internal standard [TNA (a)], the products [nitrile (b), thionamide (c)], the postulated glucosibarin-Fe^{2+}complex (d), and glucosibarin (e). The UV spectra of the different compounds are also shown.

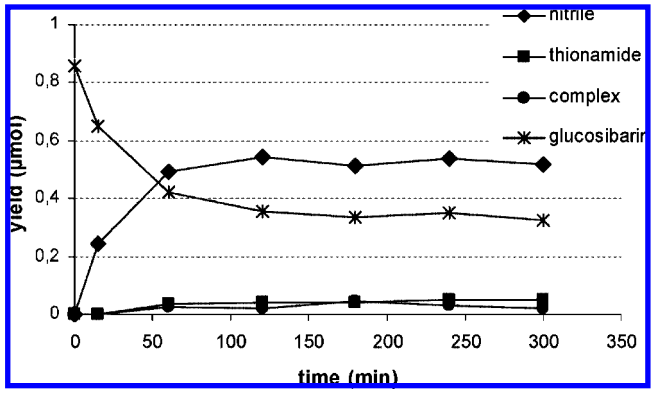

Figure 2. Rates of glucosibarin degradation and formation of the nitrile, thionamide, and the glucosibarin-Fe^{2+}complex in the presence of 1 M excess of Fe^{2+} with respect to glucosibarin. Each point is the average of two runs.

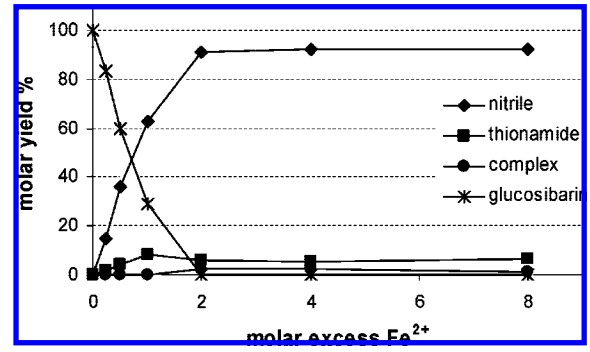

Figure 3. Molar yield (in %) of nitrile, thionamide, the Fe^{2+}-glucosibarin complex, and glucosibarin after 24 h reaction with different molar excesses of Fe^{2+}. Each point is the average of three replicates.

Fe^{2+} molar excess, the Fe^{2+}-glucosinolate complex achieved maximum concentration at around 180 min.

The nitrile was, quantitatively, the major product formed, accounting for nearly 90% of the total pool of degradation products after 24 h, independent of the Fe^{2+}:glucosinolate ratio (Figure 3). Nitrile was reported as the major product of the nonenzymatic Fe^{2+}-catalyzed degradation of progoitrin at room temperature and pH 5.2 with 10 M excess Fe^{2+}.[29] On the other hand, Austin et al.[15] reported thionamide as the major product from epi-progoitrin and glucobarbarin at 7 M excess Fe^{2+}, room temperature, and pH 5.3, although the yield decreased with incubation time. They also found that, at 95 °C and with lower concentrations of Fe^{2+}, the proportion of nitrile increased with respect to thionamide. We cannot provide an explanation as to why nitrile was the major transformation product in our experiments. However, in contrast to the methods used in previous studies,[13–16,29] the MECC method presently used allowed us to follow the reaction online;[28] thus the risk for artifact production or product degradation associated with the extraction of compounds from the reaction medium was eliminated.

Complete glucosinolate degradation at room temperature has been reported to occur only at 6 to 8 M excess Fe^{2+},[14,29] whereas at higher temperatures (95 °C), equimolar amounts are sufficient.[14,16] In the present study, 2 M excess Fe^{2+} degraded all glucosibarin in 24 h at 20 °C (Figure 3). Fe^{2+} readily oxidizes to Fe^{3+} in aqueous solutions as pH increases,[30] and in our experiments, we found that approximately half of the total iron in the stock solutions was in the oxidized form at pH 5. This shows the importance of determining the exact amount of Fe^{2+} in the reaction medium and may explain the differences found between the present and previous studies regarding the Fe^{2+} molar excess needed for total degradation of the glucosinolate.

Increasing the concentration of Fe^{2+} increased the velocity of the reaction, and in the presence of an 8 M excess Fe^{2+}, only 30% of the initial amount of glucosinolate remained after 10 min (data not shown), representing a 3-fold increase in the degradation rate with respect to 0.25 M excess Fe^{2+}. The increased velocity of reaction by increasing concentrations of Fe^{2+} has been shown both in vitro[16] and in vivo.[31] Increasing the molar excess of Fe^{2+} from 0.5 to 2 increased the concentration of the complex in the mixture, although not proportionally to the increase of Fe^{2+} (data not shown). From 2 to 8 M excess Fe^{2+}, however, the concentration of the complex in the mixture remained constant (Figure 3).

Similar results were obtained when other glucosinolates were tested. Both gluconasturtiin and progoitrin were shown to be degraded by Fe^{2+}. Nonenzymatic hydrolysis of gluconasturtiin gave rise only to nitrile, and as expected, no thionamide was formed because of the absence of a hydroxyl group in the side chain. The expected thionamide was observed in the progoitrin hydrolysis, as previously shown.[15,16,29]

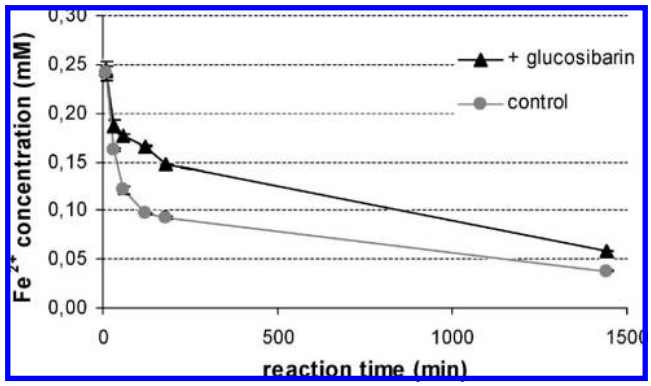

Figure 4. Variation in the concentration of Fe^{2+} over time (24 h incubation) in a control sample (no glucosinolate added) compared to a 2:1 Fe^{2+}:glucosinolate sample.

Fe^{3+} has been reported to promote nitrile formation in vivo, although to a lesser extent than Fe^{2+};[31] however, the in vitro results are contradictory. $FeCl_3$ promoted the decomposition of sinigrin to allyl cyanide,[16] but did not degrade epi-progoitrin.[14] In our experiments, $FeCl_3$ did not degrade any of the glucosinolates when tested by the MECC method under the same conditions as the $FeSO_4$ experiments.

Identification of the Reaction Products. Glucosinolate degradation by Fe^{2+}-mediated catalysis has been postulated to occur through the formation of a complex.[16] The additional peak that appeared during glucosibarin hydrolysis (Figure 1, d) was repeatedly observed at different Fe^{2+} concentrations as well as when glucosinolates other than glucosibarin were used. At concentrations of $Fe^{2+} \leq 20$ mM (equal or lower than 1 M excess), the peak disappeared after 24 h, although it remained in the mixture when 2 M excess Fe^{2+}, or more, was added (Figure 3).

To identify this compound, high-voltage electrophoresis (HVE)[10,32] was performed. After incubation, the reaction medium to which Fe^{2+} was added did not contain any residue of intact glucosibarin. Instead, a compound with no electrophoretic mobility that still retained the sulfur atom was present (data not shown). Preparative HVE allowed for the isolation of the neutral compounds formed in the reaction, but MECC of the sample prior to NMR revealed that the Fe^{2+}-glucosinolate complex had been degraded during purification. Apart from the expected nitrile, the neutral fraction of the HVE electropherogram was shown to contain thioglucose. ^{1}H NMR spectroscopy showed the complex pattern at δ 3.2–3.9 that is characteristic of glucose plus a doublet at δ 4.4 for C-1′, whereas ^{13}C NMR spectroscopy showed the following chemical shifts (δ): C-1′ 89.7, C-2′ 77.3, C-3′ 77.5, C-4′ 71.5, C-5′ 80.6, C-6′ 62.9 (DEPT). The ^{13}C NMR resonances found were in agreement with those obtained with the use of a commercial sample of thioglucose (data not shown) and with those described previously for its Na salt.[33]

The presence of thioglucose instead of glucose as a product of the Fe^{2+}-catalyzed glucosinolate degradation has previously been reported.[16] Youngs and Perlin[16] found only trace amounts of glucose after the degradation of sinigrin by Fe^{2+}, and half the amount of the sugar unit was recovered as bis(β-D-glucopyranosyl) disulfide.

Oxidation of Fe^{2+}. The presence of glucosibarin prevented the oxidation of Fe^{2+} to Fe^{3+} in the first hours of the reaction when a 2:1 ratio of Fe^{2+}:glucosinolate was used (Figure 4). At this Fe^{2+} molar excess, the difference between the Fe^{2+} concentration in the samples, with and without glucosinolate, decreased with the incubation time. In an aqueous aerobic solution under weakly acidic and alkaline conditions, oxygen can convert Fe^{2+} to Fe^{3+} spontaneously because of the redox potential of iron.[30] Our results show that the transient complex formed between the glucosinolate and Fe^{2+} has the ability to shield the Fe^{2+} and prevent its oxidation. A recent report showed that the Fe^{2+}-chelating activity of broccoli (reported as an EDTA equivalent) was the highest among a number of vegetables tested.[34]

Mechanism of Reaction. Fe^{2+} did not degrade the desulfo form of glucosibarin when tested identically as the other intact glucosinolates in the MECC assay. This shows that the presence of the sulfate group in the glucosinolate is decisive for the formation of the complex, as previously stated.[16] Figure 5 shows the proposed mechanism for the nonenzymatic Fe^{2+}-catalyzed glucosinolate degradation. The ligands in the glucosinolate-Fe^{2+} complex for the formation of the nitrile (Figure 5, left) are expected to be the S in the thioglucose and the O in the sulfate group;[16] the O at C-2 of the glucose could also participate in the complex. For the formation of the thionamide (Figure 5, right) the ligands are expected to be the O of the hydroxyl group at C-2 and the N in the sulfate group. The mechanism of reaction for thionamide formation involves the breakage of the thioglucoside bond between the S and the glucose (as for the myrosinase-catalyzed glucosinolate hydrolysis; Figure 5), and it requires the delivery of two redox equivalents for the formation of the thionamide. Breakage of this bond could also explain the trace amounts of OZT found. Nitrile formation involves the breakage of the bond between the glucosinolate carbon C-0 and S in the thioglucoside bond (Figure 5), which also requires the delivery of two redox equivalents liberating thioglucose.

Youngs and Perlin[16] suggested that the complex of Fe^{2+} with 2 mol of sinigrin provides a route for a concerted electron transfer, one phase of which involves the formation of the S−S bond between two thioglucoses. In our experiment, thioglucose and not the disulfide form was found in the reaction medium after 24 h. We propose that the reducing conditions in our experiments protect the thioglucose from oxidation to the disulfide form.

Practical Implications. Our experiments have shown that nitriles are the quantitatively major products formed nonenzymatically from intact glucosinolates at mild conditions of temperature and pH in the presence of as little as 0.25 M excess Fe^{2+}. Fe^{2+} and intact glucosinolates are often present simultaneously during the analysis, handling, processing, and consumption of glucosinolate-containing materials. Thus, the possibility of nitrile formation through Fe^{2+}-catalyzed degradation of intact glucosinolates should be acknowledged and its implications considered when assessing the biological effects of glucosinolate-containing materials.

Experimental Section

General Experimental Procedures. Disodium hydrogenphosphate was purchased from Riedel-de Häen (Seelze, Germany), ascorbic acid from Bie & Berntsen (Rødovre, Denmark), and ferrous sulfate heptahydrate from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, MA). Sodium cholate, taurine, trigonellinamide (TNA), ferric chloride hexahydrate, HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, sodium salt), trichloroacetic acid (TCA), chlorhydric acid, hydroxylamine monohydrochloride, and ferrozine chromogen [3-(2-pyridyl)-5,6 bis(4-phenylsulfonic acid)-1,2,4-triazine, disodium salt] were purchased from Sigma-Aldrich (Steinheim, Germany). The intact glucosinolates (glucosibarin, progoitrin, and gluconasturtiin) and the desulfo glucosinolate (desulfoglucosibarin) used in the experiment were from our laboratory collection.[35,36]

MECC analyses were performed in a Hewlett-Packard HP^{3D} capillary electrophoresis (CE) system (Agilent, Waldbronn, Germany) that was equipped with a diode array detector. For data processing, we used a HP Vectra 5/100 mHz Pentium with HP ChemStation Rev. B. 01.03.

^{1}H and ^{13}C 1D NMR spectra were recorded at room temperature on a Bruker Advance 400 NMR spectrometer using TMS as reference.

Measurement of Fe^{2+} Ions in the Solutions. Prior to utilization in the MECC assay, each new Fe^{2+} stock solution was analyzed for Fe^{2+} and total iron to determine the exact volume of solution that was needed in the assay. The method that we used is based on the reaction with the chromogene ferrozine,[37] modified from Kapsokefalou and Miller,[38] and adapted for microcale use in 96-well microplates. Fe^{2+} was analyzed by adding 100 μL of nonreducing protein precipitate

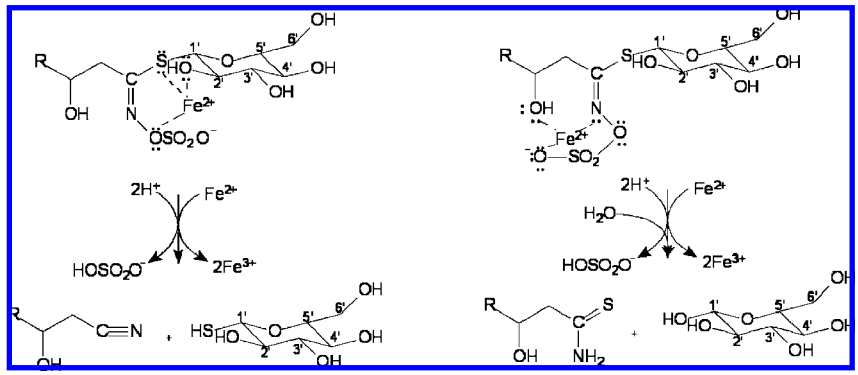

Figure 5. Proposed mechanism for nitrile (left) and thionamide formation (right) through the Fe^{2+}-catalyzed degradation of glucosinolates containing a hydroxyl group at C-2 in the side chain. The figures include only one of the two glucosinolates supposedly present in the complex.[16] See text for details.

solution (non-RPP, 1 g of trichloroacetic acid (TCA), 1 mL of 37% HCl adjusted to 10 mL with water) to 200 μL aliquots. Total iron was analyzed by adding 100 μL of reducing protein precipitate solution (RPP, 1 g of TCA, 1 mL of 37% HCl, 0.5 g of hydroxylamine monohydrochloride adjusted to 10 mL with water) to 200 μL aliquots of sample or to ferrous sulfate in 2-fold dilutions starting at 1 mM $FeSO_4$ for quantification. The aliquots, which contained either non-RPP or RPP, were left overnight at room temperature and centrifuged (2575*g*; 10 min) prior to use. Duplicates of 100 μL supernatants were placed in microtiter wells and then mixed with 200 μL of HEPES buffer (0.3 M, pH 9.9) and 25 μL of ferrozine chromogen solution (5 mg/mL in water). The absorbance was measured in a microplate reader (Bio kinetic reader EL 340 Microplate; Bio-Tek Instruments; Software: KC3; KinetiCalc for Windows, version 1.5) at 570 nm immediately after ferrozine addition for the quantification of Fe^{2+} and after 1 h for determination of total iron content.

MECC Reaction Procedure. The formation of the nitrile, the thionamide, and the complex from the different glucosinolates investigated was followed online with the method developed by Bellostas et al.[28] The MECC run buffer was composed of 35 mM sodium cholate, 100 mM disodium phosphate, 500 mM taurine, and 2% 1-propanol, and the pH was kept at 8.2. A solution of 0.8 M citric acid was adjusted to pH 5.5 ($\pm$0.1), and ferrous sulfate was added to a concentration of 0.4 M. The final pH of the ferrous sulfate stock solution ranged in all cases between 4.5 and 5. The content of Fe^{2+} in this solution was determined by the procedure described above, which allowed us to calculate the volume that was needed to have the desired μmoles of Fe^{2+} in the reaction medium. This solution was prepared freshly every time a new determination was conducted. The reaction medium was composed of TNA (as an internal standard; 20 μL, 100 mM), glucosinolate (20 μL, 50 mM), Milli-Q water (6.5 μL), and acetate buffer (3.5 μL, 100 mM, pH 5), to which different volumes of the above-mentioned $FeSO_4$ solution were added. The final pH of the reaction medium was in no case lower than 4.5. The measurements were conducted in duplicate. Concentrations of glucosibarin and nitrile (phenylacetonitrile instead of 3-hydroxyphenylproprionitrile was used) were determined by the use of concentration–response curves of the pure compounds at 206 nm in MECC (cholate buffer, 30 °C). The *E* value for TNA at 206 nm was calculated by UV spectroscopy (Shimadzu MPS-2000 UV–visible light spectrophotometer). Molar response factors of the complex and the thionamide were assumed to be identical to that of the parent glucosinolate at 206 nm.

Identification of the Degradation Compounds. HVE[10,32] was performed to determine the presence of the Fe^{2+} complex. Two aliquots of the MECC reaction medium (described above), one with Fe^{2+} (2 M excess) and the other one without Fe^{2+}, were incubated overnight at 20 °C. Previous to HVE, the sample to which Fe^{2+} was added was checked by MECC to ensure that the complex was present. The HVE was carried out in Whatman 3 MM paper in a flat plate unit. The samples were applied with a capillary to the paper in three replicates with increasing volumes (one, three, and six applications). Sinigrin was used as a reference compound. The run buffer was glacial acetic acid, formic acid, and water (4:1:45) at pH 1.9, and the electrophoresis was run for 1 h at 3 kV. The presence of glucosinolates was determined by silver nitrate staining.[10,32]

Preparative HVE was conducted to isolate the neutral degradation products. The reaction was conducted in a large volume by mixing glucosibarin (3 mg) with twice the molar amount of Fe^{2+} at pH 5 and allowing it to stand overnight at room temperature. The reaction mixture was checked by MECC for the presence of the complex previous to preparative HVE. Once HVE was run, the band where the neutral compounds appeared was washed out with water, the wash-out evaporated, and the residue redissolved in D_2O prior to ^1H and ^{13}C NMR spectroscopy.

Measurement of the Oxidation of Fe^{2+} in the Presence of Glucosinolate. The reaction mixture consisted of glucosibarin (40 μL, 10 mM) and acetate buffer (1 mL, 100 mM, pH 5), and the volume was adjusted to 4 mL with MilliQ-water. The control sample consisted of acetate buffer (1 mL, 100 mM) and Milli-Q water (3 mL). To both samples, $FeSO_4$ (7.2 μL, 100 mM) was added, and the pH was adjusted to 5. Aliquots (200 μL) were collected for measurement of Fe^{2+} in solution (vide supra), at times corresponding to 0, 10, 30, 60, 120, and 180 min and 24 h.

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