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Preparation and Biological Activity of Four Epiprogoitrin Myrosinase-Derived Products

Stefania Galletti,† Roberta Bernardi,† Onofrio Leoni,† Patrick Rollin,‡ and Sandro Palmieri*,†

Istituto Sperimentale per le Colture Industriali, Italian Ministry of Agricultural and Forestry Politics, Via di Corticella 133, I-40129 Bologna, Italy, and Institut de Chimie Organique et Analytique, Université d'Orléans, B.P. 6759, F-45067 Orléans, Cedex 2, France

(*5R*)-5-Vinyl-1,3-oxazolidine-2-thione, (*2S*)-1-cyano-2-hydroxy-3-butene, and two diastereoisomeric *erythro*-(*2S*)- and *threo*-(*2S*)-1-cyano-2-hydroxy-3,4-epithiobutanes were prepared in pure form starting from (2*S*)-2-hydroxybut-3-enyl glucosinolate (epiprogoitrin). This glucosinolate was isolated in almost pure form using ripe seeds of *Crambe abyssinica* and then hydrolyzed under different conditions. The hydrolysis was carried out using either myrosinase immobilized on nylon, to produce (*5R*)-5-vinyl-1,3-oxazolidine-2-thione, or the endogenous myrosinase contained in defatted crambe meals, to produce the other epiprogoitrin-derived products. After purification and physicochemical characterization, all four myrosinase degradation products were tested for their biological activity. A bioassay on *Lactuca sativa* was chosen as a simple test to determine their apparent action on living tissues. (*5R*)-5-Vinyl-1,3-oxazolidine-2-thione negatively affected mainly root growth, whereas (*2S*)-1-cyano-2-hydroxy-3-butene affected the early phase of germination, and both (*2S*)-1-cyano-2-hydroxy-3,4-epithiobutane diastereoisomers appeared to negatively affect both germination and root growth at doses 5−10 times lower than those of (*2S*)-1-cyano-2-hydroxy-3-butene or (*5R*)-5-vinyl-1,3-oxazolidine-2-thione.

Keywords: *(2S)-2-Hydroxybut-3-enyl glucosinolate; myrosinase; (5R)-5-vinyl-1,3-oxazolidine-2-thione; (2S)-1-cyano-2-hydroxy-3-butene; (2S)-1-cyano-2-hydroxy-3,4-epithiobutanes; Crambe abyssinica*

INTRODUCTION

Glucosinolates are plant secondary metabolites of 15 families of dicots, and they are particularly abundant in Brassicaceae. These compounds have a common structure but a varying aliphatic, aromatic, or heteroaromatic aglycon chain. In their native form, glucosinolates have a low biological activity, whereas their derived products (isothiocyanates, thiocyanates, nitriles, and epithionitriles), obtained by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1)-catalyzed hydrolysis, constitute an important group of bioactive molecules of vegetable origin.

Myrosinase is a widespread enzyme contained mainly in seeds and tissues of Brassicaceae and, in particular, *Sinapis alba* seeds are at present the main source of this enzyme. *S. alba* myrosinase (*1*−*3*) is a glycoprotein containing 3 disulfide bridges per subunit and 21 carbohydrate residues distributed in 10 glycosylation sites on the surface. In water solution, myrosinase is a dimer with two identical subunits, stabilized by a Zn^{2+} ion with tetrahedral coordination.

The myrosinase−glucosinolate system is always present at different concentrations in all Brassicaceae organs, where, when activated following tissue damage, it plays a defensive role against generalized herbivores, as well as being an essential agent in host-plant recognition by specialized pathogens (*4*−*7*). This system can generate aglycons, which spontaneously rearrange to give compounds with a cytotoxicity depending on their chemical structure. The role of glucosinolates and their derived products in plant resistance has been known for a long time (*8*, *9*). More recently, Mithen et al. (*10*), Angus et al. (*11*), and Mari et al. (*12*, *13*) demonstrated the role of the myrosinase−glucosinolate system in protecting rapeseed from *Laeptosphaeria maculans*, wheat from *Gaeumannomyces graminis*, and fruit against a number of postharvest pathogenic fungi. In addition, Manici et al. (*14*) demonstrated the strong fungitoxic activity of some glucosinolate-derived products against important soil-borne pathogens in vitro. This last property has brought those compounds to light as a convincing alternative for the control of fungal diseases.

The main glucosinolate contained in ripe seeds of *Crambe abyssinica*, (2*S*)-2-hydroxybut-3-enyl glucosinolate (epiprogoitrin) (**1**) (Figure 1), can be readily extracted from defatted crambe meal with a good yield and high purity (*15*). In addition, it is possible to carry out a high-yielding chemo- and stereocontrolled hydrolysis of **1** to obtain glucose-free, (2*S*)-1-cyano-2-hydroxy-3-butene (**2**) and/or (5*R*)-5-vinyl-1,3-oxazolidine-2-thione (**4**), using free or immobilized myrosinase (*15*, *16*) (Figure 1). At a pH of ~6.5, the myrosinase-catalyzed hydrolysis of **1** produces the corresponding short-lived isothiocyanate, which quickly generates **4** through a cyclization mechanism (*17*). The same enzymatically catalyzed hydrolysis, carried out at pH 5 in the presence of Fe^{2+} and cysteine, provides either **2** alone (*18*−*20*) or together with diastereoisomeric *threo*-

* Author to whom correspondence should be addressed [e-mail sandro.palmieri@iol.it; telephone and fax (39)-051-6316851].

† Italian Ministry of Agricultural and Forestry Politics.

‡ Université d'Orléans.

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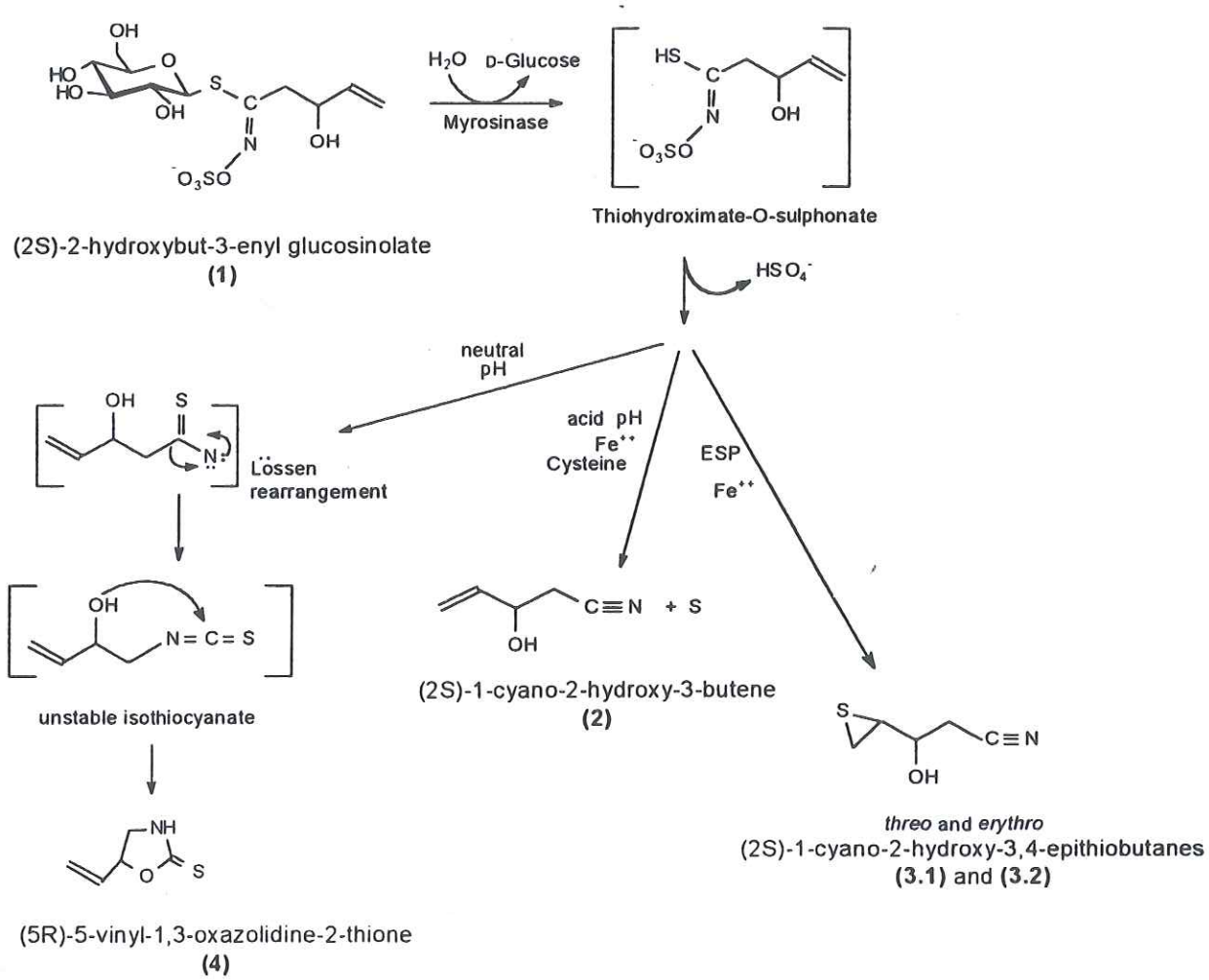

Figure 1. General pathway of myrosinase-catalyzed hydrolysis of epiprogoitrin.

and *erythro*-(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutanes (**3.1** and **3.2**, respectively), when the epithiospecifier protein, a myrosinase cofactor, is present in the reaction mixture (Figure 1) (*21*–*23*).

All epiprogoitrin-derived products have one (**2** and **4**) or two (**3.1** and **3.2**) stereogenic centers, which are important structural features from both chemical and biological points of view. In addition, using a novel chiral discrimination methodology, involving a structurally modified β-cyclodextrin (*24*), we could ascertain that **4** was obtained in enantiomerically pure form (*15*).

In a previous study, we established that some glucosinolate-derived products affected the germination process of three weeds, namely, *Chenopodium album* L., *Portulaca oleracea* L., and *Echinochloa crus-galli* (L.) Beauv. (*25*). Furthermore, Brown and Morra (*26*) pointed out an antigerminative activity of glucosinolate-derived products present in *Brassica napus* meals, and Vaughn and Berhow (*27*) identified **2** as responsible for the weed control activity of defatted crambe meal. However, because **2** is not the only epiprogoitrin-derived product produced in crambe meal, this study planned a series of germination experiments to assess the biological activity of the epiprogoitrin-derived products, **2**–**4**, produced for this purpose, purified, and identified using spectrometric techniques. As first, we chose the germination of *Lactuca sativa* as a simple and reproducible biological test to compare the cytotoxicity of these molecules in order to evaluate their potential as natural weed control products.

MATERIALS AND METHODS

Plant Material. Crambe plants (*C. abyssinica* cv. Mario) were grown in Bologna (Italy) during the spring 1996. Ripe crambe seeds with a moisture content of <8% were freed of impurities, milled, and defatted with *n*-hexane.

Epiprogoitrin. This glucosinolate was isolated starting from crambe ripe seeds. We applied a procedure previously set up in our laboratory for some other glucosinolates (*28*), based on the general method disclosed by Thies (*29*) for isolating sinigrin and glucotropaeolin. A typical preparation gives 20 g of pure **1** starting from 1 kg of seeds. The HPLC analyses of desulfo derivatives (*30*), coupled with polarographic determinations of total glucosinolate content (*31*), showed that **1** was almost homogeneous, as confirmed by NMR spectrometry.

Production of Epiprogoitrin-Derived Molecules. The epiprogoitrin-derived products, namely, **2**, **3.1**, **3.2**, and **4**, were produced as follows:

2, **3.1**, and **3.2** were produced by exploiting the endogenous myrosinase−epithiospecifier protein−epiprogoitrin system naturally contained in crambe seed, following the method described by Daxenbichler et al. (*32*) with some modifications. The hydrolysis was carried out at 9 °C, stirring for 90 min a slurry of defatted crambe meal (10 g) and 30 mL of 0.1 M acetate buffer, pH 5.0, containing 2.5 mM Fe^{2+} and 5 mM cysteine. Pure **1** (2.7 g) was also added in successive small amounts.

Crude extract proteins were precipitated by adding ethanol up to 70% of the total volume and leaving the mixture at 4 °C overnight. After centrifugation, the supernatant solution was concentrated at 50 °C in vacuo, until ethanol was completely removed, and then recentrifuged. Afterward, **2**, **3.1**, and **3.2** were extracted using ethyl ether (extraction ratio = 1:1) in a

separatory funnel six times. The organic solution was concentrated in the rotary evaporator, and a small amount of water was added before the ethyl ether was completely removed. The aqueous solution was loaded into an RPC18 HR16/10 column and then eluted with distilled water, using a GradiFrac system (Pharmacia). Fractions of 3−5 mL were collected and analyzed with a GC-MS to evaluate the contents of **2**, **3.1**, and **3.2**.

In this way, **2**, **3.1**, and **3.2** were isolated in homogeneous form. **2** was extracted from the aqueous solution, using CH_2Cl_2 (extraction ratio = 1:1), and the organic solution was dried over anhydrous Na_2SO_4. After filtration, the solution was concentrated, first in the rotary evaporator and then under a nitrogen stream, until complete solvent removal. **2** was then stored at 4 °C. **3.1** and **3.2** were concentrated in vacuo with the rotary evaporator, and stored in water solution under nitrogen at −20 °C, due to their poor stability when kept in dry form. All of the produced compounds were characterized by GC-MS, ^{1}H NMR, and/or HPLC.

4 was prepared using the myrosinase immobilized on Nylon 6,6 with a small continuous-flow bed reactor containing 10 g of nylon (*16*). The product was dried using a Büchi model RE 121 rotary evaporator at 50 °C under vacuum and then solubilized with CH_2Cl_2. The solution was dried over anhydrous Na_2SO_4, filtered, and concentrated first in the rotary evaporator and then under a nitrogen stream, until solvent removal was complete. Finally, **4** was solubilized in a minimum volume of boiling water and maintained at 4 °C. After about a week, it generally crystallized, producing transparent needles. The crystals were recovered and washed three times with cold water. The crystallization process was repeated twice. Finally, the crystals were air-dried and stored at 4 °C.

Analyses of Epiprogoitrin-Derived Products. The epiprogoitrin-derived products were analyzed using GC, Fisons Carlo Erba model Mega 5330, equipped with a 30 m × 0.32 mm capillary column Restek Rtx 5. The flow rate of the carrier gas (He) was 1.8 mL min^{-1} (split ratio = 1:40). The column temperature was 40 °C at the start and 220 °C at the end with a rate of 10 °C min^{-1}; the temperature of the injector (split) and detector (FID) was 240 °C.

For the quantitative analyses of epiprogoitrin-derived products by GC, phenyl isothiocyanate was used as internal standard. The response factor of **4** was that reported by Brown et al. (*33*), whereas response factors for **2**, **3.1**, and **3.2** were those reported by Leoni et al. (*34*).

GC-MS analyses were carried out using a Hewlett-Packard GCD system model G1800A equipped with a 30 m × 0.25 mm capillary column HP-5MS. The flow rate of the carrier gas (He) was 1 mL min^{-1}, and 1 μL of sample was injected in splitless mode. The column temperature was 40 °C at the start and 220 °C at the end with a rate of 10 °C min^{-1}. The temperatures of the injector and detector were 250 and 280 °C, respectively.

Finally, **4** was also analyzed by HPLC (*35*), using a Hewlett-Packard chromatograph model 1090L equipped with a diode array as detector and a 200 × 4.6 mm column HP ODS Hypersil C18, 5 μm.

Germination Test. Three replicates of 50 seeds of *L. sativa* cv. Romana (Blumen Seed Co.) were placed in plastic Petri dishes (9 cm diameter) on filter paper moistened with 3 mL of aqueous product solution, covered, and immediately sealed with Parafilm. The doses, of **2**, **3.1**, **3.2**, and **4**, chosen on the basis of preliminary trials, were as follows: 0 (deionized water), 0.8, 4.0, 8.0, 12.0, 16.0, 20.0, and 24.0 mM; 0, 0.8, 1.6, 2.4, and 3.2 mM; 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mM; 0, 0.4, 0.8, 4.0, 8.0, 16.0, 24.0, and 32.0 mM, respectively. Treated dishes were incubated at 20 ± 1 °C and at 8/16 h day/night cycles. On the seventh day, the percentages of normal and abnormal seedlings were recorded, following the ISTA rules for seedling evaluation suggested for lettuce (*36*, *37*); the root length was measured on a sample of 10 seedlings per replicate, and the different kinds of abnormalities were recorded.

RESULTS AND DISCUSSION

Production and Purification of Epiprogoitrin-Derived Products. In some preliminary experiments, we established that maintaining defatted crambe meal (laboratory-scale prepared) in 0.1 M acetate buffer, pH 5 at 37 °C, without additives induced fast hydrolysis of the endogenous **1**, giving approximately 39% of **2**, 26% of **3.1**, 32% of **3.2**, and 3% of **4** according to Tookey (*21*). However, with specific trials aimed at increasing the yields of **3.1** and **3.2**, the production of **4** could be reduced by >90%. In fact, working at pH 5 in the presence of 2.5 mM Fe^{2+} and 5.0 mM cysteine, we practically obtained only **2**, **3.1**, and **3.2**. In addition, when the myrosinase-catalyzed hydrolysis was carried out at lower temperature (9 °C), the relative amounts of these products remained about the same, even when previously purified **1** was added to the meal up to a limit that was evaluated at ~10 times that of the endogenous **1**. A greater amount of **1** enhanced only the production of **2** and **4**, without increasing the amounts of **3.1** and **3.2**.

Table 1. Yield of (2*S*)-1-Cyano-2-hydroxy-3-butene (2) and (2*S*)-1-Cyano-2-hydroxy-3,4-epithiobutane Diastereoisomers (3.1 and 3.2) Obtained by Myrosinase-Catalyzed Hydrolysis of Epiprogoitrin[a]

product	total amount in the crude extract (mmol)	total amount in the final water solution (mmol)	yield (%)
2	2.8	2.2	79
3.1	1.8	1.3	72
3.2	2.2	1.5	68

[a] The starting reaction mixture contained 0.6 mmol of endogenous epiprogoitrin, which was then added in pure form up to 6.3 mmol.

Table 1 shows the yield of a typical preparation of **2**, **3.1**, and **3.2** obtained when a total amount of 6.9 mmol of **1** was hydrolyzed: 0.6 mmol was endogenous, whereas the additional 6.3 mmol was added in pure form. At the end of the experiment, the reaction products were extracted, collected, and analyzed by GC-MS and NMR. Spectrometric data (not shown) compared with those reported in previous papers (*20*, *38*−*40*) made it possible to identify the epiprogoitrin-derived products, showing that **2**, **3.1**, and **3.2** were produced in the mixture and no other compounds were identified in the extraction solutions.

It is well-known that to obtain **3.1** and **3.2** enzymatically, a myrosinase cofactor named epithiospecifier protein is necessary. This protein was isolated and characterized in our laboratory (*22*), and we observed that, especially when in pure form, it showed poor stability. For this reason, we found it easier to produce **3.1** and **3.2** with defatted crambe meal as a natural and simple production system for those intriguing molecules. Some preliminary trials showed that it was impossible to produce **3.1** and **3.2** without **2** coproduction by merely carrying out the reaction at the optimal pH and in the presence of reducing agents. In addition, the low stability of **3.1** and **3.2**, especially when they are in dry form, was another drawback in the isolation process. We consider that the lower stability of **3.1** and **3.2**, in concentrated solutions but mostly in anhydrous conditions, could be the explanation for the lower purification recovery that we observed for **3.1** and **3.2** as compared to **2** (Table 1). Although several trials were carried out to improve the stability of **3.1** and **3.2**, the only procedure that made it possible to obtain acceptable results consisted in maintaining **3.1** and **3.2** in aqueous solution and, for greater safety, at low temperature under nitrogen.

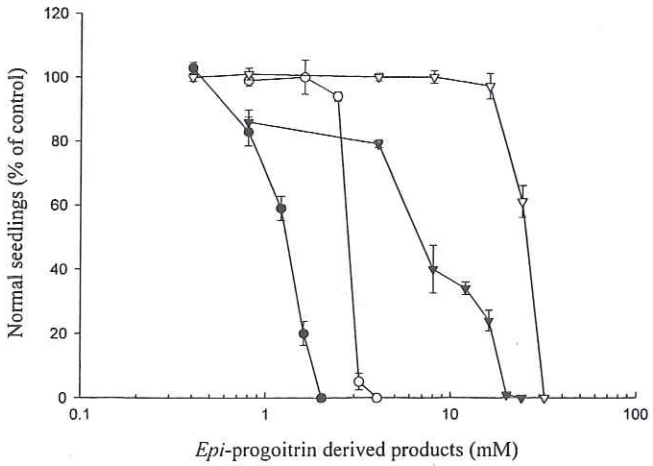

Figure 2. Effect of different doses of the four epiprogoitrin degradation products (▼, **2**; ○, **3/1**; ●, **3/2**; ▽, **4**) on seed germination of *L. sativa* reported as percent of normal seedlings on the control, after 7 days of germination on filter paper at 20 °C. Vertical bar indicates the standard error of the mean of three replicates.

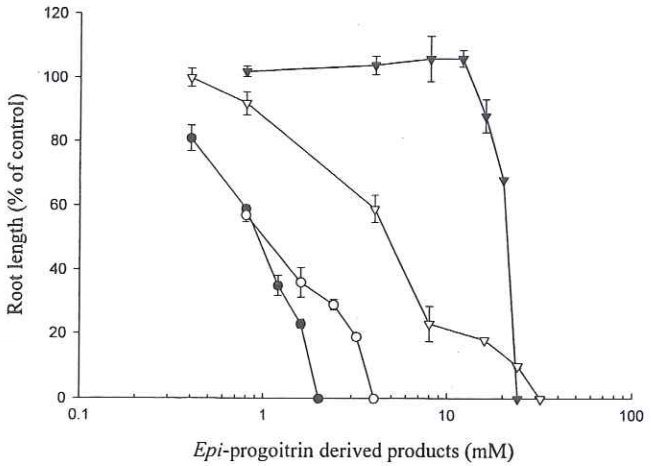

Figure 3. Effect of different doses of the four epiprogoitrin degradation products (▼, **2**; ○, **3/1**; ●, **3/2**; ▽, **4**) on the root length of *L. sativa* normal seedlings reported as percent of control, after 7 days of germination on filter paper at 20 °C. Vertical bar indicates the standard error of three replicates.

Finally, although the myrosinase-catalyzed hydrolysis at pH 6.5 was very efficient and **1** was almost completely hydrolyzed to **4**, the final yield of the procedure was only ~70%. The low yield was mostly due to the crystallization process, which, however, made it possible to obtain this compound in pure form, as determined by HPLC, GC-MS, and ^{1}H NMR analyses.

Stability of the Four Epiprogoitrin-Derived Products. Among the epiprogoitrin-derived products, **4** is the most stable both in solution and in dry or crystallized form. All nitriles are less stable than **4**, and, among them, **2** is generally more stable than **3.1** and **3.2**, although in water solution both epithionitriles were stable for weeks, without producing any further degradation product. This observation made it possible to carry out the germination tests and the sure assessment of their biological activity.

Germination Tests. Figure 2 shows the effect of different doses (0.4−32.0 mM) of the four compounds derived from **1** on lettuce (*L. sativa* cv. Romana) seed germination. This effect was measured as the mean percentage of normal seedlings recorded after 7 days of incubation. Product concentrations are reported in a logarithmic scale and the germination data as a percentage of the control, which was 94.0% (±5.3). In Figure 2 it is possible to note that **4** had little effect up to the 16.0 mM dose, whereas at the 24.0 mM concentration it caused a sharp reduction in normal seedling percentage. Germination was completely inhibited with 32.0 mM **4**, whereas **2** showed a higher inhibitory activity: Its effect was already detectable at 0.8 mM and gradually increased up to 4.0 mM. At 8.0 mM **2** caused a marked inhibitory effect, which progressively increased, producing almost complete inhibition at a concentration of 20.0 mM. However, the most effective compounds in inhibiting lettuce seed germination were **3.1** and **3.2**. In particular, **3.1** already showed inhibitory activity at 2.4 mM, and a 3.2 mM dose was sufficient to completely inhibit germination. The negative effect of **3.2** began at 0.8 mM, and a decrease of ~40% in normal seedlings was detected at 1.2 mM. A concentration of 2.0 mM of **3.2** was sufficient to provide total inhibition, thus showing the highest activity among the tested epiprogoitrin-derived products.

Figure 3 shows the decrease in root growth, measured at the end of the experiment. Again in this case, concentrations were reported in a logarithmic scale and root length data as a percentage of the control, which measured 29.0 mm (±0.7). On the contrary of what was noted for germination, we observed that **4** already had a marked effect on root elongation at 4.0 mM, producing a reduction of >40% in root length. At 8.0 mM **4** caused a greater reduction, which gradually increased with higher doses. On the other hand, **2** had no effect on root elongation up to 16.0 mM, whereas **3.1** showed an increasing effect as a function of the dose applied, starting from the lowest concentration (0.8 mM), in contrast with what was observed for germination, which was not affected up to the 2.4 mM dose. The effect of **3.2** on root growth was similar to that on germination, gradually increasing with the doses but starting at 0.4 mM. In controlling root growth, **3.1** and **3.2** appeared to be the most effective compounds. Their activities looked very similar, even though **3.1** seemed to be less active than **3.2** in showing a slightly lower effect in root elongation at higher doses.

In addition, different kinds of abnormalities were recorded: **4** induced branched roots at low doses and absence of root hairs at the highest doses assayed (24.0 and 32.0 mM). In particular, it did not really prevent germination because at the end of the experiment all seeds succeeded in germinating, although at the maximum concentration tested all seedlings were classified as abnormal due to the presence of dark rotten tissues. On the other hand, **2**, **3.1**, and **3.2**—already at low doses—induced the necrosis of the root apex and/or absence of cotyledons. Increasing doses produced imbibed seeds with broken coats, caused by the beginning of radicle protrusion.

These findings clearly demonstrate the biological activity of all four purified epiprogoitrin-derived products and highlight their different activities and modes of action. In fact, **4** essentially inhibited root growth, whereas the effect of **2** occurred in the early germination phases, blocking radicle protrusion through the seed coats. Finally, **3.1** and **3.2** showed the most interesting activities, because they were effective at doses 5−10 times lower than those of **2** and appeared to inhibit both germination and root growth. The greater activity of

these molecules could be linked to their intrinsic molecular structure. The strained episulfide bridge in **3.1** and **3.2**, which develops a specifically strong chemical reactivity of those molecules, could be the determinant element in their biological activity. In addition, the differentiated effectiveness of **3.1** and **3.2** is not surprising, as it is quite common in nature that two stereoisomeric forms display different biological properties.

The high antigerminative activity shown by **3.1** and **3.2** suggests the usefulness of extending the study of their biological activity to other fields with the idea of exploiting them as intermediates for fine chemical purposes. Although in a previous study we demonstrated that **4** also moderately inhibits the germination of some weeds (*25*), the results of this study suggest that it could be interesting to investigate the possible existence of a synergism among the different epiprogoitrin-derived products and evaluate the effectiveness of different "cocktails" of epiprogoitrin-derived products as potential antigerminative formulations.

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