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Direct Antioxidant Activity of Purified Glucoerucin, the Dietary Secondary Metabolite Contained in Rocket (*Eruca sativa* Mill.) Seeds and Sprouts

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Rocket (*Eruca sativa* Mill. or *Eruca vesicaria* L.) is widely distributed all over the world and is usually consumed fresh (leafs or sprouts) for its typical spicy taste. Nevertheless, it is mentioned in traditional pharmacopoeia and ancient literature for several therapeutic properties, and it does contain a number of health promoting agents including carotenoids, vitamin C, fibers, flavonoids, and glucosinolates (GLs). The latter phytochemicals have recently gained attention as being the precursors of isothiocyanates (ITCs), which are released by myrosinase hydrolysis during cutting, chewing, or processing of the vegetable. ITCs are recognized as potent inducers of phase II enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, epoxide hydrolase, etc.), which are important in the detoxification of electrophiles and protection against oxidative stress. The major GL found in rocket seeds is glucoerucin, GER (108 ± 5 μmol g^{-1} d.w.) that represents 95% of total GLs. The content is largely conserved in sprouts (79% of total GLs), and GER is still present to some extent in adult leaves. Unlike other GLs (e.g., glucoraphanin, the bio-precursor of sulforaphane), GER possesses good direct as well as indirect antioxidant activity. GER (and its metabolite erucin, ERN) effectively decomposes hydrogen peroxide and alkyl hydroperoxides with second-order rate constants of $k_2 = 6.9 \pm 0.1 \times 10^{-2}$ M^{-1} s^{-1} and $4.5 \pm 0.2 \times 10^{-3}$ M^{-1} s^{-}, respectively, in water at 37 °C, thereby acting as a peroxide-scavenging preventive antioxidant. Interestingly, upon removal of H$_2$O$_2$ or hydroperoxides, ERN is converted into sulforaphane, the most effective inducer of phase II enzymes among ITCs. On the other hand, ERN (and conceivably GER), like other ITCs, does not possess any chain-breaking antioxidant activity, being unable to protect styrene from its thermally (37 °C) initiated autoxidation in the presence of AMVN. The mechanism and relevance of the antioxidant activity of GER and ERN are discussed.

KEYWORDS: Antioxidant; rocket; *Eruca sativa* (Mill.); *Eruca vesicaria* (L.); glucosinolate; isothiocyanate; glucoerucin; erucin

INTRODUCTION

Rocket comprises a number of species of the *Brassicaceae* (*Cruciferae*) family belonging to the *Eruca* (Miller) and *Diplotaxis* (DC.) genera. *Eruca sativa* (Mill.) or *Eruca vesicaria* (L.) has its origin in the Mediterranean region (*1*) but is widely distributed all over the world (*2*). It is mostly harvested from the wild or cultivated as an edible vegetable for the distinct spicy flavor of young leaves. Rocket seeds are also used for the production of oil and for appreciated pungent taste sprouts.

Mentions of its medical use can be found throughout ancient literature. Rocket was believed to have aphrodisiac properties; such a virtue is mentioned by Virgil, and in the first century AD, Lucius Columella affirms "*excitet ut Veneri tardos eruca maritos*", translated as "the rocket excites as the lovers embrace the lazy husbands" (De Re Rustica, X: 108). Both Dioscorides (*3*) and Galen (*4*) recommended eating seeds for increasing semen production; for these properties, rocket was forbidden in gardens of monasteries (*5*), despite the fact that both the Bible (Second Kings 4:39−40) and Pliny (Historia Naturalis) had
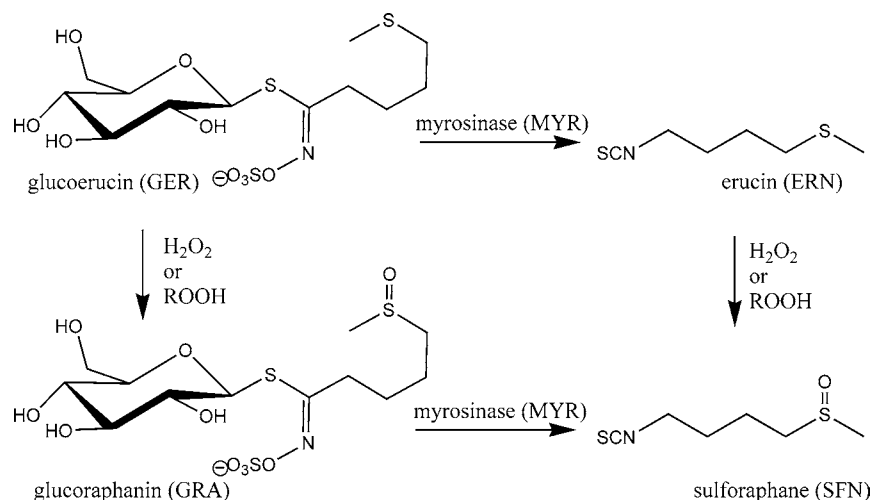
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Scheme 1

reported its benefits in eye treatment. Rocket has also been used in the traditional pharmacopoeia for various purposes: antiphlogistic, astringent, depurative, diuretic, digestive, emollient, tonic, stimulant, laxative, and rubefacient (*6*).

Like other cruciferous vegetables, rocket contains a range of health-promoting phytochemicals including carotenoids, vitamin C, fibers, flavonoids, and glucosinolates (GLs) (*7*). The latter phytochemicals have recently garnered great interest for their potential role in the maintenance of human health. In particular, many papers associate a highly significant cancer risk reduction with increasing *Brassicaceae* consumption (*8, 9*), and it has been speculated that the isothiocyanates (ITCs), obtained from myrosinase hydrolysis of GLs (by chewing, cutting, or processing the vegetable), are in great part responsible for the protective effects of Brassica vegetables (*10, 11*). The prevailing mechanism identified for the protective activity of ITCs is the induction of phase II enzymes [e.g., glutathione transferases (GSTs, EC 2.5.1.18), NAD(P)H:quinone reductase (QR, EC 1.6.99.2), epoxide hydrolase, and heme oxygenase], which are important in the detoxification of electrophiles (*12*). More than 120 different GLs have been identified, and their structures share a common core of a β-D-glucopyrano moiety linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester and a variable aglycon side chain derived from the α-amino acid biosynthetic precursor, which allows their classification in three groups: aliphatic, aromatic, and indole GLs (*13*). Several methionine-derived GLs, which constitute the largest group of GLs, bear in their side chain an additional sulfur atom at different oxidation states (sulfide, sulfoxide, or sulfone functions), which is retained in the ITC-derived compounds produced via hydrolysis by myrosinase (EC 3.2.3.1) (MYR). Since Zhang et al. (*10*) reported a comparative study of QR and GST inductions in mouse tissue by alkyl thiofunctionalized ITCs, differing in the oxidation state of sulfur, several hundred papers have focused on sulforaphane (SFN, 4-methylsulfinylbutyl ITC), which is considered the most active phase II enzyme inducer among analogues. SFN has also been attributed indirect antioxidant activity, which would arise from induction of GSTs, QR, and heme oxygenase, with increased levels of reduced glutathione (GSH), of reduced coenzyme Q (QH$_2$), and of bilirubin (*14*). Broccoli and particularly broccoli sprouts are the richest easily available sources of glucoraphanin (GRA), the GL bio-precursor of SFN (*11, 15, 16*), and this has significantly increased the interest for these vegetables over the last years. Despite the increasing interest in SFN, the availability of large amounts of pure compounds for clinical investigation constitutes a major limit. Indeed, the content of GRA in broccoli seeds or sprouts

represents 1−2% of dry weight, and it is associated with relevant amounts of other GLs (e.g., glucoiberin, glucoerucin, 4-hydroxy-glucobrassicin, progoitrin, etc.); this impairs its isolation and purification in large quantities. Our previous finding that glucoerucin (GER) could undergo a high yielding transformation into GRA by chemoselective oxidation using hydrogen peroxide (Scheme 1) (*17*) provided a possible solution to this problem and boosted our interest for GER and its ITC-derivative, erucin (ERN, 4-methylthiobutyl ITC).

Although ERN has received up to now poor attention due to the lesser phase II enzyme inducer activity as compared to SFN (*10*), our finding suggests that, unlike SFN and ITC in general (*14*), it might possess direct as well as indirect antioxidant activity.

With the aim to assess that consumption of vegetables containing the title GL could afford protection against the oxidative damage, we report here the determination of GER content in rocket seeds and sprouts, together with a detailed kinetic investigation on the reactivity of GER and ERN with hydrogen peroxide (H$_2$O$_2$), alkyl hydroperoxides (ROOH), and peroxyl radicals (ROO•).

MATERIALS AND METHODS

Materials. Solvents were of the highest grade commercially available and were used as received. 2,2′-Azobis(2,4-dimethylvaleronitrile), AMVN (Wako Pure Chemicals Ind. Ltd.), was stored at −20 °C. *RRR*-α-Tocopherol (Aldrich) was purified as previously described (*18*). 2,6-Di-*t*-butyl-4-methylphenol (Aldrich) was crystallized from hexane. Styrene (Aldrich, 99+%) was distilled under reduced pressure and percolated twice trough silica and once through activated basic alumina. The other chemicals were commercially available (Sigma-Fluka-Aldrich) and were used as received.

Seed and Sprout Samples. Rocket (*Eruca sativa* Mill.) ripe seeds and 5 day-old sprouts were supplied by Suba and Unico, Longiano, Forlì (Italy). GER content in the samples was assessed in triplicate by the EU official method (ISO 9167-1) (*19*), based on the HPLC analysis of desulfo-GLs obtained through the removal of the sulfate group of GLs via sulfatase-catalyzed hydrolysis. Seeds and freeze-dried sprouts were reduced to a fine powder, and about 400 mg aliquots were extracted by adding 5 mL of boiling 70% ethanol plus 200 μL of sinigrin (29.5 mM) as internal standard. The mixture was homogenized for 5 min at 75 °C using an U-Turrax (IKA T25) homogenizer, and the extracts were centrifuged. The residue was re-extracted with further 5 mL of boiling 70% ethanol and recentrifuged. Supernatants were combined to give a final volume of 10 mL. Each extract (1 mL) was loaded onto a minicolumn filled with 0.6 mL of DEAE-Sephadex A-25 anion-exchange resin (Amersham Biosciences, Milano) conditioned with 25 mM acetate buffer, pH 5.6. After being washed with 3 mL of buffer,

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150 μL of purified sulfatase (*20*) was loaded into the minicolumn and was left overnight at room temperature. The desulfo-GLs was then eluted with 3 mL of distilled water and finally injected into HPLC. Desulfo-GLs were analyzed using a Hewlett-Packard Model 1100 HPLC system with an Inertsil ODS3 column (250 × 3 mm, 5 μm). Chromatography was performed with 1 mL/min flow rate at 30 °C by eluting with a gradient of water (A) and acetonitrile (B) as follows: isocratically 1% B for 1 min, linear gradient to 22% B for 21 min, and linear gradient to 1% B for 3 min. Elution of desulfo-GLs was detected by a diode array, monitoring the absorbance at 229 nm. The amount of GER was determined using sinigrin as the internal standard and the relative response factor (*21*).

Extraction and Purification of GER. GER was purified from rocket (*Eruca sativa* Mill.) ripe seeds. Rocket seeds were first ground to a fine powder and defatted in hexane. The solvent was removed, and the defatted meal was then treated with boiling 70% ethanol to produce a quick deactivation of endogenous MYR and to extract the intact GL. The isolation of GER from the extract was carried out by one-step anion-exchange chromatography, as previously described (*22, 23*). The purity was further improved by gel-filtration removal of contaminants, which was performed using a XK 26/100 column packed with Sephadex G10 chromatography media (Amersham Biosciences), connected to an FPLC System (Pharmacia). The mobile phase was water at a flow rate of 2.0 mL min^{-1}, and the eluate absorbance was monitored at 254 nm. Fractions were assayed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method (*19*), and those containing the GL (>95%) were collected and freeze-dried. GER was characterized by ^{1}H and ^{13}C NMR spectrometry, and the absolute purity estimated by HPLC was close to 95%.

Preparation of ERN. ERN was produced via MYR catalyzed hydrolysis of GER, performed in 0.1 M phosphate buffer pH = 6.5 at 37 °C. MYR was isolated from *Sinapis alba* L. seeds (*24, 25*), and the enzymatic solution had a specific activity of 65 units/mg of soluble protein, where one MYR unit was defined as the amount of enzyme able to hydrolyze 1 μmol/min sinigrin at pH 6.5 and 37 °C.

The total conversion of 1.5 g of pure GER into ERN by 50 MYR units was confirmed by HPLC analysis of the desulfo-derivative (*19*), which allowed us to monitor the reduction until there was a complete disappearance of GER in the reaction mixture. The hydrolysis product was obtained after three consecutive extractions with dichloromethane. The combined extracts were concentrated to a few milliliters in a rotary evaporator at 45 °C, and finally the solvent was completely removed under a stream of nitrogen to afford 465 mg (82% yield) of pure ITC. The ERN structure was ascertained by GC–MS spectrometry using a Hewlett-Packard GCD G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5 (*26*).

Preparation of Reference Compounds GRA and SFN. GRA was produced from GER by chemoselective oxidation of the sulfide group into the corresponding sulfoxide, producing a mixture of *S*-epimers (*17*). GRA was then isolated from the reaction mixture by anion exchange chromatography and gel filtration as reported previously for GER and characterized by ^{1}H and ^{13}C NMR spectroscopy.

SFN was prepared in racemic form by MYR catalyzed hydrolysis of hemi-synthetic GRA. SFN was purified following the procedure previously used for ERN, and the structure of the compound was confirmed by GC-MS spectrometry using a Hewlett-Packard GCD G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5 (*26*).

Product Studies for Oxidation of GER and ERN. GER (1 mM) was incubated in water with a 4-fold excess of H_2O_2 or *t*-butylhydroperoxide at room temperature for 2–6 h, and the reaction mixture was analyzed by HPLC–MS using electrospray ionization (ESI) in a Waters 2695 separation module (equipped with autosampler) coupled to a Micromass ZMD ESI–MS spectrometer with the following instrumental settings: injection volume 20 μL; column, C18 (Waters X-Terra-MS, 3 mm × 150 mm, 3.5 μm); eluent 90:10 methanol/water; flow rate, 0.5 mL/min; splitting ratio, 10:1; ESI type, negative ions; desolvation gas (N_2), 600 L/h; cone flow (skimmer), 60 L/h; desolvation temperature, 350 °C; capillary voltage, 3.0 kV; cone voltage, 40 V; hexapole extractor, 3.0 V; and RF lens, 0.3 V. Calibration curves were obtained for each analyte using the same instrumental settings with authentic samples dissolved in water. Similarly, ERN (4-methylthiobutyl

ITC) was reacted with a 4-fold excess of H_2O_2 or *t*-butyl hydroperoxide in methanol. Analysis of the reaction mixture was performed by GC–MS using a Hewlett-Packard 5890 series II gas-chromatograph coupled with a HP5971 mass selective detector. The most appropriate instrumental settings were found to be the following: injection split ratio, 50:1; injection volume, 1.0 μL; column, J&W DB-5MS (30 m × 0.25 mm × 0.25 μm film thickness); carrier gas (H_2) flow, 1.0 mL/min; and temperature programming, 40 °C (hold 5 min) to 250 °C (hold 5 min) at 5 °C/min.

Kinetic Measurements with Hydrogen Peroxide and Alkyl Hydroperoxides. GER ($1-5 \times 10^{-4}$ M) was incubated with hydrogen peroxide ($1-5 \times 10^{-2}$ M) or *t*-butyl hydroperoxide (1×10^{-2} to 0.2 M) in water at 298 or 323 K using a thermostated water bath equipped with a sealed magnetic microstirrer. Aliquots of the reaction mixture were sampled at time intervals and analyzed by ESI–MS in a Micromass ZMD ESI–MS spectrometer, with direct injection in the electrospray source through a syringe pump (Harvard Apparatus Pump II) at a flow rate of 15 μL/min. The most appropriate instrumental settings were determined in a preliminary set of experiments: ESI type, negative ions; desolvation gas (N_2), 60 L/h; cone flow (skimmer), 6 L/h; desolvation temperature, 150 °C; capillary voltage, 3.0 kV; cone voltage, 40 V; hexapole extractor, 3.0 V; and RF lens, 0.3 V. Calibration curves were obtained both for GER and for GRA using authentic samples, under identical instrumental settings. The peak area of signals at *m/z* 420 and 436 were used, respectively, for the two glucosinolates giving $A = 5855 + 743722C$ ($r^2 = 0.9986$) for *m/z* 420 and $A = -56 + 471691C$ ($r^2 = 0.9991$) for *m/z* 436. Given the higher response factor of the instrument to *m/z* 420, the decay of GER was used to measure the pseudo-first-order kinetic constants. Measurements were repeated at least with three different starting concentrations of peroxide, and for each concentration, at each temperature experiments were done in triplicate.

Autoxidation Experiments. Autoxidation experiments were performed as previously described (*27, 28, 29*) in a two-channels oxygen uptake apparatus, based on a Validyne DP 15 differential pressure transducer that has already been described elsewhere (*30*). The entire apparatus was immersed in a thermostated bath that ensured a constant temperature within ±0.1 °C. In a typical experiment, an air-saturated chlorobenzene solution containing the antioxidant (2.5×10^{-5} to 5.0×10^{-4} M) was equilibrated with the reference solution containing only an excess of α-tocopherol (1×10^{-3} to 1×10^{-2} M) in the same solvent at 30 °C. After equilibration, a concentrated chlorobenzene solution of AMVN (final concentration 5×10^{-2} to 5×10^{-3}M) was injected in both the reference and the sample flasks, and the oxygen consumption in the sample was measured, after calibration of the apparatus, from the differential pressure recorded with time between the two channels. Initiation rates, R_i, were determined for each condition in preliminary experiments by the inhibitor method using α-tocopherol as reference antioxidant: $R_i = 2[\alpha\text{-tocopherol}]/\tau$. Blank experiments were performed by following the autoxidation of styrene in the absence of any antioxidant, while reference experiments were performed using α-tocopherol (1.0×10^{-5} M) or 2,6-di-*t*-butyl-4-methylphenol (BHT, 5.0×10^{-5} M) as standard antioxidants.

RESULTS

GER was purified on the gram scale to a high purity degree with good yields, starting from seeds that contained a strongly predominant individual GL, using a simple procedure (*22, 23*). The starting material used contained 108 ± 5 μmol g^{-1} d.w. GER, representing about 95% of the total GLs. Although the GER content was inferior in rocket freeze-dried sprouts that also contained its oxidized form GRA, it still retained 79% of the total GLs content, as shown in Figure 1.

Identification of purified GER was assessed by NMR spectroscopy, and the proton chemical shift assignments were in agreement with previously reported data (*31*).

Incubation of GER dissolved in water with a 4-fold excess of H_2O_2 at room temperature for 6 h resulted in complete disappearance of the starting material as judged by the HPLC–MS analysis of the reaction mixture. The compound was
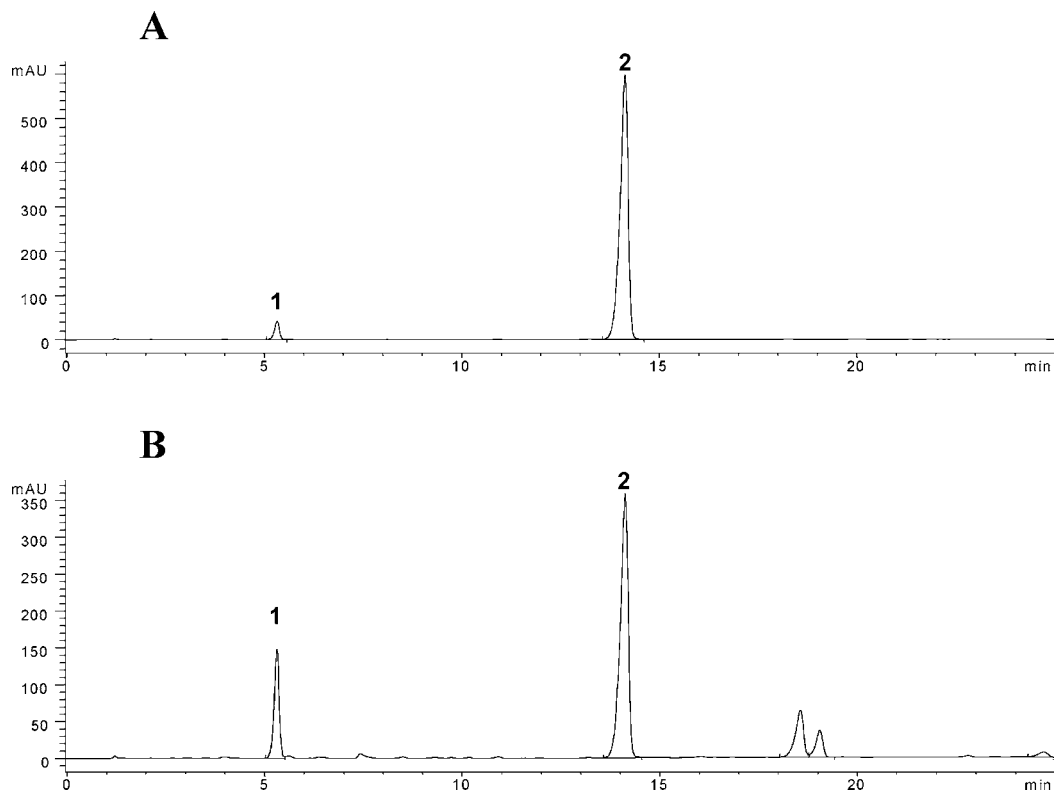
Figure 1. HPLC chromatograms (according to ISO 9167-1) of GLs present in rocket (*Eruca sativa* Mill.) seeds (**A**) and freeze-dried sprouts (**B**): 1, glucoraphanin and 2, glucoerucin.
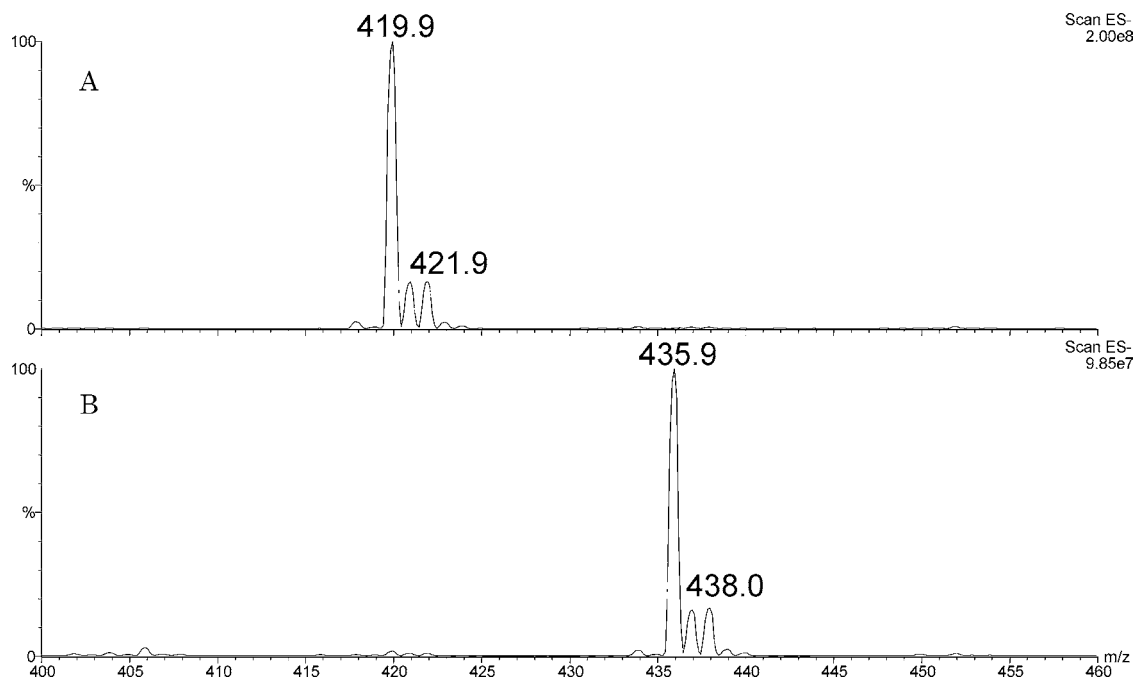

Figure 2. ESI–MS spectra of the reaction mixture of 1 mM GER and 4 mM hydrogen peroxide in water (**A**) immediately after addition and (**B**) after 6 h incubation at room temperature.

completely (97% by HPLC−MS) converted into a product giving, in ESI−MS spectrometry, a pseudo-molecular ion peak ([M − H]⁻) at *m/z* 436 in negative ionization mode (i.e., at an *m/z* value higher by 16 amu than that recorded for the starting GL (*m/z* 420, see Figure 2)). The reaction product was identified as GRA by comparison with a reference sample, previously isolated and characterized by ¹H NMR, in agreement with previous reports (*17*). Similarly, the reaction of the corresponding ITC, ERN, in methanol with an excess of H₂O₂ yielded

SFN (see Scheme 1) with 94% conversion, measured by GC−MS analysis of the reaction mixture. Clearly, with both starting materials (the GL or the ITC), regioselective oxidation of the methylthio moiety occurs to yield quantitatively the corresponding methyl sulfoxide. In contrast, the subsequent oxidation step (i.e., conversion of the methyl sulfoxide into methyl sulfone) was not observed under the experimental conditions employed.

Kinetic Studies with Peroxides. The reaction of GER with hydrogen peroxide and *t*-butyl hydroperoxide in water was
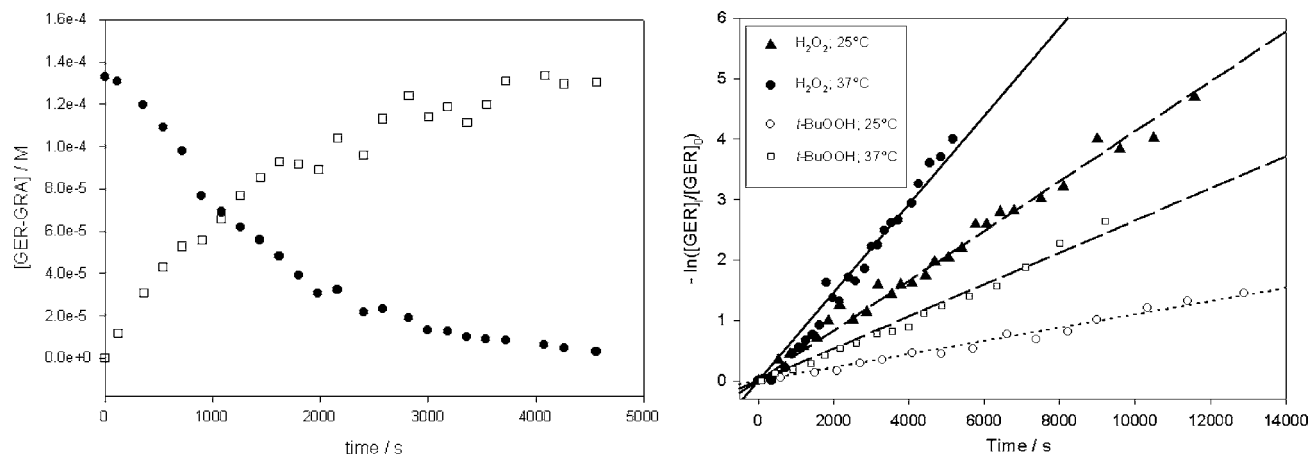
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Figure 3. (Left) decay of the signal at m/z 420 (GER, ●) and growth of the signal at m/z 436 (GRA, □) during ESI–MS analysis of the reaction mixture of glucoerucin 1.33×10^{-4} M with H_2O_2 (1.17×10^{-2} M) in water at 37 °C. (Right) first-order plots of typical time-evolution traces of the concentration of GER (1.5×10^{-4} M) in water incubated with 1.2×10^{-2} M H_2O_2 at 37 °C (●) and 25 °C (▲) or with 0.06 M t-butyl hydroperoxide at 37 ° (□) and 25 °C (○).

Table 1. Second-Order Rate Constants (k_2) for the Reaction of GER with t-Butyl Hydroperoxide and Hydrogen Peroxide in Water[a]

peroxide	k_2 (M^{-1} s^{-1})	
	298 K	310 K
t-BuOOH	$(2.0 \pm 0.3) \times 10^{-3}$	$(4.5 \pm 0.2) \times 10^{-3}$
H_2O_2	$(3.3 \pm 0.2) \times 10^{-2}$	$(6.9 \pm 0.1) \times 10^{-2}$

[a] Errors represent standard deviation.

investigated at 25 and 37 °C, under pseudo-first-order conditions by the potassium salt of the glucosinolate ($1-5 \times 10^{-4}$ M) being incubated with a 100-fold excess of the peroxide. For convenience, the reaction was followed at time intervals by direct injection of the reaction mixture in the ISI−MS spectrometer through a syringe pump. The reaction course was monitored from the disappearance of signal at m/z 420 ([M − H]$^-$ ion of GER) and appearance of the signal at m/z 436 ([M − H]$^-$ of GRA) in negative ionization mode. Preliminary studies allowed adjustment of the optimal instrumental setting for direct analysis of the reaction mixture under conditions in which the two pseudo-molecular ions were the only signals due to the glucosinolates. Calibration of the spectrometer response with authentic samples was performed using either the MS signal intensity or the integrated signal peak, which afforded almost identical results. As can be seen from Figure 3, the decay of GER and growth of GRA, monitored through the time evolutions of signals at m/z 420 and 436, respectively, afforded nice first-order complementary kinetic traces (i.e., the rate of disappearance of the signal due to GER was almost identical to the rate of formation of GRA). Since decay traces usually showed lower scattering of the mass signal, for convenience they were chosen to calculate the second-order rate constants. Measurements were always performed under pseudo-first-order conditions with various amounts of starting GL and peroxide. Results are collected in Table 1.

Kinetic Studies with Peroxyl Radicals. These studies were performed by the thermally initiated autoxidation of styrene being investigated either in the absence or in the presence of the antioxidant. The reaction was carried out in a closed system under controlled conditions at 30 °C in air-saturated solutions of a standard oxidizable substrate (styrene) in chlorobenzene. The autoxidations were initiated with 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and were followed by the oxygen consumption being monitored with an automatic recording gas

absorption apparatus, using a differential pressure transducer ($27-30$). Control experiments were performed using α-tocopherol or BHT as standard antioxidants.
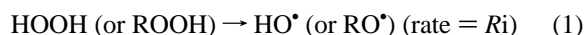
Autoxidations in the presence of α-tocopherol (1.0×10^{-5} M) showed a neat inhibition period during which the oxygen consumption was almost completely suppressed, followed by an uninhibited autoxidation kinetic when the antioxidant had been completely consumed. On the other hand, BHT (5.0×10^{-5} M) only decreased the rate of oxygen consumption, thereby acting as retardant rather than an inhibitor of the autoxidation. These findings were in excellent agreement with the known behavior of such reference antioxidants (28, 32).

With these experimental settings, we tested the antioxidant activity of ERN (4-methylthiobutyl ITC), chosen for convenience due to the higher solubility in organic solvents as compared to its precursor GER. In none of the autoxidation experiments performed in the presence of ERN in the concentration range 1.0×10^{-5} to 5.0×10^{-4} M was inhibition or retarding of the initiated autoxidation observed. Clearly, ERN, and conceivably GER, do not possess any chain-breaking antioxidant activity under the experimental conditions employed.

DISCUSSION

Antioxidants comprise a broad and heterogeneous family of compounds that share the common task of interfering with (stop, retard, or prevent) the oxidation (or autoxidation) of an oxidizable substrate. In a biological environment, such as a living organism, the most relevant autoxidation process is lipid peroxidation, a well-known and thoroughly investigated process, which involves a free radical chain reaction (33).
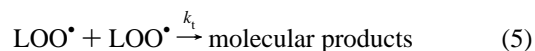
Initiation

$$\text{HOOH (or ROOH)} \rightarrow \text{HO}^{\bullet} \text{ (or RO}^{\bullet}\text{) (rate} = R\text{i)} \quad (1)$$

$$\text{HO}^{\bullet} \text{ (or RO}^{\bullet}\text{)} + \text{LH} \rightarrow \text{HOH (or ROH)} + \text{L}^{\bullet} \quad (2)$$

Propagation

$$\text{L}^{\bullet} + \text{O}_2 \xrightarrow{\text{diff.}} \text{LOO}^{\bullet} \quad (3)$$

$$\text{LOO}^{\bullet} + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}^{\bullet} \quad (4)$$

Termination

$$\text{LOO}^{\bullet} + \text{LOO}^{\bullet} \xrightarrow{k_t} \text{molecular products} \quad (5)$$

In this simple scheme, LH represents a lipid molecule, and L$^\bullet$ is the corresponding carbon-centered radical generated by abstraction of a hydrogen atom. The first step for lipid peroxidation is the production of a reactive radical species (e.g., by the decomposition of hydrogen peroxide or a hydroperoxide in alkoxyl or hydroxyl radicals). Immediately upon formation, in the subsequent propagation step, the lipid radical reacts at a diffusion-controlled rate with molecular oxygen to form a peroxyl radical, which is the actual chain-carrying species. Antioxidants can be classified on the basis of their mechanism of interference with the peroxidation process as preventive antioxidants or chain-breaking antioxidants: the former (e.g., catalase, metal chelators, UV filters) mainly interferes with the initiation process (i.e., retard or stop the initial formation of radical species), while the latter (e.g., α-tocopherol, ascorbate, flavonoids) actually blocks or slows the autoxidation by competing with the propagation reactions (i.e., they react with peroxyl radicals faster than the oxidizable substrate (usually polyunsaturated fatty acids in bio-membranes) to form species that do not propagate the oxidation chain (*34, 35, 36*)). In addition to these direct antioxidants, other compounds that do not possess relevant antioxidant activity in vitro, but can stimulate and increase the efficacy of the physiological antioxidant defenses in vivo, are usually classified as indirect antioxidants.

Isothiocyanates such as SFN and ERN are known to possess indirect antioxidant activity through induction of GSTs, QR, and heme oxygenase (*14*); however, while this mechanism is the only to be expected for SFN or its precursor, the present investigation clearly shows that GER possesses good direct, albeit preventive, antioxidant activity. This is due to its ability to decompose hydroperoxides and hydrogen peroxide, which are most often responsible for the initiation step, as well as being signaling mediators that lead to activation of redox sensitive transcription factors and responsive genes (*37*). Results from **Table 1** can be compared with the reactivity of hydrogen peroxide with glutathione, phenylaminoethyl sulfide, and fenylaminoethyl selenide (two recently developed synthetic antioxidants), whose rate constants at 37 °C have been reported as 1.01, 4.2×10^{-4}, and 4.7×10^{-2} M^{-1} s^{-1}, respectively (*38*).

As the active moiety of GER in this preventive antioxidant activity is the methylthio group, which undergoes oxidation into methyl sulfoxide, similar antioxidant activity is expected to be possessed by its biologically active metabolite ERN. Interestingly, the byproduct of this hydroperoxide-scavenging process is GRA, (i.e., the bioprecursor of SFN (see **Scheme 1**), the most effective chemopreventive ITC of the family (*10*)).

On the other hand, it is worth noting that ERN and conceivably any other ITC of the family (as well as their GL precursors) do not possess any chain-breaking antioxidant activity. This finding is not entirely unexpected when considering the structure of these compounds. Isothiocyanates have been shown to react with silyl or stannyl radicals (*39*) and with alkyl radicals (*40*) to yield a very reactive carbon centered radical. To be useful as chain-breaking antioxidants, this reaction should, however, compete with the propagation process and yield a new radical that is unable to propagate the chain: this is obviously not the case. Furthermore, it should be noted that, in oxygen rich environments, peroxyl radicals, rather than alkyl radicals, are the chain-propagating species (vide supra), and no reaction of peroxyl radicals with isothiocyanates has been so far reported. The other moiety in the ERN structure that would be potentially reactive toward peroxyl radicals—CH$_2$ hydrogens alpha to the sulfide—is expected to react too slowly (e.g., the rate constants for the reaction of benzylphenyl thioether or tetrahydrothiophene with *t*-butylperoxyl radicals are 0.16 and 0.1 M^{-1} s^{-1}, respec-

tively (*41*)), furthermore yielding an alkyl radical able to propagate the autoxidation.

Oxidative stress is deeply involved in cancer development, and oxygen-centered radicals play a role in apoptosis. It is well-known that higher amounts of the reactive oxygen species, but particularly hydrogen peroxide, are produced in some cancer cells (*42*). Hydrogen peroxide is widely recognized as being extremely important in signal transduction by acting as a signaling molecule in the mitogen-activated protein kinase pathway, which results in the activation of redox-sensitive transcription factors and a responsive gene that promotes cell survival, growth, and proliferation (*37*). However, while the constitutive high production of hydrogen peroxide in cancer cells is needed to promote their proliferation, additional amounts of hydrogen peroxide above a certain threshold cause cell cycle arrest and/or apoptosis (*37*). An extensive body of evidence demonstrates that high concentrations of ITC inhibit cancer cell proliferation by inducing the formation of intolerable amounts of reactive oxygen species, thus leading to caspase-mediated apoptosis, whereas at lower concentrations, these compounds activate protein kinases mediating the expression of defensive and survival genes, such as GSTs and QR (*43*). A recent investigation has shown that ERN induces apoptosis selectively in human leukemia cells, but not in nontransformed T lymphocytes, thereby being a promising new agent in cancer therapy (*44*).

In light of the previous information, it is possible, although speculative, that the ability of ERN to induce apoptosis selectively in cancer cells, unlike SFN (*45*), may be at least partially related to its unusual capability to act as a hydroperoxide-scavenging preventive antioxidant. Further investigation is in progress to disclose possible relationships between the fine antioxidant/prooxidant balance of ERN and its selective proapoptotic behavior.

Our current investigation boosts the interest on the role in the human diet of rocket, GER, and ERN. In a recent study, Gill et al. (*46*) found that the extract of cruciferous and legume sprouts not only exerted significant in vitro antigenotoxic activity against H$_2$O$_2$ damage in HT29 human colon cells but also protected against H$_2$O$_2$ insult of peripheral blood lymphocytes of volunteers, after they consumed about 100 g of sprouts for 2 weeks. As a matter of fact, specific dietary approaches based on broccoli sprouts have been recently investigated and proved effective in reducing oxidative stress and cardiovascular problems (*47*). Similar or even higher benefits to human health are to be expected from the consumption of rocket or rocket sprouts. Indeed, one additional argument in favor of rocket is that this vegetable is more often consumed fresh rather than boiled like other vegetables such as broccoli. This leaves MYR activity at its best and significantly increases the content of ITCs, which have been found six times more bioavailable than GLs (*48*).

CONCLUSION

It has recently been reported that leaves of *E. sativa* contain 4-mercaptobutyl GL as the major GL among nine, while GER is present only in low amounts (*49*). In the present study, we have shown that rocket seeds and sprouts contain GER as main GL, in large amounts in comparison to leaves (mature plants), thereby paralleling previous findings on other *Brassicaeae* GLs content (*11*). When eaten raw, rocket sprouts that are actually available on the market release large amounts of ERN through endogenous MYR activity.

GER and ERN possess good direct antioxidant activity as hydroperoxide-scavenging preventive antioxidants. Upon reaction with hydroperoxides, they produce GRA and SFN, respec-

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tively. Therefore, ERN is potentially capable of protection from oxidative stress by three mechanisms: (i) through induction of phase II enzymes, (ii) by scavenging hydrogen peroxide and alkyl hydroperoxides accumulated in cells and peripheral blood, and (iii) by acting as a precursor of SFN, a potent inducer of detoxifying enzymes. The lack of activity as a chain-breaking antioxidant suggests its use in combination with other health-promoting nutrients such as α-tocopherol or ascorbate. This maximization of the protection toward oxidative stress can be easily obtained through ordinary consumption of fresh uncooked rocket, an ancient underutilized vegetable crop (*50*).

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