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# Dihydroasparagusic Acid: Antioxidant and Tyrosinase Inhibitory Activities and Improved Synthesis

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Supporting Information

**ABSTRACT:** Dihydroasparagusic acid (DHAA) is the reduced form of asparagusic acid, a sulfur-containing flavor component produced by *Asparagus* plants. In this work, DHAA was synthetically produced by modifying some published protocols, and the synthesized molecule was tested in several in vitro assays (DPPH, ABTS, FRAP-ferrozine, BCB, deoxyribose assays) to evaluate its radical scavenging activity. Results show that DHAA is endowed with a significant in vitro antioxidant activity, comparable to that of Trolox. DHAA was also evaluated for its inhibitory activity toward tyrosinase, an enzyme involved, among others, in melanogenesis and in browning processes of plant-derived foods. DHAA was shown to exert an inhibitory effect on tyrosinase activity, and the inhibitor kinetics, analyzed by a Lineweaver−Burk plot, exhibited a competitive mechanism. Taken together, these results suggest that DHAA may be considered as a potentially active molecule for use in various fields of application, such as pharmaceutical, cosmetics, agronomic and food.

**KEYWORDS:** *Asparagus sp., dihydroasparagusic acid, dimercaptanics, in vitro antioxidant activity, polyphenol oxidase, sulfur-containing compounds, tyrosinase inhibition, Lineweaver−Burk (L-B) plot*

## INTRODUCTION

Plants of the genus *Asparagus*, belonging to the Liliaceae, are widely distributed and cultivated in the Mediterranean area. Among them, the species *Asparagus officinalis* is an important ingredient of the Italian and Mediterranean diet and is largely used in traditional medicine for its diuretic and depurative effects. The main secondary metabolites produced include saponins,[1] saccharides,[2] and acetylenic and sulfur-containing compounds.[3,4] Studies on *A. officinalis* extracts have highlighted how they may have a wide range of biological activities including antioxidant,[5] antidiabetic,[6] antitumor,[7] antifungal,[8] diuretic,[9] and immunostimulant.[10]

Dihydroasparagusic acid (DHAA) is the reduced form of asparagusic acid, which is produced in several species of *Asparagus*,[11,12] and has been the first dithiolane isolated from a natural source. The peculiarity of these compounds is due to the fact that they are synthesized in intact plant cells, thus representing an exceptional case of formation of sulfur-containing flavor components. Indeed, normally, sulfur compounds in vegetables are formed by enzymatic or chemical splitting of nonvolatile precursors, like *S*-alkylcysteine sulfoxides and glucosinolates during crushing of the plant material. The highest amount of asparagusic acid is present in the region around the apexes of the asparagus shoots, the edible part of the plant,[13,14] whereas the reduced form is present only in trace amounts, due to the fast conversion into other metabolites.[12] Among the naturally occurring 1,2-dithiolanes, the most widely distributed is $\alpha$-lipoic acid (LA), an essential coenzyme in many organisms.[15]

Organic sulfur derivatives with sulfhydryl functional groups (−SH), such as reduced glutathione (GSH) and dihydrolipoic acid (DHLA), are well-known for their importance as antioxidants, and reduced levels of thiol compounds are known to be associated with various disorders such as liver failure, coronary artery disease, stroke, and neurological disorders.[16,17] Although there are several reports of a wide array of biological activities and therapeutic properties of LA and DHLA,[18−21] the potential of DHAA has been poorly explored. Since its discovery, only a few studies have been carried out on the biological activities of this substance and its oxidized form (asparagusic acid). In particular, they were demonstrated to act as growth inhibitors for various plants and nematodes[22,23] while stimulating growth and pyruvate oxidation in *Streptococcus faecalis* and asparagus mitochondria;[24,25] DHAA was also studied as a chelating ligand for a series of iron and nickel complexes.[26]

On the basis of the antioxidant activities displayed by LA and its reduced form DHLA,[27] the latter structurally related to DHAA, and due to the lack of information about this compound, our attention was focused first on the determination of the in vitro antioxidant activity of this dimercaptanic acid through different methods. In particular, the ABTS and DPPH tests were used to determine its radical scavenging capacity, and the FRAP−ferrozine (FRAP-FZ) test was used to

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evaluate the reducing power. Moreover, to gain more insight into the antioxidant capacity of DHAA, two other antioxidant tests involving the use of biological radicals were carried out: the deoxyribose assay and the β-carotene bleaching (BCB) test.

Several sulfhydryl compounds, such as reduced glutathione, L-cysteine, *N*-acetyl-L-cysteine, and LA, were reported to inhibit the activity of tyrosinase.[28,29] This copper-containing enzyme (EC 1.14.18.1), also known as polyphenol oxidase (PPO),[30] is widely distributed in nature and catalyzes two distinct reactions in melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone.[31] This enzyme is responsible not only for the melanization process in animals, but also for browning of plant-derived foods and mushrooms during postharvest handling. In most fruits, vegetables, and beverages, in fact, the browning process is due to an enzymatic component, in which tyrosinase plays a key role by catalyzing the oxidation of phenolic compounds to the corresponding quinones, and by a nonenzymatic one, which can be prevented by antioxidants. An important role in the developmental and defensive functions of insects has also been reported for tyrosinase, because it is involved in the insect molting process and adhesion of marine organisms.[32,33] Thus, natural tyrosinase inhibitors represent an alternative approach for medicinal and cosmetic products, in agriculture, in the food industry, and in controlling insect pests.[34,35] On the basis of this rationale, the effect of synthetic DHAA was also tested on in vitro tyrosinase activity, and inhibition kinetics was also analyzed to understand the inhibition mechanism.

The aim of the present work was to investigate the antioxidant and in vitro tyrosinase inhibitor activities of synthetically prepared DHAA. An improved protocol for the chemical synthesis of this molecule is described. This will facilitate the production of high amounts of the molecule in view of its potential uses in various fields of application. The mechanism of inhibition of tyrosinase by DHAA is discussed.

## MATERIALS AND METHODS

**General Experimental Procedures.** Solvents, reagents, and substances were purchased from Sigma-Aldrich (St. Louis, MO, USA), Acros Organics (Geel, Belgium), ABCR (Karlsruhe, Germany), and Carlo Erba Reagenti (Milano, Italy) and were used without further purification.

Thin layer chromatography (TLC) was performed using silica gel SiF254 (Merck, Darmstadt, Germany). Plates were developed with chloroform/methanol at various percentages and sprayed with phosphomolybdic reagent, followed by heating at 120 °C.

NMR spectra were registered on a Varian Mercury 300 MHz and a Bruker Avance 400 MHz using $CDCl_3$ as deuterated solvents and TMS as internal standard.

MS spectra were performed on a Q-TOFMICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, in the negative ion mode. The rate of sample infusion was 10 μL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Biological assays were performed using a microplate reader, Victor X3 Perkin-Elmer (Perkin-Elmer Inc., Boston, MA, USA) and data analyzed with the software Work Out 2.5 or in a Jasco V-530 spectrophotometer (Jasco Europe, Cremella, Italy).

**Synthetic Procedure.** *β-β′-Diiodoisobutyric Acid (II)/Iodomethylacrylic Acid (II′).* Diethyl bis(dihydroxymethilmalonate) (**I**; 8.80 g ($4 \times 10^{-2}$ mol) was refluxed at 115−120 °C with 37.0 mL of HI 57%. During the first 45−60 min, the reaction was carried out with a continuous air flux to eliminate the volatile materials, which interfere with the desired reaction, and then the mixture was refluxed for 6 h. The reaction mixture crystallized spontaneously at room temperature and then was kept at 4 °C overnight to improve the crystallization process. The precipitate was collected by filtration on paper (Whatman Blue) and the filtrate refluxed again at 115−120 °C. This procedure was repeated three times, recovering 5.50 g of mixture from the overall process. The mean composition of the mixture of iodoacrylic and diiodoisobutanoic acids was 3:1, respectively (mean molar yield ∼ 57%). $^1$H NMR, $CDCl_3$, 300 MHz: (**II**) δ 3.47 (4 H, m, $-CH_2I$), δ 2.85 (1H, m, CH); (**II′**) δ 4.08 (2H, s, $-CH_2I$), δ 6.04 (1H, s, $=CH_2$a), δ 6.37 (1H, s, $=CH_2$b). $^{13}$C NMR $CDCl_3$, 75 MHz: (**II**) δ 174.9 (−COOH), δ 48.4 (CH), δ 4.2 ($-CH_2I$); (**II′**) δ 173.8 (−COOH), δ 130.0 ($=CH_2$), δ 128.5 (=C=), δ 5.9 ($-CH_2I$).

*β-β′-Ditioacetylisobutyric acid (III).* Four grams of the mixture **II**/**II′** was completely solubilized under magnetic stirring using an 0.8 M KOH solution, added dropwise. This reaction was carried out in anhydrous conditions under $N_2$ flux. A $CH_3COSK/CH_3COSH$ solution was freshly prepared and added dropwise, maintaining the temperature at 55 °C. The free thioacetic acid was formed in situ from thioacetate by adding a stoichiometric quantity of 2 N $H_2SO_4$. This quantity was calculated on the iodomethylacrylic acid amount present in the mixture. In 4.0 g of mixture (1:3) there were 1.393 g of diiodo derivative ($4.1 \times 10^{-3}$ mol) and 2.607 g of iodoacrylic derivative ($1.23 \times 10^{-2}$ mol). The quantity of $CH_3COSK$ used was 5.94 g ($5.4 \times 10^{-2}$ mol), solubilized in 15 mL of demineralized water, and to this solution was added 12 mL of 2N $H_2SO_4$ to re-form $2.4 \times 10^{-2}$ mol of free thioacetic acid. The reaction was monitored over 20 h, and as long as the acrylic compound was present, it was still possible to readd thioacetic acid. When the reaction was complete, the reaction mixture was cooled to room temperature and acidified with 2 M $H_2SO_4$. *β-β′*-Dithioacetylisobutyric acid, separated as an oil, was extracted with chloroform (45 mL, three times). The organic solution was dried on anhydrous sodium sulfate and filtered. Chloroform was eliminated under reduced pressure to obtain 3.75 g ($1.59 \times 10^{-2}$ mol) of pale yellow oil (mean molar yield ∼ 97%). $^1$H NMR, $CDCl_3$, 300 MHz: (**III**): δ 3.25−3.12 (4H, m, $-CH_2S-$), δ 2.95−2.86 (1H, m, −CH=), δ 2.35 (6H, s, $CH_3COS-$). $^{13}$C NMR $CDCl_3$, 75 MHz: (**III**): δ 194.7 ($CH_3COS-$), δ 177.2 (−COOH), δ 44.8 ($-CH_2S-$), δ 30.1 (CH), δ 29.0 ($-SCOCH_3$).

*DHAA.* Three grams ($1.27 \times 10^{-2}$ mol) of **III** was salified with 16 mL of 0.8 M KOH solution ($1.27 \times 10^{-2}$ mol). After complete dissolution of the oil, 56.5 mL of 0.8 M KOH solution was added under continuous stirring and under nitrogen atmosphere at 45 °C. About 7 h was required for the reaction to be complete, then, after cooling at room temperature, the reaction mixture was acidified with 2 N $H_2SO_4$, obtaining an oily suspension. This was extracted with chloroform (60 mL, three times), and the organic layer was collected, dried on sodium sulfate, and filtered. The organic solvent was removed under reduced pressure, giving an oil. This was maintained under vacuum overnight, to eliminate the remaining acetic acid. After this step, DHAA crystallized spontaneously when seeded with some crystals of DHAA from a previous synthesis, recovering 1.82 g of pure compound ($1.19 \times 10^{-2}$ mol, yield ∼ 94%). $^1$H NMR, $CDCl_3$, 300 MHz: (DHAA) δ 3.00−2.80 (5H, m, overlapped signals), δ 1.52 (2H, t, *J* 8.5 Hz, $-CH_2-SH$). $^{13}$C NMR $CDCl_3$, 75 MHz: (DHAA) δ 178.3 (−COOH), δ 50.8 (CH), δ 24.0 ($-CH_2SH$).

**DPPH and ABTS Assays.** The DPPH assay was performed according to the method of Brand-Williams et al.[36] with some modifications. Stock solutions of DHAA were prepared in water to obtain different final concentrations (from 5 to 40 μM in the assay) to calculate the $IC_{50}$ value. One and a half milliliters of a 0.05 mM DPPH methanol solution was added to different concentrations of DHAA and allowed to react at room temperature. The assay was performed in a final volume of 2 mL. After 20 min, the absorbance (Abs) values were measured at 517 nm and converted into percentage antioxidant activity using the following formula:

$$\text{scavenging capacity\%} = [1 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100]$$

The DPPH solution plus methanol was used as a negative control, whereas TR, AA, and BHA at different concentrations (from 5 to 40 μM) were used as reference antioxidant compounds. The $IC_{50}$ values

were calculated by logarithmic regression of plots, where the *x*-axis represents DHAA or reference compounds concentrations and the *y*-axis the average percentage of scavenging capacity from three independent experiments with duplicate samples.

The ABTS assay was performed according to the method of Arnao et al.[37] with some modifications. $ABTS^{\bullet+}$ radical was generated by mixing a 2 mM ABTS solution with 7 mM $K_2S_2O_8$ and incubating in the dark for 24 h at room temperature. Before usage, the $ABTS^{\bullet+}$ solution was diluted (1−25 mL methanol) to obtain an Abs value of 0.7 at 734 nm. Upon addition of 1 mL of the diluted $ABTS^{\bullet+}$ solution to 10 μL of reference compounds or DHAA stock solutions (from 5 to 40 μM), the Abs at 734 nm was recorded after 1 min. The final TEAC value of DHAA was calculated by comparing $ABTS^{\bullet+}$ decolorization with that of Trolox. The $IC_{50}$ value was calculated as described above.

**FRAP−Ferrozine Assay.** The colorimetric method used to determine the capacity of DHAA to reduce ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$) was obtained according to the method of Berker et al.[38] with some modifications.

A calibration curve was created for the $Fe^{2+}$/ferrozine (FZ) complex using increasing concentrations of $FeCl_2$ (between 10 and 100 μM) and 0.5 mM FZ (in distilled water) in a final volume of 1 mL. A blank was prepared with FZ only. An amount of 0.2 mL of a previously prepared mixture containing $FeCl_3$ (1 mM) and FZ (5 mM) was added to 0.1 mL of reference compound at different concentrations (from 0.05 to 20 μM) or DHAA at different concentrations (from 15 to 65 μM), and the volume was brought to 1 mL with distilled water. The Abs was read using the microplate reader at 570 nm after 5 min of incubation at room temperature. The Abs of the $FeCl_3$/FZ mixture was subtracted from that obtained with reference compounds or DHAA. To build a dose−response curve, the amount of $Fe^{2+}$ produced by different compound concentrations was calculated from the calibration curve. Results are expressed in micromolar FRAP, according to the original method, which represents the $Fe^{2+}$ micromolar produced by 0.1 mM of the substance under investigation. On the basis of the dose−response curve of TR, TEAC values were calculated. The assay was performed in duplicate and repeated three times.

***β*-Carotene Bleaching Test.** Prevention of the autoxidation of emulsified linoleic acid was determined by modifying the method of Mikami et al.[39] Briefly, 20 μL of different DHAA or reference compounds stock solutions (50, 100, 150, and 200 μM) was added to the microplate wells (Costar 3599) in duplicate. Then, 10 μL of linoleic acid, 47 μL of Tween 40, and 2.5 mL of *β*-carotene (2 mg/mL in chloroform) were placed in a flask. After removal of chloroform with nitrogen gas, 22.5 mL of distilled hot water (50 °C) and 2.5 mL of 0.1 M sodium phosphate buffer (pH 6.8) were added to the same flask and shaken well. An amount of 0.2 mL of the linoleic acid−*β*-carotene emulsion were transferred rapidly to each well, kept under constant temperature (50 °C), and the Abs at 490 nm monitored for 60 min. $H_2O$ (20 μL) and reference compounds (20 μL) at different concentrations (0.25, 0.5, 1, 1.5, and 2.5 mM) were used as negative and positive controls, respectively.

The difference in Abs at 50 and 5 min ($\Delta = Abs_{50\ min} - Abs_{5\ min}$) was calculated. Results are expressed in terms of percentage bleaching inhibition of the initial linoleic acid−*β*-carotene emulsion by the test samples according to the following equation:

$$\%\text{bleaching inhibition} = [1 - (\Delta Abs_{sample}/\Delta Abs_{negative\ control}) \times 100]$$

$IC_{50}$ values were calculated by logarithmic regression in terms of micromolar. The antioxidant activity was also expressed as TEAC, by comparing $IC_{50}$ values obtained for DHAA, AA, and BHA with that TR.

**Deoxyribose Assay.** 2-Deoxy-D-ribose is degraded when exposed to hydroxyl radicals generated by the Fenton reaction. The degradation can be detected by heating the products with 2-thiobarbituric acid (TBA-0.5% in 1 N NaOH, w/v), under acidic conditions using trichloroacetic acid (TCA, 2.5%, w/v). The effects of DHAA on deoxyribose oxidation were assessed as described by Gutteridge et al.[40] with some modifications. The reaction mixture, containing 64 μL of EDTA (5 mM), 64 μL of $FeCl_3$ (5 mM), and 64 μL of $H_2O_2$ (2.5 mM), was incubated at 37 °C for 15 min. Then, $H_2O$ and a blend of deoxyribose (140 mM) and ascorbic acid (5 mM) was added to obtain a final volume of 0.64 mL. Different concentrations of DHAA (from 0.1 to 2 mM) or reference compounds (from 0.01 to 0.5 mM) were added, and the mixture incubated at 37 °C for 1 h. After the addition of TBA and TCA, the mixture was incubated for 15 min at 90 °C and the Abs was read at 490 nm in a microplate reader. The $IC_{50}$ and TEAC were calculated as described above.

**Tyrosinase Assay.** The tyrosinase inhibition assay was performed according to the method of Masamoto et al.[41] Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Nine units of enzyme (1 unit being defined by the producer as the $\Delta A_{280}$ of 0.001 $min^{-1}$ at pH 6.5 at 25 °C in 3 mL of reaction mix containing L-tyrosine) and DHAA at different concentrations (30−200 μM) were incubated for 5 min in 0.1 M sodium phosphate buffer, pH 6.8, in 0.1 mL final volume. The specific activity of the enzyme is 1715 U/mg. L-DOPA (final concentration = 3 mM) was added to start the reaction, and the formation of dopachrome was immediately monitored for 5 min at 490 nm in a microplate reader under a constant temperature of 30 °C. ΔAbs values were calculated in the first 240 s and referred to 1 min.

The percentage inhibition of enzyme activity was calculated by the following formula:

$$\%\text{inhibition} = [1 - (\Delta Abs/min_{sample}/\Delta Abs/min_{negative\ control}) \times 100]$$

$IC_{50}$ (concentration necessary for 50% inhibition of enzyme activity) was calculated by constructing a linear regression curve showing DHAA concentrations on the *x*-axis and percentage inhibition on the *y*-axis. A negative control was obtained by adding water instead of DHAA. A positive control was performed using kojic acid, a well-known tyrosinase inhibitor.

A Lineweaver−Burk (L-B) plot was constructed to calculate the kinetic parameters ($K_m$ expressed in mM and $V_{max}$ in μkat) of the enzymatic reaction without and with DHAA at the $IC_{50}$ concentration (0.13 mM). Different L-DOPA concentrations were used in the range from 0.095 to 6 mM; the rate of the enzymatic reaction, expressed in microkatals, was calculated from ΔAbs 120 to 240 s to avoid the lag phase, considering dopachrome ε at 490 nm = 3.6201 $mM^{-1}$ $cm^{-1}$ and a light path length of 0.8 cm. $K_{iapp}$ was calculated by the equation

$$K_{m\,app} = K_m\left[1 + \left(\left[I\right]/K_{i\,app}\right)\right]$$

where $K_{m\ app}$ is the apparent $K_m$ in the presence of DHAA.

**$Fe^{2+}$ Chelating Assay.** The ferrous ion-chelating potential was investigated according to the method of Decker and Welch[42] with some modifications, wherein the $Fe^{2+}$-chelating ability was calculated by measuring the ferrous iron−FZ complex at 560 nm in a microplate reader. Briefly, the reaction mixture, containing different concentrations of DHAA (from 10 μM to 5 mM), $FeCl_2$ (0.2 mM), and FZ (0.4 mM), was adjusted to a total volume of 0.2 mL with $H_2O$, shaken well, and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 560 nm against a blank. EDTA (from 10 μM to 5 mM) was used as positive control. The ability of the test compound to chelate ferrous ions was calculated using the following equation:

$$\text{chelating activity (\%)} = [1 - (\Delta Abs_{sample}/\Delta Abs_{negativecontrol}) \times 100]$$

**Statistical Analysis.** Values are expressed as the mean ± SD of three independent experiments with samples in triplicate. Statistical analysis was performed using Graph Pad Prism 4 software (La Jolla, CA, USA) by one-way analysis of variance (ANOVA), considering significant differences at $P$ values of <0.05.

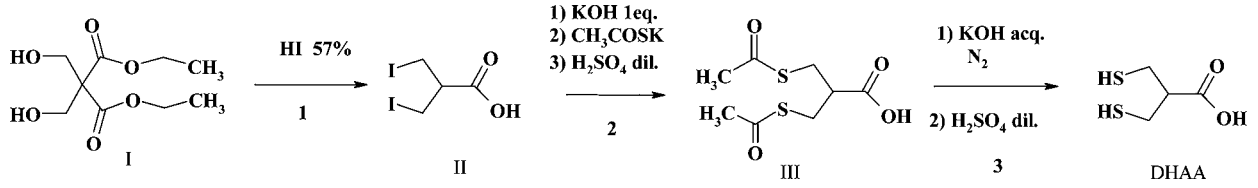


**Figure 1.** Projected scheme for DHAA synthesis.

## RESULTS AND DISCUSSION

Synthetic DHAA was obtained, after revisiting some preexistent protocols, by following the reaction by $^1$H NMR spectroscopy. In particular, the method reported by Yanagawa was adopted to obtain the first intermediate,[43] *β*-*β*′-diiodoisobutyric acid (II) from diethyl bis-(dihydroxymethylmalonate) (**I**, Figure 1). Subsequently, as reported by Schotte and Störm,[44] intermediate **II** was subjected to a nucleophilic substitution by thioacetate, leading to the formation of the dithioacetylated derivative (III), which, after hydrolysis, formed DHAA (Figure 1).

During the first step of this synthetic procedure, the formation of an acrylic byproduct (**II**′) took place (Figure 2),

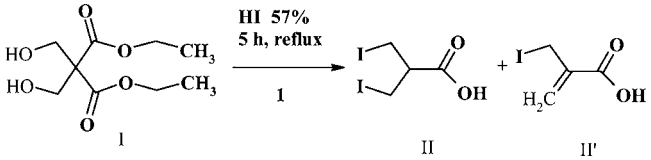


**Figure 2.** Formation of iodoacrylic byproduct.

which involves a drastic reduction of the final yield. In Yanagawa's paper this aspect was not considered,[43] but in a previous study on the effects of mineral acids on diethyl bis(dihydroxymethylmalonate) (**I**), the consistent formation of the iodoacrylic derivative was reported.[45] In the same paper, the instability of these halogenated derivatives (**II** and **II**′) was pointed out, so they were used in the following reactions as soon as possible. Although the formation of this byproduct could not be avoided, it may be at least minimized (7:3 ratio) by modulating some conditions, such as temperature and reaction time; a mean yield of about 57% of the mixture was obtained using the conditions reported under Materials and Methods.

Therefore, to increase the yield, we considered another synthetic procedure based on the use of this acrylic byproduct as intermediate, to whose double-bond thioacetic acid can be added.[46] On this basis, we modified the reaction conditions to exploit the byproduct as a reaction intermediate, avoiding any preliminary purification of the mixture obtained from the first step (Figure 3).

The presence of both thioacetate ion and thioacetic acid in the reaction mixture leads to the simultaneous substitution of halogen atoms and the addition of thioacetic acid to the double bond. The reaction can be easily monitored by $^1$H NMR spectroscopy. In fact, by observing the spectrum, one can add the required amount of thioacetic acid and potassium thioacetate, because the integrals relative to a single olefinic proton belonging to the acrylic compound and of the single methine proton belonging to the dihalogenated derivate reflect their relative abundance (see the Supporting Information, Figure S1). Therefore, the analysis of NMR data allows one to check the progression of the reaction and, on this basis, to add more reagents if required to displace the reaction and thus maximize product yield (see the Supporting Information, Figure S2). In particular, it was often necessary to readd thioacetic acid when the acrylic compound was still present, after several hours of reaction, especially with large amounts of starting material. This is probably due to the instability of thioacetic acid in an aqueous medium and its transformation into acetic acid (which does not get added to the double bond). This synthetic procedure was also transferred to gram scale, resulting in a quasi-quantitative yield from the last two steps. Indeed, yields of 97 and 94% were obtained from thioacetylation and hydrolysis reactions, respectively. In particular, the hydrolysis reaction gave an unexpectedly good result because, as previously reported by Yanagawa,[43] when the hydrolysis reaction was performed following Schotte's method, no satisfactory yield was often obtained due to the instability of the final product in alkaline medium.[44] On the contrary, we obtained an average yield of 94%, and this result is probably due to the mild conditions used during the reaction, namely, a low-concentration alkaline solution, and a nitrogen atmosphere, which avoids oxidation (see the Supporting Information, Figures S3 and S4).

The synthetic procedure represents a great advantage for molecules such as DHAA. In fact, according to Tressl et al.,[12] who investigated the biosynthesis of sulfur-containing acids in *Asparagus*, DHAA was found only in the enzyme-inhibited aroma extracts of the plant, thus suggesting that it is formed in the intact plant cells and might be one possible intermediate in the biosynthesis of asparagusic acid. Similar results were reported by Yanagawa et al.,[22] who isolated trace amounts of DHAA and other sulfur compounds during an investigation of plant growth regulating substances from extracts of *A. officinalis*. Moreover, the yield obtained with the chemical synthesis described here was much higher than that reported by Jansen,[11] who prepared the molecule by chemical reduction of asparagusic acid extracted from its natural source (and obtained

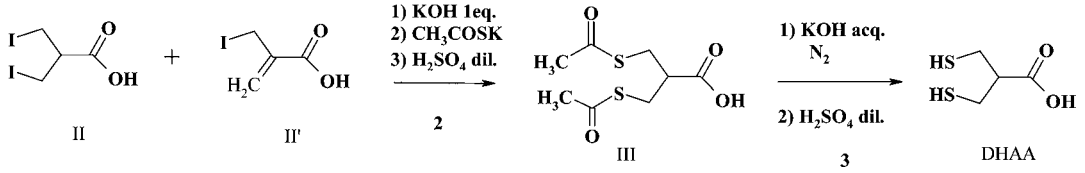


**Figure 3.** Modified synthesis of DHAA.


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only 15.21 g from 1400 kg of asparagus butts, yield = 1.1 × $10^{-5}$%). Another advantage derived from the chemical synthesis is to overcome the problem arising from the variability in endogenous content observed among different varieties of asparagus.[12]

With regard to the biological activities, DHAA showed a good radical scavenging capacity, comparable to that of well-known antioxidant compounds, such as AA, BHA, and TR.

With regard to DPPH and ABTS tests, a dose−response curve for DHAA is shown in Figure 4. $IC_{50}$ values for DHAA

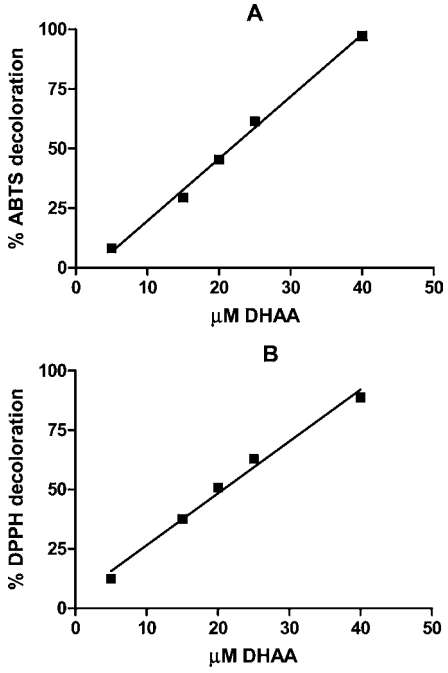


**Figure 4.** Dose−response curve of DHAA for ABTS (A) and DPPH (B) assays. $IC_{50}$ values were calculated from the following equation: $y = ax + b$, where $a = 2.6033$, $b = -6.5437$, $R^2 = 0.9955$ for ABTS; $a = 2.1835$, $b = 4.670$, $R^2 = 0.9877$ for DPPH. Data are the means of three independent determinations.

obtained in these assays were 20.76 and 21.72 μM, respectively, which are very close to those of AA and TR (Table 1). In fact, TEAC values, defined as the millimolar concentration of a TR solution having activity equivalent to that of a 1 mM solution of the substance under investigation,[47] turned out to be very close to 1. It is noteworthy to observe that the TEAC value of DHAA in the ABTS assay was similar to that reported for other SH-like antioxidants, such as 1,4-dithioerythritol, *N*-acetylcysteine, GSH, cysteine, and homocysteine (HCYSS), assayed by other authors using the same test and higher than that of DHLA.[48,49] Even if the ABTS and DPPH in vitro assays are often used for measuring antioxidant activity, they may not always be the best way to get information about some types of antioxidants. In particular, for S−S type antioxidants such as glutathione (GSSG), LA, and HCYSS, the antioxidant capacity was undetectable or very low using these tests, thus not reflecting their action in vivo.[48,49]

Consequently, the FRAP-FZ test was also performed. This test was modified with respect to the original as concerns the pH value, and a physiological pH was used instead of an acidic one.[50] In fact, the reducing capacity of some antioxidant compounds, such as DHAA, may be suppressed under acidic conditions due to their protonation, and this may indeed explain the generally low antioxidant capacity found by Grüngor et al. with the FRAP test.[49] Conversely, with the FRAP-FZ test used in this work, DHAA showed a strong redox reactivity toward $Fe^{3+}/Fe^{2+}$, revealing itself, also in this assay, as potent as antioxidant reference compounds, in particular, BHA and TR (TEAC = 0.99). Results obtained with the BCB test revealed that DHAA was also able to prevent lipid peroxidation through a chain-breaker mechanism, showing an $IC_{50}$ value similar to that of the other reference antioxidant compounds (Table 1).

Finally, DHAA was tested using the deoxyribose assay for evaluating the scavenging capacity toward the hydroxyl radical produced by the $H_2O_2/[Fe\text{-}EDTA]^{3+}$/AA system and detected by deoxyribose degradation. Unlike all of the other tests, DHAA showed a very low activity ($IC_{50}$ = 1.37 ± 0.04 mM; TEAC = 0.02, data not shown), whereas TR turned out to be rather active ($IC_{50}$ = 23.51 ± 2.23 μM). This result is in line with that reported by Scott et al.,[51] who observed a prooxidant effect of DHLA on deoxyribose degradation induced by $H_2O_2/FeC1_3$/AA. A low scavenging activity of dithiol compounds toward the hydroxyl radical was also found by Mazor et al.,[48] whereas Suzuki reported a positive result for DHLA as hydroxyl scavenger, even though a different detection method was used.[52] With regard to DHAA's ability to chelate free $Fe^{2+}$ ions, it was evaluated spectrophotometrically by the $Fe^{2+}$-chelating assay, but no such activity was found (data not shown).

In vitro tyrosinase activity was evaluated using L-DOPA as substrate and following its oxidation to dopachrome spectrophotometrically. Using this method, DHAA showed a particularly high inhibitory activity, and a reaction kinetics typical of thiolic compounds was observed (Figure 5).[53] Indeed, when DHAA was added, there was a lag before any increase in absorbance, and this lag period was longer with higher concentrations of DHAA, whereas addition of kojic acid, used as a positive control, did not show a similar trend (Figure 5). As reported by Negishi and Ozawa,[54] the lag time observed when tyrosinase activity was followed in the presence of thiol compounds is probably due to a nonenzymatic reaction in which the *o*-quinone radicals produced from L-DOPA by the

**Table 1. Antioxidant Activity of DHAA Assayed by DPPH, ABTS, BCB, and FRAP-FZ Tests**[a]

| | DPPH | | ABTS | | BCB | | FRAP-FZ | |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ | TEAC | $IC_{50}$ | TEAC | $IC_{50}$ | TEAC | μM FRAP | . |
| DHAA | 20.76 ± 4.0 a | 1.07 | 21.72 ± 3.0 a | 0.93 | 14.50 ± 2.7 abc | 1.45 | 236.41 ± 12 a | 0.99 |
| TR | 22.25 ± 2.5 a | 1 | 20.19 ± 1.9 ab | 1 | 21.05 ± 3.0 b | 1 | 239.36 ± 20 a | 1 |
| AA | 17.87 ± 2.3 ab | 1.25 | 22.35 ± 2.8 a | 0.90 | 13.04 ± 2.5 ac | 1.61 | 175.48 ± 14 b | 0.73 |
| BHA | 13.60 ± 1.5 b | 1.64 | 14.49 ± 2.8 a | 1.39 | 15.45 ± 2.8 abc | 1.36 | 226.65 ± 15 a | 0.95 |

[a]Data are expressed as $IC_{50}$ values (μM) for DPPH, ABTS, and BCB assays, and as μM FRAP values for FRAP-FZ assay. Different letters, within the same column, indicate significant differences at $p < 0.05$. Results are means ± SD of three independent experiments with three replicates.

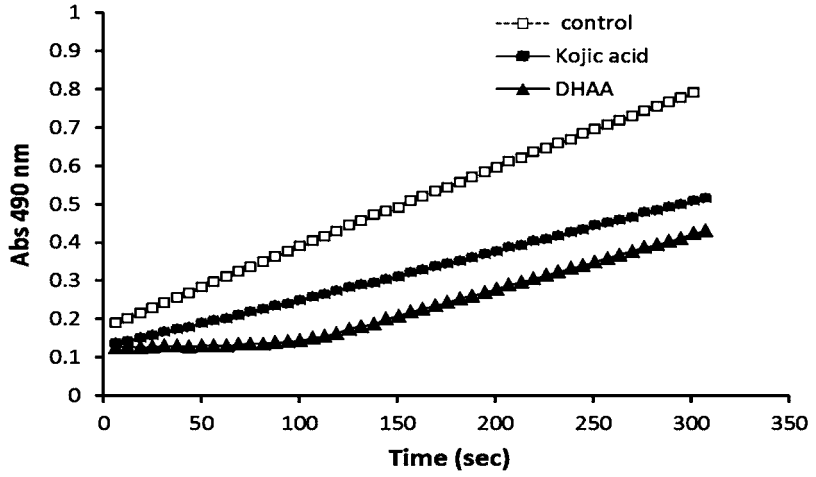


**Figure 5.** Inhibitory effect on the rate of the oxidation of L-DOPA by mushroom tyrosinase in control (□), kojic acid (■), and DHAA (▲). The enzyme and DHAA were incubated for 5 min, and assay was performed with 3 mM L-DOPA concentration.

enzyme are converted back to the original structure. After this lag phase, a reaction occurs between quinones and the chemical species derived from DHAA during the previous reaction, leading to the formation of colorless conjugates. A similar result was also reported by Tsuji-Naito et al., who demonstrated that the sulfhydryl groups of DHLA covalently react with DOPA-quinone to form lipoyl DOPA conjugates.[55] Thus, thiol compounds were able to prevent the polymerization of phenolics, which results in browning, through this non-enzymatic mechanism.

Using a 3 mM concentration of L-DOPA, the $IC_{50}$ value of DHAA, calculated from the dose−response curve (Figure 6),

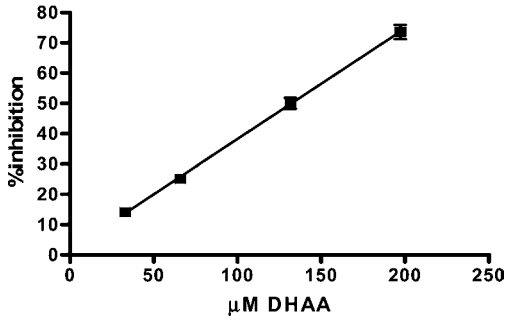


**Figure 6.** Inhibition of in vitro tyrosinase activity in the presence of increasing DHAA concentrations. Enzyme assay was performed using 3 mM L-DOPA as substrate. Data represent the mean ± SD of three independent experiments with samples in triplicate.

was 0.13 mM. This value turned out to be very close to that reported for L-cysteine by Kermasha et al.[53] ($IC_{50}$ = 0.15 mM), even though the assay conditions used by these authors were slightly different. The inhibitory activity of DHAA, expressed in terms of $IC_{50}$ values, was about 9 times lower than the one obtained with kojic acid ($IC_{50}$ = 14.7 ± 2.0 μM), but it is important to consider that these two molecules act through different mechanisms.[56] To investigate in more detail this aspect, a L-B plot was built by monitoring the reaction in the presence of different L-DOPA concentrations, keeping a DHAA concentration equivalent to the $IC_{50}$. The L-B plot (Figure 7) shows that only the $K_m$ value was modified by the presence of the inhibitor, increasing from 0.81 mM (without inhibitor) to 4.23 mM in the presence of DHAA, whereas the

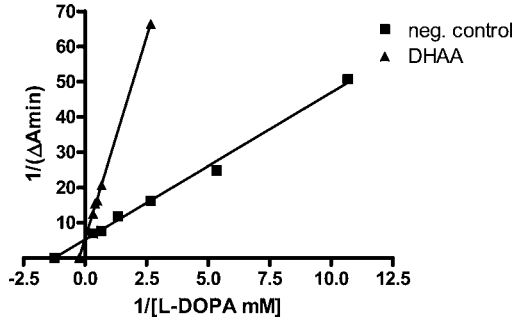


**Figure 7.** Lineaweaver−Burk plots of mushroom tyrosinase and L-DOPA without (■) and with (▲) 0.13 mM DHAA. The enzyme and DHAA were incubated for 5 min, and assay was performed with an L-DOPA concentration range from 0.095 to 6.0 mM.

$V_{max}$ remained unchanged, thus suggesting that DHAA inhibited tyrosinase activity competitively, without the inactivation of the enzyme. A similar L-B plot was obtained for a water-soluble LA derivative.[57] The kinetic parameters of the enzyme assay in the presence or absence of DHAA are shown in Table 2. To confirm the lack of irreversible

**Table 2. Kinetic Inhibition Parameters of DHAA on Mushroom Tyrosinase**

| | enzyme | enzyme−DHAA |
|---|---|---|
| $IC_{50}$ | | 0.13 mM |
| $K_m$ | 0.81 mM | 4.23 mM |
| $V_{max}$ | 0.198 Δ$A$/min (0.228 μkat) | 0.193 Δ$A$/min (0.223 μkat) |
| $K_{i\ app}$ | | 0.275 mM |

inactivation of the enzyme by DHAA, the assay was performed by increasing the incubation time from 5 to 30 min, and results were compared with a nonincubation assay. No differences in the inhibitory activities were observed among 0, 5, and 30 min of incubation (data not shown), thus suggesting that no irreversible inactivation occurs. With regard to kojic acid, a mixed mechanism of inhibition was found (data not shown), confirming previously reported results.[56]

In conclusion, the protocol for the synthesis of DHAA developed in this work results in higher yields compared to the


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previously reported protocols. Results obtained from antioxidant tests demonstrate that this molecule may be considered as a new potentially useful ingredient or preservative in the preparation of functional foods, food supplements, and nutraceuticals by virtue of its considerable antioxidant power. Compared to other sulfhydryl compounds, such as LA and DHLA, DHAA has the advantage of being soluble in aqueous solutions: this makes it more readily exploitable in different applications. The inhibitory activity exerted on PPO (tyrosinase) activity and thus on the polymerization of quinones occurring during browning represents an added value for this molecule and increases its potential in the food industry and for medicinal, agronomic, and cosmetic uses.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

AA, ascorbic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BCB, β-carotene bleaching; BHA, *tert*-butyl-4-hydroxyanisole; DHAA, dihydroasparagusic acid; DHLA, dihydrolypoic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; FZ, ferrozine; GSH, glutathione; HCYSS, homocysteine; LA, α-lipoic acid; TEAC, Trolox equivalent antioxidant capacity; TR, Trolox

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