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Production, Stability, and Antioxidative and Antimicrobial Activities of Two L-Ascorbate Analogues from *Phycomyces blakesleeanus*: D-Erythroascorbate and D-Erythroascorbate Glucoside

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D-Erythroascorbate (D-EAA), a five-carbon analogue of L-ascorbate (L-AA), and D-erythroascorbate monoglucoside (D-EAAG) are accumulated in *Phycomyces blakesleeanus* grown on glucose (99.5 and 1084 μg/g mycelial dry weight, respectively) and also excreted into the culture medium. Both compounds showed UV spectral properties and ionization constants similar to those of L-AA. D-EAAG was much more stable to aerobic oxidation than D-EAA and L-AA at acidic pH. D-EAAG is synthesized from D-erythroascorbate by a mycelial glucosyltransferase activity that uses UDP-glucose as glucose substrate donor with K_m = 2.5 mM and 41.3 μM for D-EAA. This glucosyltransferase activity was maximal in the stationary growth phase in parallel with maximal production of D-EAAG. The presence of D-arabinose or D-arabinono-1,4-lactone in the culture medium produces the maximal accumulation of D-EAA and D-EAAG (about 30- and 4-fold with respect to that obtained in glucose culture). Both compounds showed greater antioxidant activity than L-AA and other standard antioxidants, with a capacity similar to that of L-AA to inhibit the growth of *Escherichia coli*.

KEYWORDS: D-Erythroascorbate; D-erythroascorbate monoglucoside; physicochemical properties; stability; UDP-glucosyltransferase activity; antioxidant activity; *Phycomyces blakesleeanus*; filamentous fungus

INTRODUCTION

D-Erythroascorbate (D-EAA) is a C_5 ascorbate analogue similar in structure and physicochemical properties to ascorbate (*1*). We have previously reported that D-EAA and a D-erythroascorbate glucoside (D-EAAG) are naturally molecules occurring in *Phycomyces blakesleeanus*, a Zygomycete fungus (**Figure 1**), providing evidence that both compounds protect the glutathione pool and β-carotene from hydrogen peroxide-induced depletion, suggesting that they can act as antioxidants in vivo (*2*). The presence of D-EAA and the absence of L-ascorbate have been reported in two other fungal groups, the Ascomycetes and Basidiomycetes (*3*, *4*), suggesting that D-EAA is characteristic of the true fungi and that the ability to synthetize it must have evolved in the common ancestor of the three fungal groups. D-EAA can be replaced by ascorbate in several industrial processes (*5*), but does not have antiscorbutic activity in humans (*6*). Ascorbic acid can be used as a common antioxidant in many food systems and also as a cosmetic ingredient for skin care (*7*). However, the instability of ascorbic acid against oxidative environments with the consequent losses in antioxidative activity is disadvantageous for its applications. It has been described that glycosylated derivatives of ascorbic acid show effective antioxidative activity with enhanced oxidative stability (*8*).

In the present study, the molecular mass, UV spectral properties, and oxidative stability of D-EAAG synthetized by *P. blakesleeanus* are described, and the antioxidative activity of D-EAA and D-EAAG against commercial antioxidants is also evaluated. Glycosylascorbic acids have been obtained by transglycosylation with α-glucosidases and maltogenic amylase (*8*, *9*). Here we report the existence of a glucosyltransferase activity responsible for the glycosylation reaction of D-EAA to form D-EAAG. Finally, we have analyzed the capacity of *P. blakesleeanus* to synthetize D-EAA and D-EAAG under different growth conditions that demonstrate the ability of this fungus to produce D-EAA and D-EAAG in amounts larger than those described in other microorganisms.

MATERIALS AND METHODS

Materials, Strains, and Growth Conditions. All carbohydrates used, D-aldoses, D-cetose (D-fructose), D- and L-pentoses, and dissacharides (sucrose and maltose); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2′,diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactones and 2,4,6-tris(2,4,6-tripyridyl-2-triazine) (TPTZ) were obtained from Fluka Chemie A.G. (Buchs, Switzerland).

P. blakesleeanus (strain NRRL 1555, genotype (−), wild type) was grown routinely in liquid minimal medium (SIV) as described by

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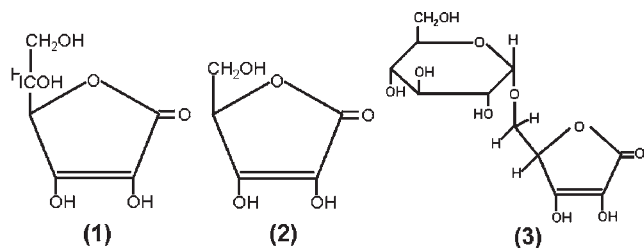

Figure 1. Chemical structures of L-ascorbate (**1**), D-erythroascorbate (**2**), and D-erythroascorbate monoglucoside (**3**).

Baroja-Mazo et al. (*2*). For experiments of production of D-EAA and D-EAAG, (1) several carbohydrates were used as carbon source or (2) the precursor was added to the culture medium with glucose 2% (w/v) at a concentration of 10 mM at the onset of growth or at the indicated time of growth on glucose. Mycelia were obtained by filtration at different stages of growth. Growth of *P. blakesleeanus* was determined as a function of mycelial dry weight. Aliquots of culture were taken and passed through a glass wool filter. They were kept at 50 °C until their weights were constant.

Ten strains of *Staphylococcus aureus*, 3 collection strains (ATCC 25923, CCTM La 2812, and ATCC 27664) and 7 foodborne isolates (6 from bulk tank ewe's milk and 1 from rabbit carcasses), and 10 strains of *Escherichia coli* foodborne isolates from bulk tank ewe's milk provided by Dr. M. R. Garcia Armesto, Dr. J. M. Rodriguez-Calleja, and Dr. I. Caro Canales, respectively, from the Department of Food Hygiene and Food Technology (University of León, León, Spain) were used in this study. Stock cultures were maintained in eppendorfs with brain–heart infusion broth (BHI; Oxoid Ltd., Basingstoke, Hampshire, U.K.) in the presence of 30% (v/v) glycerol at −40 °C. A microdilution test assay was used to determine the MICs of D-EAA, D-EAAG, and other selected antioxidants according to the method of Barry (*10*). MIC values were determined as the lowest concentrations that inhibited growth of the strain tested. At least two trials on different days were carried out in duplicate for each strain and compound.

Extraction, Quantification, and Identification of D-EAA and D-EAAG. *P. blakesleeanus* mycelium grown in liquid medium under the indicated conditions in each experiment was used to purify D-EAA and D-EAAG as described by Baroja-Mazo et al. (*2*). The routine identification of D-EAA and D-EAAG was carried out by high-performance liquid chromatography (HPLC) using an Alliance Waters 2690 liquid chromatograph equipped with an Array 996 photodiode detector (Waters) and an anionic exclusion column Supelcogel C-610H (300 × 7.8 mm) with a Supelcogel C-610 coupled precolumn (50×4.6 mm) (Sigma Chemical Co.). Separation and identification of D-EAA and D-EAAG were achieved by isocratic elution with 0.75 mM H_2SO_4 at a flow rate of 1 mL/min. D-EAA and D-EAAG were detected by their absorbance at 254 nm. The peaks of D-EAA and D-EAAG were identified by their retention times, 8.30 ± 0.15 and 6.15 ± 0.20 min, respectively, as described by Baroja-Mazo et al. (*2*). The areas of the peaks were determined by using the Empower software and the quantification of D-EAA and D-EAAG from an L-ascorbate standard curve (0.025–5 mM).

Mass Spectrometry Analysis. A Q-Tof *micro* (Waters Corp.) tandem mass spectrometer with an ESI LockSpray source in negative ion mode was used for the analysis of D-EAA and D-EAAG. Samples were introduced in the mass spectrometer by direct infusion using as mobile phase 50% methanol and 1 mM ammonium acetate in high purity grade water. The mobile phase flow rate was between 5 and 20 μL/min. Ionization parameters were optimized by direct infusion of standard solutions and were as follows: capillary voltage, 2.2 kV; sample cone voltage, 25 V; extraction cone voltage, 1.0 V; multiplier voltage, 2500 V; source temperature, 80 °C; and desolvation temperature, 120 °C. Nitrogen was used as cone (50 L/h), nebulizing, and desolvation gas (200 L/h). Argon was used as collision gas (vacuum pressure = 2.6×10^{-7} mbar) with a low collision energy of 2 eV, which did not cause fragmentation of the analytes for MS spectra. MS/MS analyses were carried out using the same settings for ionization, and the following deprotonated molecular ions were used for the fragmentation experiments: glucose ($[M - H]^-$ *m*/*z* 179, collision energy, 4 and 30 eV); chlorine adduct of glucose ($[M + Cl]^-$, *m*/*z* 215, collision energy, 4 and 12 eV); D-EAA ($[M - H]^-$ *m*/*z* 145, energy collision, 8 and 32 eV); and D-EAAG ($[M - H]^-$ *m*/*z* 307, collision energy, 8 and 44 eV). Data were acquired with MassLynx 4.0 software (Waters Corp.) in continuous mode, from *m*/*z* 50 to 400 to obtain complete fragmentation patterns.

Spectrophometric Analysis and Stabilities of D-Erythroascorbic Acid and D-Erythroascorbic Glucoside in Aqueous Solution. Ultraviolet (UV) absorption spectra were measured in solutions at different pH values on a Shimadzu UV-260 double-beam spectrophotometer, and extinction coefficients were determined. Ionization constants were determined by the changes in inflections of curves of λ_{max} versus pH (*1*). To determine the aerobic stabilities of D-EAA and D-EAAG, the solutions (final concentrations of each at 0.23 and 0.7 mM in 3 mL of buffer) of D-EAA and D-EAAG were prepared in 0.1 M sodium citrate buffer, pH 4, and 0.1 M sodium phosphate buffer, pH 6 and 8, respectively, in open-topped glass tubes (15 × 85 mm, 15 mL). The tubes were placed in a constant-temperature bath at 25, 35, or 45 °C. The decline in UV absorbance at 251 nm (pH 4), 263 nm (pH 6), and 261 nm (pH 8) for D-EAA and at 255 nm (pH 4) and 265 nm (pH 6 and 8) for D-EAAG was determined to follow losses of both compounds.

Glucosyl Transferase Activity Assay and Analysis of Reaction Product. The glucosyltransferase assay contained 9 mM UDP-glucose, 0.2 mM D-EAA, and *P. blakesleeanus* mycelial extract (after passing through a PD-10 column) in 50 mM sodium phosphate buffer, pH 7, containing 14 mM 2-mercaptoethanol (MSH) and 2 mM dithiothreitol (DTT) in a final reaction volume of 600 μL. The reaction mixture was incubated at 30 °C for 30 min, and then 500 μL of the reaction mixture was loaded onto an anion exchange column (Amprep-trimethylaminopropyl SAX, 1 mL bed volume), washing this with 1 mL of H_2O and eluting the reaction product with 500 μL of 60 mM formic acid. The reaction product was analyzed by HPLC (as described above) and identified as D-EAAG.

Antioxidant Activity Determination. The antioxidant activity of D-EAA, D-EAAG, and the standard antioxidants ascorbic acid, gallic acid, resveratrol, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) was determined according to three procedures: (1) The FRAP method was conducted according to the procedure of Benzie and Strain (*11*). A solution of 10 mM TPTZ and 20 mM ferric chloride was diluted in 300 mM sodium acetate buffer, pH 3.6, at a ratio of 1:1:10. One hundred microliters of standards or D-EAA (25 μM) or D-EAAG (25 μM) was added to 3 mL of the TPTZ solution, and absorbance at 593 nm was determined after 15 min of reaction. A Trolox standard curve was obtained in a concentration range of 0–30 μM. (2) The ABTS assay was carried out as described by Ozgen et al. (*12*), with minor modifications. ABTS stock solution (7 mM) with 2.45 mM potassium persulfate was prepared in 20 mM sodium acetate buffer, pH 4.5, and was allowed to stand for 12–16 h at room temperature in the dark, remaining stable for several weeks under these conditions. On the day of analysis, the ABTS solution was diluted with the same buffer to an absorbance of 0.7 at 734 nm. For the spectrophotometric assay, 1 mL of the ABTS solution and 10 μL of antioxidant (100 μM) were mixed, and the absorbance at 734 nm was registered for 6 min at 30 °C. A Trolox standard curve was obtained in a concentration range of 0–30 μM. (3) The ability to scavenge the DPPH radical was measured as described by Brand-Williams et al. (*13*) with modifications. The assay mixture contained 1 mL of 200 μM $DPPH^\bullet$ (dissolved in 100% ethanol) and 1 mL of antioxidant (2 μM) in 100 mM Tris-HCl buffer, pH 7.4. The mixture was mixed and left to stand in the dark at room temperature for 20 min, after which the absorbance at 517 nm was determined. A Trolox standard curve was obtained in a concentration range of 0–100 μM. All data were expressed as Trolox equivalents from the three assay methods.

RESULTS AND DISCUSSION

Molecular Mass, UV Spectral Properties and Stability of D-Erythroascorbate Glucoside from *Phycomyces blakesleeanus*. We have previously described the characterization and biosynthesis of D-EAA and the presence of a D-EAAG in *P. blakesleeanus*, as well as some properties of the glucosylated form (*2*). Here, we have analyzed a sample of the D-EAAG purified (as described under Materials and Methods) by ESI mass spectrometry using a Q-Tof micro mass spectrometer (Water Corp.) equipped with an ESI LockSpray source. The ESI mass spectra acquired in the

Table 1. UV Spectral Properties and Ionization Constants of D-Erythroascorbate Glucoside (D-EAAG) Obtained from *Phycomyces blakesleeanus*

	pH 2.0		pH 4.0		pH 6.0		pH 7.0		pH 8.0			
	λ_{max} (nm)	ε (mM^{-1} cm^{-1})	λ_{max}(nm)	ε (mM^{-1} cm^{-1})	λ_{max}(nm)	ε (mM^{-1} cm^{-1})	λ_{max}(nm)	ε (mM^{-1} cm^{-1})	λ_{max}(nm)	(mM^{-1} cm^{-1})	pK_{a1}	pK_{a2}
D-EAAG	243[a]		255	11.2	265	19.8	262	18.6	265	19.0	3.70	12.40
D-EAA	243[b]	10[b]	251	17.2	263	16.5	264	23.2	261	24.1	3.96	12.10
L-AA	243[b]	10[b]	254	13.7	258	11.3	258	12.0	260	15.1	4.03	11.92

[a] From Baroja-Mazo et al. (*2*). [b] From Shao et al. (*1*).

negative ion mode showed an intense molecular ion $[M - H]^-$ signal at *m/z* 307, which is in line with the molecular formula of $C_{11}O_{10}H_{16}$ ($M = 308$) corresponding to the molecular mass of D-EAAG in which the glucosyl moiety is composed by a single glucose unit. This observed molecular ion is in agreement with the observed ion at *m/z* 331.1 by ESI-MS analysis in the positive ion mode from the fungus *Sclerotinia sclerotiorum* corresponding to M + H of the Na salt of the galactopyranosyl-D-erythroascorbate (*14*) and is also similar to that obtained with the glucopyranosyl-D-erythroascorbate from the basidiomycete *Hypsizigus mamoreus* (*4*). The fragmentation pattern of the ion with *m/z* 307 obtained by the MS/MS procedure involves formation of an intense fragment ion at *m/z* 127 that could correspond to loss of 180 mass units to glucose moiety from the $[M - H]^-$ ion. It has been reported that in flavonoid glycosides the cleavage at the glycosidic O linkage with a concomitant H arrangement leads to the elimination of dehydrated monosaccharide residues (*15*). On the other hand, when a solution of D-EAAG was hydrolyzed with 3 M HCl for 15 min at 98 °C, the MS spectra of the hydrolysate showed $[M - H]^-$ and $[M + Cl]^-$ signals at *m/z* 145 and 215 associated with the molecular mass of D-EAA and the chlorine adduct of glucose, respectively, as compared with the ESI mass spectra obtained for the purified D-EAA and glucose under the same conditions. The MS/MS fragmentation patterns of the ion peaks at *m/z* 145 and 215 obtained in the negative ion mode were similar in both cases. A signal at *m/z* 145 was also obtained by negative ion electrospray mass spectrometry by Spickett et al. (*16*) in the characterization of D-EAA from *S. cerevisiae*.

The glycosidic residue is sometimes crucial for the activity of the biological compounds, and in other cases glycosylation provided an increase in its hydrophilicity (mainly affecting membrane transport) and stability against UV light, heat, and oxidation (*17*). Glycosylated ascorbic acids have been synthesized by using the transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase, with maltotriose showing effective antioxidative activity with enhanced oxidative stability (*8*). Thus, we have analyzed the effect of the glucosylation of D-EAA testing the UV spectral properties, ionization constants, and stability against chemical oxidation in aqueous solution under aerobic conditions of the D-EAAG.

Table 1 illustrates the pH effect on UV spectral properties and ionization constants of the D-EAAG from *P. blakesleeanus*. D-EAAG showed a bathochromic shift and hyperchromic effect when the pH was increased from acidic to basic values. λ_{max} shifted toward a longer wavelength, about 20 nm, when the pH was increased from 2 to 8, and ε (molar extinction coefficient) was increased from 11.2 to 19.0 mM^{-1} cm^{-1} when the pH rose from 4 to 8. As can be seen in **Table 1**, these properties resembled those of D-EAA and L-ascorbate (L-AA). On the other hand, a glucoside of L-AA, 6-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-6G) (*9*), shows spectral data similar to those observed with L-AA, D-EAA, and D-EAAG. However, 2-*O*-substituted AA derivatives showed a characteristic bathochromic shift of about 5 nm to the shorter wavelength at both pH 2 and 7, whereas the hypochromic effect was observed only in 3-*O*-substituted AA (*8*, *18*). D-EAAG and D-EAA display two acidic protons of pK_a values similar to pK_a values for the 3- and 2-hydroxyls of L-AA (**Table 1**). It has been described that the substitution of the 2-OH of L-AA increased the acidity of the 3-OH, resulting in a decrease in the pK_a value from 4.2 to 3.11−3.40, whereas substitution at 3-OH produces a drop in the pK_a value of the 2-OH from 11.8 to 6.22−7.9 (*18*). The spectral properties of D-EAAG from *P. blakesleeanus* as well as the pK_a values that we obtained are similar to those of L-AA, indicating that the glucosyl unit is attached to C-5 of D-EAA (**Figure 1**).

Table 2. Stability of D-Erythroascorbate (D-EAA) and D-Erythroascorbate Glucoside (D-EAAG) under Aerobic Conditions[a]

		half-life (h)			
		D-EAA		D-EAAG	
pH	temperature (°C)	0.23 mM	0.70 mM	0.23 mM	0.70 mM
4	25	40.9 ± 3.90	stable for 156 h	stable for 156 h	stable for 156 h
	35	16.2 ± 2.70			
	45	15.2 ± 0.06			
6	25	64.3 ± 7.72	89.0 ± 11.40	182.4 ± 1.75	257.2 ± 37.54
	35	28.5 ± 0.06	31.6 ± 2.30	91.1 ± 21.94	229.7 ± 56.81
	45	15.6 ± 2.34	11.7 ± 1.20	9.7 ± 0.83	28.1 ± 7.7
8	25	69.1 ± 10.37	164.0 ± 23.00	6.2 ± 0.83	16.3 ± 0.45
	35	35.0 ± 0.11	38.9 ± 1.55	3.7 ± 0.01	13.5 ± 0.91
	45	31.7 ± 7.00	17.0 ± 0.60	1.5 ± 0.11	2.5 ± 0.17

[a] The half-lives were determined in 0.1 M sodium acetate buffer, pH 4, and 0.1 M sodium phosphate buffer, pH 6 and 8, as described in the text. Values are the mean of at least two experiments in duplicate ± standard deviation of the mean.

We evaluated the stability of D-EAAG and D-EAA for comparison in aqueous solution under aerobic conditions at three different pH values, 4, 6, and 8 and three different temperatures, 25, 35, and 45 °C, respectively. Under all of the tested conditions, oxidative degradation of both compounds follows first-order kinetics. On the basis of analysis by nonlinear regression of plots of percent residual concentration versus incubation time we calculated the half-lives of D-EAA and D-EAAG under the aerobic conditions described in **Table 2**. As can be seen, D-EAAG was much more stable at acidic pH values than at basic pH. D-EAAG maintained its spectrophotometric activity completely for almost 7 days at pH 4 for all of the temperatures studied in the concentration range between 0.033 and 0.11 mg/mL. D-EAA was more stable at pH 8 than D-EAAG, whereas the opposite occurs at acidic pH values. Shao et al. (*1*) stated that L-AA and D-EAA are more stable at pH 4 and 8 than at pH 6, as was observed for D-EAA at the high concentration used in this study. On the other hand, the increase in initial concentration significantly decreased the oxidative degradation rate of both compounds, which means that D-EAA and D-EAAG stability can be substantially increased by increasing their concentrations. This behavior can be explained because the concentration of both compounds is varied while the oxygen concentration remains constant. It is known that ascorbate oxidation is dependent upon the oxygen concentration (*19*,*20*).

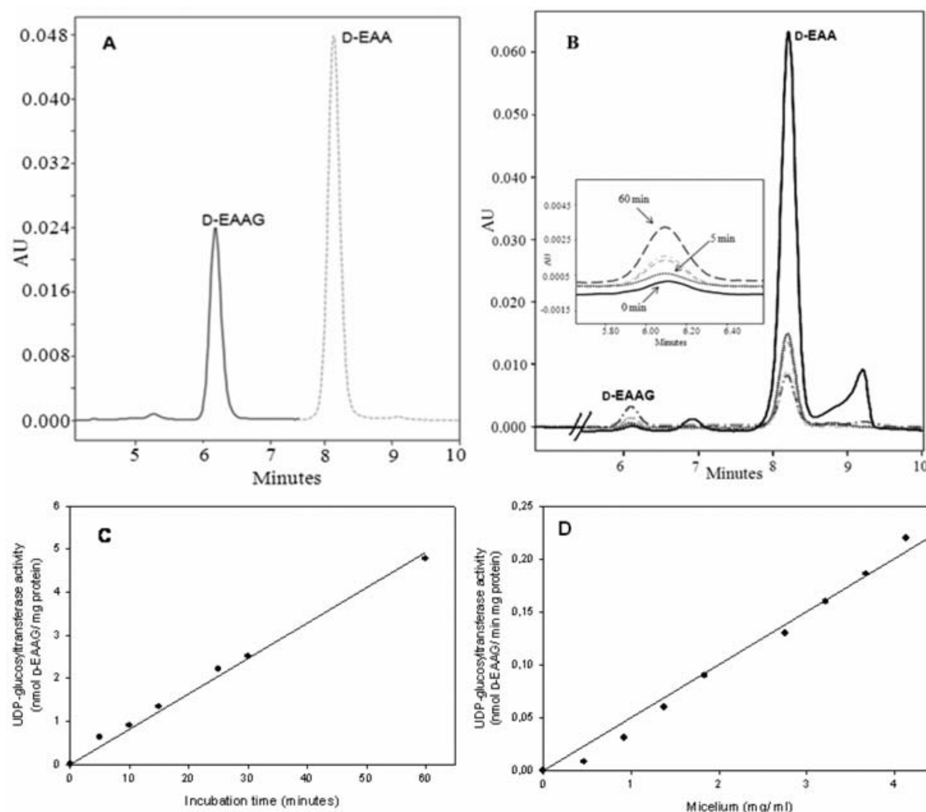

Figure 2. Glucosyltransferase activity from *P. blakesleeanus* mycelium: (**A**) HPLC chromatogram of purified D-EAA and D-EAAG; (**B**) HPLC chromatogram of glucosyltransferase reaction product with D-EAA and UDP-glucose as susbtrates [(inset) D-EAAG formation at different reaction times]; (**C**, **D**) incubation time and mycelial dependence of glucosyltransferase activity. Glucosyltransferase activity and HPLC chromatograms were carried out as described under Materials and Methods.

The effect of temperature on the aerobic oxidation of D-EAA and D-EAAG is expressed by the activation energy value (E_a) of the oxidative degradation process, obtained from linear plots of log k versus $1/T$, k being the oxidative degradation constant. At pH 6, we obtained E_a values of 14.7 and 24.1 kcal/mol, whereas at pH 8 these values were 25.3 and 15.4 kcal/mol for D-EAA and D-EAAG, respectively. An E_a value of 14.8 kcal/mol has been described for aerobic oxidation of ascorbic acid in commercial water (*20*), whereas E_a values of 3.3, 9.0, and 13.3 kcal/mol have been reported for ascorbic acid degradation in tomato juice (*21*), in pasteurized packed table olives (*22*), and in pasteurized orange juice (*23*). Thus, because the pH values of many food systems, such as fruit jams and jellies, fruit and vegetable juices, and cheeses, are in the acidic pH range, we think that D-EAAG can be the focus of attention due to its possible antioxidant role in many food systems.

As can be see in **Table 2**, D-EAAG was much more unstable that D-EAA at pH 8. In the presence of molecular oxygen and trace levels of transition metals (Cu^{2+}, Fe^{3+}), it has been proposed that the oxidation of ascorbic acid involves the formation of a metal−ascorbate monoanion−molecular oxygen complex (*19*). Shao et al. (*1*) proposed that the metallic ions present are probably more highly chelated by the phosphate ions in the buffer at pH 8 than at pH 4 or 6, and this effect could explain the greater stability of D-EAA and ascorbic acid at alkaline pH value, because the oxidation of ascorbic acid by molecular oxygen with various metal chelate compounds of Cu(II) and Fe(III) as catalysts occurs at much slower rates than the corresponding metal ion catalyzed reactions (*24*). However, this effect is not observable in D-EAAG oxidation at pH 8, suggesting that the presence of glucose in the molecule at alkaline pH value does not have a stabilizing effect. Thus, we analyzed the oxidative degradation of D-EAA (0.23 mM) in 0.1 M sodium phosphate buffer, pH 8, in the presence of an equimolar amount of glucose, at 25 °C. The presence of glucose diminished about 5-fold the half-life of D-EAA (from 69.2 to 14.8 h). It is possible that at alkaline pH values, glucose facilitates the formation of a metal−D-EAAG monoanion−molecular oxygen complex, opposing thus the chelating effect of phosphate ions and favoring the oxidative degradation of D-EAAG under these conditions.

Enzymatic Production of D-Erythroascorbate Glucoside. In general, glycoside formation involves the presence of glycosyltransferase activity (GTs; EC 2.4.*x*.*y*), which in the vast majority uses sugar donors containing diphosphate leaving groups, with UDP/TDP leaving groups as the most common (for a review see ref *25*). We have found glucosyltransferase activity in mycelia of *P. blakesleeanus* grown in a liquid minimal medium with 2% (w/v) glucose as carbon source in darkness and 13 mM asparagine as nitrogen source. The glucosyltransferase activity was determined in the forward direction using UDP-glucose as sugar substrate donor for D-EAAG formation. As can be seen in **Figure 2A**, incubation of D-EAA with 60-h-old *P. blakesleeanus* mycelial extract in the presence of 9 mM UDP-glucose resulted in the time-dependent formation of D-EAAG as determined by HPLC. The retention time of the reaction product (6.15 ± 0.2 min) corresponds to D-EAAG by comparison with the retention times obtained for authentic D-EAAG and D-EAA (**Figure 2B**) purified as described by Baroja-Mazo et al. (*2*). In addition, UV spectra of the reaction product from D-EAA were indistinguishable from the corresponding authentic D-EAAG. As the incubation time increased, the peak area corresponding to D-EAAG increased with a concomitant decrease in the peak area corresponding to

Table 3. Contents of D-EAA and D-EAAG in *P. blakesleeanus* Growing on Different Carbon Sources in the Dark with 13 mM Asparagine as the Nitrogen Source

	μg/g of dry weight[a]			
	D-EAA		D-EAAG	
carbon source	24 h	48 h	24 h	48 h
D-glucose	78 ± 10.3	4.7 ± 0.3	35 ± 3.4	470 ± 37.3
D-sorbitol	157 ± 13.2	nd	186.5 ± 18	337 ± 29
D-xylose	100 ± 12.6	nd	55 ± 3.7	538 ± 56.5
D-fructose	47 ± 5.1	48.8 ± 4.3	173.4 ± 10.4	458 ± 44.9
L-arabinose	32 ± 1.4	nd	184.4 ± 5.7	145 ± 9.5
D-galactose	0.55 ± 0.05	140 ± 12.4	111 ± 14.4	287 ± 31.4
D-mannose	47 ± 5.6	2 ± 0.13	78 ± 7.6	346 ± 38.1
sucrose	74.8 ± 7.3	nd	131 ± 18.1	427 ± 41.2
maltose	42.9 ± 1.5	4.7 ± 0.2	87 ± 5.1	151.5 ± 14.5
D-mannitol	88 ± 5.2	nd	37.8 ± 3.5	nd
D-glucose + D-arabinose[b]	119 ± 8.7	63 ± 7.4	69.4 ± 4.4	1442 ± 129
D-glucose + L-fucose[b]	94.7 ± 6.6	1.76 ± 0.15	60.5 ± 5.1	1011 ± 149
D-glucose + L-gulono-1,4-lactone[b]	20.4 ± 1.8	4.2 ± 0.4	30.5 ± 1.8	746 ± 9.7
D-glucose + L-galactone-1,4-lactone[b]	56 ± 5.0	5.5 ± 0.38	53 ± 6.1	566 ± 55.1

[a] Values are the mean of at least two experiments in duplicate ± standard deviation of the mean. nd, not detected. [b] D-Arabinose (10 mM), L-fucose (10 mM), L-gulono-1,4-lactone (10 mM), or L-galactono-1,4-lactone (10 mM) was added at the onset of growth on D-glucose 2% (w/v).

D-EAA substrate. Assays in the absence of the UDP-glucose donor substrate or in the absence of mycelial extract yielded no product (data not shown). On the other hand, in the enzymatic mycelial extract used, prepared as described under Material and Methods, no D-EAA or D-EAAG presence was detected by HPLC analysis.

The glucosyltransferase activity shows a linear dependence on the quantity of mycelial extract present in the incubation mixture (**Figure 2D**) and also a linear dependence on the incubation reaction time up to 60 min (**Figure 2C**). Thus, the enzymatic activity measured under these experimental conditions corresponding to an initial velocity of 78.2 pmol of D-EAAG/min/mg of protein. The addition of Mg^{2+} (13.5 mM) to the reaction mixture has no effect upon the extent of D-EAA glucosylation, similar to what has been described for UDP-glucose:flavonoid 3-*O*-glucosyltransferase from *Vitis vinifera* (*26*) and for UDP-glucose: anthocyanin 3′,5′-*O*- glucosyltransferase from *Clitoria ternatea* (*27*). We have not detected either the reverse reaction, that is, the generation of D-EAA from UDP and D-EAAG, or trans-glucosylation reaction using maltose as sugar donor substrate for D-EAAG formation under our experimental conditions. The formation of glucosyl derivatives of L-ascorbic acid with mammalian α-glucosidase by enzymatic tranglucosylation from maltose to L-ascorbic acid (*9*) has been described.

No significant glucosyltransferase activity was present at the onset and during the exponential growth phase of *P. blakesleeanus*, increasing at the onset of the stationary growth phase and reaching maximal activity at 72 h of growth and then decreasing from this time. This maximal glucosyltransferase activity was in parallel with the production of D-EAAG, which shows the higher levels also in the stationary growth phase (data not shown).

The kinetics of D-EAA glucosyltransferase were studied for both donor and acceptor substrates. Hyperbolic saturation curves were obtained for both substrates, from which Lineweaver−Burk transformation gave an apparent K_m values of 2.5 mM for UDP-glucose and 41.3 μM for D-EAA, with apparent V_{max} values of 0.11 and 0.07 nmol D-EAAG/min/mg of protein, respectively. The apparent K_m value obtained for UDP-glucose is similar to those reported for other glucosyltransferase from *V. vinifera* (*26*) and *C. ternatea* (*27*).

D-Erythroascorbate and D-Erythroascorbate Glucoside Production from *P. blakesleeanus*. D-EAA and D-EAAG accumulated during the exponential and stationary phases of mycelial growth, respectively, when *P. blakesleeanus* was grown on 2% (w/v) glucose as carbon source (*2*). We have determined the accumulation of D-EAA and D-EAAG in mycelium of *P. blakesleeanus* when it was grown in liquid minimal medium with the following carbon sources: 2% (w/v) D-mannose, 2% (w/v) D-galactose, 2% (w/v) D-fructose, 1.8% (w/v) L-arabinose, 2% (w/v) sucrose, 2% (w/v) maltose, 2% (w/v) D-xylose, 2% (w/v) D-mannitol, and 2% (w/v) D-sorbitol. On the other hand, *P. blakesleeanus* did not grow with 2% (w/v) L-xylose, 1.8% (w/v) D-arabinose, 2% (w/v) L-gulono-1,4-lactone, 2% (w/v) L-galactono-1,4-lactone, or 1.8% (w/v) L-fucose, as the carbon source. Thus, the effect of these sugars on the accumulation of D-EAA and D-EAAG was determined from *P. blakesleeanus* cultures obtained with 2% (w/v) D-glucose plus 10 mM of each of them individually added at the onset of growth. In all cases, asparagine (13 mM) was the nitrogen source. The effect of all these carbon sources on the accumulation of D-EAA and D-EAAG was determined in 24- and 48-h-old mycelia of *P. blakesleeanus* corresponding to the second half of the exponential growth phase and the onset of the stationary growth phase, respectively, when 2% (w/v) D-glucose was the carbon source.

Table 3 shows the contents of D-EAA and D-EAAG in *P. blakesleeanus* growing on the different carbon sources used in this study under the conditions indicated above. D-EAA and D-EAAG were detected in 24-h-old mycelia under all conditions. With respect to 48-h-old mycelia, D-EAA was not detected in L-arabinose, D-xylose, D-mannitol, sucrose, and D-sorbitol cultures, and D-EAAG was not detected in D-mannitol cultures. It is noticeable that D-EAA accumulates 10- 13-, and 30-fold, respectively, in comparison with D-glucose cultures in 48-h-old mycelia grown with D-fructose, D-arabinose, and D-galactose. The cultures on D-galactose and D-fructose showed a larger lag in comparison with the D-glucose cultures. Thus, at 48 h, the D-galactose cultures are at a stage of growth that could be equivalent to accumulation on glucose cultures at 24 h of growth. The production of D-EAA and D-EAAG when *P. blakesleeanus* grew on L-arabinose as carbon source may be due to the fact that L-arabinose catabolism is closely linked to the D-xylose pathway in filamentous fungi (*28*), involving a series of redox transformations from which L-arabinose is converted into L-xylulose, which is reduced to xylitol and which can be oxidized to D-xylose by a xylose reductase. This hypothesis is supported by the higher accumulation of D-EAA in *P. blakesleeanus* grown with D-xylose.

Table 4. Effect of D-Arabinose and Light on the Maximal Mycelial Content of D-EAA and D-EAAG

growth conditions	μg/g of mycelial dry weight[a]	
	D-EAA	D-EAAG
D-glucose[b]	99.5 ± 5.8	1084.2 ± 40.3
D-glucose in light[c]	125.4 ± 1.4	3755.0 ± 221
+D-arabinose[d]	2664.5 ± 15.7	3997.0 ± 758
+D-arabinose[e]	3180.0 ± 90.0	4683.0 ± 586

[a] Values are the mean of at least two experiments in duplicate ± standard deviation of the mean. [b] Growth on D-glucose 2% (w/v) in the dark as the control. [c] Growth on D-glucose 2% (w/v) in white light (0.5 W/m^2). [d] After 60 h of growth on D-glucose 2% (w/v) in the dark, 10 mM D-arabinose was added to the culture following growth under the same conditions for almost 36 h. [e] After 60 h of growth on D-glucose 2% (w/v) in light, 10 mM D-arabinose was added to the culture following growth under the same conditions for almost 36 h.

Table 5. Maximal Accumulation of D-EAA and D-EAAG in the Culture Medium When *P. blakesleeanus* Grows under the Described Conditions[a]

growth conditions	culture medium content (μg/mL)		mycelial content (μg/mL)	
	D-EAA	D-EAAG	D-EAA	D-EAAG
D-glucose[b]	0.45	1.80	0.52	52.22
+D-arabinose[c]	43.10	13.04	6.12	281.50
+D-arabino-1,4-lactone[c]	102.1	15.25	57.0	272.61

[a] D-EAA and D-EAAG were determined by HPLC as described under Materials and Methods. For comparison, the mycelial content was expressed in μg/mL by assuming a water content of 90% for *Phycomyces* sporangiophore (*32*). [b] 48 h of growth on D-glucose 2% (w/v) in the dark. [c] After 12 h of growth on D-glucose 2% (w/v) in light, 10 mM D-arabinose or 10 mM D-arabino-1,4-lactone was added, and the cultures were kept under these conditions for another 36 h.

The maximal amount of D-EAA present in glucose-grown mycelia at 30 h of growth of *P. blakesleeanus* in darkness (100 ± 5.8 μg/g of mycelial dry weight) is much higher than that reported for *S. cerevisiae* (1.67 ± 0.26 μg/g of dry weight) (*5*) and for mycelium of *S. sclerotiorium* (< 40 μg/g of dry weight) (*29*). The presence of 10 mM D-arabinose increases the amount of D-EAA produced by *P. blakesleeanus* (about 120 μg/g of mycelial dry weight). This value is close to the amount of D-EAA produced by the functional expression of *S. cerevisiae* D-arabinono-1,4-lactone oxidase in *E. coli* (178 μg/g of wet cells) (*30*) and lower than *S. cerevisiae* grown in the presence of D-arabinose (200.8 ± 21.2 μg/g of dry weight) (*5*). However, *P. blakesleeanus* also produced D-EAAG, and thus the total amount of both compounds exceeded the content reported for *S. cerevisiae* by about 8-fold.

The presence of D-arabinose (10 mM) at the onset of growth increases the amounts of D-EAA and D-EAAG present in the cultures (**Table 3**), and because only D-EAA and D-EAAG are accumulated in these conditions (*2*), we analyzed the D-arabinose effect when it was added at 12, 24, 48, and 60 h of growth on 2% (w/v) D-glucose after following the incubation for almost 36 h. In general, the addition of D-arabinose at the different times of glucose culture appears to have a greater effect on the level of D-EAA, the maximum being reached when D-arabinose is added at the stationary growth phase. **Table 4** shows the maximum levels of D-EAA and D-EAAG obtained in the presence of 10 mM D-arabinose. The maximum content of D-EAA increases 27−32-fold in comparison with that obtained in D-glucose culture as the control, whereas the maximum D-EAAG level was only about 4-fold that of the control culture. It appears to be evident that the synthesis of D-EAAG is more dependent on the glucosyltransferase activity responsible for the glucosylation of D-EAA, whereas the synthesis of the latter is naturally more influenced by the presence of D-arabinose, which is the substrate for D-arabinose dehydrogenase that produces the immediate precursor of D-EAA, D-arabino-1,4-lactone. The addition of D-arabino-1,4-lactone (10 mM) to the culture medium did not have a significant effect on the maximum levels of either compound. *P. blakesleeanus* shows responses to the light that involve changes in metabolism, growth, and positive phototropism (*31*). Thus, we tested the effect of light on D-EAA and D-EAAG production in the absence or presence of D-arabinose in the culture medium. The presence of light, in the absence or presence of D-arabinose, resulted in a slight increase in the maximum levels of D-EAA and D-EAAG (**Table 4**).

We also tested the effect of the nitrogen source on D-EAA and D-EAAG production when asparagine was replaced by the following compounds in *P. blakesleeanus* cultures: glycine, alanine, glutamic acid, aspartic acid, leucine, and ammonium chloride, all at 26 mM, arginine (9 mM), and urea (13 mM) plus Zn^{2+} (10^{-4} M). D-EAA accumulation was maximal at the onset of the exponential growth phase (18 h) with all nitrogen sources studied, except glycine and leucine. In both cases *P. blakesleeanus* cultures showed a large lag in the growth curve (2 days for glycine), and this could be the reason of no detection of D-EAA in these conditions. The higher accumulation of D-EAA was obtained with urea plus zinc ions with values 8- and 13-fold higher than those obtained from control cultures. It has been reported that *P. blakesleeanus* growth is much better on glucose−urea media when cultures are supplied with 10^{-4} M zinc ions than glucose−asparagine (for revision see ref *32*). In all cases D-EAAG accumulates in the highest amounts at 96 h of growth with values similar to those shown by control cultures with asparagine. These results seem to indicate that D-EAA production was more dependent on the nitrogen source than D-EAAG formation and reaffirm the association between mycelial growth and D-EAA as we have previously reported (*2*).

Finally, we had already reported that *P. blakesleeanus* excretes into D-EAA and D-EAAG growth medium (*2*). **Table 5** shows the maximal accumulation of D-EAA and D-EAAG in the culture medium. Assuming a water content of 90% for *Phycomyces* sporangiophore (*32*), we calculated the mycelial content (expressed in μg/mL) of both compounds for comparison. As can be seen, *P. blakesleeanus* excreted D-EAA and D-EAAG into the culture medium, although it is clear that the mycelial content of D-EAAG is much greater than that excreted into the culture medium, whereas the content of D-EAA is higher in the culture medium than inside the mycelium, except in the control on D-glucose. The addition of D-arabinose or D-arabino-1,4-lactone to the culture medium increased mycelial content of D-EAA with a parallel increase in its content in the culture medium, but the increase in mycelial D-EAAG observed under the same conditions is not accompanied by a parallel increase in D-EAAG into the culture medium. Specific transport systems for ascorbate and dehydroascorbate in animal, human, and plant cells has been described (*33*, *34*), indicating that simple diffusion plays only a very minor role. Therefore, assuming the existence of a similar transport system in *P. blakesleeanus*, it seems clear that it prefers D-EAA over D-EAAG. High-affinity transporters have been described for both ascorbate and dehydroascorbate (*33*, *34*), suggesting that they potentially regulate the level and redox status in the cell. Obviously, more studies are needed to establish the existence of a similar transport system in *P. blakesleeanus*, which was responsible for the excretion of both D-EAA and D-EAAG into the culture medium.

Evaluation of the Antioxidative and Antimicrobial Activities of D-Erythroascorbate and D-Erythroascorbate Glucoside. We determined the antioxidant activities of D-EAA and D-EAAG in order to discuss the possibility of their being used as antioxidants. As it is well-known that the numerous methods available for assessing

Table 6. Antioxidant Activity of D-EAA, D-EAAG, and Standard Antioxidants Measured by DPPH, ABTS, and FRAP Assays

	μM Trolox equivalent		
antioxidant	DPPH	ABTS	FRAP
D-EAA	2.80	1.80	2.50
D-EAAG	4.91	0.93	2.50
L-AA	1.00	1.17	1.12
gallic acid	4.80	3.73	2.09
resveratrol	1.05	3.25	0.30
BHT	1.15	0.69	0.30
BHA	1.48	1.12	1.11

Table 7. MIC[a] Values of D-EAA, D-EAAG, and Other Selected Antioxidants

		MIC (μg/mL)		
compound	bacterium	range	MIC_{50}	MIC_{90}
BHA	*S. aureus*	200−300	200	280
	E. coli	1600−6400	3200	6400
gallic acid	*S. aureus*	87.5−1600	125	1600
	E. coli	3200−6400	4800	6400
ascorbic acid	*S. aureus*	1600−2400	1600	2,400
	E. coli	6400	6400	6400
D-EAA	*S. aureus*	6400	6400	6400
	E. coli	6400	6400	6,400
D-EAAG	*S. aureus*	1600−>6400	1600	>6400
	E. coli	>6400	>6,400	>6,400

[a] MIC is minimal inhibitory concentration.

antioxidant activity have different sensitivities, we adopted three assays that include two radical scavenging assays (ABTS and DPPH) and a FRAP assay to monitor the ferric reducing power. As a comparison, we also analyzed the antioxidant activities of three natural antioxidants (ascorbic acid, gallic acid, and resveratrol) and two synthetic antioxidants (BHT and BHA). The results of the antioxidant activity obtained for D-EAA, D-EAAG, and standard antioxidants are summarized in **Table 6**. As can be seen, the three assays gave different values for all antioxidants tested, but in any case, D-EAA showed higher antioxidant capacity than ascorbic acid by any of the three test methods, whereas D-EAAG showed higher antioxidant capacity than ascorbic acid when tested by the DPPH and FRAP methods and slightly lower capacity than ascorbic acid when measured by the ABTS method. D-EAA and D-EAAG have identical antioxidant capacities by the FRAP method, whereas the antioxidant capacity of D-EAA is twice that of D-EAAG by the ABTS method and the reverse happens using the DPPH method. Pearson's correlation coefficients among all antioxidant assays were calculated. The three types of tests are positively correlated, correlation between FRAP and DPPH values being the strongest (coefficient = 0.820). We believe that these results open a promising way for the possible use of D-EAA and D-EAAG as antioxidants in the food, pharmaceutical, and cosmetics industries.

In addition, 10 strains of *S. aureus* and 10 strains of *E. coli* were used in this study to test the possible antimicrobial activities of D-EAA and D-EAAG. The antimicrobial effect produced by BHA, gallic acid, and L-ascorbic acid for comparison is included in the analysis. We determined the MIC ranges, the mean value of MICs at which 50% of strains of *S. aureus* and *E. coli* were inhibited (MIC_{50} values), and the mean value of MICs at which 90% of these strains were inhibited (MIC_{90} values) (**Table 7**). D-EAA and D-EAAG showed MIC values similar to those of ascorbic acid for inhibiting the growth of *E. coli* (6400 μg/mL), but higher than those of L-ascorbic acid for *S. aureus*. In general, MIC values for these three compounds are higher than those of gallic acid and BHA against *S. aureus* (14−32 times), whereas they are virtually unchanged for *E. coli*, and could therefore be used as substitutes for BHA and gallic acid in inhibiting the growth of Gram-negative bacteria. The MIC_{50} values for L-AA, D-EAA, and D-EAAG were identical to those of MIC_{90} for *E. coli* strains against these compounds. However, they are less effective against *S. aureus* than BHA and gallic acid. Obviously, further extensive work should be performed to establish precisely the antimicrobial potency of D-EAA and D-EAAG.

ABBREVIATIONS USED

D-EAA, D-erythroascorbate; D-EAAG, D-erythroascorbate glucoside; L-AA, L-ascorbate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2′,diphenyl-1-picrylhydrazyl; TPTZ, 2,4,6-tris-2,4,6-tripyridyl-2-trizine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; AA-6G, 6-*O*-α-D-glucopyranosyl-L-ascorbic acid; MIC, minimal inhibitory concentration.

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