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5-*O*-(α-D-GALACTOPYRANOSYL)-D-*GLYCERO*-PENT-2-ENONO-1,4-LACTONE: CHARACTERIZATION IN THE OXALATE-PRODUCING FUNGUS, *SCLEROTINIA SCLEROTIORUM*

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Key Word Index—D-erythroascorbic acid; 5-*O*-(α-D-galactopyranosyl)-D-erythroascorbic acid; oxalic acid biosynthesis; sclerotia; *Sclerotinia sclerotiorum*.

Abstract—Extracts of sclerotia from *Sclerotinia sclerotiorum*, a fungal phytopathogen, contain two electrochemically-active constituents, D-*glycero*-pent-2-enono-1,4-lactone (trivial name: D-erythroascorbic acid), and a previously unidentified compound, here characterized as 5-*O*-(α-D-galactopyranosyl)-D-*glycero*-pent-2-enono-1,4-lactone on the basis of its physical and chemical properties and its two hydrolytic products, D-galactose and D-erythroascorbic acid. Treatment of this galactoside with alkaline hydrogen peroxide produces oxalic acid as observed earlier with erythroascorbic acid.

INTRODUCTION

The biosynthetic origin of oxalic acid in plants and fungi has been ascribed variously to isocitrate, oxaloacetate, glycolate/glyoxalate and L-ascorbic acid [1]. In *Sclerotinia sclerotiorum*, a phytopathogenic fungus, evidence has been presented to show that D-*glycero*-pent-2-enono-1,4-lactone (trivial name, D-erythroascorbic acid), an electrochemically-reactive analog of L-ascorbic acid, also functions as a precursor of oxalic acid [2]. Murakawa *et al.* found D-erythroascorbic acid in *Candida* species and proposed a pathway: D-arabinose → D-arabinono-1,5-lactone → D-arabinono-1,4-lactone → D-erythroascorbic acid [3]. The final enzymatic step in this process has been characterized [4].

Soluble extracts of *S. sclerotiorum* contain a second electrochemically reactive compound which readily separates from erythroascorbic acid by HPLC on an anion-exchange resin column. This compound, previously described as unknown No. 1 (unk1) [2], has chemical and biochemical properties resembling L-ascorbic acid and D-erythroascorbic acid. The present paper describes the purification and characterization of this compound and examines its potential role as a precursor of oxalic acid.

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RESULTS AND DISCUSSION

*Separation of unk*1 *and erythroascorbic acid*

Both compounds were recovered from aqueous extracts of sclerotia, a condensed hyphal form of *S. sclerotiorum* [5], and were separated by anion chromatography [2]. Pooled fractions were characterized by HPLC using amperometric detection. Unk1 and erythroascorbic acid eluted at 8.5 and 11.6 min, respectively, under selected conditions.

*Analysis of unk*1 *and its comparison to erythroascorbic acid*

Both compounds produced infrared spectra with similar characteristic absorption peaks at approximately 1685 and 1760 cm^{-1}, closely matching the lactone double bond ($\nu_{C=C}$) and carbonyl-based ($\nu_{C=O}$) stretching assignments of erythroascorbic acid at 1660 and 1740 cm^{-1}, respectively [6]. Similarities between absorption spectra of erythroascorbic acid and unk1 suggested that unk1, like erythroascorbic acid, possessed the unsaturated

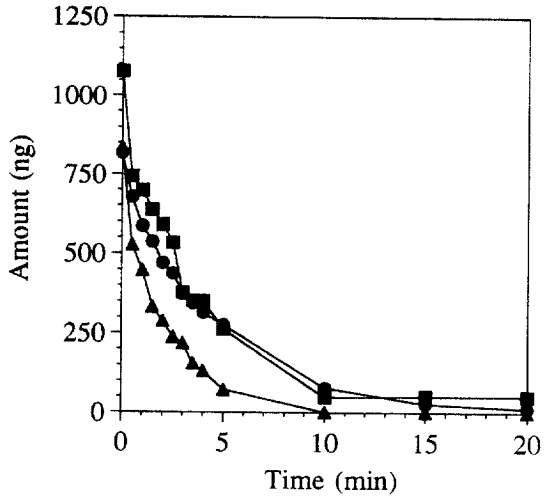


Fig. 1. Oxidation of L-ascorbic acid (▲), D-erythroascorbic acid (●) and unk1 (◆) by ascorbate oxidase (EC 1.10.3.3).

1,4-lactone character of an ascorbic acid analog [6]. The crystal structure of D-erythroascorbic acid has recently been reported as two slightly different molecular orientations in the crystallographic unit as compared to L-ascorbic acid with four similar ones per unit [7].

With ESI-MS analysis, the ion observed at m/z = 331.1 corresponded to M + H of the Na salt of unk1, consistent with the formula $C_{11}H_{15}O_{10}Na$. The ion at m/z 313 was consistent with a loss of water from M + H $[C_{11}H_{13}O_9Na]^+$ and the ion at m/z 348 would correspond to $[M + NH_4]^+$ where M was $C_{11}H_{15}O_{10}Na$. Glucopyranosyl-D-erythroascorbic acid from a Basidiomycete, *Hypsizigus marmoreus*, had a similar protonized molecular peak [8].

Unk1 and D-erythroascorbic acid were readily oxidized by ascorbate oxidase (EC 1.10.3.3) at a rate similar to that of L-ascorbic acid (Fig. 1).

Acid hydrolysis of unk1

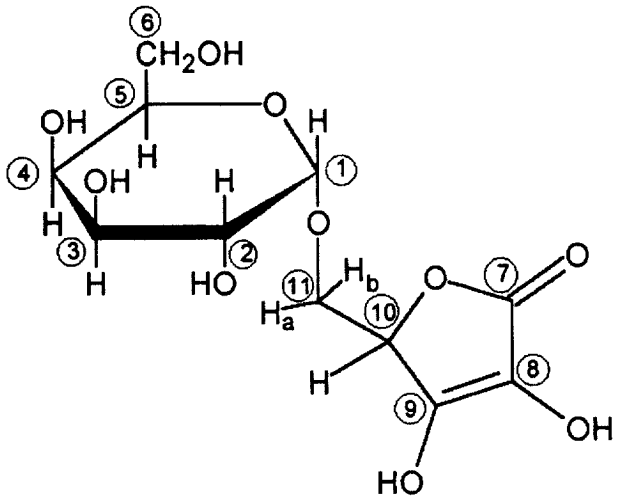
Treatment of unk1 with 1 M HCl at 95° for 1 h released erythroascorbic acid and an unknown sugar. Erythroascorbic acid (retention time, 11.6 min) was separated from the sugar on an anion exchange HPLC column using amperometric detection. The sugar was detected by refractive index and had a retention time (9.6 min), identical to that of galactose. ^{1}H NMR spectra of authentic galactose and the glycosyl moiety from unk1 were virtually identical. ^{1}H NMR of unhydrolyzed unk1 provided evidence that the galactosyl moiety was attached to C5 of the aglycone and that the sugar had an α-D-galactopryanosyl configuration. This was obtained by applying ^{1}H NMR coupling constants data from Fig. 2 to simulations for both aglycone and galactosyl moieties. Simulation of coupling constants data corresponding to the α-D-galactosyl moiety of unk1 clearly superimposed on the galactosyl portion of unhydrolyzed unk1. A similar analysis of the aglycone moiety accounted for the remaining signals in unhydrolyzed unk1.

A detailed description of NMR experiments used to establish the complete structure of unk1 follows: ^{13}C NMR (D_2O) showed the presence of 11 carbons, of which 3 were quaternary carbons appearing at 175.8, 158.5 and 119.9 ppm suggesting the presence of an α–β unsaturated ester or amide functionality. DEPT analysis [9] of the protonated carbons showed 6 methine carbons and 2 methylene carbons at 63.3 and 100.8 ppm. The chemical shifts of the sp3 carbons suggested that all were attached to heteroatoms with one likely being an acetal carbon (100.8 ppm).

^{1}H NMR spectrum (D_2O) was complicated by an unusually narrow chemical shift dispersion with only 2 protons being well resolved at 4.87 and 4.83 ppm. The spectrum was further complicated by small coupling constants making proton–proton correlation spectroscopy difficult. Two separate spin systems could be delineated via ^{1}H–^{1}H COSY and ^{1}H–^{1}H TOCSY experiments [10]. One of the spin systems involved the proton at 4.87 (dd, J = 2.5, 4.1) and two protons at 3.89 and 3.83 ppm. The second spin system involved the proton at 4.83 ppm (broad singlet) and the protons at 3.67 ppm (broad singlet), 3.68 ppm (broad singlet), 3.85 ppm (broad singlet), 3.77 (broad triplet), and two apparent singlets at 3.59 and 3.60 ppm. The protonated carbons were correlated to the directly attached protons via an HMQC experiment [11] which showed the protons at 3.59 and 3.60 ppm as well as 3.83 and 3.89 ppm were geminal methylene protons.

The NMR data seemed to suggest a glycoside in which the sugar was a hexose and the aglycone consisted of a $C_5H_5O_4$ fragment. Careful analysis of ^{1}H–^{13}C HMBC data [12] collected at long range J_n values of 7 and 4 Hz gave the structure shown in Fig. 2 which was consistent with the ^{1}H–^{1}H correlation data and the one bond ^{1}H–^{13}C correlation data. Strong HMBC correlations at both long range J values between the methylene protons on C11 and the anomeric carbon at 100.8 ppm, as well as the correlation between the anomeric proton at 4.83 ppm and the carbon at 67.3 ppm, unambiguously established the linkage between the sugar and the aglycone at C1 and C11. Comparison of the ^{13}C spectrum of synthetically-produced erythroascorbic acid [6] with the chemical shifts of the aglycone showed excellent agreement with the exception of C11 of the natural product which showed a downfield shift of 5.6 ppm relative to the hydroxymethyl carbon of erythroascorbic acid. The downfield shift provides further evidence of the attachment of the erythroascorbic acid moiety to the sugar at C11.

The identity of the sugar as galactose came primarily from the hydrolysis of the natural product and subsequent isolation of the sugar. ^{1}H NMR



Carbon Atom	^{13}C Chemical Shift, ppm	^{1}H Chemical Shift,* ppm	Coupling Constants, Hz	Pertinent HMBC, carbon no.
1	100.78	4.83 br s	$J_{1,2} = 1.3$	
2	71.65	3.67 br s	$J_{2,3} = 8.0$, $J_{2,1} = 1.3$	
3	70.55	3.68 br s	$J_{3,2} = 8.0$, $J_{3,4} = 1.5$	
4	71.50	3.85 br s	$J_{4,5} = 1.5$, $J_{4,3} = 1.5$	5
5	73.36	3.77 br t	$J_{5,6a} = 6.0$, $J_{5,6b} = 6.3$, $J_{5,4} = 1.5$	4, 3
6	63.33	H_{6a} 3.60 H_{6b} 3.59	$J_{6a,6b} = -10.5$, $J_{6a,5} = 6.0$ $J_{6b,6a} = -10.5$, $J_{6b,5} = 6.3$	5, 4 5, 4
7	175.79			
8	119.87			
9	158.51			
10	78.34	4.87 dd	$J_{10,11a} = 2.5$, $J_{10,11b} = 4.1$	11, 7, 9, 8
11	67.28	H_{11a} 3.89 dd H_{11b} 3.83 dd	$J_{11a,10} = 2.5$, $J_{11a,11b} = 11.8$ $J_{11b,10} = 4.1$, $J_{11a,11b} = 11.8$	10, 9, 1 9

* br s = broad singlet, dd = doublet of doublet, br t = broad triplet

Fig. 2. Structure of unk1 as (A) 5-*O*-(α-D-galactopyranosyl)-D-*glycero*-pent-enono-1,4-lactone and (B) its chemical shifts and coupling constants analyses from NMR data of unk1.

spectra of the isolated sugar fraction were compared with commercial galactose which had been treated in accordance with the isolation protocol (see Experimental) and were found to be superimposable. It is interesting that upon formation of the glycoside with erythroascorbic acid the chemical shifts of galactose protons become less dispersed. This lack of dispersion causes the proton spectrum to become very strongly coupled necessitating second order analysis of the J values and chemical shifts. Simulation of the galactose spin system in an iterative fashion starting with values of chemical shifts derived from the HMQC data and J values from single frequency decoupling studies yielded a set of chemical shift and J values which was found to be a suitable match to the ^{1}H spectrum of the sugar portion of the natural product.

The small coupling constant ($J_{1,2} = 1.3$ Hz) between H1 and H2 of the galactose moiety was clearly indicative of an α anomeric configuration for the glycoside. Further evidence for the α configuration was found in the NOE difference spectrum upon irradiation of the anomeric proton to give a strong positive enhancement of the H2 proton. A lesser enhancement of the H11a proton of the erythroascorbic acid moiety was taken as further evidence of the attachment of erythroascorbic acid to the anomeric position of the sugar via the oxygen at the C11 position. Irradiation of the H5 proton of the galactose moiety gave clear positive NOE's to

the H4, H3 and H6a protons establishing the axial nature of the oxygen at C4 (i.e. H5 and H4 as well as H4 and H3 are *syn*). Irradiation of the degenerate H2 and H3 protons yielded strong enhancements to the H1 and H4 protons as well as a weak enhancement of the H5 proton.

Based on these results, unk1 was assigned the structure 5-*O*-(α-D-galactopyranosyl)-D-*glycero*-2-enono-1,4-lactone (Fig. 2).

Alkaline oxidation of 5-O-(α-D-*galactopyranosyl*)-D-*erythroascorbic acid by* H_2O_2

Ascorbic acid is oxidatively cleaved to oxalic acid and L-threonic acid by alkaline H_2O_2 [13–15]. Erythroascorbic acid also undergoes this peroxygenative process to yield oxalic acid and D-glyceric acid [2]. Treatment of 5-*O*-(α-D-galactopyranosyl)-D-erythroascorbic acid with alkaline H_2O_2 produced oxalic acid which was recovered from the reaction and characterized by HPLC with ion conductivity detection (data not given). The nature of the second product was not examined.

The possibility that oxalic acid biosynthesis in *S. sclerotiorum* is analogous to ascorbic acid-derived oxalic acid formation in higher plants [16–18] emerged from the discovery that ascorbic acid is rare or absent in yeasts and fungi. An analog, erythroascorbic acid, is present and can be peroxygenatively cleaved to yield oxalic acid [2]. *S. sclerotiorum* contains not only D-erythroascorbic acid but also 5-*O*-(α-D-galactopyranosyl)-D-erythroascorbic acid and both are potential substrates for oxalic acid production. In fact, glycosides of ascorbic acid analogs occur in some fungi lacking free erythroascorbic acid [8, 19]. This raises an interesting question regarding the functional form of the ascorbic acid analog involved in oxalic acid production in fungi. Is it a glycoside rather than a free ascorbic acid analog? If so, are free ascorbic acid analogs mere by-products released either by hydrolysis of the glycoside during metabolism and/or during the extractive process?

α-Galactosylation of erythroascorbic acid is a potential mechanism for storage, targeting and/or stabilization for transport of this ascorbic acid analog. Sclerotia contain higher concentrations of the galactoside compared to the mycelium of *S. sclerotiorum* [2]. Sclerotia are primary sites of stored energy and they supply nutrients that are required for myceliogenic or carpogenic germination as well as for post-germinative maintenance of this multihyphal structure [5]. Our discovery that the sugar residue involved in glycosylation is D-galactose may have profound significance as regards desiccation tolerance, targeting and transport. In higher plants, α-galactosides serve such roles quite effectively [20, 21].

Attachment of the galactosyl residue to C5 of erythroascorbic acid may also bear on the functional role of this glycoside. Unlike substitution at C2, as in L-ascorbic acid 2-*O*-α-glucoside [22] which renders the glycoside non-reducing, the fungal galactoside retains its characteristic reducing properties as well as its stability.

EXPERIMENTAL

Preparation of sclerotial extracts of S. sclerotiorum

Sclerotia were grown in quantity using a bulk culture method [23]. Following harvest, sclerotia were washed ×6 with sterile distilled H_2O, dried in a laminar flow hood, lyophilized and stored at −20°. Two bulk culture preparations provided 60 g of lyophilized sclerotia. Sclerotial extracts were prepared by grinding 10 g of lyophilized sclerotia in an electric coffee mill and suspending the powder in 50 ml of 5 mM dithiothreitol which contained 0.5% polyvinylpolypyrrolidone. Insoluble material was removed by centrifugation (17000*g*, 1 h, 4°). Soluble proteins were removed by diluting the supernatant with 4 volumes of cold MeOH and centrifuging to pellet insoluble material. MeOH was removed under reduced pressure at 30° and the extract was resuspended to its original volume of 50 ml with 5 mM dithiothreitol. This process was repeated until all 60 g of sclerotia had been extracted.

Separation of unk1 and erythroascorbic acid

The processed sclerotial extract was run through tandem ion exchange columns (1 × 14 cm) of Dowex 50 × 8 H^+, 200–400 mesh and Dowex 1 × 8 (formate), 200–400 mesh. Unk1 and erythroascorbic acid bound to the latter and were eluted with a formic acid gradient following a slightly modified earlier procedure [24]. Compounds of interest were detected at 254 nm. Pooled peak fractions were further characterized on a Bio-Rad HPX-87H anion HPLC exchange column (250 × 4.6 cm) using 4 mM H_2SO_4 as mobile phase at 0.6 ml/min, 35°, with amperometric (0.6 V) and UV_{210} nm detection. 5-*O*-(α-D-Galactopyranosyl)-D-erythroascorbic acid and erythroascorbic acid had retention times of 8.5 and 11.6 min, respectively. Separated components were lyophilized, resuspended in 5 mM dithiothreitol at a concentration of approximately 10 mg/ml and again run through the ion exchange resin procedure as a final means of purification. Pooled fractions corresponding to unk1 and erythroascorbic acid were assayed by HPLC, lyophilized, resuspended at a concentration of approximately 0.7 mg ml^{-1} and distributed into vials which were flash-frozen at −70°, lyophilized and stored at −20°.

Oxidation by ascorbate oxidase

Ten units of ascorbate oxidase (Sigma Chem. Co.) was added to 500 μg of substrate (L-ascorbic acid, D-erythroascorbic acid or unk1) in 0.1 M

citrate phosphate buffer, pH 5.6, to a total volume of 700 μl. Aliquots (25 μl) were removed at selected intervals and the reaction was terminated by addition of 50 μl of 30% CF_3COOH. Each sample was filter-centrifuged (0.45 μm), further diluted to a final volume of 1 ml with distilled H_2O and assayed by HPLC using amperometric detection.

Mass spectral analysis

Samples were run by infusion at 3 μl min^{-1} in a solvent mixture of MeOH:H_2O:HOAc (0.1%) on a Finnigan LCQ by positive ion electrospray (ESI-MS).

NMR spectral analysis

NMR spectra were obtained on a Bruker AMX 300 spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C and on a Varian Inova 500 spectrometer operating at 499.8 and 125.7 MHz for ^1H and ^{13}C, respectively. ^1H chemical shifts were referenced to residual HDO that was assigned to 4.65 ppm at 25°. ^{13}C chemical shifts were referenced to external p-dioxane that was assigned a chemical shift of 66.8 ppm. The sample of the natural product consisted of 2 mg of the glycoside dissolved in 250 μl of D_2O containing 0.7% v/v CD_3COOD which was placed in a microcell NMR tube which was constructed of glass matched in magnetic susceptibility to water (Shigemi). DEPT, ^1H–^1H COSY, DQFCOSY, TOCSY, ^1H–^{13}C HMQC and HMBC spectra were all taken using standard pulse programs. Two different HMBC data sets were collected utilizing long range J_{CH} values of 4 and 7 Hz.

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REFERENCES

1. Franceschi, V. R. and Loewus, F. A., in *Calcium Oxalate in Biological Systems*, ed. S. R. Khan. CRC Press, Boca Raton, FL, 1995, pp. 113–130.
2. Loewus, F. A., Saito, K., Suto, R. K. and Maring, E., *Biochemical and Biophysical Research Communications*, 1995, **212,** 196–203.
3. Murakawa, S., Sano, S., Yamashita, H. and Takahashi, T., *Agricultural and Biological Chemistry*, 1977, **41,** 1799–1800.
4. Huh, W.-K., Kim, S.-t., Yang, K.-S., Seok, Y.-J., Hah, Y. C. and Kang, S.-O., *European Journal of Biochemistry*, 1994, **225,** 1073–1079.
5. Willetts, H. J. and Bullock, S., *Mycological Research*, 1992, **96,** 801–816.
6. Shao, Y.-Y., Seib, P. A., Kramer, K. J. and Galen, D. A., *Journal for Agricultural and Food Chemistry*, 1993, **41,** 1391–1396.
7. Wang, X.-Y., Seib, P. A., Paulstelis, J. V., Seib, L. L. and Takusagawa, F., *Journal of Carbohydrate Chemistry*, 1995, **14,** 1257–1263.
8. Okamura, M., *Journal of Nutritional Science and Vitaminology*, 1994, **40,** 81–94.
9. Bendall, M. R., Dodrell, D. M. and Pegg, D. T., *Journal of the American Chemical Society*, 1981, **103,** 4603–4605.
10. Bax, A. and Freeman, R., *Journal of Magnetic Resonance*, 1981, **44,** 542–561.
11. Bax, A. and Subramanian, S., *Journal of Magnetic Resonance*, 1986, **67,** 565–569.
12. Bax, A. and Summers, M. F., *Journal of the American Chemical Society*, 1986, **108,** 2094–2096.
13. Herbert, R. W., Hirst, E. L., Percival, G. V., Reynolds, R. J. W. and Smith, F., *Journal of the Chemical Society*, 1933, 1270–1289.
14. Isbell, H. S. and Frush, H. L., *Carbohydrate Research*, 1979, **72,** 301–304.
15. Kwon, B.-M., Foote, C. S. and Khan, S. I., *Journal of the American Chemical Society*, 1989, **111,** 1854–1860.
16. Saito, K. and Loewus, F. A., *Phytochemistry*, 1992, **31,** 3341–3344.
17. Saito, K., *Phytochemistry*, 1996, **41,** 145–149.
18. Saito, K., Ohmoto, J. and Kuriha, N., *Phytochemistry*, 1997, **44,** 805–809.
19. Okamura, M., *Journal of Nutritional Science and Vitaminology*, 1998, **44,** 25–35.
20. Obendorf, R. L., *Seed Science Research*, 1997, **7,** 63–74.
21. Bachmann, M. and Keller, F., *Plant Physiology*, 1995, **109,** 991–998.
22. Murakami, K., Muto, N., Fukazawa, K. and Yamamoto, I., *Biochemical Pharmacology*, 1992, **44,** 2191–2197.
23. Nelson, B., Duval, D. and Wu, H.-I., *Phytopathology*, 1988, **78,** 1470–1472.
24. Wagner, G. and Loewus, F. A., *Plant Physiology*, 1973, **52,** 651–654.