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METABOLISM OF 4-HYDROXYBENZALDEHYDE, 3-BROMO-4-HYDROXYBENZALDEHYDE AND BROMIDE BY CELL-FREE FRACTIONS OF THE MARINE RED ALGA *ODONTHALIA FLOCCOSA*

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Key Word Index—*Odonthalia floccosa*; Rhodomelaceae; red alga; biosynthesis; bromophenols; lanosol.

Abstract—Cell-free fractions from *Odonthalia floccosa* incubated with 4-hydroxybenzaldehyde-[U-^{14}C], 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] and $^{82}Br^-$ formed the dibromo-dihydroxybenzaldehyde derivatives of the bromophenols (brominated benzylalcohols) which were also identified as naturally occurring products.

INTRODUCTION

Members of the family Rhodomelaceae contain a variety of simple bromophenols, of which lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) is the most commonly encountered. *Odonthalia floccosa*, a member of this family, is known to contain lanosol [1, 2]. It has been suggested that lanosol and its aldehyde are artifacts of isolation derived from dipotassium 2,3-dibromo-5-hydroxybenzyl-1',4-disulfate [3]. The form in which bromophenols occur in algae is not known although they may exist as both the free phenol and sulfate ester [4].

It has been demonstrated previously [5, 6] that L-tyrosine is metabolized in *O. floccosa* to 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde through a series of intermediates and it has been proposed that lanosol is synthesized by such a pathway.

This study is an attempt to determine if 4-hydroxybenzaldehyde is an intermediate in the formation of bromophenols from tyrosine. Thus a series of cell-free incubations using ^{14}C-labeled 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzaldehyde, and $^{82}Br^-$ were utilized. In this paper we report the identification of 3 bromophenols (as benzylalcohols) and the formation of their aldehydes from 4-hydroxybenzaldehyde.

RESULTS AND DISCUSSION

Identification of bromophenols

GLC of a silylated sample of authentic lanosol gave two peaks, one of which had a MS corresponding to lanosol–TMSi; the other minor peak did not contain bromine. TLC of lanosol revealed more spots than GLC peaks of the same compound. The following bromophenols were recovered from *O. floccosa* extracts by GLC and were identified by the MS of their TMSi derivatives through a comparison with published data and authentic lanosol–TMSi: lanosol–TMSi (MW = 512), R_t = 32 min; lanosol, methylether–TMSi (MW = 454), R_t = 30.4 min; 3-bromo-4,5-dihydroxybenzyl alcohol–TMSi (MW = 434), R_t = 23.26 min; 3,5-dibromo-4-hydroxybenzyl alcohol-TMSi (MW = 424), R_t = 24.2 min. No other brominated phenols or simple phenolic compounds were detected. Lanosol, which was also identified from a two-dimensional TLC of the acidified extract, is the major bromophenol of *O. floccosa*. 3-Bromo-4,5-dihydroxybenzyl alcohol and 3,5-dibromo-4-hydroxybenzyl alcohol are present in concentrations less than 5% (based on GC–MS) of those of lanosol and are natural products, while the methylether of lanosol is an artifact of isolation [7].

Identification of intermediates from incubations

The R_f values for the various compounds chromatographed in the solvent system have been reported previously [5, 6]. Radioactive compounds identified by TLC-autoradiography of the 4-hydroxybenzaldehyde-[U-^{14}C] incubations were 3,5-dibromo-4-hydroxybenzaldehyde, 3-bromo-4-hydroxybenzaldehyde, 4-hydroxybenzaldehyde and a spot in the 4-hydroxybenzoic acid/2,3-dibromo-4,5-dihydroxybenzaldehyde region. The compound is probably 2,3-dibromo-4,5-dihydroxybenzaldehyde since an increase in radioactivity is seen at pH 5.4 in the presence of KBr and H_2O_2 which is indicative of bromoperoxidase [8], the enzyme believed to be responsible for both the ring hydroxylation (pH_{max} 5.4) and bromination (pH_{max} 6.7). Neither protocatechuic acid nor 3-bromo-protocatechuic acid was identified. Incubation mixtures containing H_2O_2/KBr resulted in significantly darker spots than those observed with other incubations indicating greater conversion to the intermediates and showed radioactivity in the region of lanosol (R_f 0.14). Definite identification of lanosol

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could not be made due to poor resolution. Radioactivity migrated with ubiquinone (R_f 0.98) with incubations containing ATP and H_2O_2/KBr.

The radioactive compounds determined by TLC autoradiography of the 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] incubations containing only KBr and H_2O_2 were 3,5-dibromo-4-hydroxybenzaldehyde, 3-bromo-4-hydroxybenzaldehyde and a spot corresponding to the 3-bromo-4-hydroxybenzoic acid/3,5-dibromo-4-hydroxybenzyl alcohol zone. This last spot was detected in all the incubations. No 3-bromoprotocatechuic acid was detected and no significant radioactivity was observed at the origin.

The $^{82}Br^-$ incubations yielded the 3-bromo derivative of the 4-hydroxy substrate. Based on spot densities it appears that the aldehydes are far better substrates for bromination than the alcohol. Incubations at pH 5.4 produced significantly more product than those at pH 7. The incubations (pH 5.4) with 4-hydroxybenzaldehyde as the substrate also yielded 3,5-dibromo-4-hydroxybenzaldehyde. The incubation (pH 5.4) with 3,4-dihydroxybenzaldehyde as the substrate also yielded 2,3-dibromo-4,5-dihydroxybenzaldehyde. Traces of radioactivity were seen (R_f 0.99) in all incubations. Surprisingly, both bromination and hydroxylation of the substrates did not occur in the $^{82}Br^-$ incubations. This is anomalous since one would expect such compounds in the [^{14}C] incubations. It is possible that the presence of ammonium hydroxide, the solvent from $NH_4{}^{82}Br$, inhibited the hydroxylase activity of the bromoperoxidase.

The formation of 3,5-dibromo-4-hydroxybenzyl alcohol could not be reliably determined in either [U-^{14}C] incubations since the compound itself, 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenzoic acid have similar R_f values, 0.60, 0.58, and 0.58, respectively. It is, however, considered likely that this radioactive spot in the 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] incubation is the acid since 3,5-dibromo-4-hydroxybenzyl alcohol was not detected in the 4-hydroxybenzaldehyde-[U-^{14}C] or $^{82}Br^-$ incubations. The formation of 3-bromo-4-hydroxybenzoic acid from L-tyrosine-[U-^{14}C], via 4-hydroxy benzaldehyde, to 4-hydroxybenzoic acid has been detected in planktonic algae [9], whereas cell-free incubations of *O. floccosa* with L-tyrosine-[U-^{14}C] yielded 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] only [6]. The formation of 3-bromo-4-hydroxybenzoic acid in the 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] incubations, while not being identified as a natural product, is unexpected. The identification of the substituted benzaldehydes, especially the aldehyde of lanosol, as products certainly does not support the contention that all bromophenols are artifacts of extraction from corresponding sulfate salts.

The results of the incubations suggest the pathway illustrated in Fig. 1. The aldehydes of the naturally occurring products (benzyl alcohols) were formed but not the alcohols themselves, except perhaps for lanosol. The reduction of the aldehydes to the alcohols is assumed to be a reaction catalysed by an aromatic alcohol dehydrogenase. Such enzymes have been identified and characterized in bacteria [10], fungi [11] and higher plants [12] but not in algae. Four incubations containing NADPH and favoring the activity of an aromatic dehydrogenase did not yield the alcohols. This negative result, however, does not disprove the existence of this enzyme in *Odonthalia* since conditions employed may have inactivated the enzyme.

The formation of the sulfate esters could not be established with the TLC conditions used since standards (lanosol dipotassium disulfate salt) did not migrate. It is possible that the radioactivity detected at the origin represents the sulfate esters. However, the presence of $MgSO_4$, ATP and adenosine 5'-phosphosulfate (APS), the last two compounds being intermediates in the formation of 3'-phosphoadenosine-5'-phosphosulfate (sulfate reduction) which is the substrate for sulfotransferase, did not visibly increase the radioactivity at the origin, nor did it produce any other radioactive spots not present in the other incubations. It is considered unlikely that C-1 sulfate esters were formed since the benzyl alcohols were not detected.

The proposal that bromophenols in red algae are derived from L-tyrosine via 4-hydroxybenzaldehyde is supported by our previous studies [5, 6] and by the identification of 3,5-dibromo-4-hydroxyphenyl pyruvic acid, 3,5-dibromo-4-hydroxyphenyl acetic acid in addition to the more common bromophenols in *Halopitys incurvus* [13], and the isolation of 4-hydroxybenzaldehyde from 2 marine red algae [14, 15] which do not produce bromophenols.

Bromination by cell-free fractions

The results of the various assays are given in Table 1. Similar data have been reported previously [6]. The ability of the various fractions to brominate 4-hydroxybenzaldehyde to 3-bromo-4-hydroxybenzaldehyde is evident and is presented for the first time.

The 100 g fractions contained the majority of the chlorophyll, confirming the presence of chloroplasts [6]. Microscopic examination of this fraction indicated very slight contamination, the nature of which was not determined. No microbial contamination was observed. Bromoperoxidase activity is associated with the 100 g fraction which suggests its location in the chloroplast. Various qualifications must be added to such determinations involving chloroplasts. These include the problems of using small amounts of labeled precursor [16], the possible presence of microsomes and the difficulty in obtaining undamaged organelles from this alga. These qualifications have been previously discussed [6].

Catalase activity (H_2O_2 disappearance) was greatest in the 5000 g fraction with a significant amount of activity in the 39 000 g fraction, suggesting the presence of peroxisomes. Cytochrome *c* oxidase activity is high in both the 39 000 g fraction and supernatant indicating the presence of mitochondria. This activtiy is not believed to be due to an 'ascorbate oxidase' since such a control (ascorbate alone) was performed.

Other evidence supports the proposal that bromophenols are formed in the chloroplast: (1) the formation of 3-bromo-4-hydroxybenzaldehyde from L-tyrosine by chloroplast fractions of *O. floccosa* [6], (2) the isolation of bromophenols from the cyanophycean *Calothrix brevissima* [7], (3) the formation of 4-hydroxyphenylpyruvate from L-tyrosine by an L-amino acid oxidase bound to the thylakoids of the blue–green alga *Anacystis nidulans* [17], (4) the determination of bromoperoxidase as a particulate

Table 1. Analysis of fractions obtained by differential centrifugation

Sample	Chlorophyll	Protein	Catalase activity*		Cytochrome c oxidase activity*		3-Bromo-4-hydroxybenzaldehyde formation from 4-hydroxybenzaldehyde-[U-^{14}C]	
	% of total of homogenate	% of total of homogenate	% of total activity in homogenate	Relative sp. act. (×100)	% of total activity in homogenate	Relative sp. act. (×100)	% of total dpm of homogenate	Relative dpm/mg protein (×100)
100 g	73.9	23.3	10.5	8	5.7	18.0	81.6	100
5000 g	15.5	12.0	66.2	100	7.9	47.8	8.6	20.5
39 000 g	6.1	5.6	23.0	75	6.7	88.2	9.7	50.0
Supernatant	4.5	5.6	0	0	79.8	100	—	—

* 'Relative sp. act.' signifies the sp. act. (as dp/mg protein) relative to the highest value which is designated as 100.

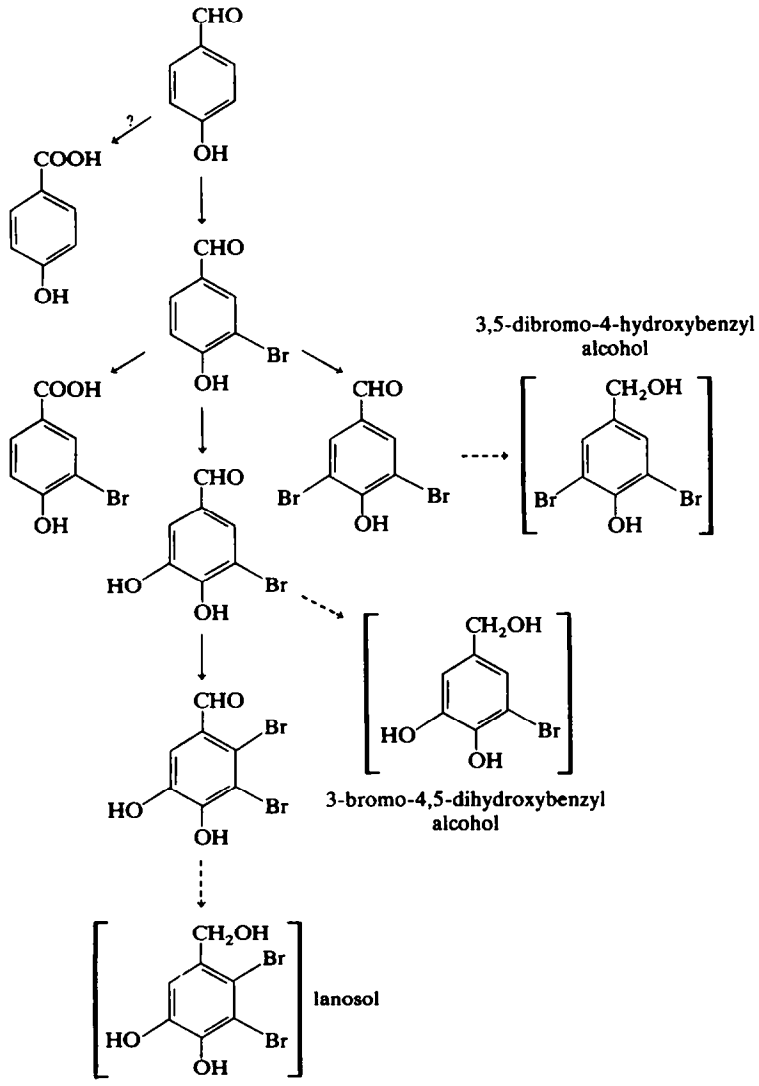

Fig. 1. Metabolism of 4-hydroxybenzaldehyde. Those compounds in brackets are the natural products but were not identified in labeling studies. Question mark represents an inconclusive metabolic step.

enzyme from the red algae *Cystoclonium purpureum* [8] and (5) the localization of bromine in the chloroplasts of *Lenormandia prolifera* [18].

EXPERIMENTAL

Extraction and identification of bromophenols. Cleaned and macerated *O. floccosa* (500 g) was boiled under reflux for 4 hr in 4 l. of 90% MeOH and the process repeated with 4 l. 80% MeOH. The combined MeOH fractions were filtered, concd under vacuum and the aq. soln filtered through Celite which had been prewashed with 2 l. boiling dist. H_2O. Any sulphate esters were cleaved by the addition of HCl to a concn of 0.05 M and boiling for 10 min. The resulting soln was extracted with EtOAc until the EtOAc extract (6. l.) was colorless. The soln was evapd under vacuum to $\frac{2}{3}$ vol., frozen to remove H_2O, dried (Na_2SO_4), filtered and reduced to 100 ml by evapn under vacuum. An aliquot (10 ml) of the soln was evapd to dryness under N_2. The residue was desiccated for 24 hr over P_2O_5 and silylated by the addition of 50 μl MeCN and 50 μl BSTFA with 1% TMCS. The sample (1.5 μl) and a sample of lanosol (also silylated) were subjected to GC–MS with a 2 m silanized glass column packed with 3% SP-2250 on Chromosorb W. Injector port and detector temps. were 250°, with a flow rate (He) of 42 ml/min. R_ts were determined over a temp. program of 100°–240° at 8°/min at 240°. The presence of lanosol in the acidified extract was also determined by 2D-TLC [6]. Lanosol and the bromophenols were quantitated from the GLC trace.

Radioactive chemicals. $NH_4{}^{82}Br$, (New England Nuclear, Boston, MA) in 1 N NH_4OH, sp. act. 2.14 mCi/mg Br, concn 0.96 mCi/ml, with a total Br content of 0.45 mg/ml had a radionuclidic purity of greater than 99%. 4-Hydroxybenzaldehyde-[U-^{14}C] and 3-bromo-4-hydroxybenzaldehyde-[U-^{14}C] were obtained for use from previous L-tyrosine-[U-^{14}C] incubation mixtures [5, 6], which had been stored frozen. These were combined, evaporated under vacuum to dryness, resuspended in 1 ml EtOAc and subjected to TLC in A ($CHCl_3$–EtOH–HOAc, 8:1:1:) [6]. Two bands corresponding to 3-bromo-4-hydroxybenzaldehyde and 4-hydroxybenzaldehyde were isolated, eluted with EtOH, concentrated, and separately rechromatographed in B (toluene–ethyl formate–97% HCO_2H, 5:4:1) [6]. The process was repeated, except that the EtOH solns were not subjected to TLC, to give an activity of 2×10^6 dpm/ml and 6.12×10^4 dpm/ml for 4-hydroxybenzaldehyde and 3-bromo-4-hydroxybenazldehyde, respectively. An aliquot (15 μl) of both solns was chromatographed (system B) and counted for purity and another (50 μl) of 4-hydroxybenazldehyde was silylated and subjected to GC–RC [6]. 4-Hydroxybenzaldehyde-[U-^{14}C] was determined to be greater than 97% pure, while the 3-bromo-4-hydroxybenzaldehyde was determined to be greater than 96% pure. No visible contamination was detected.

Incubations. O. floccosa (Esp.) Falk was homogenized, fractionated and analysed as described previously [6]. The algal material was cut into small pieces, homogenized in a Virtis-45 homogenizer at max speed for 3 min with 10% (w/w) sucrose in 0.02 M glycylglycine buffer, pH 7.5 at 4°, filtered through 4 layers of cheesecloth and subjected to a sequential series of 30 min centrifugations at 100 g, 5000 g and 39 000 g. The pellets from each step were recovered and resuspended in the buffered sucrose.

The incubation mixtures with $^{82}Br^-$ contained (final concn): 4 mM H_2O_2, 2 mM KBr, 0.27 μM $NH_4{}^{82}Br$, 0.01% Triton X-100, 0.02 M 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol or 3,4-dihydroxybenzaldehyde, and 0.02 M KPi buffer pH 5.4 or 7 and 100 μl of a 100 g fraction (final vol. 1 ml). The incubations were carried out for 2 hr at 30° with shaking followed by centrifugation (10 000 g, 10 min). The supernatant (50 μl) was spotted, with suitable cold standards for TLC in system A, after which an autoradiograph was produced through a 24-hr exposure to X-ray film [6].

The following incubation mixtures (final vol. 1 ml) were prepared with 20 μl of Triton X-100 (0.5%) soln, 25 μl of either ^{14}C-labeled compound and 100 μl of the 100 g resuspended pellet (final concn): (a) ATP, neutralized (0.02 M) and $MgSO_4$ (0.02 M) in 0.05 M Tris buffer pH 7; (b) APS (0.02 M), $MgSO_4$ (2 mM) in 0.05 M Tris buffer pH 7; (c) H_2O_2 (2 mM), KBr (2.5 mM) in KPi buffer, 0.02 M pH 5.5 and 7; (d) NADPH (0.05 mM) in KPi buffer, 0.02 M pH 7; (e) ATP, $MgSO_4$, H_2O_2, KBr, NADPH (in the above concns) in Tris buffer pH 7 with 4-hydroxybenzaldehyde-[U-^{14}C] only. After 2 hr at 30° with shaking, the incubations were centrifuged, lyophilized, dissolved in EtOH (75 μl) and subjected to TLC (system A), together with 25 μl of both labeled compounds. The plates were exposed to film for 1 month (autoradiography).

The resuspended pellets from the centrifugations (100 μl) were incubated with 4-hydroxybenzaldehyde-[U-^{14}C] in mixture (c), pH 5.5 for 1 hr with shaking. The reaction was stopped with TCA, centrifuged and lyophilized [6]. EtOH extracts were subjected to TLC in system A [6] with standards. The spots corresponding to 3-bromo-4-hydroxybenzaldehyde were recovered and counted for radioactivity.

Assay of cell-free fractions. Chlorophyll *a* was determined according to the method of ref. [19] and protein using that of ref. [20]. The possibility of phenolic interference with the protein assay is considered unlikely since no significant difference is seen between values obtained from the 39 000 g supernatant that was dialysed and that which was not. Also the only phenolic compounds identified were the 3 bromophenols present in a concn of less than 0.06%.

Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm [21]. Cytochrome *c* oxidase activity was determined by measuring the rate of O_2 consumption in the presence of TMPD and Na ascorbate in the dark [22]. A mixture of 1.9 ml 0.01 M KPi (pH 7) was added to the O_2 electrode chamber with 20 μl of 0.5% Triton X-100 and 25 μl of the sample. TMPD (40 μl of 5 mM) and ascorbate (20 μl of 50 mg/ml) were also added and the rate of O_2 uptake measured in the dark. Ascorbate alone was also added and the rate of O_2 uptake measured and the values subtracted from above to account for any 'ascorbate oxidase' activity.

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