FORMATION OF 3-BROMO-4-HYDROXYBENZALDEHYDE FROM L-TYROSINE IN CELL-FREE HOMOGENATES OF *ODONTHALIA FLOCCOSA* (RHODOPHYCEAE): A PROPOSED BIOSYNTHETIC PATHWAY FOR BROMINATED PHENOLS

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1. Introduction

Simple bromophenols have been identified in a number of red algae [1,2], the brown algae *Fucus vesiculosus* [3] and the cyanophycean *Calothrix brevissima* [4]. The greatest quantity and diversity of these compounds, however, occurs in members of the family Rhodomelaceae (Rhodophyceae), which includes *Odonthalia floccosa.* Lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) is the most common bromophenol encountered, and is often isolated as the dipotassium salt of the disulfate ester [5]. Lanosol is the principal bromophenol of *O. floccosa* [6]. On structural grounds tyrosine may be considered as a likely precursor for the bromophenols, and Landymore [7] has demonstrated the in vivo formation of 3-bromo-4-hydroxybenzoic acid from L-tyrosine by the diatom *Navicula incerta* and the haptophycean *Isochrysis galbana.* We report here the in vitro formation of 3-bromo-4-hydroxybenzaldehyde by a cell-free system from *Odonthalia floccosa.*

2. Materials and methods

The following non-radioactive standards, all as 1% solutions in ethanol, were used: From Sigma Chemical Co. (St. Louis, MO), 4-hydroxybenzoic acid, 4-hydroxymandelic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxycinnamic acid (*p*-coumaric acid), L-tyrosine and 2,5-dihydroxyphenylacetic acid (homogentisic acid); from Aldrich Chemical Co. (Milwaukee, WI), 4 hydroxybenzylalcohol, 4-hydroxybenzaldehyde, 4-methoxybenzaldehyde (*p*-anisaldehyde), 3,4-dihydroxybenzaldehyde (protocatechualdehyde) and 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid); from ICN-K & K Labs (Irvine, CA) 3,4-dihydroxybenzoic acid (protocatechuic acid). Pfaltz and Bauer (Stamford, CT) supplied 2,3-dibromo-4,5-dihydroxybenzaldehyde. Lanosol was generously supplied by Dr J. S. Craigie.

Bromination of the respective hydroxyaldehydes or acids with equimolar Br_2 at room temperature for 24 h was performed in a suitable solvent: Chloroform for 4-hydroxybenzaldehyde; diethyl ether for 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, and ethanol for 3,4-dihydroxybenzaldehyde. Bromination of 4-hydroxybenzaldehyde with twice the molar amount of Br_2 under similar conditions produced 3,5-dibromo-4-hydroxybenzaldehyde. Reduction of 3-bromo-4-hydroxybenzaldehyde with sodium borohydride yielded 3-bromo-4-hydroxybenzyl alcohol. 4-Hydroxyphenylacetaldehyde was synthesized from D,L-synephrine (Sigma Chemical Co.) according to the described procedures [8]. The chromatographic systems (TEF and CAE) described below were used for purification and monitoring the purity of the products. Melting points, for identification and additional purity checks, were performed on

Abbreviations: BSTFA, *N, O-bis*-trimethylsilyl-trifluoroacetamide; FAD, flavin adenine nucleotide; GLC–RC, gas liquid chromatography-radiocounting; NADP, nicotinamide adenine dinucleotide 2'-phosphate; PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; TLC, thin layer chromatography; TMCS, trimethylchlorosilane; UV, ultraviolet

Table 1

Compound	M.p. (a)	TLC (b) $R_F \times 100$			GLC retention times in min with program 100°C at 5°C min^{-1} of silated derivatives
		TEF	CAE	BAW	
	°C				
L-tyrosine	–	4	0	–	(c)
p-coumaric acid	–	52	48	25	19.7
4-hydroxyphenylpyruvic acid	–	46	12 (d)	–	22.0
4-hydroxyphenyllactic acid	–	29	10	–	19.4
2,5-dihydroxyphenylacetic acid	–	35	11	–	18.2
4-hydroxyphenylacetaldehyde	–	48	54	28	–
4-hydroxyphenylacetic acid	–	46	39	19	13.5
3,4-dihydroxyphenylacetic acid	–	38	22	–	18.2
4-hydroxymandelic acid	–	26	8	–	16.1
4-hydroxybenzaldehyde	–	51	58	21	7.9
4-hydroxybenzoic acid	–	50	46	20	13.5
3,4-dihydroxybenzoic acid	–	42	29	–	18.2
4-hydroxybenzyl alcohol	–	43	27	–	10.6
3,4-dihydroxybenzaldehyde	–	42	37	7	13.5
3-bromo-4-hydroxyphenylacetic acid	102–104 (107)	52	59	31	18.2
3-bromo-4-hydroxybenzaldehyde	123–125 (124)	55	69	–	12.2
3-bromo-4-hydroxybenzoic acid	174–176 (177)	53	58	32	18.2
3-bromo-4-hydroxybenzyl alcohol	123–124 (128)	48	43	–	15.5
3-bromo-4,5-dihydroxybenzaldehyde	227–229 (230)	50	41	13	17.3
3,5-dibromo-4-hydroxybenzaldehyde	178–180 (179–180)	68	80	–	16.5
2,3-dibromo-4,5-dihydroxybenzaldehyde	–	52	48	21	21.3
lanosol	–	49	14	–	22.9
p-anisaldehyde	–	65	83	–	–

(a) Experimentally determined. Literature value in parenthesis
(b) Chromatographic solvent systems as in text
(c) Does not come off column after 20 min upon reaching 300°C
(d) Estimated because of extreme tailing when preceded by TEF in first dimension

the final crystallized material and given (table 1) as corrected values. In all cases the yields were close to theoretical. The purity of the L-tyrosine [U-^{14}C] (ICN, Irvine, CA) and L-tyrosine [-^{14}COOH] (Amersham Searle, Des Plaines, IL) was verified on 10 μl samples via the two dimensional chromatographic system, described below, and monitoring the plate by radioautography. Additional verification was provided by GC–RC of the trimethylsilylated (silated) derivative (see below). The reported purity from the suppliers was greater than 98%. No radioactive impurities, especially 4-hydroxybenzaldehyde, were detected.

Odonthalia floccosa was collected at Shell Beach, Sonoma County, CA, thoroughly cleaned of invertebrates and epiphytes and rinsed for 20 min with agitation in running distilled water with ice at 10°C to remove micro-organisms, especially diatoms. Microscopic examination (1000 X) of the thallus showed no algal or fungal contaminants. All subsequent procedures were carried out at 4°C. 160 g wet weight of *O. floccosa* were homogenized with a Virtis-45 homogenizer (45 000 rev./min) in 240 ml of 25% w/w sucrose, 0.02 M glycylglycine buffer, pH 7.5. The homogenate was filtered through two layers of cheese cloth and one layer miracloth. Incubation conditions: The following optimal incubation mixture was used for the duplicate incubations:

(a) 10 μl of 100 μCi/ml, 463 mCi/mmol L-tyrosine [U-^{14}C] or 10 μl of 100 μCi/ml, 54 μCi/mmol L-tyrosine [-^{14}COOH].

(b)	Cofactors	Final Concentration
	FAD	5×10^{-5} M
	NADP	5×10^{-5} M
	$MnBr_2$	6.25×10^{-4} M
	α-ketoglutaric acid	1×10^{-3} M

(c) 20 μl of 0.5% Triton X-100
(d) 100 μl of homogenate
(e) 0.02 M potassium phosphate buffer pH 7.0.

Total volume 1ml

A control mixture was boiled for 2 min immediately after preparation and prior to incubation. The control and test mixtures were incubated with shaking in a water bath for 60 min at 30°C.

Identification of intermediates: The sample mixtures were centrifuged for 20 min at 39 000 × *g* and the supernatants recovered. One set of supernatants was lyophilized, while 50 μl of each of the other set was spotted with cold standards on separate activated silica gel G (Brinkman, Burlingame, CA) thin layer plates (20 × 20 cm, 0.5 mm thick) in the following ascending systems. First dimension: toluene:ethyl formate:formic acid (97%) (TEF) 5:4:1, v/v/v [9]; second dimension: chloroform:acetic acid (glacial): ethanol (CAE) 18:1:1, v/v/v. For separation of possible intermediates with similar R_F values in the above system, the chloroform:acetic acid:ethanol solvent was used in the first dimension and the organic phase from a benzene:acetic acid (glacial):water (BAW) 10:7:3, v/v/v mixture [7] was used in the second dimension.

Spots were visualized with short wavelength UV illumination or by a 0.05% aqueous solution of Fast Blue B. The plates were dried overnight and exposed to Kodak no screen X-ray film. Exposure time for the L-tyrosine [U-^{14}C]experiments was two weeks and five weeks for the L-tyrosine [-^{14}COOH]experiments. Additional identification of intermediates was also achieved by GLC–RC (Nuclear Chicago Selectra System 5000 GC- and RC). For gas chromatography a 6 ft silanized glass column packed with 3% (w/w) SE-30 on 80 mesh acid washed chromosorb W (Supelco, Inc., Bellefonte, PA) was used. Retention times were determined over a temperature program from 100°C to 300°C at 5°C min^{-1}. Injector port and detector temperatures 270°C and 300°C respectively. Argon was used as the carrier gas with 8.5:1 split for the radiocounter, and a flow rate at the detector of 67 cc/min.

The lyophilized supernatant was desiccated over phosphorus pentoxide for 24 h, redissolved in 20 μl acetonitrile and silated with 20 μl BSTFA with 1% TMCS. A solution of non-radioactive standards was also silated. An aliquot mixture of 5 μl sample and 2 μl standards was used for column injection.

3. Results

Microscopic examination of the *Odonthalia* homogenate revealed chloroplasts and various microbodies (presumptive peroxisomes, proplastids and mitochondria) and cell debris.

This layer chromatographic R_F values are based on 6 or more chromatographic runs (table 1).

4-hydroxyphenylacetaldehyde, 3-bromo-4-hydroxyphenylacetic acid, 3-bromo-4-hydroxybenzoic acid and 4-hydroxybenzaldehyde have similar R_F values with the first two-dimensional system employed but 4-hydroxybenzaldehyde can be separated using the other system. 3,4-dihydroxybenzaldehyde and 4-hydroxyphenylacetic acid are also separated on the other system.

Radioactive spots from the uniformly labeled L-tyrosine incubation, co-chromatographed with 3-bromo-4-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 4-hydroxymandelic acid, 4-hydroxyphenylacetic acid and 4-hydroxyphenylpyruvic acid on TLC. Three labeled spots were not identified. The specifically labeled L-tyrosine [-^{14}COOH]incubation produced a spot which corresponded with 4 hydroxyphenylpyruvic acid on TLC. Two of the three unidentified labeled spots from the L-tyrosine [U-^{14}C] incubation were also observed in the L-tyrosine [-^{14}COOH]incubation. Radioactivity corresponded with the spots located by UV and by spray reagent.

Those compounds identified by GLC–RC from the L-tyrosine [U-^{14}C]incubation were the same as those identified by TLC; in addition a radioactive peak corresponded with 3,5-dibromo-4-hydroxybenzaldehyde (table 1). The L-tyrosine [-^{14}COOH] incubation yielded labeled 4-hydroxyphenylpyruvic acid. L-tyrosine is retained by the column even after 20 min at 300°C, presumably because of incomplete silation.

4. Discussion

The formation of 3-bromo-4-hydroxybenzaldehyde from L-tyrosine is evident. The identification of 3,5-dibromo-4-hydroxybenzaldehyde was by GLC–RC only. This compound was not detected by TLC, probably due to the small amount produced by the incubations.

The compounds identified in this study are shown in fig.1. Significant compounds that were not detected:

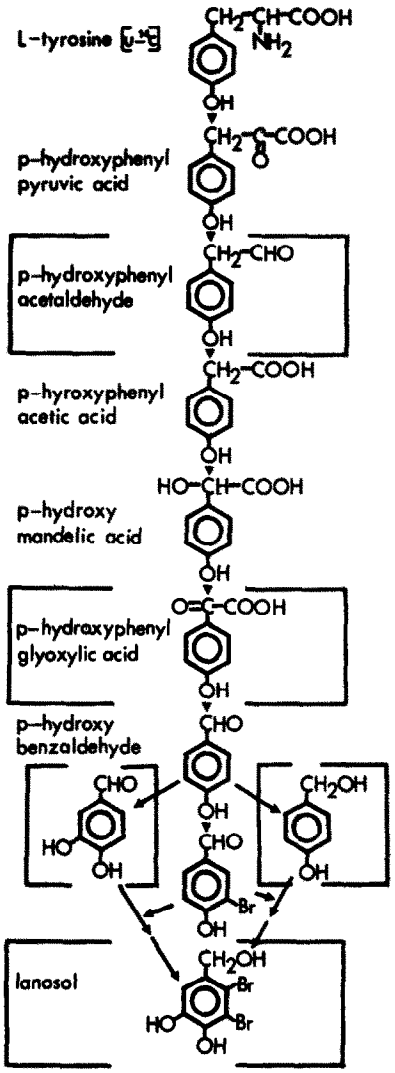

Fig.1. Proposed biosynthetic pathway for lanosol in *Odonthalia floccosa*. Compounds in parentheses have not been identified.

(1) 4-hydroxycinnamic acid (*p*-coumaric acid); this indicates that TAL was inactive or absent in *O. floccosa*. The existence of PAL and TAL in marine red algae is unresolved. Both enzymes were not detected in *Rhodymenia palmata* and *Polysiphonia lanosa* [10], the latter producing large quantities of bromophenols [2]. Lunularic acid, believed to be derived from cinnamic acid [11], has been detected in *Polysiphonia urceolata* and *Chondrus crispus* [12], the former producing bromophenols [2].

(2) Homogentisic acid; 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid, which are both common substrates for ring fission [13].

(3) 4-hydroxyphenylacetaldehyde and 4-hydroxyphenylglyoxylic acid which are plausible intermediates. The latter is probably very unstable and readily decarboxylated to 4-hydroxybenzaldehyde [7] and hence its existence cannot be determined.

(4) 4 hydroxybenzyl alcohol and 3-bromo-4-hydroxybenzyl alcohol; only oxidative reactions occurred in the incubations.

(5) 3-bromo-4-hydroxyphenylacetic acid; this suggests that bromination occurs after the C_6-C_1 moiety is formed.

Two of the three labeled spots not identified on the TLC plate must have the basic C_6-C_3 structure since they are observed in both the [U-^{14}C] and [-^{14}COOH]experiments. They are very polar as indicated by their R_F values.

Based on these results and the fact that *O. floccosa* contains lanosol, a pathway is proposed for its biosynthesis via L-tyrosine and 3-bromo-4-hydroxybenzaldehyde (fig.1). It is possible that this pathway may not be the main route for the synthesis of bromophenols but rather an auxiliary mode of tyrosine degradation. It is, however, interesting that the usual substrates for ring fission were not detected. The same basic pathway is seen in *Isochrysis galbana* and *Navicula incerta*, forming 3-bromo-4-hydroxybenzoic acid, but not 3-bromo-4-hydroxybenzaldehyde [7]. 4-Hydroxybenzaldehyde has been isolated from the marine red algae *Marginisporeum aberrans* [14] and *Dasya pedicellata* [15], indicating that this pathway may also be present in these organisms, and is in fact the pathway to the bromophenols in the red algae.

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