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4-Hydroxybenzoic acid: a likely precursor of 2,4,6-tribromophenol in *Ulva lactuca*

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Abstract

The green marine alga *Ulva lactuca* is known to contain simple bromophenols, especially 2,4,6-tribromophenol, but the precursor of these compounds in the alga is not known. With the aim of identifying potential precursors, the alga was analyzed for the presence of phenolic compounds. The compounds identified by gas chromatography–mass spectrometry were phenol, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenyllactic acid, 4-hydroxybenzaldehyde, 3,5-dibromo-4-hydroxybenzoic acid and 2,4,6-tribromophenol. Free L-tyrosine and free L-phenylalanine were also present in the alga. Furthermore, a crude enzyme extract from the alga, which contained bromoperoxidases, was used to brominate a range of phenolic compounds and the formation of bromophenols was monitored. The compounds forming 2,4,6-tribromophenol were phenol, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzyl alcohol and 2-hydroxybenzyl alcohol. 4-Hydroxybenzoic acid is designated as the most likely precursor of 2,4,6-tribromophenol in *U. lactuca* and a pathway for its formation from L-tyrosine, via 4-hydroxyphenylpyruvic acid, is proposed.

Keywords: *Ulva lactuca*; Chlorophyta; Algae; Bromophenol; Bromoperoxidase; Enzyme; Bromination; Biosynthesis; Gas chromatography; Mass spectrometry

1. Introduction

Marine algae are known to produce a variety of brominated compounds, which could be formed by the action of bromoperoxidases present in the algae (Neidleman & Geigert, 1986). Bromoperoxidases are enzymes capable of brominating organic substances in the presence of bromide and hydrogen peroxide and are frequently detected in marine algae (Moore & Okuda, 1996; Hewson & Hager, 1980). For a review on their characteristics see Butler and Walker (1993).

The green marine alga *Ulva lactuca* has been shown to contain 2-bromo-, 4-bromo-, 2,4-dibromo-, 2,6-dibromo- and 2,4,6-tribromophenol (Flodin, Helidoniotis, & Whitfield, in press; Whitfield, Helidoniotis, & Drew, 1997). The concentrations of these compounds vary over the year but 2,4,6-tribromophenol is always present in highest concentrations and 4-bromophenol and 2,4-dibromophenol are dominant over 2-bromophenol and 2,6-dibromophenol (Flodin et al., in press). It has also been demonstrated that *U. lactuca* possesses bromoperoxidase activity (Hewson & Hager, 1980; Flodin et al., in press), which also shows a seasonal variation (Flodin et al., in press). This bromoperoxidase activity most probably accounts for the formation of bromophenols in *U. lactuca*. However, the precursor of the bromophenols is not known.

The aim of this study was to investigate what compound could be the precursor of bromophenols and possibly also identify the biosynthetic pathway of these compounds in *U. lactuca*. The precursor should be a phenolic compound and possibly a phenolic acid. Accordingly, the alga was analyzed for the presence of such compounds, both by the extraction of untreated alga and of alga subjected to acid hydrolysis. Furthermore, several phenolic compounds were

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assayed for their ability to form bromophenols in the presence of a crude enzyme extract from the alga, bromide and hydrogen peroxide.

2. Results and discussion

2.1. Phenolic compounds in U. lactuca

Identification of free phenolic compounds in *U. lactuca* was accomplished by extracting an homogenate of the alga with ethyl acetate. The phenolic compounds identified were phenol and 4-hydroxyphenylacetic acid (4-HPAA), together with traces of 4-hydroxybenzoic acid (4-HBA), 4-hydroxybenzaldehyde (4-HBAld) and 2,4,6-tribromophenol (2,4,6-TBP) (Table 1). In addition, amino acid analysis of the algal homogenate demonstrated the presence of free L-tyrosine and free L-phenylalanine in the alga (Table 1).

After acid hydrolysis of the alga, higher concentrations of phenolic compounds were obtained and a number of new phenolic compounds were identified (Table 1). The compounds detected only after acid hydrolysis were 4-hydroxyphenyllactic acid (4-HPLA) and 3,5-dibromo-4-hydroxybenzoic acid (3,5-dibromo-4-HBA). The amounts of 4-HBA and 4-HPAA were higher after acid hydrolysis than before. These results suggest that the bulk of these compounds were not present as such in *U. lactuca* but were bound to other moieties and released upon hydrolysis. 4-HBAld and 2,4,6-TBP were detected in similar amounts regardless of method, suggesting a non-bound form of these compounds.

The concentration of 2,4,6-TBP in this alga sample had previously been determined to be 0.3 μg/g of fresh alga by a simultaneous steam distillation-solvent extraction technique (Flodin et al., in press). A reagent blank for the acid hydrolysis method was carried out and did not contain any of these compounds.

2.2. Production of bromophenols from various phenolic compounds

A crude enzyme extract from *U. lactuca*, containing a bromoperoxidase (BPO), was used to brominate a range of phenolic compounds and the formation of bromophenols was monitored. The phenolic compounds identified in *U. lactuca*, together with 4-hydroxybenzyl alcohol (4-HBAlc) and 2-hydroxybenzyl alcohol (2-HBAlc) were used as substrates for the bromination reaction (Table 2). The hydroxybenzyl alcohols were studied as they have been brominated to 2,4,6-TBP by bromoperoxidases isolated from the red algae *Corallina vancouveriensis* (Shang, Okuda, & Worthen, 1994), *Corallina pilulifera* and *Amphiroa ephedraea* (Yamada, Itoh, Murakami, & Izumi, 1985).

Each assay contained one substrate, crude enzyme extract, bromide and hydrogen peroxide in a buffer solution. To investigate if the crude enzyme extract could produce bromophenols without addition of a substrate, one assay was performed with only the crude enzyme extract, bromide and hydrogen peroxide. Two other assays were performed with a deactivated enzyme extract to demonstrate the role of the enzymes in the bromination reactions.

The results are presented in Table 2 and show that the enzymes play a vital role in the formation of bromophenols in the assays. Bromophenols could not be detected in the experiments with the deactivated enzyme extract, but were detected in all assays with an active enzyme extract. The active enzyme extract could produce small amounts of 4-bromophenol (4-BP) and 2,4,6-TBP even without the addition of a substrate,

Table 1
Phenolic compounds identified in *Ulva lactuca*. n.d. = not detected. n.a. = not analyzed. tr. = trace amounts

Phenolic compounds	Amount of phenolic compounds (μg/g fresh wt.)	
	homogenized algae[a]	acid hydrolysed algae[b]
Phenol	1.3	tr.
4-Hydroxybenzaldehyde	tr.	tr.
4-Hydroxybenzoic acid	tr.	1.7
4-Hydroxyphenyllactic acid	n.d.	2.4
4-Hydroxyphenylacetic acid	0.9	14
L-tyrosine	20[c]	n.a.
L-phenylalanine	23[c]	n.a.
2,4,6-Tribromophenol	tr.	tr.
3,5-Dibromo-4-hydroxybenzoic acid	n.d.	tr.

[a] *U. lactuca* homogenized in Milli-Q water and extracted with EtOAc.
[b] *U. lactuca* hydrolysed in 2 M HCl and extracted with EtOAc.
[c] From the amino acid analysis by the Australian Proteome Analysis Facility.

Table 2
Amounts of bromophenols in assays after one hour. The assays were made in 0.1 M K–Pi buffer at pH 7.0 in a total volume of 2 ml, with 25 μl of crude enzyme extract, substrate (0.1 mM), KBr (100 mM) and H_2O_2 (2 mM). n.d. = not detected

Substrate[a]	Amount of bromophenols in 2 ml assay (pmol ± S.D.)[a]				
	2-BP	4-BP	2,4-DBP	2,6-DBP	2,4,6-TBP
Deactivated enzyme, Br^-, H_2O_2 [b]	n.d.	n.d.	n.d.	n.d.	n.d.
Deactivated enzyme, 4-HBA, Br^-, H_2O_2 [c]	n.d.	n.d.	n.d.	n.d.	n.d.
Active enzyme, Br^-, H_2O_2 [d]	n.d.	8 ± 2	n.d.	n.d.	42 ± 2
4-HBA	n.d.	10,500 ± 100	2700 ± 100	n.d.	19,100 ± 300
4-HBAlc	n.d.	4300 ± 200	690 ± 20	n.d.	17,700 ± 900
Phenol	16,700 ± 500	15,800 ± 800	1800 ± 100	470 ± 20	14,300 ± 600
2-HBAlc	3500 ± 500	7 ± 1	480 ± 10	330 ± 10	14,200 ± 1100
4-HPAA	n.d.	120 ± 10	28 ± 1	n.d.	4300 ± 200
L-phenylalanine	n.d.	6 ± 1	n.d.	n.d.	30 ± 3
4-HPLA	n.d.	7 ± 1	n.d.	n.d.	22 ± 1
L-tyrosine	n.d.	6 ± 1	n.d.	n.d.	18 ± 1
4-HBAld	n.d.	140 ± 10	4 ± 1	n.d.	4 ± 1

[a] Abbreviations: 4-HBA, 4-hydroxybenzoic acid; 4-HBAlc, 4-hydroxybenzyl alcohol; 2-HBAlc, 2-hydroxybenzyl alcohol; 4-HPAA, 4-hydroxyphenylacetic acid; 4-HPLA, D,L-3-(4-hydroxyphenyl)lactic acid; 4-HBAld, 4-hydroxybenzaldehyde; 2-BP, 2-bromophenol; 4-BP, 4-bromophenol; 2,4-DBP, 2,4-dibromophenol; 2,6-DBP, 2,6-dibromophenol; 2,4,6-TBP, 2,4,6-tribromophenol.
[b] Boiled crude enzyme extract, Br^- and H_2O_2 in buffer. Reacted for 1 h.
[c] Boiled crude enzyme extract, 4-HBA, Br^- and H_2O_2 in buffer. Reacted for 1 h.
[d] Crude enzyme extract, Br^- and H_2O_2 in buffer. Reacted for 1 h.

demonstrating the presence of a natural precursor of these compounds in the crude enzyme extract.

The assays with added substrates showed that the compounds able to produce 2,4,6-TBP were 4-HBA, 4-HBAlc, phenol, 2-HBAlc and 4-HPAA (Table 2). The conversion of these substrates to 2,4,6-TBP varies from 2% for 4-HPAA to 10% for 4-HBA.

In addition to the formation of 2,4,6-TBP from these compounds, 4-HBA, 4-HBAlc and 4-HPAA also formed 4-BP and 2,4-dibromophenol (2,4-DBP). When 2-HBAlc was used as the substrate all bromophenols, except 4-BP, were formed. These results show that the formation of bromophenols from these compounds involve direct substitution of their functional group by bromine.

Bromophenols were not detected in the assays with L-phenylalanine, L-tyrosine and 4-HPLA. However, 4-HBAld produced small amounts of 4-BP and 2,4-DBP, but appeared to have a quenching effect on the production of 2,4,6-TBP from the natural precursor present in the crude enzyme extract (Table 2).

2.3. Production of other brominated compounds

The extracts from the above assays were also analyzed by GC–MS in the full-scan mode to detect reaction products other than the bromophenols. Several brominated products were identified and were mostly the products of a direct bromination of the substrate: 3-bromo-4-HBA and 3,5-dibromo-4-HBA from 4-HBA, 3-bromo- and 3,5-dibromo-4-hydroxybenzyl alcohol from 4-HBAlc, 3-bromo-, 5-bromo- and 3,5-dibromo-2-hydroxybenzyl alcohol from 2-HBAlc, 3-bromo-4-hydroxyphenyllactic acid from 4-HPLA and 3-bromo- and 3,5-dibromo-4-hydroxybenzaldehyde from 4-HBAld.

In the assay with 4-HPAA, 3-bromo- and 3,5-dibromo-4-hydroxyphenylacetic acid was identified, but the most abundant product was 3,5-dibromo-4-hydroxybenzyl alcohol. Subsequent bromination of this compound should account for the 2,4,6-TBP also present in this assay.

No brominated or non-brominated products originating from L-tyrosine and L-phenylalanine were detected.

2.4. Assays with two substrates present

To investigate how the formation of 2,4,6-TBP from 4-HBA would be affected in the presence of other compounds, two assays were conducted with 4-HBA together with either 4-HBAld or 4-HPAA.

The results from the assay with 4-HBA and 4-HBAld together show that 4-HBAld had a quenching effect on the formation of 2,4,6-TBP from 4-HBA. Only small amounts of 2,4,6-TBP could be detected in this assay. The only brominated products detected in high concentrations were 3-bromo- and 3,5-dibromo-4-hydroxybenzaldehyde.

The results from the experiment with 4-HBA and 4-HPAA together demonstrated that the bromination of either acid was not affected by the presence of the

other. The bromophenols were produced in similar amounts as if only 4-HBA would be present (comparable with the results from 4-HBA in Table 2). 3-Bromo-4-HBA, 3,5-dibromo-4-HBA, 3-bromo-4-hydroxyphenylacetic acid and 3,5-dibromo-4-hydroxyphenylacetic acid were formed as before. The only difference was that 3,5-dibromo-4-hydroxybenzyl alcohol was not formed in high concentrations as previously seen when only 4-HPAA was present in the assay. Instead, small amounts of 3,5-dibromo-4-hydroxybenzaldehyde was formed. Thus, the formation of 3,5-dibromo-4-hydroxybenzyl alcohol from 4-HPAA was repressed in the presence of 4-HBA.

These experiments show that the presence of two substrates in the one assay can drastically influence the products formed in enzyme catalyzed reactions. This could be critical in experiments where cell free extracts are used as these could contain inhibitory compounds that are normally not present at the reaction site within the cell.

2.5. Proposed precursors of bromophenols in U. lactuca

The following discussion is focussed on the formation of 2,4,6-TBP, as this bromophenol is present in highest concentrations in the alga. However, the formation of the other bromophenols will also be discussed briefly.

The compounds that were able to form 2,4,6-TBP were phenol, 4-HBA, 4-HPAA, 4-HBAlc and 2-HBAlc. However, of these substrates, the most likely precursor in vivo, is 4-HBA. The reasons are that this compound did not only produce high amounts of 2,4,6-TBP, but it also favored the formation of 4-BP and 2,4-DBP over 2-bromophenol (2-BP) and 2,6-dibromophenol (2,6-DBP), as observed in the living alga (Flodin et al., in press). Furthermore, 4-HBA and 3,5-dibromo-4-HBA could be detected in *U. lactuca*, which demonstrates that 4-HBA is available for bromination within the alga. The high production of 4-BP and 2,4-DBP from 4-HBA also suggests that 4-HBA is the precursor of 4-BP and 2,4-DBP in the alga.

The other substrates are less likely as precursors for various reasons. 4-HBAlc and 2-HBAlc could not be detected in the alga by any of the methods used. Neither were any of their brominated analogues detected. 4-HPAA was detected in the alga but is probably not in contact with the bromoperoxidase as neither brominated 4-HPAA nor 3,5-dibromo-4-hydroxybenzyl alcohol were detected in the alga. Phenol could be regarded as a likely precursor with the exception that this compound forms significant amounts of 2-BP. However, it could be the precursor of the small amounts of 2-BP and 2,6-DBP that are present in the alga (Flodin et al., in press).

2.6. Proposed biosynthetic pathway for the formation of 2,4,6-TBP in U. lactuca

A biosynthetic pathway for the formation of 4-HBA in marine phytoplankton has been proposed by Landymore, Antia, and Towers (1978). It involves the metabolism of L-tyrosine, via 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid and 4-hydroxybenzaldehyde. The results from the present study indicate that this is also the pathway that takes place in *U. lactuca* as most of the compounds in this pathway were detected in the alga (Fig. 1). 4-HBA is subsequently brominated to form 3,5-dibromo-4-HBA (possibly in bound form). 2,4,6-TBP can be formed either by bromination of free 4-HBA or by a simultaneous release and bromination of bound 3,5-dibromo-4-HBA. The formation of 4-bromo- and 2,4-dibromophenol probably occurs by way of the bromination of 4-HBA.

A possible reason why L-tyrosine could not be converted to 2,4,6-TBP in these experiments is that the enzyme extraction method did not facilitate the extraction of necessary enzymes or cofactors. To achieve the conversion of tyrosine to 2,4,6-TBP, cofactors such as FAD may need to be added.

The results in the present study also indicate that the degradation of L-tyrosine to 4-hydroxybenzoic acid should take place in a cell compartment separate from where the bromination reaction takes place. If 4-hydroxyphenylacetic acid or 4-hydroxybenzaldehyde was available to the bromoperoxidase, brominated analogues of these compounds should be detected in the alga. Furthermore, if 4-HBAld was present it would inhibit the production of 2,4,6-TBP from 4-HBA.

Manley and Chapman have suggested a similar pathway for the biosynthesis of 2,3-dibromo-4,5-dihydroxybenzyl alcohol (lanosol) by the red alga *Odonthalia floccosa* (Manley & Chapman, 1978). However, the cell free homogenate that was used in their experiments could not convert L-tyrosine to lanosol. Instead 3-bromo-4-hydroxybenzaldehyde was formed. The results from the present study suggest that the formation of 3-bromo-4-hydroxybenzaldehyde, or other degradation products, in their experiments could influence the outcome and inhibit the formation of lanosol.

The most favored mechanism for the formation of 4-HBA in higher plants is the degradation of 4-hydroxycinnamic acid (*p*-coumaric acid), which in turn is derived from L-phenylalanine or L-tyrosine (Gross, 1985). However, this pathway is not likely to be present in *U. lactuca* as 4-hydroxycinnamic acid was not detected.

Further studies are needed to investigate in what form 4-HBA is present in the alga and if the bound form of 4-HBA can produce 2,4,6-TBP.

3. Experimental

3.1. Collection of U. lactuca

The green marine alga *U. lactuca* was collected in January 1998 at Turimetta Head, just north of Sydney on the eastern coast of Australia. The alga was collected in the intertidal zone at low tide, transported on ice to the laboratory and stored at −20°C.

3.2. Phenolic compounds by acid hydrolysis

A method for the detection of phenolic acids in plant tissues and soil was used with a few modifications (Heimler & Pieroni, 1994). The alga was thawed and cleaned from sand, other algae or animals mixed with the *U. lactuca*. The alga was patted dry between paper tissues and cut into small pieces with a pair of scissors. Three grams of cut alga was then refluxed for one hour in 20 ml of 2 M HCl. The solution was allowed to cool and filtered through a Whatman filter (No. 541). An aliquot (8 ml) of the filtrate was extracted with 2 ml of EtOAc with a MIXXOR extractor. After the extraction, 1 ml of the EtOAc phase was collected and 3,5-dimethyl-2,4,6-trichlorophenol was added as internal standard (10 μl of 0.5 mg/ml EtOAc). The sample was dried by freezing out the water over night, passing the solution through a small column of dry Na_2SO_4, and finally evaporating it to dryness under a gentle stream of nitrogen. The phenolic compounds were redissolved in 300 μl of EtOAc and silylated by adding 20 μl of BSTFA with 10% TMCS. The extract was analyzed by GC–MS on the same day as derivatization. A reagent blank was also carried out by repeating the method without the alga.

The phenolic compounds were identified by GC–MS against authentic compounds by matching retention times and mass-spectra of their silylated analogues. The quantifications were made against 3,5-dimethyl-2,4,6-trichlorophenol-TMSi in the full scan mode with the assumption of a response ratio of 1:1.

3.3. Free phenolic compounds

The same procedure was used as for the acid hydrolysis method, except that the alga was homogenized

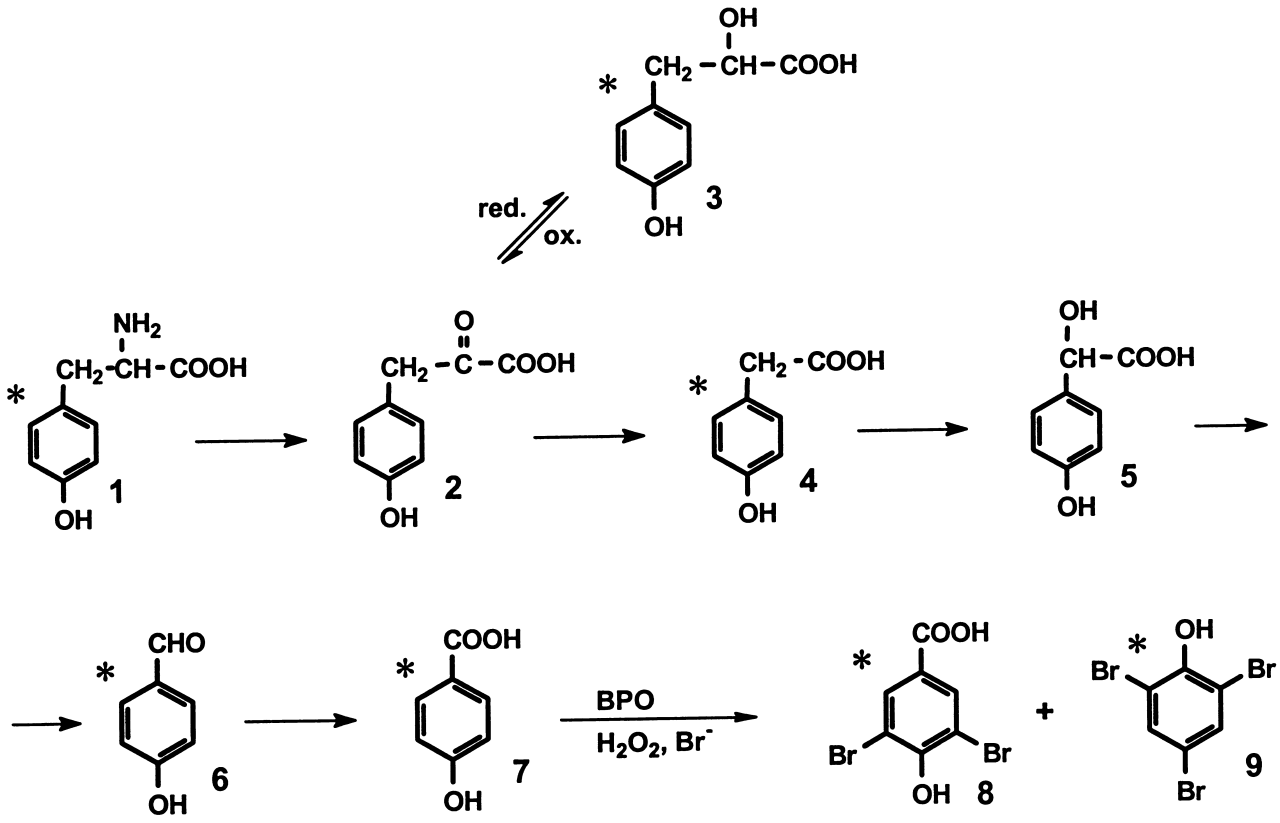

Fig. 1. Proposed biosynthetic pathway for the formation of 2,4,6-tribromophenol in *U. lactuca*. The pathway from tyrosine to 4-hydroxybenzoic acid is based on the study by Landymore, Antia, and Towers (1978). The compounds marked with (*) were detected in *U. lactuca*. Compounds: (1) L-tyrosine; (2) 4-hydroxyphenylpyruvic acid; (3) 4-hydroxyphenyllactic acid; (4) 4-hydroxyphenylacetic acid; (5) 4-hydroxymandelic acid; (6) 4-hydroxybenzaldehyde; (7) 4-hydroxybenzoic acid; (8) 3,5-dibromo-4-hydroxybenzoic acid; (9) 2,4,6-tribromophenol.

in Milli-Q water instead of boiled in HCl before filtration and extraction with EtOAc.

3.4. Free amino acid analysis

Five grams of alga was homogenized in 20 ml of Milli-Q water with an Ultra Turrax homogenizer. The slurry was squeezed through eight layers of cheese cloth and the filtrate was analyzed for free amino acids. The analysis was performed at the Australian Proteome Analysis Facility.

3.5. Preparation of crude enzyme extract

A crude enzyme extract was prepared from the alga to be used in the experiments of formation of bromophenols from various phenolic compounds. The enzyme extraction method was developed in order to obtain highest yield of bromoperoxidase activity from *U. lactuca* (Flodin et al., in press). The enzyme extract used in these experiments was, in average, able to brominate 2.9 μmol of monochlorodimedone/min/g of alga in a standard assay (Flodin et al., in press). The use of a crude enzyme extract also allowed the presence of other enzymes that could be needed for the conversion of the substrates to bromophenols.

Frozen alga was thawed and cleaned from sand, other algae or animals mixed with the *U. lactuca*. Four grams of cut alga were then homogenized on ice with an Ultra Turrax for four min in 20 ml of Milli-Q water. The slurry was squeezed through eight layers of cheese cloth and glycerol (2 g) was added to an aliquot of the filtrate (8 g). This crude enzyme extract had a pH of 6.8 and was stored at -6° before used for the bromination tests within a day of extraction.

3.6. Assays with one substrate

Potential precursors of bromophenols in *U. lactuca* that were assayed were L-tyrosine, L-phenylalanine, 4-HPLA, 4-HPAA, 4-HBA, 4-HBAld, 4-HBAlc, 2-HBAlc and phenol. All substrates were made up in Milli-Q water at concentrations ranging from 1 to 10 mM, depending on their solubility in water.

The assays were performed in 0.1 M K–Pi buffer at pH 7.0 in a total volume of 2 ml in the presence of 25 μl of crude enzyme extract, 0.1 mM substrate, 100 mM KBr and 2 mM H_2O_2. The reaction conditions were chosen for optimal bromoperoxidase activity (see Flodin et al., in press). One assay was carried out in the same way but without the addition of a substrate. Two other assays were conducted in the presence of 25 μl of a deactivated enzyme extract, 100 mM KBr and 2 mM H_2O_2, with one of them also containing 0.1 mM 4-HBA. The deactivation of the crude enzyme extract was accomplished by boiling for 10 min. All assays were made in triplicates. The reaction was terminated after one hour by the addition of four drops of 10% H_2SO_4. The acidified solution was then extracted with 0.5 ml of CH_2Cl_2 using a MIXXOR extractor. After the extraction, 250 μl of the CH_2Cl_2 phase was collected and 2,6-dibromophenol-d_3 was added as internal standard (10 μl of 10 μg/ml *iso*-octane). Any water present was removed by holding the solution at -15° overnight followed by the addition of small amounts of dry Na_2SO_4. The dried solution was transferred to a new vial and the phenolic compounds were silylated by the addition of 10 μl of BSTFA with 10% TMCS. The compounds were identified and quantified by GC–MS on the same day as the derivatization.

To detect the more hydrophilic compounds, the acidified solutions from the above assays were further extracted with 0.5 ml of EtOAc. An aliquot (250 μl) of the EtOAc phase was collected and the solution was dried and silylated as described under Section 3.2.

The bromophenols were identified and quantified as TMSi derivatives in the selected ion monitoring mode against calibration curves of each compound with 2,6-dibromophenol-d_3 as internal standard. Positive identifications were based on matching retention times with that of authentic compounds and appearance of the correct isotopic ratios of the selected ions. Target ions as TMSi derivatives were as follows: 2,6-dibromophenol-d_3, m/z 312, 314; monobromophenols, m/z 244, 246, 229, 231; dibromophenols, m/z 307, 309, 322, 324; tribromophenol, m/z 400, 402, 404, 406, 387, 389. Other brominated compounds in the reaction mixtures were identified in the full-scan mode by manual interpretation of their mass-spectra. The substitution patterns for the bromine atoms were suggested on the basis of activated sites in the aromatic ring. 3,5-Dibromo-4-HBA was available as a reference compound.

3.7. Assays with two substrates

The experiments with two substrates present were performed in the same way as with one substrate. The substrates assayed together were 4-HBA and 4-HBAld, and 4-HBA and 4-HPAA.

3.8. GC–MS parameters

GC–MS was used for the identification and quantification of bromophenols and phenolic acids in the alga. The GC was equipped with a non-polar bonded phase capillary column (HP-5 trace analysis (5% PH 95% ME Siloxane) 25 m × 0.2 mm × 0.33 μm film thickness). Helium was used as the carrier gas at a constant velocity of 30 cm/s. Injection temp. was 250° and one μl of the sample was injected with a split ratio of 1:20.

The column temp. was initially held at 40° for 2 min, then programmed from 40° to 280° at 20°/min before holding this temp. for 20 minutes. The MS detector was operating in the EI mode (ionization 70 eV at 170°).

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