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Brominated precursors of Tyrian purple (C.I. Natural Violet 1) from *Plicopurpura pansa*, *Plicopurpura columellaris* and *Plicopurpura patula*

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ABSTRACT

Tyrian purple (C.I. Natural Violet 1) and its precursors have enjoyed much attention as various species of gastropods of the Muricidae family. However, few investigations have concerned the dye's precursors, namely *Plicopurpura columellaris* and *Plicopurpura patula*. Derivatization and NMR revealed that the purple pigment in *Plicopurpura pansa* is 6,6′-dibromoindigo. ^{1}H and ^{13}C NMR enabled tyrindolinone (6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3H-indol-3-one), a methanethiol adduct of tyrindoleninone from *P. pansa*, to be identified. GC/MS was used to identify the precursors of C.I. Natural Violet 1 from *P. pansa*, *P. columellaris* and *P. patula*. Tyrindoleninone (6-bromo-2-methylsulsulfanyl-3*H*-indol-3-one) was present in the milk and the hypobranchial gland of *P. pansa* only whereas an indole derivative, 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one), was present in the hypobranchial gland of *P. patula* and *P. columellaris* but not in *P. pansa*.

1. Introduction

Natural dyes extracted from animal and plant species have been used intensively in human activities throughout history. Of such dyes from animal origin, many are derived from the hypobranchial glands of several gastropods of the family Muricidae, the most famous being Tyrian purple (C.I. Natural Violet 1) [1,2] which is obtained from species of the genera *Murex*, *Bolinus*, *Purpura*, *Plicopurpura* and *Thais*. Depending on the species, the dye can be obtained in different purple and blue hues, due to the presence of brominated and non-brominated compounds [2]. *Plicopurpura pansa* is unique as it does not have to be sacrificed in order to obtain the dye in so far as it can be "milked" periodically [3–5].

In 1880, Schunck investigated the purple dye obtained from *P. pansa* and showed that it was like indigo, but not identical to it. In 1909, Friedlander identified the purple product from the Mediterranean *Murex (Bolinus) brandaris* as 6,6′-dibromoindigo [2]. In *P. pansa* the major component of the purple dye was identified by HPLC as 6,6′-dibromoindigo **5** (90%), along with 6-bromoindigo (9%), and 6,6′-dibromoindirubin (1%) by Withnall et al. [6].

The tyrindoleninone **3** (red) and tyrindolinone **6** (yellow) intermediates in the production of the purple dye were identified, with TLC with 6-bromoisatin **7** as another product from different muricid species [Baker and Duke, 1971 in 2; 7; Hiyoshi and Fujise 1992 in 2; 8; 9]. LC/MS has been used to identify Tyrian purple precursors such as tyrindoxyl sulfate **1** and tyrindoxyl **2** in *Dicathais orbita* [10].

GC/MS has been useful in the identification of 6,6′-dibromoindigo **5** precursors as well as other brominated compounds in some muricids; Benkendorff et al. [11,12] found several known and unknown brominated compounds.

Nuclear magnetic resonance (NMR) has been used to characterize the intermediates precursors of natural and synthetic dyes. Tyriverdin **4** structure, the former precursor of 6,6′-dibromoindigo **5** was obtained by chemical synthesis [13]. On the other hand 6,6′-dibromoindigo **5** has been synthesised from different compounds such as: 6-bromoindole and 4-methyl-aniline [14,15]. The metabolic

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path of 6,6′-dibromoindigo **5** is already known. The organism produces a sulfatase (purpurase), that eliminates sulfate ion to give tyrindoxyl **2**, when exposed to oxygen it changes to tyriverdin **4** which, in the presence of sunlight, gives the permanent form 6,6′-dibromoindigo **5** (Fig. 1) [8].

The purpose of the "milk" in gastropods is not yet fully understood, speculations includes its role in feeding where it may be secreted to anaesthetize prey, or as a stimulant to facilitate detachment of the gastropod from its surroundings. It may be used in reproduction, since the secretory activity, in some muricids, increases during breeding season [Fretter and Graham, 1994 in 16]; there are indole derivatives in egg masses and there appears to be an influence of genital ducts on indigoid biosynthesis during the reproductive season [16]. Indole derivatives of 6,6′-dibromoindigo **5** have been found in different muricids and it has been demonstrated that these derivatives can have antimicrobial activity [11,12]. It has been reported that 6-bromoindirubin is a strong GSK inhibitor [17]. Several investigations on the hypobranchial glands of the Muricidae family have revealed that the number and nature of precursors involved in the production of Tyrian purple differs among species [12]. Consequently, it is possible that different precursors could be found in the species of *Plicopurpura*.

The genus *Plicopurpura* has only three species, *P. pansa*, *Plicopurpura columellaris* and *Plicopurpura patula*, the former two live in the Pacific and *P. patula* lives in the Atlantic; these three species are predators of the intertidal rocky shore and they also produce Tyrian purple and expel it, but only *P. pansa* yields enough "milk" to use it to dye. *P. pansa* is the species that the Mixtecan people from Oaxaca, Mexico use to dye the cotton to make their clothes, and handcrafts. The Tyrian purple and its precursors have been studied in *P. pansa* but not in *P. columellaris* and *P. patula*. In this study, we characterized the 6,6′-dibromoindigo **5** and the

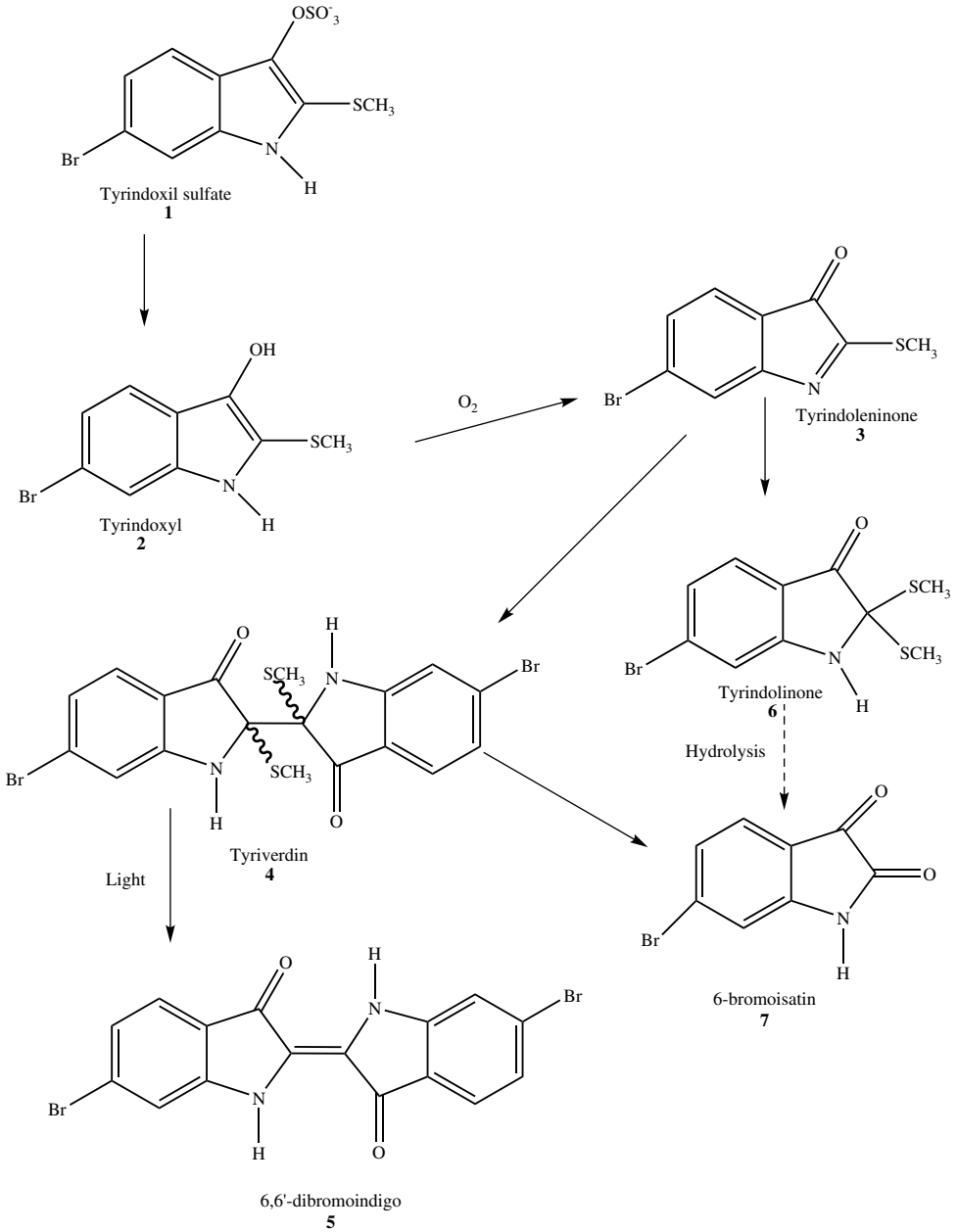

Fig. 1. The generation of the 6,6′-dibromoindigo (taken from Cooksey 2001 [2]).

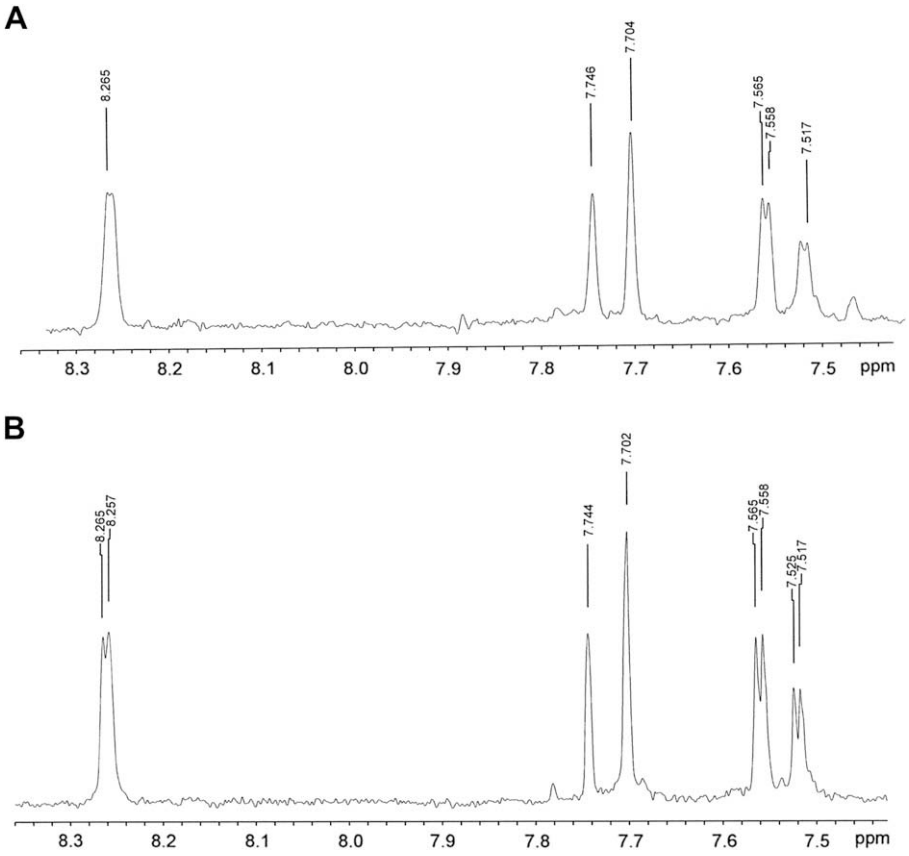

Fig. 2. ^{1}H NMR spectrum of synthetic and natural 6,6′-dibromoindigo isolated from milk of *P. pansa* (200 MHz, $CDCl_3$ and TMS as internal standard).

precursors along with another volatile indole derivatives on *P. pansa* from the milk and hypobranchial gland in order to use them to characterize the 6,6′-dibromoindigo **5**, the precursors and the indole derivatives from *P. columellaris* and *P. patula*.

2. Experimental

2.1. Extraction of natural dye

50 ml of the glandular secretion (“milk”) was obtained from a total of 150 organisms of *P. pansa* at Peñitas, Michoacán, Mexico. It was obtained stimulating the operculum, pressing softly with the thumb, and collecting the “milk” into a dark bottle in order to avoid photochemical oxidation. A portion of the “milk” collected was exposed 8 days to sunlight to obtain 6,6′-dibromoindigo **5** crystals. Crystals were refluxed in ethanol for 2 h and a second reflux with ethyl benzoate to obtain pure purple crystals [18].

Hypobranchial glands from fresh *P. pansa* (8 organisms from Peñitas, Michoacán), and frozen *P. columellaris* (15 organisms from Tenacatita, Jalisco) and frozen *P. patula* (14 organisms from Piedra Escondida, Quintana Roo, all beaches from Mexico) were dissected out from snails and macerated with methanol–chloroform (1:1 v/v), the next steps were the same as in the “milk” extract. The identification of brominated compounds was accomplished using GC/MS.

2.2. Chemical synthesis

The chemical synthesis of 6,6′-dibromoindigo **5** was synthesised according to the procedure of Tanoue et al. [14].

2.3. N-N′-*Bis(trifluoroacetyl) derivatives*

The purple crystals were refluxed with 2 ml of chloroform and 2 ml trifluoroacetic anhydride for 4 h until the initial violet solution became orange. Complete evaporation gave a brown solid, which was dissolved in $CDCl_3$ and the proton NMR spectra were recorded immediately using a Varian model Mercury Plus at 400 MHz, with tetramethylsilane as internal standard.

2.4. Isolation of precursors

The “milk” collected from the snails was soaked in methanol/chloroform (1:1 v/v), 12 h, then decanted and the solvent replaced.

Table 1
Volatile brominated compounds in extracts from the hypobranchial gland of *P. pansa*, *P columellaris* and *P. patula*.

Compound	Rt	Major fragments	*P. pansa* (milk)	*P. pansa*	*P. columellaris*	*P. patula*
6-Bromo-2-methoxy-3*H*-indole-3-one **8**	7.43	MI 239, 241 mf 222, 224, 168, 170	+	+	+	+
6-Bromoindalin-2-one **9**	10.31	MI 211, 213			+	+
Tyrindoleninone **3**	11.25	MI 255, 257 mf 240, 242, 212, 214, 182, 184, 133, 75	+	+		
6-Bromo-2-methylsulfinyl-3*H*-indole-3-one	13.84	MI 271, 273, mf 224, 226, 168, 170	+			
6-Bromoisatin **7**	14.11	MI 225, 227 mf 177, 179, 170, 172	+			

Table 2
Unidentified brominated indoles from the glandular extract (milk) of *P. pansa*, and hypobranchial gland of *P. pansa*, *P. columellaris* and *P. patula*.

Molecular Ion	Rt	Major fragments	*P. pansa* (milk)	*P. pansa*	*P. columellaris*	*P. patula*
229, 231	7.44	197, 199, 170, 172, 28		+	+	+
239, 241	7.57	229, 231, 197, 199, 170, 172	+			
253, 255	8.70	224, 226, 168, 170	+			
271, 273	8.81	256, 258, 224, 226, 208, 210	+			
255, 257	9.82	224, 226, 168, 170	+			
223, 225,	10.98	211, 213, 182, 184			+	
255, 257	11.03	240, 242, 227, 211, 213, 182, 184, 171, 101				+
257, 259	11.08	229, 231, 211, 213, 197, 199, 182, 184			+	+
255, 257	11.93	245, 247, 240, 242, 198, 200, 170, 172	+			
255, 257	12.12	239, 241, 211, 213, 182, 184	+			
317, 319, 321[a]	12.44	302, 304, 306, 224, 226, 168, 170	+			
271, 273	13.84	224, 226, 168, 170	+			
225, 227	14.12	197, 199, 170, 172	+			
237, 239	14.64	208, 210, 44, 32, 28			+	+
255, 257, 259[a]	16.10	240, 242, 244, 210, 212, 182, 184			+	+
299, 301	18.89	257, 259, 242, 244	+			
289, 291	21.23	261, 263, 154	+			

[a] Dibromo compound.

This was repeated three times with a last soak being overnight. The extracts were then mixed and evaporated to dryness in a rotary evaporator and resuspended in chloroform (5 ml). The chloroform extract was separated by column chromatography using a silica gel column (Sigma 70–230 mesh, 4 × 60 cm). The column was eluted with chloroform. The fractions were further separated on preparative TLC plates (Silica Gel 60) using dichloromethane/hexane (1:1 v/v).

2.5. Analysis of precursors

The structure of the isolated precursor was determined by GC/MS (Hewlett Packard GC mod. 5972, with a HP5MS (crosslinked 5% PH ME Siloxane)) 30 m × 0.25 mm × 0.25 μm film thickness, coupled to a low-resolution mass analyzer (Hewlett Packard mod. 5972 MSD). The operating conditions were: initial temperature 150 °C for 3 min and then increased at a rate of 4 °C/min to a final temperature of 300 °C which was maintained for 20 min. Helium was used as the carrier gas with a constant flow of 1 ml/min; one microliter of sample was injected with a split ratio of 50:1. The electron beam energy in the mass spectrometer was 70 eV and the source temperature was 230 °C.

3. Results and discussion

Chemical synthesis of 6,6′-dibromoindigo **5** gave 47% yield, slightly lower than that reported by Tanoue et al. [14]. The synthesis of 6,6′-dibromoindigo **5** was used to confirm the natural dye, this compound is insoluble in any solvent at ambient temperature, but it has been found that it is conveniently converted to the *N*,*N*′-bis(trifluoroacetyl) derivative by a treatment with trifluoroacetic anhydride [18].

The *N*,*N*′-bis(trifluoroacetyl) derivatives from synthetic and natural dye obtained from "milk" collected from *P. pansa* snails, were chemically indistinguishable. Both derivatives showed the three proton multiplets in the ^{1}H NMR spectrum δ 8.26, d, $J = 1.3$ Hz, 1H, Ar**H**; δ 7.72, d, $J = 8.3$ Hz, 1H Ar**H**; δ 7.54, dd, $J = 8.3$,1.5 Hz, 1H, Ar**H** (Fig. 2). We did not obtain the ^{1}H NMR of the dye in the hypobranchial glands of *P. columellaris* and *P. patula* due to the low concentration of crystals of 6,6′-dibromoindigo **5**.

The analysis of glandular secretion ("milk"), obtained from *P. pansa,* by column chromatography revealed seven colored compounds and one insoluble red compound, which did not pass through the silica column as Benkendorff et al. [12] found in frozen

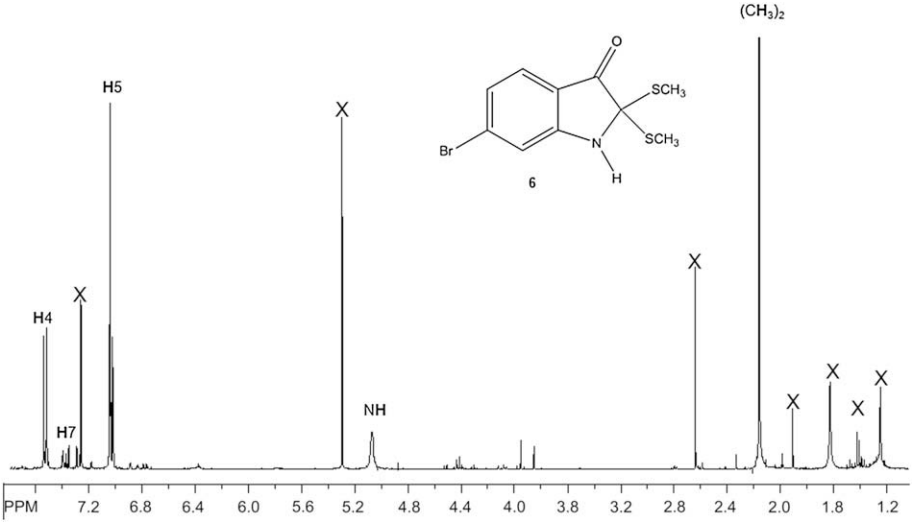

Fig. 3. ^{1}H NMR spectrum of 6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one (tyrindolinone) isolated from milk of *P. pansa* (400 MHz, $CDCl_3$ and TMS as internal standard).

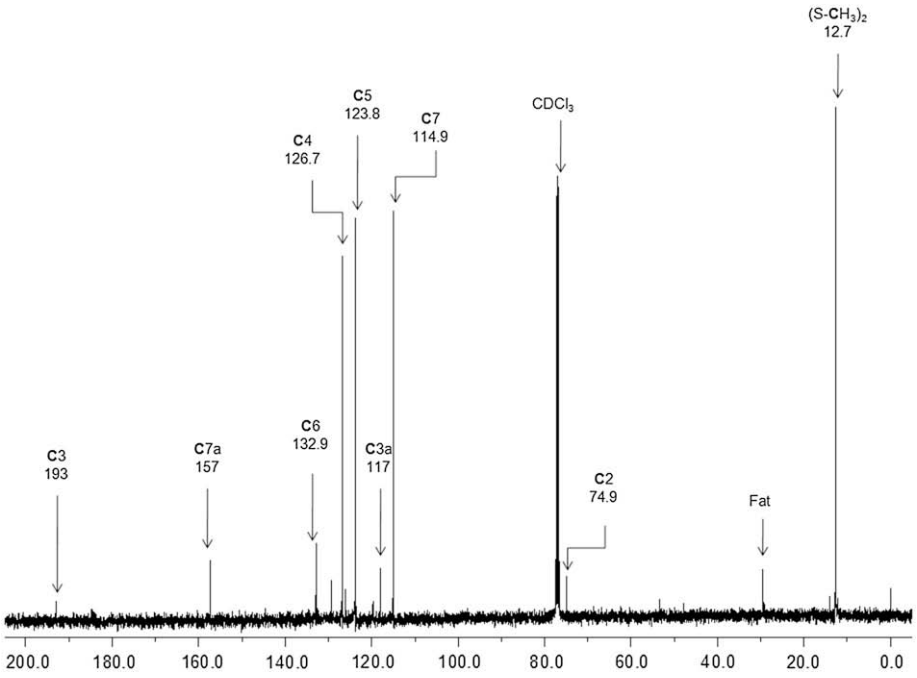

Fig. 4. ^{13}C NMR spectrum of 6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one (tyrindolinone) isolated from milk of *P. pansa* (400 MHz, $CDCl_3$ and TMS as internal standard).

eggs of *D. orbita* and *Trunculariopsis trunculus*. The components from the second and fourth bands were successfully isolated from a chloroform extract by thin-layer chromatography using dichloromethane and hexane (1:1, v/v). The second band broke down on silica to produce five bands: the orange component was identified as tyrindoleninone **3** by GC/MS, which was present only in *P. pansa* (Table 1). Benkendorff et al. [12] found that egg capsules of all muricids contain tyrindoleninone **3**, which is a metabolite that has antimicrobial activity.

GC/MS analysis of the "milk" from *P. pansa* revealed four compounds that could be identified as tyrindoleninone **3**, 6-bromoisatin **7**, 6-bromo-2-methylsulfinyl-3*H*-indole-3-one and 6-bromo-2-methoxy-3*H*-indole-3-one **8** (Table 1); the last two were identified by comparison with the MS data given by Benkendorff et al. [12]. There were also eleven brominated compounds detected in trace amounts, which were determined as brominated in base of the mass spectra pattern (Table 2). Two dibromo indole compounds were distinguished due to the distinctive triplet in the mass spectra present in the dibromo compounds (Table 2). Tyrindoleninone (6-bromo-2-methylsulfanyl-3*H*-indol-3-one) **3** was the most abundant compound with a retention time of 11.25 min; and M^+ *m*/*z* 255, 257, (^{79}Br, ^{81}Br) major fragments 242, 240,133 and 75. When the sample of the isolated tyrindoleninone **3** was analyzed by NMR spectroscopy, two weeks after the initial GC/MS analysis, the tyrindoleninone **3** had changed to tyrindolinone (6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one) **6**, the ^1H NMR demonstrated the presence of tyrindolinone **6**, showing a singlet at δ 2.168 corresponding to the ($SC\mathbf{H}_3)_2$, protons (6H), with additional peaks at δ 7.53, d, $J = 8.3$ Hz, 1H Ar**H**; δ 7.019, d, $J = 8.3$ Hz, 1H Ar**H**; δ 7.05, m, 1H Ar**H**; δ 5.08, brs, 1H, N**H** (Fig. 3). The carbons in the ^{13}**C** NMR spectrum of tyrindolinone **6** resonated at 400 MHz, the signals were: δ 12.738 ($\mathbf{C}H_3)^2$, δ 74.96 (**C**-2), δ 114.897 (**C**-7), δ 117.968 (**C**-3a), δ 123.784 (**C**-5), δ 126.741 (**C**-4), δ 132.913 (**C**-6), δ 157.276 (**C**-7a) and δ 193.065 (**C**-3) (Fig. 4). These data confirm the

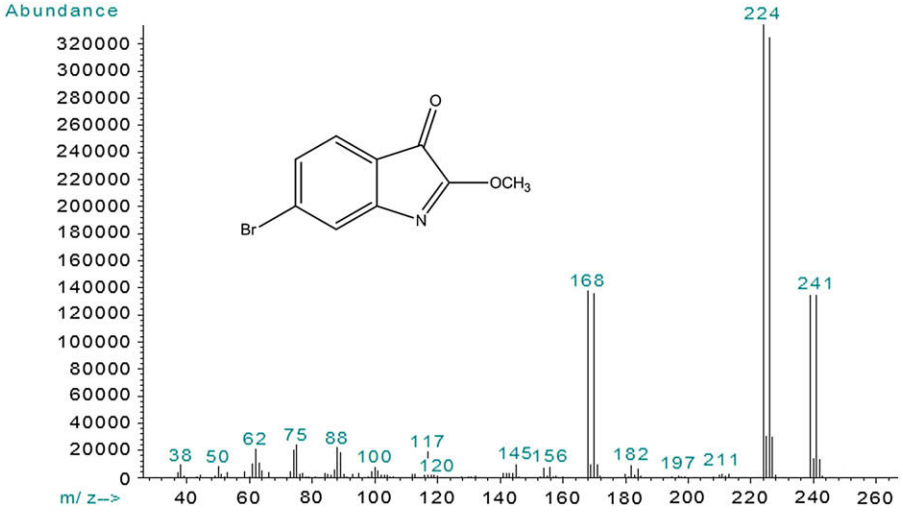

Fig. 5. Mass spectrum of 6-bromo-2-methoxy-3*H*-indole-3-one.

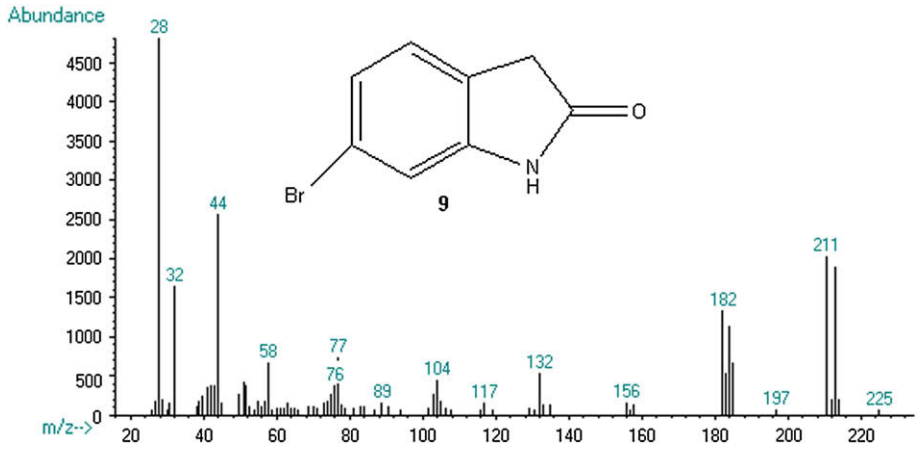

Fig. 6. Mass spectrum of 6-bromo-1,3-dihydro-2*H*-indole-2-one (6-bromoindalin-2-one).

presence of 10 C′s which correspond to tyrindolinone **6**, while tyrindoleninone **3** has 9 C′s.

6-Bromoisatin (6-bromo-1*H*-indole-2,3-dione) **7** was found in the milk of *P. pansa* in small amounts and identified by GC/MS with a retention time of 14.11 min and M^+ *m*/*z* 225, 227 (^{79}Br, ^{81}Br); major fragment ions were observed at *m*/*z* 197, 170 and 63. Benkendorff et al. [11] proposed that 6-bromoisatin **7** is a by-product of the decomposition of tyriverdin **4**. However we found tyrindoleninone **3** as a major compound and this finding may support the idea of Cooksey and Withnall [8], who proposed that 6-bromoisatin **7** may be a product of the hydrolysis of tyrindolinone **6** (dashed arrow in Fig. 1). None of the hypobranchial extracts of *P. pansa*, *P. columellaris* and *P. patula*, or the "milk" of *P. pansa*, showed peaks at *m*/*z* 418, 420, 422 which are characteristic of tyriverdin **4**. We concluded that the absence of this compound could be the result of the low solubility of tyriverdin **4** in all solvents.

Another compound found in the "milk" of *P. pansa* and in the hypobranchial glands of the three species, was 6-bromo-2-methoxy-3*H*-indol-3-one **8** (Table 2) which had a retention time of 7.43 min and a M^+ *m*/*z* 239, 241, major fragments 224, 226, 168,170 (Fig. 5). This compound was reported by Benkendorff et al. [12] in egg masses of *D. orbita*, *Ceratostoma erinaceum* and *T. trunculus* and it was suggested that 6-bromo-2-methoxy-3*H*-indol-3-one **8** could be a by-product due to the use of methanol as solvent.

On the other hand, in the hypobranchial gland of *P. columellaris* and *P. patula* an indole derivative was present, the mass spectra library matched to 5-bromoindalin-2-one (5-bromo-1,3-dihydro-2*H*-indole-2-one), but as in 5-bromoisatin, this is more likely to be 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one) **9**, since most, if not all, the compounds have the bromine atom in sixth position. Benkendorff et al. [12] reported 6-bromoindoxyl (6-bromo-1,2-dihydro-3*H*-indole-3-one) in egg masses of some muricids, and M^+ *m*/*z* 211,213, with major fragments 102, 104. Our compound, with a retention time of 10.31 min and M^+ *m*/*z* 211, 213, with major fragments 182, 184 (M–CO), and a small fragment 156, 158 (M–CH_2NH) which increases the *m*/*z* 28 fragment (Fig. 6), confirm that the compound was the 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one) **9**. This compound was found as a result of the pyrolysis of 6,6′-dibromoindirubin in Tyrian purple obtained from *Murex trunculus* [19].

We detected other unidentified volatile brominated compounds in the extracts of hypobranchial glands from *P. columellaris* and *P. patula* but not in *P. pansa* by comparing the mass spectra with the ones reported by Benkendorff et al. [12] (Table 2).

Secondary metabolites differ from primary metabolites in having a restricted distribution in the animal and plant kingdoms. They can be found in only one species or a taxonomically related group of a species, whereas the basic primary metabolites are found throughout the kingdoms [20]. Their direct role in plant metabolism is not yet well documented. However, their ecological role, herbivore interaction, and chemotaxonomy have been well established [21]. In the last 30 years, nearly 10,000 different natural products have been isolated from marine organisms. These compounds have eventually served as leads for the development of modern nucleoside drugs for antiviral chemotherapy [17,22].

Chemotaxonomy has contributed to species classification when the organisms have morphological similarities, as had been shown in lichens [23], fungus [24], and marine species [25]. Comparing the secondary profile of a species, it is possible to observe a group characterized by the presence of one compound and another group by the presence of one type of metabolite could be different. So the presence of a kind of metabolite could be used, in addition to morphological data, to define species. *P. pansa* had been considered as the same species as *P. columellaris* [26], and as a subspecies of *P. patula* [27], afterwards, studying the morphology of the radulae the three species were reported as different species [28]. The results presented in this paper support the idea proposed by Castillo [28].

4. Conclusion

The chemical nature of the precursors and brominated indoles of the purple dye in the hypobranchial gland and the extracts of the glandular secretion from *P. pansa*, were identified according to their known fragmentation patterns. The tyrindoleninone **3** was a major compound in *P. pansa*. On the other hand, the presence of 6-bromoindaline-2-one **9** was detected in the hypobranchial gland of *P. columellaris* and *P. patula* but not in *P. pansa*.

According to the results presented here, the three species had different brominated indole derivatives supporting the morphological [28] and molecular data [29] that distinguish the three species.

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