Variation of 2,3,4-tribromopyrrole and its sodium sulfamate salt in the hemichordate *Saccoglossus kowalevskii*

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ABSTRACT: We quantified spatial and temporal patterns of brominated pyrrole variation in the common marine hemichordate worm *Saccoglossus kowalevskii*. Using fast-atom bombardment mass spectrometry we found a novel sodium sulfamate salt ($C_4HBr_3NO_3SNa$) of 2,3,4-tribromopyrrole (TBP) that predominated over 2,3,4-TBP (11 % vs 0.6 % ash-free dry wt). There was no relationship between the total amounts of the 2 compounds. The sulfamate salt was concentrated in the hepatic region (50 %), a possible site of synthesis or storage. 2,3,4-TBP was highest (1.5 %) in the proboscis and tail, which are exposed to predation. No seasonal pattern was apparent for the sulfamate salt over a 30 mo sampling period. 2,3,4-TBP exhibited temporal increases, possibly associated with spawning. The sulfamate salt was not detectable by GC/MS techniques, raising the possibility that sulfonation may be more common than realized. The sulfamate salt may serve as the non-autotoxic stable precursor to the more volatile 2,3,4-TBP.

KEY WORDS: Bromopyrrole · Sulfamate · *Saccoglossus* · Hemichordate · Organohalogen · Chemical variation · Marine natural products

INTRODUCTION

Quantitative and qualitative variation in the secondary metabolite composition of marine invertebrate species occurs on multiple levels. Research in numerous systems, including sponges (Makarieva et al. 1981, Thompson et al. 1983, 1987, Cambie et al. 1988, Molinski & Ireland 1988, Karuso et al. 1989, Sennett et al. 1992), octocorals (Tursch et al. 1978, Williams et al. 1988, Harvell & Fenical 1989, Wylie & Paul 1989, Paul 1992, Harvell et al. 1993), bryozoans (Walls et al. 1991, Anthoni et al. 1990), molluscs (Williams et al. 1986, Kernan et al. 1988, Faulkner et al. 1990, Avila et al. 1990, 1991, Di Marzo et al. 1991), polychaetes (Fielman 1989, Goerke & Weber 1990, Goerke et al. 1991), echinoderms (Lucas et al. 1979) and tunicates

(Lane & Wilkes 1988, Lindquist et al. 1992), has demonstrated that substantial chemical variation occurs over a range of spatial scales, including within organisms (μm to cm), among organisms (cm to m), and over geographic areas (1 to 10^3 km). In contrast, temporal variation of secondary metabolites among marine invertebrate species has not been as well studied, although some temporal variation appears to be associated with spawning activity or other discrete events (Sammarco & Coll 1988, Coll et al. 1989, Goerke & Weber 1991, Green et al. 1992).

We investigated variation of secondary metabolites in a member of the marine phylum Hemichordata. The occurrence of secondary metabolites, particularly halogenated compounds, is especially common among the hemichordate worms (class Enteropneusta). Hyman (1959) first noted their presence as a characteristic 'iodoform' odor. Volatile brominated and chlorinated compounds have been isolated from at least 11 enteropneusts: *Balanoglossus biminensis* (Ashworth & Cormier 1967), *Ptychodera flava laysanica* (Higa &

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Scheuer 1977), *P. flava*, *Glossobalanus* sp., *B. carnosus*, *B. misakiensis* (Higa et al. 1980), *Glossobalanus* sp. (Higa et al. 1985), *Saccoglossus kowalevskii* (King 1986, Woodin et al. 1987), *Ptychodera* sp. (Higa et al. 1987), the deep-sea species *Stereobalanus canadensis* (Jensen et al. 1992), and *P. bahamiensis* (Corgiat et al. 1993). The metabolites identified from these sources have included brominated phenols and quinones, brominated and chlorinated indoles, bromocyclohexenones, and bromopyrroles. Concentrations of these compounds are ca 0.2% of animal wet weight (King 1986) and may range from 2 to 10% of animal dry weight (King 1986, Higa et al. 1987, Barron 1990).

Our experimental approach was to quantify spatial and temporal patterns of halocarbon chemical variation in the common marine hemichordate, *Saccoglossus kowalevskii* (Agassiz, 1873) (class Enteropneusta, family Harrimaniidae). This species is one of the most abundant and wide-spread hemichordate worms. *S. kowalevskii* is found on both the western and eastern shores of the Northern Atlantic (Hyman 1959). In estuarine habitats it is a characteristic infaunal organism and can occur in densities on the order of hundreds per m² (Van Dolah et al. 1984, Carey & Farrington 1989, Gypson 1989, Sarda 1991). Previous chemical investigations have identified the major secondary metabolites in *S. kowalevskii* as halogenated pyrroles (Woodin et al. 1987, Barron 1990) or bromophenols (King 1986, but see King et al. 1994 for species revision).

We have used *Saccoglossus kowalevskii* as a model system to address the following questions: (1) what is the extent of chemical variation (type and concentration) within and among individuals in a given population? and (2) how do the observed patterns of variation change over time? We then interpreted these patterns relative to what is known of the ecology of this organism and in light of the wealth of information surrounding chemical variation in plant and insect systems.

MATERIALS AND METHODS

Isolation and identification of compounds. Preliminary gas chromatography/electron impact mass spectrometry (GC/EIMS) and proton nuclear magnetic resonance spectroscopy (¹H NMR) experiments demonstrated that the halogenated metabolites from *Saccoglossus kowalevskii* in Delaware, USA, could be obtained from methanol extracts. The possibility of artifacts resulting from extraction and storage

in methanol was addressed by comparing the organo-halogen content of methanol and acetone extracts. A rapid and robust high pressure liquid chromatography (HPLC) method was created to handle the bulk of the analyses and to minimize sample handling. The best separation of crude methanol extracts was obtained via HPLC using a Rainin Dynamax Lichrosorb cyano (CN) column (5 µm, 0.45 cm i.d. × 25 cm length) with a 2.5 cm guard column and solvent parameters as in Fig. 1. Compounds were detected with a Knauer variable wavelength UV detector (Model 731.87) at 235 nm, the calculated λ_{max} for tribromopyrrole (Silverstein et al. 1981).

Two peaks of interest were identified from HPLC separations of crude methanol worm extracts (Fig. 1). To obtain material suitable for absolute identification and for construction of standard curves, the following preparative-scale isolation methods were developed. The first peak of interest (A) was a predominant component of the methanol extracts. Purification of this compound required a reverse-phase approach, since the compound appeared to degrade when using normal-phase sorbents. A methanol extract of ground worms (ca 100 g wet wt) was concentrated and separated by vacuum-liquid chromatography (VLC) on a C₁₈ sorbent (Polygosil 60 to 2540; 60 Å pore size; 25 to 40 µm irregular) with a stepwise elution of 5-10-20-30-100% acetonitrile (ACN)/H₂O (ca 20 ml each). The presence of the peak of interest in these fractions was determined by separating 200 µl of the eluent using the analytical reverse-phase HPLC method detailed in Fig. 1. The compound typically eluted in the VLC preparation at 10 to 20% ACN/H₂O as part of a yellow-

Fig. 1. HPLC run conditions with representative chromatogram. The 2 peaks of interest are to the right of the labels A and B. ABS: absorbance at 235 nm (arbitrary units); ACN: acetonitrile; HOAc: acetic acid

green band. Fractions containing the compound of interest were lyophilized, resuspended in 25% ACN/H₂O (1 to 2 ml) and filtered (0.45 μ m) to remove insoluble materials. The resulting mixture was separated on a reverse-phase semi-preparative column (Whatman Partisil 10 μ m M9 10/50 ODS-2, 9.4 mm i.d. $\times$ 50 cm length) at 3 ml min⁻¹ with a gradient run from 25 to 60% ACN/H₂O in 8 min. The central part of a peak eluting at 8 min (50% ACN/H₂O) was collected and lyophilized. The dried sample was further purified on a CN column (Rainin Dynamax Lichrosorb, 5 μ m, 0.45 cm i.d. $\times$ 25 cm length with 2.5 cm guard; conditions as in Fig. 1, minus the 0.1% acetic acid, HOAc). The center of a peak eluting at 5.6 min (55% ACN/H₂O) was collected and lyophilized. As a final purification step, the material was eluted from the CN analytical column with an isocratic run at 15% ACN/H₂O + 0.1% HOAc (1 ml min⁻¹) to yield milligram quantities of an odorless white powder after lyophilization.

The second peak of interest (B) was purified using normal phase HPLC. To obtain an extract suitable for separation on normal phase sorbents, worms (ca 80 g wet wt) were initially extracted in diethyl ether (Et₂O). The resulting crude extract was concentrated and dried with MgSO₄. Solvent strength was then reduced by the addition of a small amount of hexane (HEX). The extract was then separated by VLC on silica gel (Silica Gel 60; EM Science) using a stepwise elution of 10-20-30-40-50-100% Et₂O/HEX (ca 25 ml each). The compound of interest was confirmed by thin layer chromatography (TLC) on silica gel (EM Science Kieselgel 60 F₂₅₄) in 50% Et₂O/HEX. The compound was visualized by short- and long-wave UV and by a 4% cerium (IV) sulfate / 1 M H₂SO₄ spray (Emrich et al. 1990) before and after briefly (ca 1 min) heating at 110°C. Fractions collected at 20 to 30% Et₂O/HEX typically contained the compound of interest as evidenced by an intense purple spot (R_F = 0.34) that developed after the cerium sulfate solution was applied. These fractions were combined, concentrated, dried with MgSO₄, and separated on a silica semi-preparatory column (Whatman Partisil 10 μ m M9/50, 9.4 mm i.d. $\times$ 50 cm length; 10 to 90% Et₂O/HEX 10 min; 3 ml min⁻¹). Detection was at 235 nm. A peak eluting at 14.5 min (90% Et₂O/HEX) was collected, concentrated and rechromatographed using an isocratic run at 40% Et₂O/HEX. The major peak eluting at 14 min was collected and concentrated. Final purification was achieved by isocratic elution at 15% Et₂O/HEX on an Alltech Si-100 5 μ m analytical column with matching guard (1.0 cm length).

The purified materials were used for further elucidation of compound structure by NMR and GC/MS. ¹H and ¹³C NMR spectra were recorded in acetone-*d*₆ on either a JEOL FX90Q or a Bruker-AM 500 MHz

spectrometer. GC/EIMS were obtained on a Finnigan Model 4521C equipped with a flame ionization detector. The compounds were separated on an HP-5 fused silica capillary column (0.25 mm i.d. $\times$ 30 m length, 1.0 μ m stationary phase thickness) typically programmed with a temperature gradient of 3°C min⁻¹ (ca 30 min) with initial injector and column conditions of 250°C and 50°C, respectively. Electron impact spectra (70 eV ionization voltage) were scanned from 50 to 600 *m/z* once every second. Fast-atom bombardment mass spectrometry (FABMS) was carried out on a VG model 70SQ spectrometer using a glycerol matrix. Both positive- and negative-ion low resolution FABMS spectra (100 to 700 *m/z*) were obtained for each compound. High resolution data were obtained for negative ions only.

Intra- and interorganismal variation. Standard response curves were generated for each purified compound of interest. The response curves were based upon 5 repeat injections of 25 μ l of each standard concentration. Natural chemical variation within and among individuals was quantified during a 30 mo sampling period (January 1990 to June 1992). Collections were not possible in January 1992 due to inclement weather and tidal conditions. Each month, 10 intact worms (mean $\pm$ SD, 0.53 $\pm$ 0.31 g) were collected from the population at the Cape Henlopen sandflats, Lewes, Delaware, USA (38° 46' N and 75° 06' W). In the field, worms were divided into 3 sections: proboscis (0.04 $\pm$ 0.03 g), trunk (collar through hepatic region, 0.30 $\pm$ 0.19 g), and tail (posthepatic region, 0.19 $\pm$ 0.13 g). For higher resolution in determining intraorganismal variation, specimens were further dissected into proboscis, anterior trunk (collar, branchial, and gonad region), middle trunk (gonad and hepatic region), anterior tail and posterior tail during some months. Worms collected in 1992 were also visually identified as male or female to address sex-related differences. Sexes were easily determined in the field during most of the year by examining the color of the gametic material through the distended gonads (sperm = peach; eggs = grey). Each worm subsection was then placed in a separate 20 ml scintillation vial containing 10 ml ice-cold HPLC-grade methanol.

Upon return to the lab the vials were sealed with teflon tape to prevent evaporative loss and stored at -20°C until analysis. Compounds were extracted under these conditions by soaking worm tissue for a minimum of 1 mo prior to analysis. The compounds of interest showed no loss of peak area for as long as 22 mo in storage. Preliminary repetitive extraction procedures demonstrated that the soaking procedure was $\geq$ 98% efficient. No grinding or other manipulation was necessary and would have been detrimental due to associated tissue loss. Peak areas for the 2 compounds

were determined from the mean of replicate (2) 25 μ l injections of the crude methanol extract after centrifugation to remove particulates. Sample injections were arbitrarily considered to be acceptable replicates when the difference between their peak areas was $\leq 10\%$. Extracted wet weights (WW), dry weights (DW) (60 °C per 48 h) and ash-free dry weights (AFDW) (425 °C for 12 h) to account for sediment in the gut were measured for each subsection. These optimal times and temperatures were empirically determined from preliminary experiments.

Statistical analyses. Due to marked differences in the magnitude of variation associated with chemical content among body parts, even after transformation, and given the relatively large size of the data set, non-parametric statistics were used. The effect of body part on compound concentration (% WW, % DW, or % AFDW) was evaluated by a Kruskal-Wallis 1-way ANOVA ($\alpha = 0.05$). When an effect was detected, differences among body parts were tested using a Wilcoxon Signed Ranks multiple comparisons test (overall $\alpha_{(2)} = 0.05$ within normalization method). Sex differences in compound concentrations (% AFDW) between the trunk sections and whole bodies of male and female worms were evaluated by the Mann-Whitney U-test.

RESULTS

Isolation and identification of compounds

Crude extracts

Characterization of the crude methanol extract by ^1H NMR (90 MHz) in acetone indicated a single proton in the aromatic region at δ 7.39 (C-5 – H). No nitrogen-linked proton was observed. Comparison of these data with a known spectrum of 2,3,4-TBP in Et_2O (Emrich et al. 1990) showed a downfield shift in our sample absorbance of δ 0.81. Although pyrrole ring-proton chemical shifts are subject to solvent effects via hydrogen-bonding to electronegative atoms or dipolar interactions with aromatic π -systems (Chadwick 1990), this shift seemed excessive and warranted further investigation.

GC/EIMS data obtained for crude methanol extracts of individual worms from the Delaware population of *Saccoglossus kowalevskii* indicated a major brominated metabolite of the formula $\text{C}_4\text{H}_2\text{Br}_3\text{N}$: m/z (rel. int.) $[\text{C}_4\text{H}_2\text{Br}_3\text{N}]^+$ 307 (31), 305 (96), 303 (100), 301 (34); $[\text{C}_4\text{H}_2\text{Br}_2\text{N}]^+$ 226 (26), 224 (59), 222 (31); $[\text{C}_3\text{HBr}_2]^+$ 199 (20), 197 (43), 195 (21); $[\text{C}_3\text{HBr}]^+$ 118 (30), 116 (31); $[\text{Br}]^+$ 81 (10), 79 (11). These data were consistent with previous identification of the compound as a tribromopyr-

role (Barron 1990, Emrich et al. 1990). No bromophenol-containing worms were found. No differences in tribromopyrrole content were observed between methanol and acetone extracts. Thus, no methanol-specific alteration or significant decomposition of the compounds of interest occurred during extraction and storage in methanol.

Further EIMS analysis of the compounds identified as peaks A and B in the analytical HPLC chromatographs (Fig. 1) revealed that the 2 were chemically related. In semi-pure preparations they had identical spectra in the m/z range from 50 to 400. However, the compounds did not constitute an acid-base pair, since the addition of strong/weak acid or base did not result in their interconversion. More frequently, attempts at acid/base conversion led to decomposition as observed by decreasing HPLC and NMR peak areas and the appearance of a pink color in the samples due to liberation of bromine. HPLC analysis of a filtered (0.45 μm) seawater supernatant in which a living worm had been placed for several hours confirmed that both compounds were from the worm and not an extraction artifact.

Purified compounds

Purification of milligram quantities of the tribromopyrrole compounds enabled detection of conspicuous differences with highfield ^1H and ^{13}C NMR (Table 1). The marked downfield chemical shifts of the C-5 ring proton and altered carbon chemical shifts, suggested the presence of an electron-withdrawing group associated with nitrogen in the more polar compound (A). Significantly, the functional group appeared to lack both protons and carbon. In contrast, the less polar compound (B) exhibited a broad D_2O -exchangeable singlet at δ 11.2, integrating 1:1 with the C-5 proton and occasionally observed to couple ($J = 3.07$ Hz) with it, indicating the presence of a single hydrogen on the

Table 1. ^1H and ^{13}C NMR data obtained in acetone- d_6 for purified compounds of interest, tribromopyrrole A and tribromopyrrole B. s: singlet; bs: broad singlet

Analytical method	Tribromopyrrole	
	A	B
HPLC retention (min)	5.6	9.2
^1H NMR (δ ppm)	7.38 s	7.11 s 11.80 bs
^{13}C NMR (δ ppm)	96.89 100.81 103.24 122.72	98.33 99.90 100.51 120.60

ring nitrogen. ^{13}C NMR spectra obtained using the INEPT pulse sequence showed that the same ring carbon in both compounds was protonated (A: 122.72 ppm; B: 120.60 ppm).

Purified compound B gave a GC retention index of 1656, consistent with that of 2,3,4-tribromopyrrole (Barron 1990, Emrich et al. 1990). Purified compound A was represented by a very small poorly-shaped peak in the gas chromatograms and gave a weak EI signal; thus, it appeared to be non-volatile. Unusual amounts of an acetone dimer [$\text{CH}_3\text{COCH}_2\text{C}(\text{CH}_3)_2\text{OH}$] present in the EI spectrum for compound A suggested that a proton scavenging reaction had occurred during the analysis. At low resolution, both the positive and negative ion FABMS spectra for compound B were consistent with a tribromopyrrole [(+) FAB:MH⁺ 302; (–) FAB:MH[–] 300]. No parent peak was present in the positive ion trace for compound A. Instead, the presence of sodium was detected. Negative ion FABMS spectra for compound A contained a parent peak with a tribrominated pattern (1:3:3:1) at 80 m/z units higher than that of tribromopyrrole [(386, 384, 382, 380) vs (306, 304, 302, 300)]. A dibrominated pattern (1:2:1) for a fragment of compound A centered at an m/z ratio of 304 suggested the loss of bromine from the parent molecule. At high resolution, the measured mass of the negative ion parent peak for compound A was 381.7205. The only molecular formula consistent with all the spectral data obtained was $\text{C}_4\text{HBr}_3\text{NO}_3\text{S}^-$. This formula has a calculated mass of 381.7207. Because of the abundance of Na^+ in the positive ion

Fig. 2. Proposed structures for the 2 brominated pyrroles isolated from *Saccoglossus kowalevskii*. (A) 2,3,4-tribromopyrrole sulfamate (sodium salt). (B) 2,3,4-tribromopyrrole

FABMS we concluded that compound A was the sodium sulfamate salt of 2,3,4-tribromopyrrole with the molecular structure shown in Fig. 2.

Intra- and interorganismal variation

The purified compounds gave standard detector response curves ($\mu\text{g ml}^{-1}$) of [2,3,4-TBP sulfamate] = $0.0139 \times \text{peak area} + 0.00605$ ($r^2 = 0.998$) and [2,3,4-TBP] = $0.0121 \times \text{peak area} + 0.0113$ ($r^2 = 0.998$). The average 2,3,4-TBP sulfamate content of whole worms was almost 20 times greater than the 2,3,4-TBP content when normalized to extracted wet, dry, or ash-free dry weight (Table 2). Because of sediment present in the worms' guts, we consider the concentration patterns associated with AFDWs to be most meaningful. On an

Table 2. *Saccoglossus kowalevskii*. 2,3,4-Tribromopyrrole (TBP) and its sodium sulfamate salt in worm body parts and whole animals on a percent methanol extracted wet weight (% WW), dry weight (% DW) and ash-free dry weight (% AFDW) basis. Within a compound type and weight normalization method, means having the same superscript letter were not significantly different using Wilcoxon's Signed Ranks multiple comparisons test (overall $\alpha_{(2)} = 0.05$). Range of values is the 5 to 95 percentile range. n: number of worms; SD: standard deviation; CV: coefficient of variation

Body part	n	% WW Mean $\pm$ SD	Range	n	% DW Mean $\pm$ SD	Range	n	% AFDW Mean $\pm$ SD	Range	CV
TBP sulfamate										
Proboscis	282	0.69 ± 0.27^A	0.31–1.1	281	4.4 ± 1.6^A	1.8–7.1	280	5.9 ± 2.0^A	2.9–8.9	34.0
Trunk	285	1.1 ± 0.56^B	0.26–2.2	289	4.3 ± 3.0^A	0.90–10.4	283	12.6 ± 6.0^B	2.5–22.4	47.5
Tail	277	0.43 ± 0.37^A	0.10–1.1	281	1.2 ± 1.5^B	0.20–3.2	275	10.9 ± 5.6^C	4.5–22.7	51.3
Whole	268	0.85 ± 0.36	0.29–1.6	275	2.7 ± 1.7	0.82–6.3	263	11.2 ± 4.3	3.8–18.6	38.2
TBP										
Proboscis	266	0.20 ± 0.48^A	0.019–0.85	267	1.3 ± 2.5^A	0.13–5.7	264	1.8 ± 4.3^A	0.19–7.2	234
Trunk	278	0.028 ± 0.035^B	0.0063–0.11	281	0.11 ± 0.18^B	0.019–0.32	277	0.30 ± 0.38^B	0.015–1.0	130
Tail	277	0.055 ± 0.080^C	0.0080–0.20	277	0.13 ± 0.20^B	0.018–0.58	274	1.5 ± 2.2^A	0.10–5.8	152
Whole	251	0.047 ± 0.056	0.011–0.18	254	0.15 ± 0.20	0.035–0.58	247	0.65 ± 0.77	0.16–2.5	120
TBP + TBP sulfamate										
Proboscis	261	0.90 ± 0.54^A	0.40–1.7	260	6.4 ± 4.5^A	2.8–12.0	259	7.8 ± 4.7^A	3.6–13.6	60.2
Trunk	274	1.2 ± 0.57^B	0.30–2.2	277	4.7 ± 3.0^B	1.1–10.6	271	12.8 ± 6.1^B	2.9–22.9	47.4
Tail	269	0.48 ± 0.38^C	0.14–1.2	269	2.6 ± 2.5^C	0.64–7.9	263	12.2 ± 5.5^B	5.6–23.0	45.3
Whole	239	0.91 ± 0.37	0.32–1.6	242	3.3 ± 1.8	1.2–7.1	231	11.9 ± 4.3	4.1–19.3	36.3

Fig. 3. *Saccoglossus kowalevskii*. Intraorganismal distribution of (A) 2,3,4-TBP sulfamate, (B) 2,3,4-TBP, and (C) total tribromopyrrole content (2,3,4-TBP sulfamate + 2,3,4-TBP) on a percent ash-free dry weight (AFDW) basis in body parts from worms collected in January 1990 to June 1992. Numbers in parentheses are number of observations. Error bars are ± 1 SE of the mean

AFDW basis there was no apparent relationship between the total amount of 2,3,4-TBP and its sulfamate salt present in individual worms. A 5-fold range in the average concentration of the sulfamate salt among the majority (90%) of individuals within the population was observed (Table 2). 2,3,4-TBP values were more than 3 times as variable. Total tribromopyrrole content (2,3,4-TBP + 2,3,4-TBP sulfamate) exhibited greater than a 4-fold range.

Expressed as a percentage of the worm's extracted AFDW, the worm proboscis contained significantly less

of the sulfamate salt than either trunk or tail tissue (Fig. 3A). The sulfamate salt contents of trunks and tails were similar, although trunks contained significantly more compound. This was in contrast to the intraorganismal distribution of 2,3,4-TBP, which was significantly lower in the worms' trunks than in proboscises or tails (Fig. 3B). The 2,3,4-TBP contents of proboscises and tails were not significantly different. The total tribromopyrrole contents (2,3,4-TBP + 2,3,4-TBP sulfamate; % AFDW) of trunk and tail were not different from one another, but were significantly

Fig. 4. *Saccoglossus kowalevskii*. Intraorganismal distribution of 2,3,4-TBP sulfamate and 2,3,4-TBP on a percent ash-free dry weight (AFDW) basis in body parts of worms collected in May 1992. Number of observations = 10. Error bars are ± 1 SE of the mean. Data points plotted with the same letter were not significantly different at an overall $\alpha = 0.05$ (Wilcoxon's Signed Ranks multiple comparisons test)

Fig. 5. *Saccoglossus kowalevskii*. (A) 2,3,4-TBP sulfamate, (B) 2,3,4-TBP and (C) total tribromopyrrole content (2,3,4-TBP sulfamate + 2,3,4-TBP) of the trunk sections of male and female worms collected in 1992. Error bars are ± 1 SE of the mean. Number of animals: male = 23, female = 23

greater than the average proboscis concentration (Fig. 3C). Examination of the sulfamate salt distribution within worms on a finer scale confirmed the patterns observed above, but also revealed within the midsection a marked concentration of this compound in the hepatic region of the worms and a deficit in the branchial area (Fig. 4). In contrast, the amount of 2,3,4-TBP was low in both regions. There were no significant differences in the chemical content of male and female worms (whole worm or trunk regions) (Fig. 5), although there is a trend for the chemical content of males to be somewhat greater than that of females.

Differences in the magnitude of variation of compound concentration among body parts were also evident. These differences were apparent in both the range of values observed among the majority of individuals in the population (5 to 95 percentile range) and in the intrinsic variability (coefficient of variation) of the samples (Table 2). The variability of the sulfamate salt was highest in trunk and tail tissue. Although the greatest range of values was evident in trunk 2,3,4-TBP values, the intrinsic variability was highest for proboscises and lowest in trunk tissues. Correspondingly, the range of values observed for total tribromopyrrole content was greatest for trunks, but the intrinsic variability was greatest for proboscises.

No temporal pattern in the sulfamate salt content of whole worms was evident (Fig. 6). From fall 1990 to spring 1992, 2,3,4-TBP values appeared to increase during spring and fall (Fig. 6). These increases were generally evident across all body sections (Fig. 7).

However, because of interannual variability and the small size of this time series, the pattern could not be statistically substantiated.

DISCUSSION

Using GC/EIMS, previous studies had identified the halometabolite from *Saccoglossus kowalevskii* as a bromopyrrole of the formula $C_4H_2Br_3N$ (Woodin et al. 1987, Barron 1990). This study confirmed these findings, but additionally demonstrated by FABMS that the predominant compound present in the worm is a compound with the formula $C_4HBr_3NO_3SNa$. The proposed structure for the molecule represented by this formula is a sodium sulfamate salt (Fig. 2). We favor this structure over an oxygen-linked derivative for 2 reasons. First, this structure is consistent with the enzymology of the sulfotransferases, enzymes that can transfer a sulfonyl group directly to amines, forming sulfamates (Ramaswamy & Jacoby 1987). Second, the highly stable chemical character of the sulfamates (Greenwood & Earnshaw 1984) is consistent with the observed stability of the compound.

This study represents the first identification of a halogenated aromatic sodium sulfamate salt as the predominant secondary metabolite of a hemichordate. Occurrence of this compound was not initially apparent due to the generally low concentration of 2,3,4-TBP in the worms and the equivalence of the 2 compounds' electron impact mass spectra when crude or semi-pure

Fig. 6. *Saccoglossus kowalevskii*. Temporal variation of 2,3,4-TBP sulfamate and 2,3,4-TBP content of whole worms on a percent ash-free dry weight basis (AFDW, $n = 10 \text{ mo}^{-1}$) from January 1990 to June 1992. Error bars are ± 1 SE of the mean

extracts were screened by GC/EIMS. Clearly, initial attempts at identification of the predominant compound in *Saccoglossus kowalevskii* by GC/EIMS (Woodin et al. 1987, Barron 1990, this study) were obscured by bond cleavage and a corresponding hydrogen-scavenging reaction, which may have occurred in the high temperature injector port of the GC. An additional factor of relevance was the use of negative ion FABMS which allowed the detection of the negatively charged sulfate derivative of the tribromopyrrole. Such negatively charged ions escape ready detection by widely used conventional positive ion mass spectral techniques (i.e. EIMS). Thus, more careful preparation and screening of extracts may be necessary in future studies intending to examine chemical variation.

The difficulty of concurrent detection of the metabolites from *Saccoglossus kowalevskii* raises the possibility that similar compounds previously identified from marine invertebrates by standard techniques could be present as analogs in the animals themselves. For example, Emrich et al. (1990) isolated 2,3,4-tribromopyrrole from the marine infaunal polychaete *Polyphysia crassa* Oersted (Scalibregmidae) using acetone-hexane extraction, normal phase chromatography and GC/EIMS. Concentrations of this metabolite (via GC analysis) were found to range from 0.006 to 0.008% of the worm's wet weight. Although these low concentrations may indeed represent the actual 2,3,4-TBP total in the worms, using a similar approach

we were unable to detect the sulfamate derivative of this compound from *S. kowalevskii*. N-linked sulfur-containing functional groups could be common among the many nitrogenous secondary metabolites known from marine organisms. Until this study, sulfamates were known only from microalgal metabolites (Hall et al. 1990). Analogously, the simple halogenated phenolic metabolites from sponges, polychaetes, and hemichordates (Higa 1991) could exist within the animals as sulfate esters similar to the dipotassium salt of lanosol from the red alga *Neorhodomela larix* (Carlson et al. 1989).

In *Saccoglossus kowalevskii*, the first consistent pattern of chemical variation noted was between the 2 tribromopyrrole compounds; the sulfamate salt was much more abundant than 2,3,4-TBP in almost all worms examined (Table 2). The concentration differences may reflect inherent differences in toxicity of the compounds or their ease of storage. Organisms containing autotoxic secondary metabolites may employ at least 1 of 3 common strategies which permit sequestration: (1) physiological or biochemical tolerance; (2) compartmentalization; and (3) detoxification (Duffey 1980, Blum 1981, Karuso 1987, Rosenthal & Berenbaum 1992). Whether *S. kowalevskii* is biochemically or physiologically insensitive to high concentrations of its bromopyrroles is currently unknown. As an ionic molecule, the sulfamate salt may be more readily retained within storage vesicles analogous to those demonstrated for other organohalogen-containing

Fig. 7. *Saccoglossus kowalevskii*. Temporal variation of 2,3,4-TBP content of worm proboscis, trunk and tail tissue on a percent ash-free dry weight basis (AFDW, $n = 10 \text{ mo}^{-1}$) from January 1990 to June 1992. Error bars are ± 1 SE of the mean

marine organisms (Young et al. 1980, Westlund et al. 1981, Pallaghy et al. 1983, Pedersén & Roomans 1983, Thompson et al. 1983). The hepatic region of *S. kowalevskii*, rich in the sulfamate salt (Fig. 4), is described as a highly vacuolated region (Hyman 1959). Among terrestrial organisms, bioactive secondary metabolites are commonly stored via conjugation to sugar residues (Blum 1981, Pasteels et al. 1983, Harborne 1988, Rosenthal & Berenbaum 1992). Similarly, in the marine environment, steroidal and triterpenoid glycosides (saponins) are common among echinoderms (Burnell & ApSimon 1983, Ricco et al. 1987). In nudibranchs whose defensive secretions contain the autotoxic sesquiterpene polygodial, olepupane is thought to serve as the 'protected' acetate precursor (Cimino et al. 1985, Cimino et al. 1988). In general, however, marine organisms may favor the use of sulfate as a means of reducing the autotoxicity of their secondary metabolites because of the abundance of this ion in marine waters (SO_4^{2-} ca 2700 mg l^{-1} ; Kenish 1989). Sulfate conjugation is commonly used by

marine invertebrates in the detoxification of anthropogenic compounds (James 1987, Livingstone 1991) producing sulfate esters, sulfonic acids or sulfamates (Mulder 1981). Such sulfated secondary metabolites are known from a range of marine organisms, including micro- and macroalgae, seagrasses, sponges, molluscs, echinoderms and fishes (Baker & Murphy 1976, McMillan et al. 1980, Faulkner 1992 and references therein). Consistent with this detoxification role, naturally occurring sulfate derivatives of secondary metabolites can be less bioactive than their non-sulfated analogs (e.g. McLachlan & Craigie 1966, Burnell & ApSimon 1983, Shimizu et al. 1984, Harada et al. 1989, Hall et al. 1990).

The large reservoir of the sulfamate salt may represent the chemically stable non-toxic precursor to a more toxic and volatile 2,3,4-TBP, which could serve as a predator deterrent. If the 2,3,4-TBP in *Saccoglossus kowalevskii* has an antipredator role, then this system may constitute an activated induced defense. Among higher plants and insects, activated defenses are well known (Blum 1981, Harborne 1988, Rosenthal & Berenbaum 1992), but in marine systems, they are known only from the common tropical green seaweed *Halimeda* spp. (Paul & Van Alstyne 1992). Enzymatic conversion of the sulfamate salt to 2,3,4-TBP by *S. kowalevskii* has not yet been demonstrated, although the sulfamate salt decreases as 2,3,4-TBP increases in seawater worm homogenates over short time periods (1 to 10 min, authors' unpubl. data).

A biologically important role for 2,3,4-TBP, possibly as a predator deterrent, is suggested by its changing concentration over time (Fig. 6). Increases in the amount of 2,3,4-TBP appear to occur during spring and fall months of some years, the known spawning periods for this species (Gypson 1989). This temporal variation contributes greatly to the observed intra- and interorganismal variation (Table 2). We do not believe these increases to be due to allocation of bromopyrroles to gametes, as the trunk region of worms (the region of gamete synthesis and storage) contains less 2,3,4-TBP than other body parts (Table 2) and the apparent trend is for the trunks of females to contain less tribromopyrrole than males (Fig. 5). The observed increases in 2,3,4-TBP during spawning months may instead reflect a reduction in palatability or an increase in toxicity of somatic tissue during this period of energy allocation to gamete development. Although *Saccoglossus kowalevskii* can regenerate tissue lost to predation (Tweedell 1961), its cost of replacement and associated changes in feeding activity following tissue loss (Woodin 1984) could be detrimental to gamete production. This hypothesis is supported by the observations that concurrent increases are generally observed across all body parts and that the increases

are greatest in proboscis and tail (Fig. 7), the tissues most apparent to epifaunal predators. Confirmation of these results and conclusions will require additional chemical and biological investigation. Preliminary bioassays suggest that at 2% AFDW, 2,3,4-TBP is distasteful to fish predators (authors' unpubl. data).

Fluctuation in nutrient supplies might also explain some of the observed chemical variation. As nitrogen becomes increasingly limiting, carbon allocation to secondary metabolites has been shown to increase (Ilvessalo & Tuomi 1989, Herms & Mattson 1992). The abundant brominated pyrrole which *Saccoglossus kowalevskii* contains, however, is a nitrogenous metabolite. It would be predicted to decrease when nitrogen is in short supply. The high concentrations and lack of a seasonal pattern for the predominant compound (2,3,4-TBP sulfamate) suggests that at the population level, nitrogen is in sufficient supply to sustain both primary and secondary metabolism in these worms throughout the year. Whether any of the observed interorganismal variation in field-collected worms could be attributed to competition or inherent differences in resource acquisition is unknown.

The marked differences in the intraorganismal distribution patterns observed for the 2 tribromopyrrole compounds were unexpected. Whereas the sulfamate salt was concentrated in the hepatic region of the worms, 2,3,4-TBP was highest in the worms' proboscises and tails (Table 2). *Saccoglossus kowalevskii* lives in a helical U-shaped burrow from which these body parts are exposed to predation during feeding and defecation. Significantly, both have considerable regenerative capacity (Tweedell 1961), suggesting an adaptation to partial predation. Extracts containing the halogenated compounds from hemichordates exhibit toxic effects in laboratory bioassays (Whitin & Azariah 1982, Azariah & Westley 1990), but the ecological relevance of these studies is unclear. Thus, it is difficult to define the bromopyrrole distributions within *S. kowalevskii* as optimizing defense. Allocation of small halogenated aromatic compounds to exposed tissues is a recurrent theme in studies examining long-lived, soft-bodied infaunal invertebrates. For example, the bromobenzyl alcohols present in *Thelepus crispus* and *T. extensus* are allocated to tentacles and tail in these terebellid polychaetes that inhabit U-shaped tubes (Goerke et al. 1991, S. A. Woodin pers. comm.). Similarly, in the head-down deposit-feeding capitellid polychaete *Notomastus lobatus*, the concentration of monobromophenols is an order of magnitude higher in the tail of the animal, which is exposed to predation during defecation (Fielman 1989). These bromophenols have shown predator-deterrent properties at ecologically relevant concentrations in laboratory and field bioassays (Woodin pers. comm.). The distribution

of 2,3,4-TBP in *S. kowalevskii* may also prove to be consistent with optimizing its predator-deterrent role. One danger to this generalization is that previous studies have based their results on wet or dry weight methods of standardization, which may not account for the presence of sediment in the animal's gut. In this study, the apparent patterns of allocation changed among wet, dry and AFDW methods of normalization (Table 2). Thus, previous authors' conclusions must be viewed as tentative or suspect and interstudy comparisons must be made with some caution.

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