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Article

Metabolomics Reveals Minor Tambjamines in a Marine Invertebrate Food Chain

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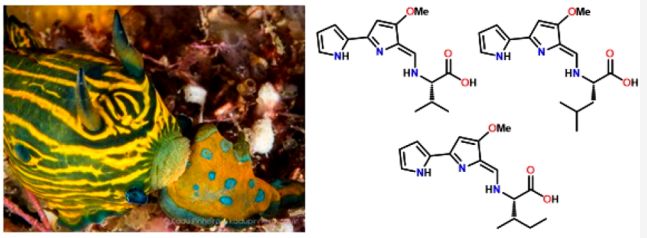
ABSTRACT: Metabolomics analysis detected tambjamine alkaloids in aqueous and EtOAc extracts of the marine invertebrates *Virididentula dentata*, *Tambja stegosauriformis*, *Tambja brasiliensis*, and *Roboastra ernsti*. Among several tambjamines, the new amino acid derivatives tambjamines M-O (**17**−**19**) were identified by Marfey's advanced analysis, UPLC-MS/MS analyses, and total synthesis. The tambjamine diversity increased from the bryozoan *V. dentata* to its nudibranch predators *T. stegosauriformis* and *T. brasiliensis* and attained a higher diversity in *R. ernsti*, the nudibranch that preys upon *T. stegosauriformis* and *T. brasiliensis*. The total tambjamine content also increases among the trophic levels, probably due to biomagnification. Tambjamines A (**1**), C (**3**), and D (**4**) are the major metabolites in the tissues of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* and are likely the main chemical defenses of these marine invertebrates.



Roboastra ernsti feeding on *Tambja stegosauriformis*

Tambjamines are bispyrrole alkaloids isolated from marine invertebrates, including nudibranchs,[1−6] bryozoans,[1,2,6,7] and ascidians.[3] Thirteen tambjamines have been isolated from marine invertebrates. Tambjamines A−D (**1**−**4**) were first isolated from the nudibranchs *Tambja abdere*, *Tambja eliora*, and *Roboastra tigris*, as well as from the bryozoan *Sessibugula transluscens*.[1,2] Subsequently, tambjamines E (**5**) and F (**6**) were reported from the ascidian *Atapazoa* sp. and from nudibranchs of the genus *Nembrotha*.[3] Later on, tambjamines G−J (**7**−**10**) were isolated from the bryozoan *Virididentula dentata* (aka *Bugula dentata*).[7] More recently, tambjamine J1 (**11**) has been reported from the nudibranch *Tambja stegosauriformis*,[6] while tambjamine K (**12**) was isolated from the nudibranch *Tambja ceutae*.[5] The isolation of tambjamine L (**13**) from *Tambja capensis* has been reported in a MSc dissertation.[8] The aldehydes of tambjamines A (**14**), B (**15**), and D (**16**) were reported as isolation artifacts.[1,2,6]

Natural tambjamines display cytotoxic[5,9,10] and DNA binding and cleavage bioactivity.[11,12] Additional activities have been reported for synthetic tambjamines, such as their function as transmembrane ion transporters[13−15] and antimalarial properties.[16] Tambjamines are implicated in defense mechanisms of marine invertebrates that accumulate these alkaloids.[1−7] Early investigations of marine invertebrates that accumulate tambjamines indicated capture and transfer of tambjamines between prey and predators.[1−7]

Tambja stegosauriformis Pola, Cervera & Gosliner, 2005;[17] *T. brasiliensis* Pola, Padula, Gosliner & Cervera, 2014;[18] and *Roboastra ernsti* Pola, Padula, Gosliner & Cervera, 2014[18] (Polyceridae, Nembrothinae), are nudibranch species recently described for the Southeastern Brazilian coastline (Figure 1).

We have previously reported several tambjamines from *T. brasiliensis*,[4] *T. stegosauriformis*, and *Virididentula dentata*.[6] A recent collection and analysis of tambjamines in *R. ernsti* mantle indicated a larger variety of these alkaloids, some of which in amounts that could only be detected by HPLC−UV−MS and UPLC−quadrupole time of flight (QToF)−MS analyses. We then performed a metabolomics analysis of tambjamine distribution in *T. brasiliensis*, *T. stegosauriformis*, *R. ernsti*, and *V. dentata* and discovered new minor tambjamines which are likely biosynthetic precursors of the known major alkaloids.

RESULTS AND DISCUSSION

V. dentata, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* were collected and immediately preserved in EtOH. The animals were subsequently extracted with MeOH. The pooled EtOH/MeOH extract was evaporated and defatted by MeOH/hexane partitioning. The MeOH fraction was evaporated and partitioned between H_2O and EtOAc. HPLC−UV−MS analysis indicated tambjamine alkaloids in both EtOAc and

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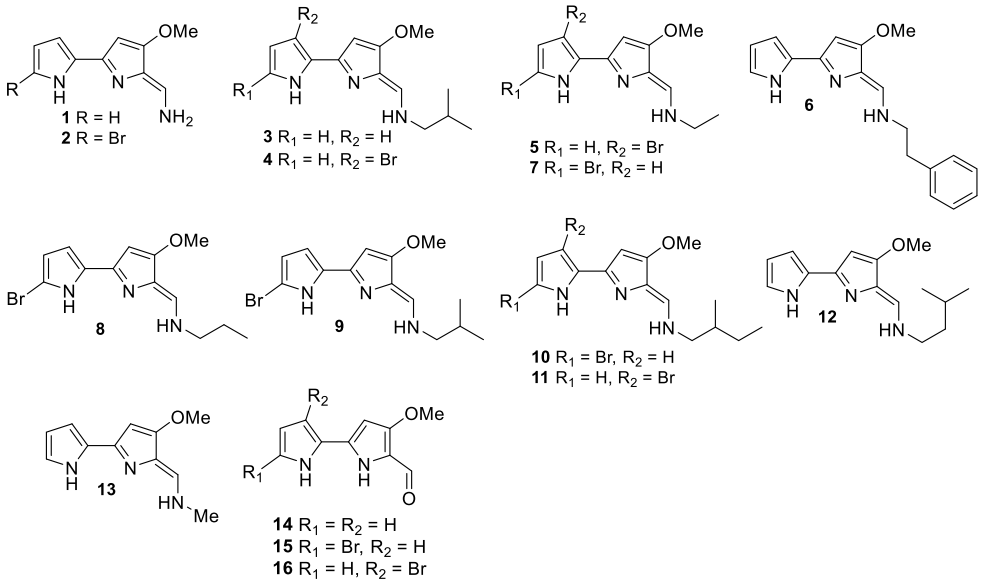

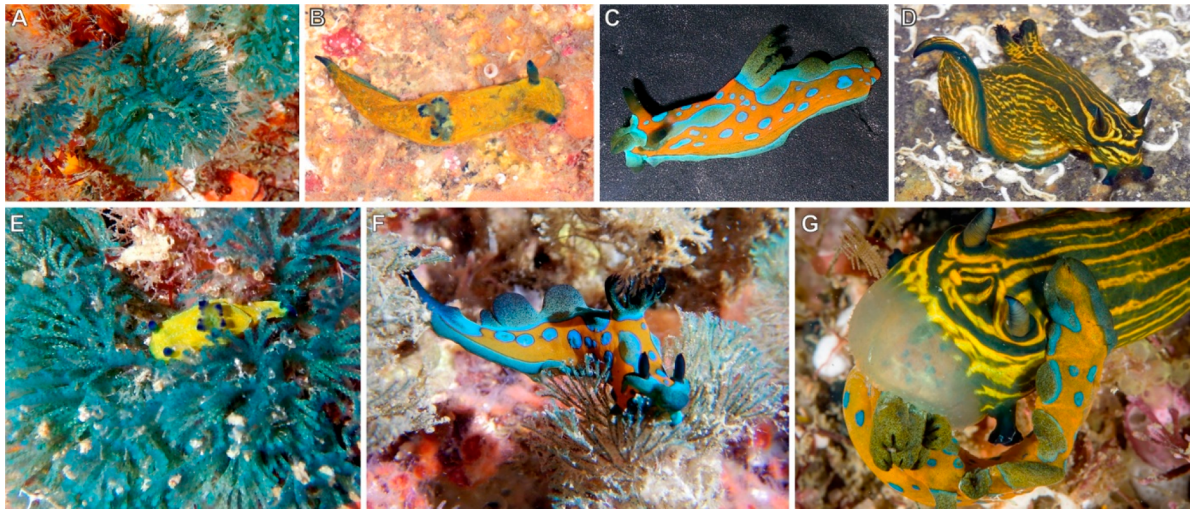

Figure 1. (A) *Virididentula dentata*. (B) *Tambja brasiliensis*. (C) *Tambja stegosauriformis*. (D) *Roboastra ernsti*. (E) *T. brasiliensis* feeding upon *V. dentata*. (F) *T. stegosauriformis* feeding upon *V. dentata*. (G) *R. ernsti* eating *T. stegosauriformis*.

H_2O fractions of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*. Tambjamines have characteristic UV−vis spectra with λ_{max} at 390 and 410 nm, with slightly variable wavelengths depending on the substituents connected to the chromophore. Tambjamines with an unsubstituted enamine group, such as **1** and **2**, have UV−vis absorptions between λ_{max} 250−255 and 385 nm. Tambjamines with a substituted enamine group (**3**−**13**) display UV absorption bands at λ_{max} 255 and 398 nm.[6] HPLC−UV−MS data of organic and aqueous fractions from extracts of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* indicated the presence of tambjamines A (**1**), C (**3**), D (**4**) or I (**9**), and K (**12**) and the aldehydes of tambjamines A (**14**) and B (**15**), by comparison with authentic standards.

Tambjamines also present a characteristic MS fragmentation pattern. When subjected to MS/MS experiments, tambjamines generate fragment ions at *m/z* 175.07, *m/z* 163.09, *m/z* 148.06, and *m/z* 131.06.[6] Organic and aqueous extracts of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* were analyzed by UPLC−QToF−MS/MS. By comparison of retention times, accurate mass measurement, and fragmentation patterns with available tambjamine standards, we confirmed the presence of tambjamines A (**1**), C (**3**), D (**4**), and K (**12**) and of the aldehydes **14** and **15** in the tissues of the nudibranchs and of **3**, **4**, **12**, and **15** in *V. dentata* (Supporting Information, Figure S74). Although tambjamines D (**4**) and I (**9**) are isomers with similar UV spectra, as well as with the same accurate mass and fragmentation pattern, **4** and **9** have slightly different retention times in UPLC−QToF−MS analyses, which allowed us to confirm the presence of **4**, but not **9**, in the samples of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*.

UPLC−QToF−MS, UPLC−QToF−MS/MS, and molecular networking analyses indicated a limited diversity of tambjamines in the organic extract of the bryozoan *V. dentata*, and no alkaloids were detected in its aqueous extract. Tambjamine A (**1**) and its aldehyde (**14**) were mainly detected in the aqueous extracts of *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* (Table 1).

The organic extract of *R. ernsti* indicated the presence of seven unknown compounds with UV absorptions and fragmentation patterns similar to known tambjamines. As these new tambjamines were present in very small amounts,

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Table 1. Occurrence of Tambjamines and Percentage (%) of Tambjamine Content in Tissues of the Bryozoan *V. dentata* and in the Mantle of the Nudibranchs *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*

organism	H_2O fraction	EtOAc fraction
V. dentata		**3** (73%), **4** (22%), **12** (4%), **15** (1%)
T. stegosauriformis	**1** (19%), **14** (2%)	**1** (43%), **3** (23%), **4** (9%), **12** (1%), **14** (2%), **15** (1%)
T. brasiliensis	**1** (20%), **4** (5%), **14** (8%), **15** (6%)	**3** (4%), **4** (51%), **12** (1%), **15** (5%)
R. ernsti	**1** (53%), **3** (4%), **4** (3%), **12** (1%), **14** (5%), **15** (4%)	**3** (9%), **4** (11%), **12** (1%), **14** (6%), **15** (3%), *m/z* 312/314 (nq),[a] *m/z* 333 (nq)

[a]nq: not quantified.

new specimens of *R. ernsti* were collected, extracted and the defatted extract was fractionated by HPLC−UV. A total of 13 fractions were obtained. Analyses by HPLC−UV−MS and UPLC−QToF−MS/MS indicated the presence of the new tambjamines M−O (**17**−**19**), as well as of tambjamines which could not be unambiguously identified (Figure 2). The structures of the new tambjamines (**17**−**19**) are proposed by analysis of spectroscopic data and confirmed by total synthesis.

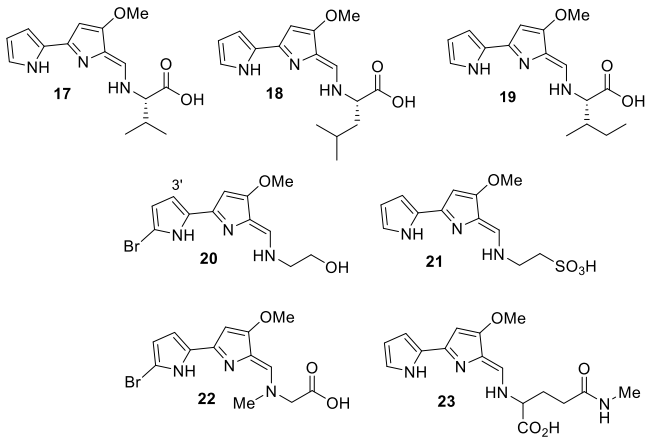

Figure 2. *R. ernsti* tambjamines M−O (**17**−**19**) identified by analysis of spectroscopic data. Structures **20**−**23** were suggested for additional tambjamines but could not be unambiguously confirmed.

High-resolution MS/MS data for the new tambjamines **17**−**19** indicated the product ion at *m/z* 190.0975 corresponding to the tambjamine core (Figure 3 and Tables S1 and S2 in the Supporting Information). Tambjamines M−O (**17**−**19**) presented MS/MS fragmentation patterns that showed losses of neutral fragments $C_5H_8O_2$, $C_6H_{10}O_2$ and $C_6H_{10}O_2$, respectively, with the addition of CO_2 relative to the fragmentation of the corresponding unbrominated **3**, **9**, and **10**. Figure 3 illustrates the comparison of fragmentation observed for tambjamine C (**3**) and M (**17**). Structures **20** and **22** were initially proposed for two additional tambjamines that presented an isotopic pattern in the HRMS spectra consistent with the presence of one bromine atom (*m/z* 312.0350 and 314.0333 $[M + H]^+$ for compound **20** and *m/z* 340.0294 and 342.0280 $[M + H]^+$ for compound **22**). Additional tambjamines detected in *R. ernsti* fractions showed an HRMS spectrum with an isotopic pattern consistent with the presence of one sulfur atom or with an even nominal mass indicating an even number of nitrogen atoms in its structure. The structures

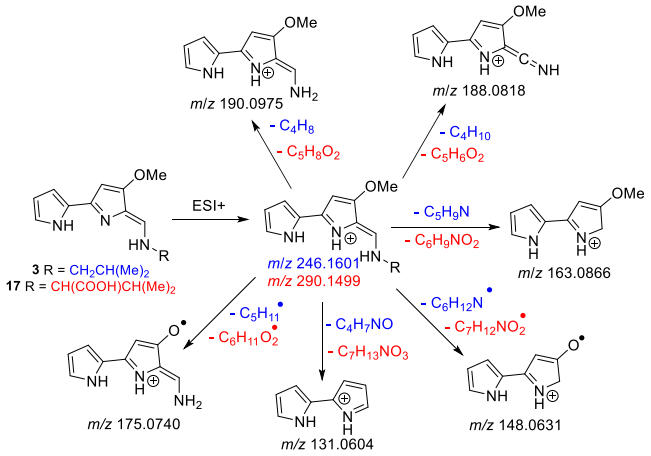

Figure 3. Fragment ions observed in HRMS/MS spectra for tambjamine C (**3**, in blue) and M (**17**, in red).

17−**23** were initially proposed for these additional alkaloids based on the MS/MS fragmentation.

The MS/MS fragmentation suggested that the new alkaloids detected in *R. ernsti* could be tambjamine amino acid derivatives. Therefore, fractions 1R-2 and 1R-4 from *R. ernsti* were subjected to the advanced Marfey's analysis as recently reported,[19] using 5-fluoro-2,4-dinitrophenyl-Nα-L-tryptophanamide (FDTA) as the derivatizing agent. The hydrolyzed and derivatized material was analyzed by UPLC−QToF−MS using a recently described method.[20] By comparison with the retention time of the corresponding L- and D-DTA derivatized amino acid standards, we confirmed the presence of a residue of L-valine in fraction 1R-2 and residues of L-leucine and L-isoleucine in fraction 1R-4, in agreement with the structures that were proposed on the basis of the MS/MS analyses.

Aiming to unambiguously confirm the structures of the new tambjamines, we performed the total synthesis of **17**−**19** and **23** using the aldehyde of tambjamine A (**14**) as starting material (Scheme 1). Bromopyrrole enamine **25** was prepared from **24** by bromination and concomitant condensation with DMF in 64% yield.[21] The product (**25**) was immediately used in a Suzuki coupling reaction with *N*-Boc-pyrrole-2-boronic acid. After removal of the Boc protecting group, the aldehyde of tambjamine A (**14**) was obtained in 82% yield.[22] Aldehyde **14** was obtained in two steps from commercially available 4-methoxy-3-pyrolin-2-one (**24**) in 52.5% overall yield.[21] For the synthesis of **20** and **22** (Scheme 1), the aldehyde of tambjamine B (**15**) was employed, prepared as reported previously.[23] Tambjamines **17**−**19** were obtained by a coupling reaction between the aldehyde of tambjamine A (**14**) and L-valine, L-leucine, and L-isoleucine, respectively. Derivative **21** was obtained by reaction between **14** and 2-bromoethylamine in EtOH, followed by the addition of Na_2SO_3. Alkaloid **23** was synthesized from **14** and *tert*-butyl-N^5-methylglutaminate. Compounds **20** and **22** were prepared by the reaction of **15** with ethanolamine and sarcosine *tert*-butyl ester hydrochloride in TFA, respectively. Synthetic tambjamines **17**−**23** were purified by column chromatography and HPLC. The structures of **17**−**19** and of **21** were confirmed by analysis of spectroscopic data, including HSQC, COSY, and HMBC data (see the Supporting Information). Unfortunately, **20** and **23** were not obtained in good purity, while **22** degraded during the purification steps.

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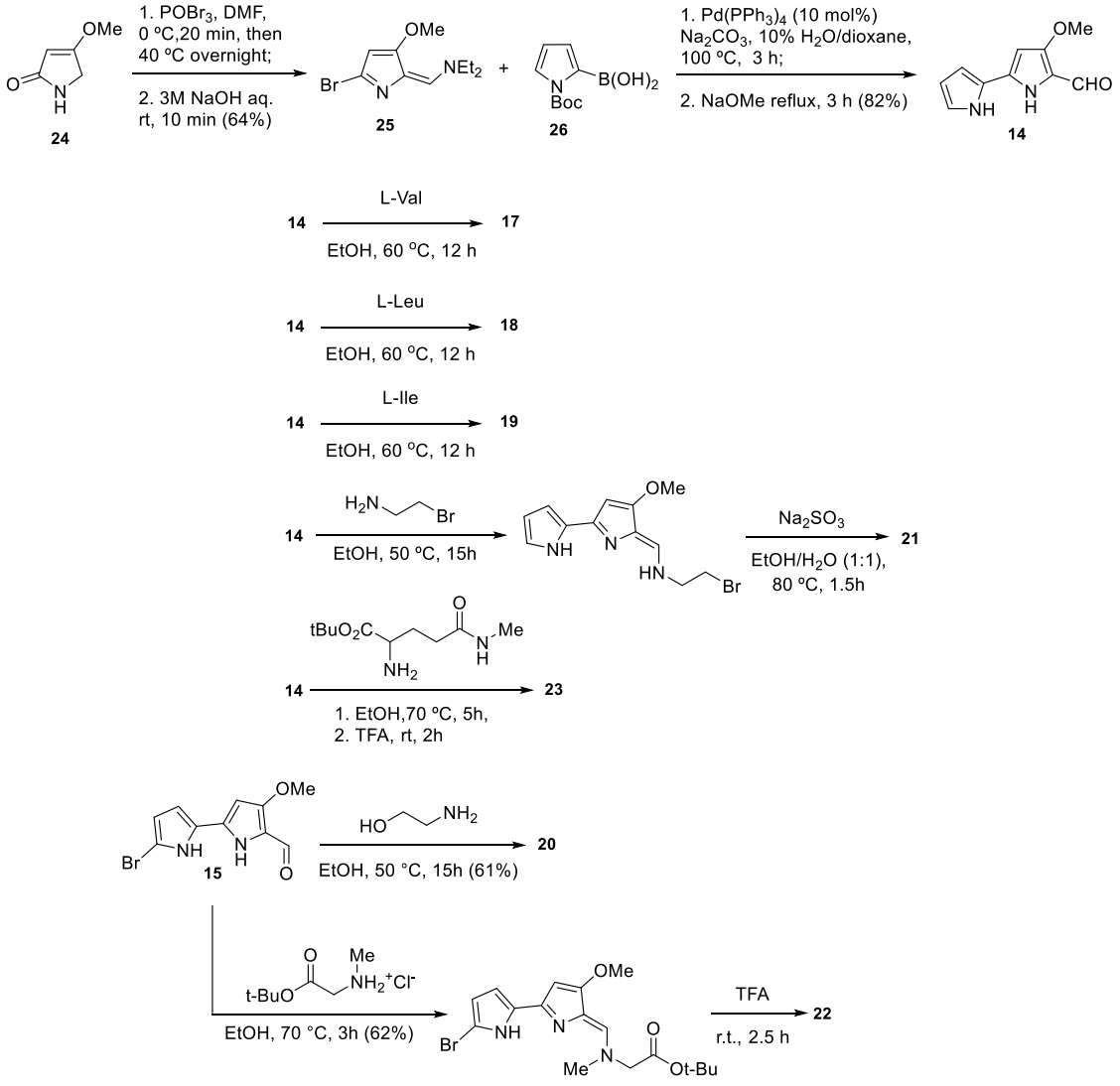
Scheme 1. Synthesis of Tambjamines M−O (17−19) and of 20−23



Synthetic tambjamines and fractions obtained from *R. ernsti* extracts were analyzed by UPLC−QToF−MS/MS to compare the retention time, accurate mass, and fragmentation patterns. The UPLC−QToF−MS/MS analyses confirmed the identity of tambjamines M−O (**17**−**19**) in fractions 1R-2 and 1R-4 from *R. ernsti*. Compound **20** did not present the same retention time to the corresponding natural compound detected in fraction 1R-1E of *R. ernsti* (Figures S53 and S55). Nevertheless, the natural and synthetic samples presented exactly the same MS/MS fragmentation pattern (Figures S54 and S56). Such results suggested an isomeric structure for the corresponding compound detected in fractions from *R. ernsti*, instead of **20**. Considering that tambjamines D (**4**) and I (**9**) are also isomeric and have similar UV spectra, the same accurate mass and exactly the same MS/MS fragmentation pattern, but slightly different retention times in UPLC−MS analyses (Table S1, Figures S8 and S9) such as compound **20** and the compound detected in *R. ernsti* fractions, an isomeric structure with a bromine atom at C-3′ is suggested for the ethanolamine tambjamine derivative detected by UPLC−QToF−MS and UPLC−QToF−MS/MS in fraction 1R-1E obtained from *R. ernsti*. Synthetic **23** did not present the same retention time and fragmentation pattern as observed for the corresponding natural tambjamine in *R. ernsti*, while **21** decomposed during the UPLC−QToF−MS/MS analyses, even using milder analysis conditions. Therefore, we have been unable to identify *R. ernsti* tambjamines that showed $[M + H]^+$ ions at *m*/*z* 298 and 340/342.

Next, we quantified the tambjamine content in the tissues of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* by HPLC with a fluorescence detector (HPLC-Flu) (Tables S5−S8). Tambjamines have fluorescence emission spectra with λ_{emiss} between 412 and 451 nm. Standard solutions of 1.00 mg/mL of tambjamines A (**1**), C (**3**), D (**4**), I (**9**), and K (**12**) and the aldehydes of tambjamines A (**14**) and B (**15**) were analyzed by HPLC-Flu. Tambjamine I (**9**) was not detected in any fraction of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*. The total tambjamine concentration in *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* is shown in Table 2.

The carnivorous *R. ernsti* feeds upon the smaller nudibranchs *T. stegosauriforms* and *T. brasiliensis*, which in turn feed upon the bryozoan *V. dentata* (Figure 1). There is an increase of tambjamine diversity (Table 1) and overall content (Table 2) among the trophic levels, from *V. dentata* to *T. brasiliensis* and *T. stegosauriformis*, then to *R. ernsti*. The content of tambjamines in *R. ernsti* is 3-, 5-, and 39-fold that of *T.*

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Table 2. Normalized Total Concentration of Tambjamines in *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*.

organism	starting material (g)	Tt conc[a]	Tt cont[b]
V. dentata	2.4	0.36 ± 0.03	0.15 ± 0.01
T. stegosauriformis	1.5	1.68 ± 0.05	1.12 ± 0.03
T. brasiliensis	0.3	0.50 ± 0.02	1.79 ± 0.09
R. ernsti	0.1	0.83 ± 0.02	5.81 ± 0.18

[a]Tt conc: total tambjamine concentration in mg/mL. [b]Tt cont: total tambjamine content per gram of organism (mg mL^{-1} g^{-1}).

brasiliensis, *T. stegosauriformis*, and *V. dentata*, respectively. The content of tambjamines in *T. stegosauriformis* and in *T. brasiliensis* is 7-fold and 12-fold that of *V. dentata* (Table 2). The increase in the concentration of the tambjamines among the trophic levels suggests that the higher accumulation in *R. ernsti* is due to biomagnification.[24] However, field experiments are needed to confirm this hypothesis. The enhanced accumulation of tambjamines in *R. ernsti* tissues enabled us to detect tambjamine amino acid derivatives **17**–**19**, as well as a tambjamine ethanolamine derivative, for the first time.

Tambjamines A (**1**), C (**3**), and D (**4**) are the major tambjamine alkaloids present in extracts of *V. dentata*, *T. brasiliensis*, *T. stegosauriformis*, and *R. ernsti*. These compounds display antibiotic, antifungal, cytotoxic, and antimitotic activities.[1,4,9,25] The biological activities of tambjamines are probably responsible for their success as chemical defenses for the organisms that accumulate these alkaloids.

Ecological functions of tambjamines were previously demonstrated in three trophic levels, among the nudibranchs *R. tigris*, *T. eliora*, and *T. abdere* and the bryozoan *Sessibugula translucens*.[2] *S. translucens* was prioritized as a food source by *T. eliora* and *T. abdere*, which, in turn, were preyed upon by *R. tigris*.[2] The Southeastern Brazilian species *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* present a predator–prey relationship in analogy. While tambjamines **1**–**4** were isolated from *R. tigris*, *T. eliora*, *T. abdere*, and *Sessibugula translucens*, indicating the accumulation of these compounds through the food chain,[2] tambjamines **1**, **3**, and **4** are the major compounds found in tissues of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*. Among *R. tigris*, *T. eliora*, *T. abdere*, and *S. translucens*, the predatory preference is related to the nature and concentration of tambjamine alkaloids.[2] The content of tambjamines in *T. eliora* is approximately 2 mg/animal, whereas in *T. abdere*, it is approximately 10 mg/animal.[2]

The Azorean nudibranch *T. ceutae* preys upon the bryozoan *V. dentata*. Both invertebrates accumulate tambjamines A (**1**), B (**2**), and K (**12**).[5] The data collected among different sets of nudibranch–bryozoan food chains indicate that tambjamines A–D (**1**–**4**) are the main chemical defenses of these animals. Given that different tambjamines present distinct levels of cytotoxic and antibiotic activity, the whole pool of tambjamines constitute an effective and sophisticated armamentarium against predators and pathogens.

The discovery of the amino acid derived tambjamines M–O (**17**–**19**) in *R. ernsti* provides further insights on the origin of these metabolites. Tambjamines **17**–**19** can be considered either as detoxification conjugates of tambjamine aldehyde A (**14**) or as biogenetic precursors of tambjamines **3**, **10**, and **12**. In this case, side chains of marine derived tambjamines would be possibly derived from proteinogenic amino acids after decarboxylation, with the exception of tambjamine H (**8**). Considering that the related microbial pyrrole alkaloids prodigiosines and prodiginines are all PKS derived,[26,27] the apparently distinct biosynthesis of these two classes of pyrrole alkaloids may suggests an evolution of closely related biosynthetic pathways in unrelated organisms and a possible additional example of biochemical redundancy.[28−32]

In conclusion, the present investigation reports metabolomics data of tambjamines from the bryozoan *V. dentata* and from the nudibranchs *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*. *Roboastra ernsti* has the amino-acid-bearing tambjamines **17**–**19**. Structures of tambjamines M–O (**17**–**19**) were assigned by UPLC–MS/MS analysis and confirmed by total synthesis. Quantification of tambjamine content indicated that tambjamines A (**1**), C (**3**), and D (**4**) are the major metabolites in the tissues of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti*. Our results show that the use of targeted metabolomics to investigate the chemistry of invertebrates connected through a food chain may help to clarify the origin of secondary metabolites involved in the adaptation of different species.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a Polartronic H Schmidt+Haensch polarimeter. Ultraviolet spectra were obtained on a UV-3600 Shimadzu UV spectrophotometer. The samples were diluted in MeOH at a concentration of 0.01 mg/mL. Infrared (IR) analyses were performed on a silica plate using a Shimadzu model IRAffinity instrument. NMR spectra were obtained at 25 °C on a Bruker Avance III spectrometer (9.4 T) operating at either 600 MHz (^{1}H) or 150 MHz (^{13}C), using TMS as a standard. HPLC–UV–MS analyses were performed on a chromatographic system consisting of a Waters 2695 Alliance control system coupled to a Waters 2696 UV–vis spectrophotometric detector with photodiode array detector and a Waters Micromass ZQ 2000 mass spectrometry detector operated by Empower software. Chromatographic separation was performed using a reversed phase C_{18} XTerra column (4.6 mm × 250 mm, 5 μm; Waters Corporation), and the mobile phase was 100% H_2O + 0.1% formic acid (solvent A) and 1:1 MeCN/MeOH + 0.1% formic acid (solvent B). The gradient elution used was as follows: from 10 to 100% of solvent B in 20 min and then 100% of solvent B from 20.1 to 26 min; from 100 to 10% of solvent B from 26.1 to 27 min and then 10% of solvent B from 27 to 35 min. The flow rate was 1.00 mL min^{-1}, and the injection volume was 20 μL. Samples were diluted in MeOH to a concentration of 1.00 g/mL. The mass spectrometer detector was set at the following conditions: capillary voltage = 3 kV; temperature of the source = 100 °C; desolvation temperature = 350 °C; ESI mode, acquisition range m/z 100–1000; gas flow without cone = 50 L/h; and desolvation gas flow = 350 L/h. UPLC–QToF–MS/MS analyses were performed on a Waters Acquity UPLC H-class liquid chromatograph coupled to a Waters Xevo G2-XS QToF mass spectrometer with an electrospray interface (ESI). The chromatographic separation was performed using a UPLC column Acquity BEH C_{18} (2.1 mm × 50.0 mm, 1.7 μm; Waters Corporation), and the mobile phase was 100% H_2O + 0.1% formic acid (solvent A) and 100% MeCN + 0.1% formic acid (solvent B). The gradient elution used was as follows: from 10 to 50% of solvent B in 6.0 min and then 50–98% of solvent B from 6.0 to 9.0 min; then, from 90 to 10% of solvent B from 9.0 to 9.1 min and then 10% of solvent B from 9.1 to 10 min. The flow rate was 0.5 mL/min. The volume of injection was between 0.1 and 6.0 μL. The column and samples were maintained at 40 °C and at 15 °C, respectively. The positive mode ESI conditions were: 1.2 kV capillary voltage, 30 V cone voltage, 100 °C source temperature, desolvation temperature of 450 °C, 50 L h^{-1} cone gas flow, and 750 L/h desolvation gas flow. For internal calibration, a solution of leucine enkephalin at 200 pg/mL was infused by the lock-mass probe with a flow rate of 10 μL/min. HPLC semipreparative

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separations were performed with a Waters instrument (600 quaternary pump and 2487 double-beam UV detector), with 0.1% formic acid in all eluents used.

Organism Collection and Extraction Procedure. The bryozoan *V. dentata* (2.4182 g dry weight) and the nudibranchs *T. stegosauriformis* (8 specimens; 1.4989 g dry weight), *T. brasiliensis* (2 specimens; 0.2819 g dry weight), and *R. ernsti* (1 specimen; 0.1421 g dry weight) were collected by scuba diving at Ilha dos Pargos, Cabo Frio, southeastern Brazil (22°51′19″S 41°54′25″W), and preserved in EtOH. In the laboratory, the organisms were removed from the EtOH and extracted with MeOH in an ultrasound bath for 30 min three times. The alcoholic extracts were pooled and evaporated. The resulting material was redissolved in 95% MeOH and partitioned with hexane three times. The MeOH extract was evaporated, ressuspended in H_2O/EtOAc, and partitioned three times. The EtOAc and the aqueous extracts of each organism were analyzed by HPLC−UV−MS, HPLC-Flu, and UPLC−QToF−MS/MS.

In another collecting expedition, four specimens of *R ernsti* were collected by scuba diving in Cabo Frio, RJ (Brazil), and preserved in EtOH. In laboratory, the nudibranchs were removed from EtOH and reextracted in MeOH. The alcoholic extracts were pooled and evaporated. The resulting material was resuspended in 95% MeOH and partitioned with hexane three times. The MeOH extract (1R, 546.2 mg) was fractionated by HPLC−UV with a semipreparative C18 Inertsustain column (10 mm × 250 mm, 5 μm, GL Science Inc.) using an isocratic elution of 50:25:25 H_2O/MeOH/MeCN (flow rate = 3.0 mL min^{-1}). Seven fractions were obtained and named 1R-1 (326.9 mg), 1R-2 (3.2 mg), 1R-3 (1.1 mg), 1R-4 (4.0 mg), 1R-5 (4.8 mg), 1R-6 (1.4 mg), and 1R-7 (1.1 mg). Fraction 1R-1 was further fractionated by HPLC−UV using semipreparative C18 Inertsustain column (10 mm × 250 mm, 5 μm, GL Science Inc.). A linear elution gradient from 20 to 80% of (1:1) MeCN/MeOH in H_2O (flow rate = 3.0 mL min^{-1}) during 30 min was used. Eight fractions were obtained and named 1R-1A (238.4 mg), 1R-1B (2.2 mg), 1R-1C (3.2 mg), 1R-1D (1.2 mg), 1R-1E (1.2 mg), 1R-1F (0.6 mg), 1R-1G (1.8 mg), and 1R-1H (0.7 mg).

Tambjamine Standards. Previously isolated tambjamines A (**1**), C (**3**), D (**4**), I (**9**), and K (**12**); the aldehydes of tambjamines A (**14**) and B (**15**);[4,6] and synthetic tambjamine amino acid derivatives **17**−**19** were used as standards.

Metabolomic Analyses by UPLC−MS/MS. Mass measurements of EtOAc aqueous extracts of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* were acquired in Continuum MS/MS mode, with acquisition time of 10 min. The ESI source was set up for a mass range between *m/z* 50−600, scan time of 0.200 s^{-1}, and collision energy ramp (for MS/MS) from 20 to 30 eV. Acquisition of mass spectra of *R. ernsti* (1R) fractions was performed in Continuum MS^e mode, acquisition time of 10 min. The ESI source was set up for mass range between *m/z* 150−1000, scan time of 0.200 s^{-1}, and collision energy of 0 eV to the function 1 and collision energy ramp from 20 to 30 eV for function 2.

Molecular Networking Analyses. UPLC−QToF−MS/MS raw data was deconvoluted, peak picked, and aligned using the UNIFI Scientific Information System version 1.8 (Waters Corporation, Milford, USA). The peak detection threshold was set to 500 counts, and the MS/MS peak intensity threshold was set to 10 counts. Processed data were exported as mascot generic format (.mgf). Molecular networking was performed using the online platform GNPS (Global Natural Products Social Molecular Networking) (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp). MS/MS peak lists resulted from the UPLC−HRMS processed data were uploaded to the UCSD GNPS FTP server and investigated via the METABOLOMICS-SNETS workflow. Standards of tambjamines and samples were organized into three groups (G1−G3). A cosine score of 0.6 and a minimum number of matched peaks of 4 were chosen for these analyses. The mass tolerances were set to 0.02 Da for precursor ions and 0.02 Da for fragment ions. Library search parameters were set to a cosine of 0.6 and a minimum of 4 matched peaks. All other settings were left as default values. The resulting network was visualized on Cytoscape 3.7.1. Nodes and edges tables were exported from Cytoscape as. csv files and then uploaded onto Gephi 0.9.2. The Gephi platform was employed to visualize which known tambjamines were found in which biological samples. To build the Gephi network, the parameters were set as follows: repulsion strength = 70 000, attraction strength = 10, size of the nodes related to tambjamine standards = 110, size of the nodes related to samples = 70, and edge thickness = 4.0. The layout Force Atlas was chosen for this analysis. Nodes were colored according to the type of sample.

Quantification of Tambjamine Content. The quantification of tambjamine content in the samples of *V. dentata*, *T. stegosauriformis*, *T. brasiliensis*, and *R. ernsti* was carried out on a Waters Alliance 2695 liquid chromatography system coupled to a Waters 2475 fluorescence photodiode array detector (Flu/PDA), controlled by Waters Empower 2 software. The chromatographic separation was performed using a Phenomenex Kinetex PFP column (4.6 mm × 250 mm, 5 μm) and the mobile phase 100% H_2O + 0.1% formic acid (solvent A) and 1:1 MeCN/MeOH + 0.1% formic acid (solvent B). The gradient elution used was as follows: from 30 to 95% of solvent B in 30 min and then 95−30% of solvent B from 30 to 31 min; then, 30% of solvent B from 31 to 40 min. The flow rate was 1 mL min^{-1}; the injection volume was 20 μL, and the column was kept at 25 °C. To detect tambjamines, the fluorescence detector was set at $\lambda_{excitation}$ = 400 nm. The $\lambda_{emission}$ was adjusted for each tambjamine. The alkaloids were identified in the samples by direct comparison with standards (fluorescence and UV spectra and retention time). The quantification of the tambjamine contents was performed by an external standard method, using different concentrations of tambjamines standards, analyzed in triplicate.

Advanced Marfey's Derivatization. Advanced Marfey's analyses were performed for fractions 1R-2 and 1R-4 as previously described.[19] Briefly, 100 μg of aliquots of fractions 1R-2 and 1R-4 were subjected to acid hydrolysis in 1 mL of 6 mol L^{-1} HCl at 100 °C under magnetic stirring overnight. Then, the solutions were cooled to room temperature and dried under vacuum. The hydrolysates were washed with 1 mL of H_2O three times to remove the residual HCl and evaporated. For the Marfey's derivatization, hydrolysates were resuspended in 500 μL of H_2O, followed by the addition of 20 μL of 1 mol L^{-1} $NaHCO_3$ and L-FDTA (1.4 equiv). The mixture was stirred and left at 40 °C for 1h. The reaction mixture was then cooled to room temperature, and the pH was neutralized with 10 μL of 2 mol L^{-1} HCl. Amino acid standards (L-leucine, L-isoleucine, L-*allo*-isoleucine, and L-valine) were derivatized using both L- and D-FDTA. Samples were analyzed by UPLC−QToF−MS using the method previously described.[20]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01043.

Data of tambjamines quantification; UPLC−MS analyses of tambjamines and of Marfey DTA derivatives; experimental procedures of synthesis; HRMS, UV, IR, ^1H NMR, ^{13}C NMR, and 2D NMR spectra of synthetic tambjamines M−O (**17**−**19** and **21**); and HPLC-Fluorescence quantification of tambjamine content (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Dr. A. Douglas Kinghorn, The Ohio State University, for his pioneering work on bioactive natural products.

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