Title

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Isolation of Bastadin-6-*O*-Sulfate and Expedient Purifications of Bastadins-4, −5 and −6 from Extracts of *Ianthella basta*

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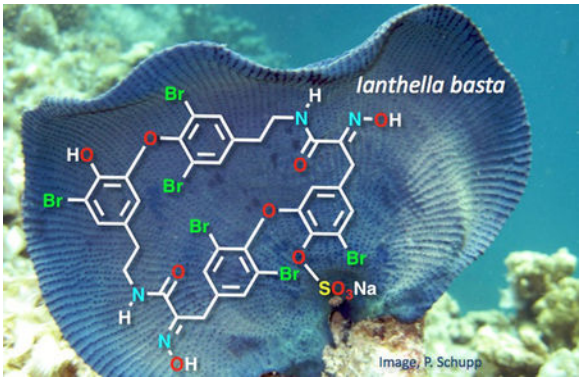
Abstract

Bastadin-6–34-*O*-sulfate ester (8) was isolated from methanol extracts of *Ianthella basta*. The structure of 8 was characterized by analysis of MS and NMR data, and conversion through acid hydrolysis, to the parent compound, bastadin-6, which was identical by HpLC, MS and NMR with an authentic sample. An improved procedure for procurement of pure samples of bastadins-4, −5 and −6 is described.

Graphical Abstract



1. Introduction

Bastadins are group of over 20 highly brominated natural products, formally derived from bromotyrosine (**1**), dibromotyrosine (**2**) or their corresponding tyramines, and classified by two hypothetical parent skeletons: ‘bastarane’ (***ia***)[†] and its constitutional isomer,

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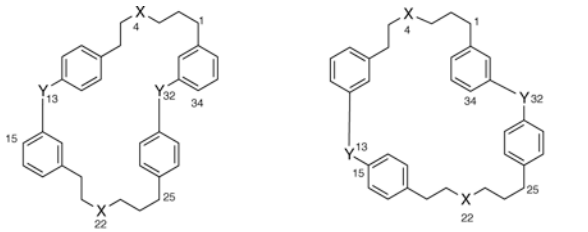
The parent names ‘bastarane’ for ***ia,*** or more informatively 13,32-dioxa-4,22 diazabastarane, ***ib,*** were proposed to unify the family of compounds and avoid awkward nomenclature. See Ref. 1a for a discussion. The name ‘isobastarane’ skeleton was coined later by Capon to describe bastadin-13, the first member with an alternate catechol ether linkage (see Ref. 1c), but it also brings order to numbering the schemes.

'isobastarane' (***iia***). The first seven bastadins including the ring opened symmetrical dimer, bastadin-3 (**3**), and macrodilactams, bastadins-4 (**4**), −5 (**5**) and −6 (**6**), were characterized over 35 years ago by Kazlauskus and coworkers from samples of the Australian marine sponge *Ianthella basta,* Pallas [1a,b] and identified with modest antibiotic properties. They gained new significance in 1995 with the finding that bastadin-5 (**5**) and bastadin-6 (**6**, Figure 1) are potent agonists for the release of Ca^{2+} ions from stores within the sarcoplasmic reticulum (SR) though modulation of heterotetrameric megaprotein (2.5 MDa) Ca^{2+} ion-channel, RyR-1.[2] The most potent agonist, bastadin-5 (5) (EC_{50} = 2.3 μM), was shown to promote release of Ca^{2+} through modulation of RyR-1[3,4] – the last stage of excitation-contraction coupling in mammalian striated muscle – through alteration of the gating kinetics of channel opening and closing.[2]



ia bastarane X = Y = CH_2
ib dioxadiazabastarane X = NH, Y= O

iia *iso*bastarane X = Y = CH_2
iib dioxadiaza*iso*bastarane X = NH

Although high-resolution cryo-EM structures of the RyR-1 complex have been reported recently,[5] the binding site of **5** is as yet unknown.

Two 'channelopathies' - malignant hyperthermia (MH)[6] and central core disease (CCD)[7] - are manifestations of different single-point autosomally dominant inherited mutations of the same gene, RYR1 in chromosome 19q13.1, that encodes RyR-1. MH is a 'cryptic' mutation that reveals itself during general anesthesia with certain inhalation anesthetics. The resulting condition − runaway hyperthermia − can be fatal to the patient if not treated immediately. CCD is congenital myopathy that is often debilitating and can lead to delayed ambulation and reduced vital capacity.

preliminary structure activity relationships of the bastadins have been described.[8] While **5** and **6** are almost equipotent RyR-1 agonists, bastadin-4 (**4**, 5,6-dehydrobastadin-5) is less potent by order of magnitude, while the constitutional isomer of **5** − bastadin-19 (**5**) with an *iso*bastarane skeleton - is essentially inactive (IC_{50} > 100 μM).[2,19] Nevertheless, **4** is valuable because it can be converted into **5** or the isotopically labeled probe, ***5-d*** (and, ostensibly, **5**-t) through selective cationic reduction (Et_3SiH or Et_3SiD, TFA) as we demonstrated earlier.[10]

Compounds **5** and **6**, and synthetic analogs inspired by their structures,[8] may be useful probes in the study of RyR-1 mutations, expanding our understanding of the mechanism of RyR-1 channel gating and Ca^{2+} release, and the possible development of therapeutic agents for the treatment of Ca^{2+} channel-related myopathies.

However, contemporary investigations into the pharmacology of **5** and **6** are limited by supply; both compounds only occur as minor components along with complex mixtures of

bastadins and their *O*-sulfate esters. The natural products are not separable on silica chromatography and are typically won through tedious, multistep reversed-phase chromatography.[11] Total syntheses of **5**[12a] and **6**[12b,c] have been reported, but they involve lengthy series of linear steps and the overall yields are typically low. Thus, practical sources of **5** and **6** are required.

in our investigations of sources of new bastadins from samples of *I. basta* collected at two locations in separated oceans − Guam in the Pacific and Exmouth Gulf, Western Australia, in the Indian Ocean −we discovered a new member of the series: bastadin-6-*O*-sulfate ester (**8**) which is the subject of this report. In addition, we describe a streamlined procedure for rapid procurement of the two most useful RyR-1 modulators, **5** and **6** from *I. basta,* and an improved protocol for conversion of **4** to the highly valued **5**.

2. Experimental

2.1. General methods

Optical rotations were measured on a JASCO P-2000 at the D-double emission line of Na°. UV-vis spectra were measured on a JASCO V-630 spectrometer. FTIR spectra were collected on thin-film samples using a JASCO FTIR-4100 fitted with an ATR accessory (ZnSe plate). 1D NMR and inverse-detected 2D NMR spectra were measured on a Bruker Avance II NMR spectrometer with a 1.7 mm $^{1}H\{^{13}C/^{15}N\}$ 600 MHz microcryoprobe. Other NMR spectra were measured on a JEOL ECA spectrometer equipped with a 5 mm $^{1}H\{^{13}C\}$ 500 MHz room-temperature probe. ^{13}C NMR spectra were measured using a Varian NMR spectrometer equipped with a 5 mm Xsens $^{13}C\{^{1}H\}$ 125 MHz cryoprobe. NMR spectra are referenced to residual solvent signals. High-resolution ESITOF analysis was carried out on an Agilent 1200 HPLC coupled to an Agilent 6230 TOFMS. Low-resolution MALDI MS measurements were made on a Bruker Biflex IV in a nitrobenzyl alcohol matrix. Low-resolution MS measurements were made on a Thermoelectron Accela UHPLC coupled to an MSQ single-quadrupole detector. Preparative, semipreparative, and analytical HPLC were performed on a JASCO PU-2086 Plus system consisting of a dynamic mixer (MX-2080–32) with UV-VIS detector (UV-2075) operating at λ 250 nm. Automated flash chromatography was carried out with a Teledyne-Isco CombiFlash system with UV (λ 250 nm) and RI detection.

2.2. Animal Material

Two collections of the sponge *Ianthella basta* were made using scuba − one in 2009 from Guam (09-IBC, sample courtesy of Peter Schupp, University of Oldenburg) and a second in 1993 from Bennett Shoal, Exmouth Gulf, Western Australia (93-07-101). The samples were stored at −20 °C until required.

2.3. Expedient Purification of Bastadins-5 and −6.

A typical isolation procedure is described here for *Ianthella basta*. Frozen lyophilized sponge (93-07-101, 92 g, dry wt.) was cut into smaller pieces (~5–10 cm) and extracted by slow stirring with CH_3OH at room temperature (2 × 600 mL, 12 h), and the combined extracts concentrated under reduced to half of the volume. The H_2O content was adjusted to 1:9

H_2O-CH_3OH, and the extract repeatedly partitioned against hexanes (2 × 400 mL) to give, after removal of solvent, 'fraction A' (1.41 g). The water content was re-adjusted to 2:3 H_2OCH_3OH and the solution partitioned against CH_2Cl_2 (2 × 400 mL) to give, after removal of solvent, 'fraction B' (2.49 g). MeOH was removed from the remaining aqueous-MeOH partition, under reduced pressure, and the remaining H_2O solution partitioned against *n*-BuOH (2 × 100 mL) to give 'fraction C' (600 mg). The residue of the aqueous phase constituted 'fraction D' (9.8 g).

Fraction B was purified by size-exclusion chromatography (Sephadex LH-20) with elution by MeOH to give six fractions (F1–6). grouped by TLC (silica F254, developed with 1:9 MeOH-CH_2Cl_2, UV visualization and staining with vanillin-H_2SO_4-EtOH, Figure 2). TLC of F-4, containing **5** and **6** (LCMS), gave a spot that characteristically stained green while F-6, containing mostly **4**, stained a yellow or orange yellow (Figure 2). Further purification of F-4 was achieved by reversed-phase preparative HPLC (Phenomenex Kinetex C_{18} column, 150 × 21.2 mm, 5μ, linear gradient, 50:50 H_2O-0.1% TFA: CH_3CN to 30:70 over 20 min, 13 mL/min flow rate, Figure 3). Pure bastadin-5 (**5**, 5.2 mg) eluted as a peak at 15.6 min, while pure bastadin-6 (**6**, 4.0 mg) eluted at 17.2 min. The peak eluting at 14.2 min, '**X**', contained a mixture of bastadins-16[15] and −19[9] that could be separated under alternative HPLC conditions (RP C_{18}). Bastadin-4 (**4**, 11.0 mg) was the major component of fraction 6. The identity of all compounds were confirmed by comparisons of their MS and ^{1}H NMR data with literature values.

2.4. *Bastadin-6–34*-O-*sulfate Ester (8)*

The MeOH extract of *I. basta* from Guam was solvent-partitioned and a portion of the *n*-BuOH-soluble partition ('fraction C') was separated by automated low-pressure chromatography (silica, gradient elution, 050% MeOH-CH_2Cl_2 over 60 min) to give nine fractions. Fraction 7 (385 mg) was further separated by HPLC (Phenomenex Luna 5μ Phenyl-hexyl, 21.2 × 250 mm, 8.5 mL.min^{-1}, 70–100% MeOH-10 mM Na_2S0_4 over 60 min). Individual HPLC fractions were freed of salts and recovered by capture on C_{18} solid-phase extraction cartridges, followed by washing with H_2O and elution of the organic compound with MeOH to give bastadin-6 (**6**, 4.5 mg), bastadin-5 (**5**, 3.2 mg) bastadin-4 (**4**, 3.8 mg) and a mixed fraction containing **8**. The latter was further purified by reversed phase HPLC (Phenyl-hexyl, 4.0 mL.min^{-1}, 60–100% MeOH-H_2O over 15 min) and a similar recovery procedure, to provide pure **8** (4.5 mg). Colorless powder; FTIR (ATR, ZnSe plate): ν 1676, 1437, 1203, 1133, 841, 801, 723 cm^{-1};^{1}H and^{13}C NMR (CD_3OD), see Table 1 (see Supporting Information for NMR data in CD_3CN). MALDI-TOF *m/z* 1222.87 $[M+Na]^+$ (calcd for $C_{34}H_{25}{}^{79}Br_3{}^{81}Br_3N_4Na_2O_{11}S$ 1222.61). EIMS *m/z* 497.8 (34%)/499.8 (99%)/501.8 (100)/503.8 (36), see Figure 4. HRESIMS *m/z* 1092.6943 $[M-SO_3+H]^+$ (calcd for $C_{34}H_{27}{}^{79}Br_6N_4O_8$ 1092.6924).

2.5. Acid Hydrolysis of 8

A mixture of **8** (0.5 mg) and 2M HCl (0.5 mL) was heated in a sealed tube at 50 °C for 30 minutes. The sample was cooled, dried under a stream of nitrogen and taken up in CD_3OD. The^{1}H NMR (500 MHz) of the solution was identical to that of authentic bastadin-6 (**6**). Analytical HPLC of **8** and authentic **6** (Dynamax Microsorb C_{18} column, 10 × 250 mm, 4.0

mL.min^{-1}, gradient: 60–100% MeOH-H_2O over 15 min, then isocratic) gave retention times of 9.9 min and 15.2 min, respectively. HPLC of the hydrolysate of **8** also gave a single peak with retention time of 15.2 min, consistent with **6**.

2.6. Cationic Reduction of Bastadin-4 to Bastadin-5.

Cationic reduction of bastadin-4 (**4**) to bastadin-5 (**5**) was carried out by an improved modification of our earlier reported procedure.[10] To a solution of **4** (3.8 mg, 3.7 μmol, 1.0 equiv) in CF_3COOH (0.5 mL) under an atmosphere of dry N_2 was added a solution of triethylsilane (5.5 μL, 37 μmol, 10 equiv) in CH_2Cl_2 (0.5 mL). The mixture was stirred vigorously for 30 min, dried under a stream of dry N_2, and the residue purified by reversed phase HPLC (Microsorb C_{18}, 3.0 mL.min^{-1}, 80:20 MeOH-H_2O+0.1% TFA) to afford recovered **4** (0.5 mg, 13%) and **5** (1.5 mg, 40%; 65% based on recovered starting material) identical with an authentic sample by MS and ^{1}H NMR.

3. Results and Discussion

3.1. Structure Elucidation of 8

Compound **8** was purified from the Guamanian sample of *Ianthella basta* using a variation of the conventional protocols we have used in the past and reported elsewhere.[9] The formula of **8** $C_{34}H_{25}Br_6N_4NaO_{11}S$ was assigned from mass spectrometric measurements [MALDI *m/z* 1222.87 $[M+Na]^+$ calcd. 1222.61 for $C_{34}H_{25}{}^{79}Br_3{}^{81}Br_3N_4Na_2O_{11}S$; HRESITOFMS *m/z* 1092.6943 $[MSO_3+H]^+$). The isotope pattern reveals the presence of Br_6, the highest number of Br atoms that can be accommodated in a bastadin skeleton (e.g. **6**). While the $^{79}Br_3{}^{81}Br_3$ isotopomer of **6** is expected to show *m/z* 1120.67 $[M+Na]^+$ the higher mass measured for **8** is reconciled by the difference ΔM= 101.94 $[SO_3+Na-H]$; therefore, **8** is an ***O***-sulfate half-ester of **6**.

The ^{1}H NMR spectrum of **8** (Table 1) showed characteristic two-proton aryl signals for each of the symmetrical 3,5-dibromotyrosine-like spin systems (δ 7.59, s, 2H, H-8/H-12; δ 7.55, s, 2H, H-27/H-31). These data could only be accommodated by the aryl-ring substitution pattern found in the hexabromo-substituted, bastadin-6 (**6**), or its unreported *iso*-bastarane (***iia***) constitutional isomer. Assignment of the catechol ether linkages between the eastern and western hemispheres of structure **8** was secured by observation of two sets of HMBC correlations ((Table 1, Figure 2) recorded in different solvents (600 MHz), CD_3OD (Table 1) and CD_3CN (see Supporting Information) to resolve equivocal assignments from overlapping signals. Cross peaks were observed between H-21 (δ 3.40, m, 2H) and H-25 (δ 3.80, s, 2H) to the C-23 amide carbonyl at (δ 164.4, s), and between H-1 (δ 3.70, s, 2H) and H-5 (δ 3.45, m, 2H) and C-3 (d, 164.0, s).

The foregoing data for **8** support the same constitution as **6**. The locations of Br substituents in the carbon skeleton of **8** were verified by electron-impact mass spectrometry (EIMS) that revealed loss of SO_3 and a major Br_3-containing fragment (*m/z* 497.82/499.8/501.82/503.82) arising from the previously noted [1] and characteristic double-heterolytic cleavage between the amide carbonyl and ketoxime C=N double bond (Figure 4). Additional fragment ions

from sequential homolytic losses of Br (*m/z* 417.8/419.8/421.8 and 339.9/341.9) were also observed in the EI mass spectrum of **8**. Therefore, **8** is a member of the 'bastarane' series, ***ia.***

Confirmation of the structure of **8** followed from its acid hydrolysis (2M HCl aqueous MeOH, 50 °C, 30 min) which gave a compound that was identical with authentic **6** by ^{1}H NMR[1] and HPLC retention time.

The structures of known bastadin sulfate half-esters have *O*-sulfate groups placed at either C-15 or C-34, or both.[9,13] In order to ascertain the position of the *O* -sulfate in **8**, the ^{13}C NMR chemical shifts of the latter were compared with those of **6**.[1,] As noted earlier by Ragan [14], Wright and co-workers[13] and others,[9] sulfation of a phenoxyl group leads to an upfield shift of the *ipso* ^{13}C signal by approximately Δδ 5 ppm and downfield shifts of *ortho* and *para* ^{13}C signals. The 13C NMR chemical shifts of bastadin-6 (**6**) were assigned by HMBC and HSQC and compared with those of **8** (Table 1 and Figure 5), and subjected to differential analysis. The C-34 quaternary carbon signal of **1** (δ 137.2, s) was shifted upfield (Δδ −4.6 ppm), while C-33 and C-35 appeared downfield (Δδ 5.7 and 9.2 ppm, respectively); therefore, the *O*-sulfate half-ester in **8** is positioned at C-34.

3.2. Optimized Procurement of Bastadins-4–6

Given the lengthy and tedious procedure required to obtain **4–6** and **8** from the Guamanian sample of *I. basta,* we invested time to optimize the purification of the former desirable compounds and reduce the number of HPLC purification steps. A sample of *I. basta* collected in 1993 in Exmouth Gulf, Western Australia, was found to be devoid of **4** but contained **5** and **6** (LCMS). Since the former complicates the separations of the latter by preparative reversed phase HPLC, we investigated high-loading purification of MeoH extracts of this sponge. In the event, gel filtration (Sephadex LH-20, MeOH elution) of the solvent-partitioned 'fraction-B', with monitoring by LCMS, delivered a single late-eluting fraction containing **5** and **6**. The latter fraction was separated in single HPLC step (reversed phase Phenyl-hexyl column, Phenomenex, H_2O-CH_3CN gradient) giving pure samples of **5** and **6**.

Although **4** is the 5,6-dehydro-derivative of **5**, conversion of **4** into **5** by catalytic hydrogenation is complicated by over-reduction and loss of Br through hydrogenolysis. Attempted homogenous catalytic hydrogenation of **4** with Wilkinson's catalyst ($(Ph_3P)_3RhCl$, H_2 >60 atm) returned only starting material.[10] Eventually, we refined an optimized procedure for procurement of high-value **5**; cationic reduction of **4** (Figure 6, Et_3SiH, CH_2Cl_2-CF_3COOH, vigorous stirring under nitrogen) gave **5** in 65% yield (based on recovered **4**) after HPLC purification (Figure 5), which significantly improves over our earlier protocol.[10]

3.3. Conclusions

The new compound, bastadin-6-34-0-sulfate ester (**8**) was isolated from a specimen of *Ianthella basta* collected in Guam. Refinement of a new purification protocol gave pure samples of highly-value bastadins-5 (**5**) and −6 (**6**) in two steps from a polar solvent-partitioned fraction. An improved conversion of **4** to **5** by cationic reduction facilitates

access to this most potent RyR-1 agonist. Compound **8** undergoes acid hydrolysis to provide **6**. Thus, these reactions of **4** and **8** follow convergent paths to deliver more of the high-value analogs **5** and **6**, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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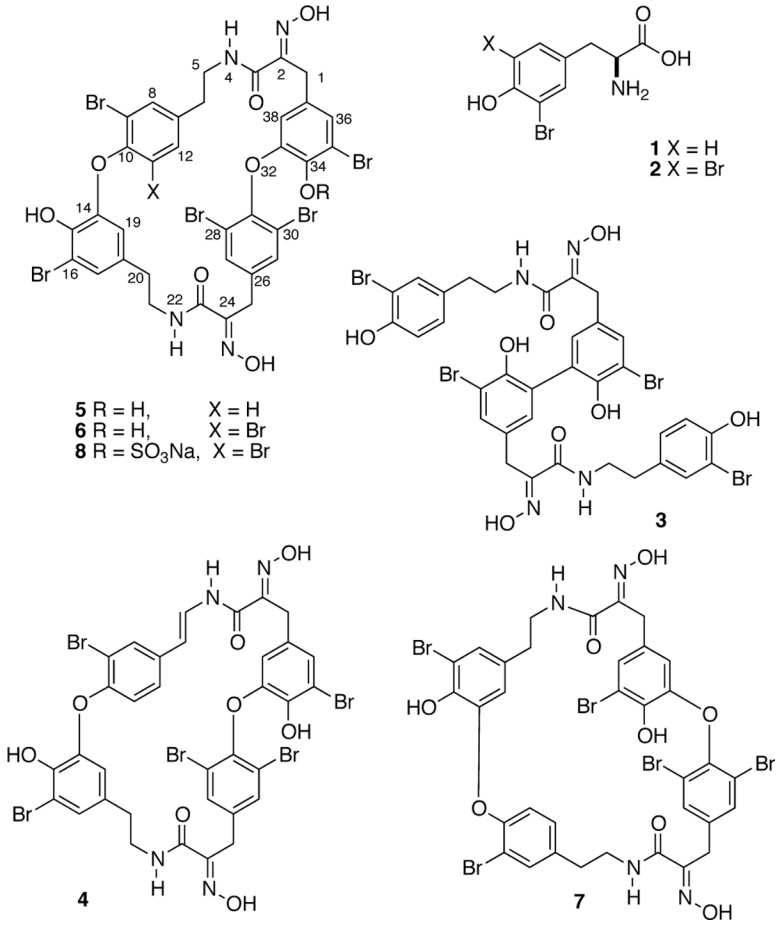

Figure 1.

Structures of bromotyrosines (**1**, **2**), bastadins −3 (**3**), −4 (**4**), −5 (**5**), −6 (**6**), −19 (**7**) and bastadin-6-*O*-34-sulfate (**8**).

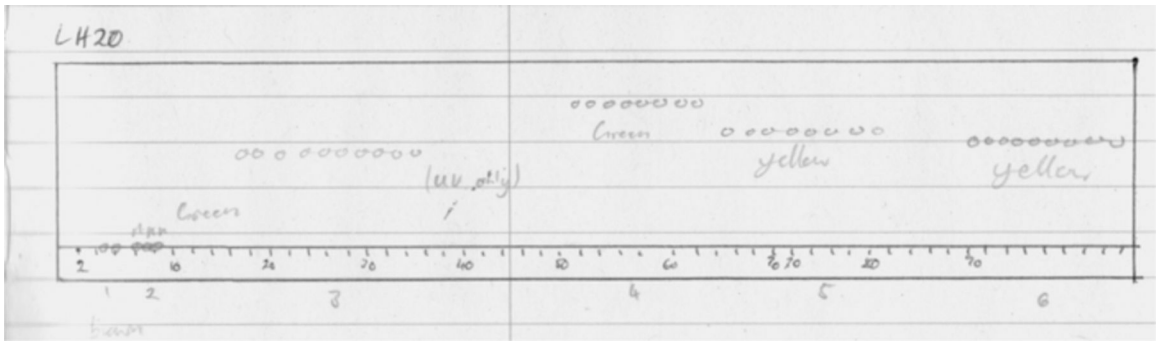

Figure 2.

TLC of fractions F1–6 from Sephadex LH-20 of an extract of *Ianthella basta* (1:9 MeOH-CH_2Cl_2, visualization with vanillin-H_2SO_4). Fraction 4 contained **5** and **6**. Fraction 6 (orange-yellow) largely pure **4**.

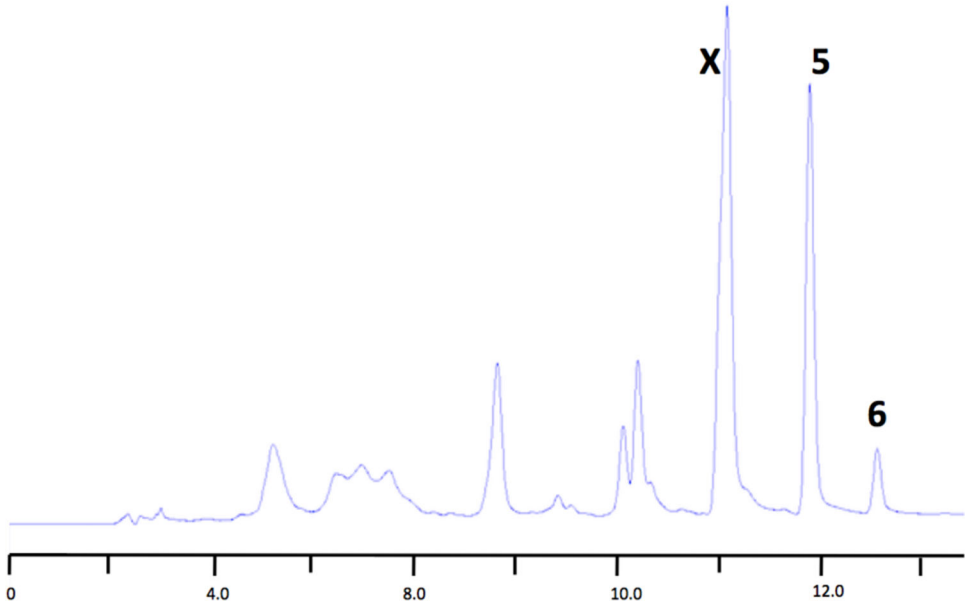

Figure 3.

RP HPLC chromatogram of Sephadex LH-20 Fraction 4 from *Ianthella basta* (UV detection, λ 250 nm) ; See Experimental and Section 2.4 for conditions.). Retention times: **X** – a mixture of bastadins-16[15], and −19[9] (t_R = 14.2 min), bastadin-5 (**5**, t_R = 15.6 min), bastadin-6 (**6**, t_R = 17.2 min).

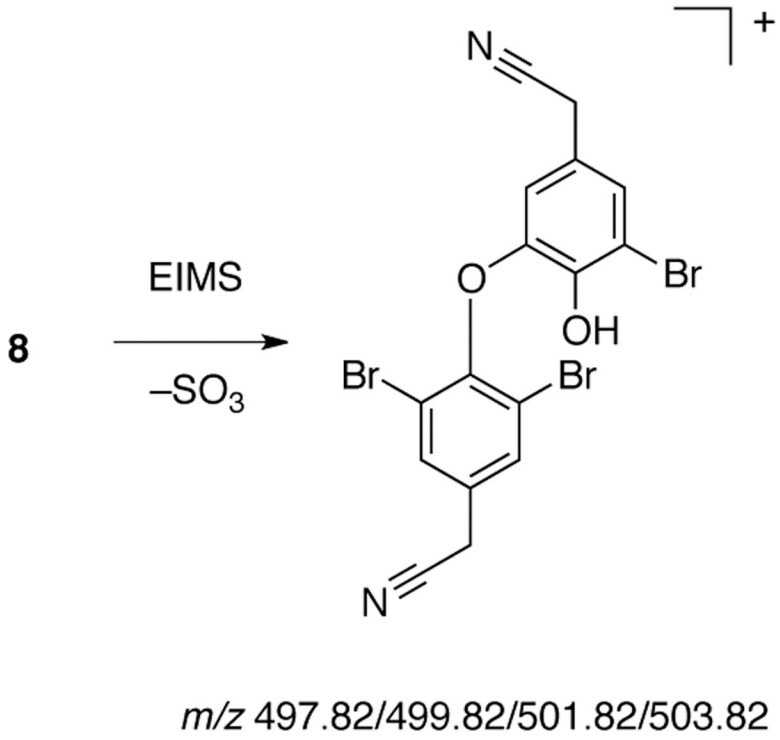

Figure 4.
ESIMS fragmentation of **8**

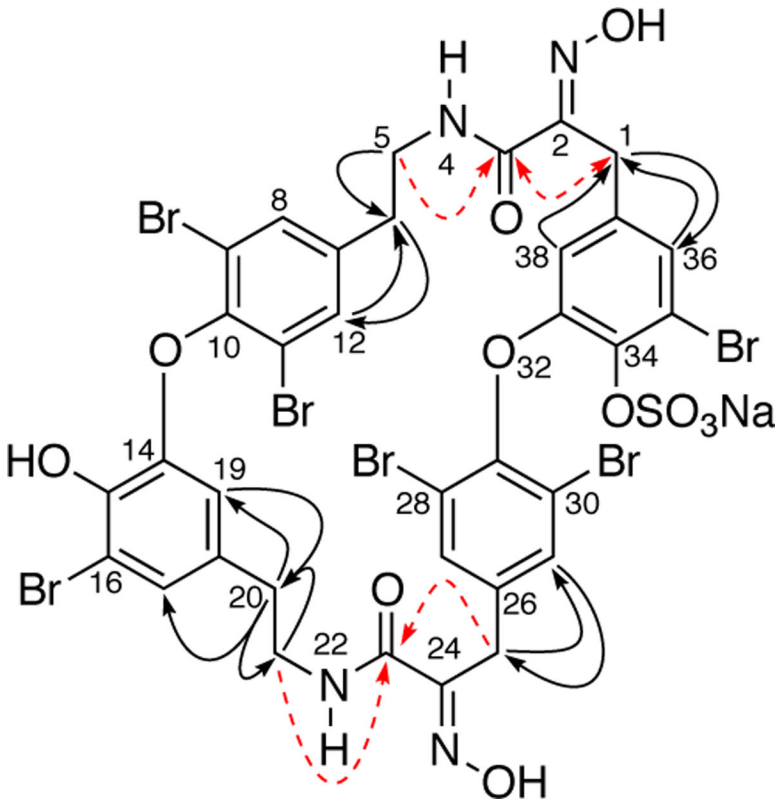

Figure 5.

HMBC data (500 MHz) of **8**. Correlations (^{1}H –> ^{13}C) observed in CD_3CN (red dashed lines, obscured in CD_3OD), and those observed both CD_3OD and CD_3CN (solid lines).

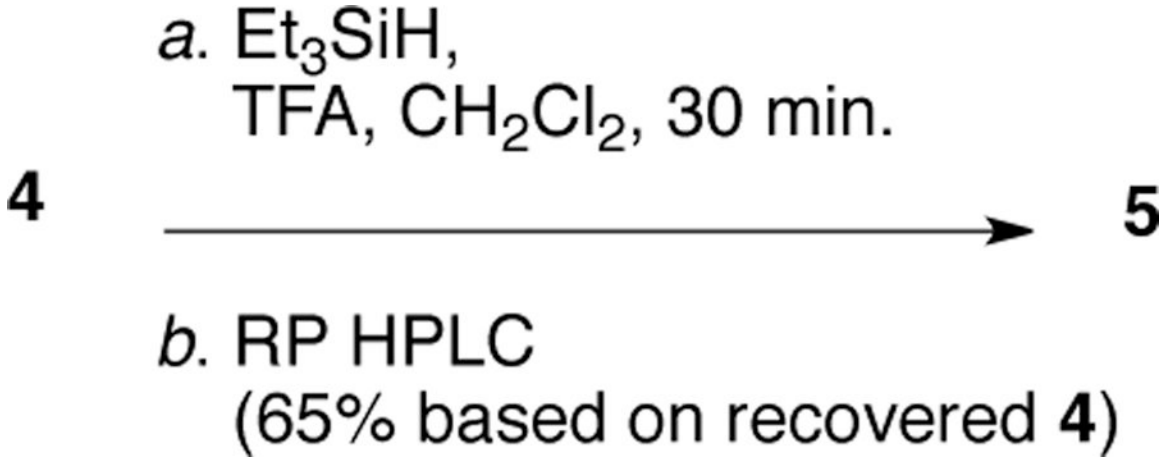

Figure 6.
Cationic reduction of **4** to **5**.

Table 1–

^{1}H and^{13}C NMR data for **8** (CD_3OD, 23 °C)

Atom	$\delta^{13}C$[a] 6	$\delta^{13}C$,[b] 8	$\delta^{1}H$ (mult, J, integ.)[c] 8	HMBC 8 (^{13}C–>^{1}H)
1	27.3	27.2	3.70 (s, 2H)	36,38
2	151.5	150.9		1
3	163.0	164.0		1,5
4				
5	38.4	40.5	3.45 (m, 2H)	6
6	33.9	33.9	2.79 (t, *J*=7.5 Hz, 2H)	5
7	140.1	140.0		5,6
8	133.6	133.4	7.59 (s, 1H)	6,10
9	117.4	117.5		8
10	146.1	147.0		8,10
11	117.6	117.5		10
12	133.6	133.4	7.59 (s, 1H)	6,8
13		–		
14	144.6	144.8		19
15	141.6	142.0		17,19
16	110.2	110.2		17
17	126.2	126.0	7.06 (d, *J*=1.9 Hz, 1H)	19,20
18	130.7	130.8		20,21
19	111.7	112.0	6.30 (d, *J*=1.9 Hz, 1H)	17,20
20	32.7	33.5	2.7 (t, *J*=7.2 Hz, 2H)	17,19,21
21	40.4	39.1	3.40 (m, 2H)	20
22				
23	163.3	164.4		21,25
24	150.4	150.4		25
25	28.7	28.3	3.80 (s, 2H)	27,31
26	137.6	137.2		25
27	133.2	133.2	7.55 (s, 1H)	25,31
28	117.1	117.6		27
29	146.1	147.9		27,31
30	117.1	117.6		31
31	133.2	133.2	7.55 (s, 1H)	25,27
32				
33	144.8	150.5		38
34	141.8	137.2		36,38
35	109.8	119.0		36
36	126.8	135.2	7.21 (d, *J*=1.9 Hz. 1H)	1,38
37	128.1	127.0		1
38	112.6	113.8	6.30 (d, *J*=1.9 Hz, 1H)	1,36

[a] recorded in DMSO-*d*6, from reference 12a.

[b] 125 MHz.

[c] 500 MHz.