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Amethystin, the Coloring Principle of *Stentor amethystinus*

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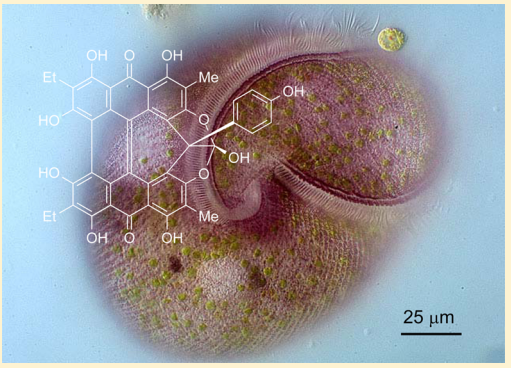
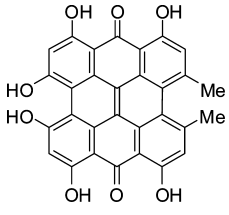
Supporting Information

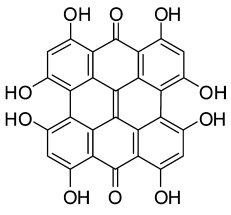
ABSTRACT: Among the ciliates, *Stentor amethystinus* stands out for its conspicuous red-violet color compared to its blue- and red-colored relatives *Stentor coeruleus* and *Blepharisma japonicum*. Rich blooms in German lakes allowed us to collect sufficient organisms to isolate the pigments and elucidate the structure of the main component amethystin (**4**) by spectroscopic methods as a carboxy derivative of blepharismin. Depending on conditions, the carboxy group appears as an orthoester or as a mixture of the orthoester and small amounts of a hydroxylactone. Derivatives of both isomeric forms were obtained by acetylation and methylation supporting the proposed structures. On reaction of amethystin with base in the presence of oxygen, oxyamethystin and, under vigorous conditions, *p*-hydroxybenzoic acid were formed. In addition to **4**, two homologues, an isomer of amethystin, and stentorin F (**1b**) were identified in the primary extract. Further, a biosynthetic scheme is proposed linking stentorin, blepharismin, and amethystin type compounds to the hypothetical protostentorin as a common intermediate.



Stentor and Blepharisma are heterotrich ciliates (protozoa) prevalent in lakes, ponds, and marine environments. Some species are brightly colored by pinkish, red, blue, violet, or brown pigments located predominantly in granules that are arranged in stripes just below the cell surface,[1,2] The compounds responsible are structurally related to hypericin, the coloring principle of St. John's Wort (*Hypericum perforatum*), which, most remarkably, is also stored in "granules" embedded in the leaves and petals of the plant,[3,4] and the fringelites, fossil pigments from a Jurassic sea lily.[4,5]



Hypericin



Fringelite D

Thus, stentorin C (**1a**), the blue pigment isolated from *Stentor coeruleus*, has been identified as the 3,3′-diisopropyl analogue[6] of fringelite D[5] with the alkylation pattern proven by total synthesis.[7] Maristentorin from *Stentor dinoferus* shows yet another, though questionable, alkylation pattern.[8a,b] In contrast, the pinkish blepharismin (**2**) from *Blepharisma japonicum* resembles protohypericin with the gap in the ring system bridged by a *p*-hydroxybenzylidene group.[9,10] From cultures kept in daylight, or on subsequent irradiation of blepharismins (**2**) in the presence of oxygen, oxyblepharismins (**3**) were isolated,[2] and its stentorin-like structure was elucidated.[11] Both compounds were found as a mixture of homologues with R^1, R^2 = Et, i-Pr, R^3 = H, Me. Surprisingly, the deep-red-colored marine ciliate *Pseudokeronopsis rubra* contains an entirely different merocyanin-like chromophore with pyrrole and pyrone end groups.[12] Yet another type of yellow-brown pigment is present in *Loxodes* species.[13] Its chemical nature is not known.

All of these pigments appear to have two functions.[14] They are apparently deterrent, paralyzing, or even toxic for predators such as other protozoa when they are ingested or released from the granules (extrusomes) by mechanical stress.[15−18] Experiments with the isolated compounds indicated antimicrobial activity[14,19] and inhibition of the development of sea urchin eggs[17c] and of the inward calcium transport of the ciliate *Euplotes*.[20] Another function of the pigments is that of photoreceptors mediating the orientation of the organisms in a light gradient.[14,21] Whereas generally negative phototaxis was observed, in the presence of algae as symbionts, positive phototaxis is also observed to allow photosynthesis. Investigation of the photosignal transduction pathway revealed the presence of stentorin associated with a 50 kDa protein[22] and of blepharismin associated with a 200 kDa protein.[23] However, no further protein data have been published so far. Other work indicates that there is no specific blepharismin-binding protein.[24] Thus, the primary signal, protons released from the excited chromophore (photoacidity),[25]

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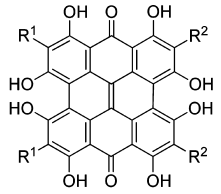
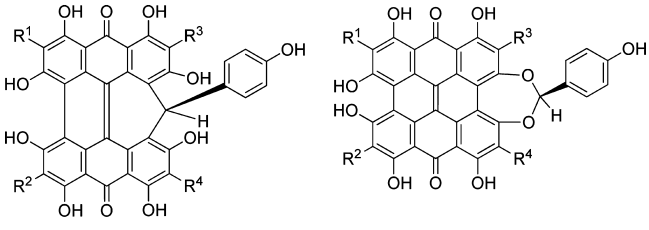
singlet oxygen,[26] or hydroxy radicals[27] would have to reach the target molecule by diffusion.



Stentorin C (**1a**) R^1 = *i*-Pr, R^2 = H
F (**1b**) R^1 = Et, R^2 = Me



Blepharismins (**2**) Oxyblepharismins (**3**)

R^1, R^2 = Et, *i*-Pr; R^3, R^4 = H, Me

In general, the ciliates represent only a small percentage of the total zooplankton, and organisms such as *Blepharisma* or *Pseudokeronopsis* have to be cultivated to generate biomass for in-depth chemical analysis or biosynthetic investigations by precursor feeding.[29] Fortunately *Stentor*, which is basically a sessile organism that occurs only sparsely in the open water, sometimes forms massive blooms that turn the water black. Such a "black-spot" bloom of *Stentor amethystinus* was reported in Lake Garda, Italy, in the summer of 2004.[31] Our work on *S. amethystinus* described below was initiated during such a bloom in Germany in 1995. At that time we became interested in *S. amethystinus* because of its conspicuous red-violet color, which is not observed in other ciliates. In fact, the visible absorption spectrum of organic extracts excluded the presence of a stentorin-like chromophore, but showed similarity to blepharismins (**2**).

RESULTS AND DISCUSSION

Large-scale collection from blooms in ponds in northeast and southwest Germany using a plankton net produced a biomass consisting of <95% of *S. amethystinus*. On contact of the wet cell mass with organic solvents such as acetone, the pigments are instantaneously released; they can be completely extracted by EtOAc. Re-extraction of the brown cell debris with various solvents, including extraction after previous acid or base hydrolysis, did not produce any more coloring matter. The organic extracts were preseparated by Sephadex LH-20 chromatography or by precipitation from ether solutions with petroleum ether, followed by silica gel chromatography. Final purification was achieved by RP-18 chromatography to yield 755 mg of amethystin (**4**) as a dark red, crystalline powder. Solutions in MeOH/H_2O were red, but changed to red-violet at pH 8. Contrary to blepharismin,[30] amethystin solutions in MeOH were perfectly stable when exposed to sunlight. The cytotoxicity of **4** against the mouse fibroblast cell line L-929 was very low, with an MIC of 37 μg/mL in the dark; however, this value decreased to 4 μg/mL in the presence of light, due to a photodynamic effect.[31]

Later collections from a small water basin in Darmstadt (Germany) in the summer and fall of 2013 gave only low yields of *S. amethystinus* together with an excess of copepods, which could be eliminated by filtration through a nylon stocking. Surprisingly, HPLC analysis of the extracts showed a very low yield of amethystin per specimen (3.3 ng) compared with the bloom of 1995, which gave an estimated >10-fold higher yield. Nevertheless, microscopic inspection of the organisms showed the typical arrangement of dark pigment granules.

The electronic spectrum of **4** is very similar to that of blepharismin (**2**),[9] with only a 7 nm bathochromic shift of the three bands in the visible region. The molecular weight was consistently determined by FABMS and HRESIMS to be 724 mu, and the elemental composition, $C_{42}H_{28}O_{12}$. This is two hydrogen atoms less and one carbon and one oxygen atom more than blepharismin C (**2**, R^1, R^2 = i-Pr, R^3, R^4 = H);[11] thus, amethystin (**4**) is a close relative of blepharismin C. From the proton NMR spectra (Table 1) **4** is symmetrical with the isopropyl groups replaced by two ethyl groups tentatively located at C-9, C-9′ and two methyl groups at C-3, C-3′. The four protons of a *p*-hydroxyphenyl group appear as a singlet at 6.11 ppm but are split into two doublets at 6.53 and 6.84 ppm in pyridine-d_5. Four hydroxy protons in *peri* positions are observed at 14.19 and 14.74 ppm and a phenylhydroxy is observed at 8.92 ppm, whereas hydroxy signals from the bay region are not observed as described also for blepharismin (**2a**).[9] An additional broad hydroxy signal at 4.10 ppm was also present. The assignment of the carbon NMR spectrum was greatly facilitated by comparison with that from ^{13}C-labeled

Table 1. NMR Spectroscopic Data of Amethystin (4) in DMSO-d_6 (75 and 300 MHz)

position	δ_C, type	δ_H (*J* in Hz)	HMBC[a]
1	122.1, C[b]		
2	161.3, C		
3	108.5, C		
4	165.4, C		
4-OH		14.19 s	3, 4, 5
5	107.3, C		
6	182.0, C		
7	100.1, C		
8	164.5, C		
8-OH		14.74 s	7, 8, 9
9	117.2, C		
10	171.4, C		
10-OH			
11	120.0, C		
12	124.1, C[b]		
13	124.7, C[b]		
14	126.9, C[b]		
15	57.3, C		
16	118.5, C		
16-OH		4.10 br s	
17	136.9, C		
18	127.8, CH	6.11 s[c]	20
19	114.3, CH	6.11 s[c]	20
20	155.9, C		
20-OH		8.92 br s	
21	8.6, CH_3	2.22 s	2, 3, 4
22	16.4, CH_2	2.76 q (7,0)	8, 9, 10, 23
23	13.3, CH_3	1.14 t (7,0)	22, 9

[a]HMBC correlations are from the proton(s) stated to the indicated carbon. [b]Tentative assignment. [c]In pyridine-d_5: 6.53 (d, *J* = 7.5 Hz), 6.84 (d, *J* = 7.5 Hz).

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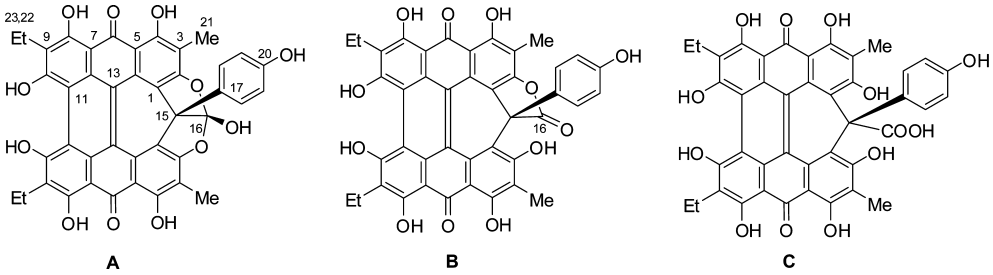

Figure 1. Possible structures of amethystin (**4**).

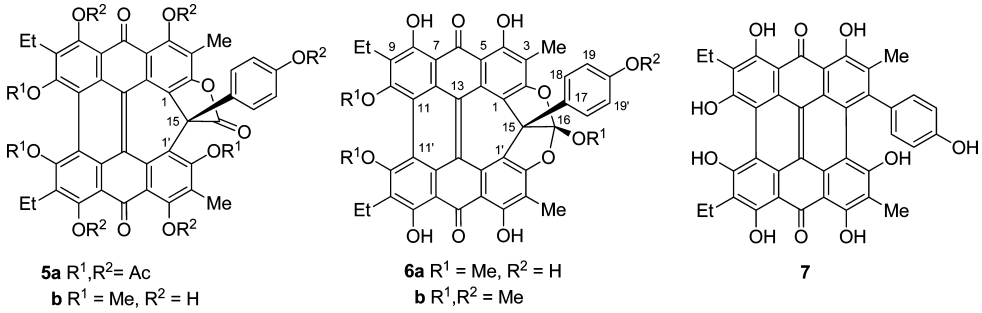

Figure 2. Chemical derivatives of amethystin (**4**).

blepharismin (**2a**).[29] A significant shift was observed for the benzylic methine carbon from 32 ppm in blepharismin to 57.3 ppm in amethystin (**4**). The C-15 proton in blepharismin is replaced in **4** by an extra quaternary carbon observed at 118.5 ppm. This together with the elemental composition leads tentatively to the orthoester structure **A** for amethystin (Figure 1). It is corroborated by an IR spectrum showing only quinone carbonyl signals. Disturbingly, with several other samples of **4**, a carbonyl band of low and variable intensity was observed at 1798 cm^{-1}, an absorbance later found in the lactone derivative **5b** (see below). We believe that, depending on the conditions of purification and crystallization, a variable small proportion of the lactone isomer **B** is formed. Similar orthoesters are well known from terpenoid natural products where hydroxy and carboxy groups in favorable steric alignment are present, as is the case with amethystin. A well-known example is tetrodotoxin, which has also been observed as the orthoester or the hydroxylactone.[32] Another ambiguity arose with the elemental analysis, which for C, H, and O agreed very well with the free acid **C** but not with **A** and **B**. However, because the IR did not show a carboxy group, the crystalline amethystin analyzed must be a monohydrate of **A**.

Three minor biosynthetic byproducts of amethystin (**4**) were partially characterized by HPLC/HRMS and proton NMR spectra. They are lower and higher homologues by CH_2 with an undefined substitution pattern, whereas an isomer in the ^1H NMR spectrum in addition to ethyl and methyl groups shows signals for an isopropyl group and an aryl hydrogen and, thus, has the same substitution pattern as blepharismin BL-4/D.[9,11] Another regular polar component in primary extracts amounting to 3−6% of amethystin was identified by HPLC/UV/HRESIMS as stentorin F (**1b**). It has the same elemental composition as stentorin C (**1a**) from *S. coeruleus*;[7] however, it has the same substitution pattern as amethystin (**4**) according to the ^1H NMR data.

After attempts to prepare crystals of sufficient quality for an X-ray structure analysis failed, independent proof of structure **4** was sought by derivatization. Thus, acetylation gave an octaacetate that in the ^1H NMR spectrum showed doubling of the alkyl and of most of the acetyl signals. Due to the ^1H NMR shift difference between the two C-methyl groups of 0.1 ppm, the asymmetry must be located in the 3,3′ position. This is in good agreement with the lactone structure **5a**, which is formally derived from the amethystin isomer **B**. Methylation with diazomethane gave trimethyl ether **5b** and as side products an isomer **6a** and tetramethyl ether **6b**. The trimethyl ether **5b** was characterized in detail by ^1H, ^{13}C, and HMBC NMR spectra (Table S1). As with the octaacetate, most proton and carbon signals were doubled. The lactone group was proven by an IR band at 1796 cm^{-1} and the chemical shift of 174.2 ppm for C-16. The assignment of carbon signals was greatly facilitated by comparison with the data from ^{13}C-labeled blepharismin[21] and by the HMBC spectrum. The four hydroxy groups in *peri* positions are expectedly hydrogen bonded, giving four individual signals in the range 13 to 14 ppm, which show far ranging correlations with all carbons of the ring system with the exception of those in the center part, C-1, C-1′, C-11 to C-14, and C-11′ to C-14′. A free hydroxy group at C-20 is indicated by a hydroxy signal at 8.29 ppm and the chemical shift of 156.2 ppm for C-20. This was proven by degradation in melting KOH to give *p*-hydroxybenzoic acid. Thus, the *O*-methylation must have occurred in the bay positions at C-10, C-10′, and C-2′, which is corroborated by their HMBC correlation with the *O*-methyl protons. The isomeric trimethyl ether must have the orthoester structure **6a**, as it shows a single set of NMR signals and no lactone carbonyl. The third methoxy group, according to its chemical shift of 3.24 ppm, and that of C-20 (155.8 ppm), are at C-16. Another byproduct of methylation is tetramethyl ether **6b**. Its structure is nearly identical to **6a** with the difference that the C-20 hydroxy is also methylated (3.68 ppm).

In order to obtain a salt of the hypothetical free acid **4C** and take NMR spectra, **4** was dissolved in aqueous 3 N sodium hydroxide. Immediately a deep blue color developed; however, no defined NMR signals could be observed, indicating that a radical reaction was occurring. After 20 h the reaction mixture was acidified and extracted with EtOAc. Two predominant products, a blue-violet one (**7**) and a red one, were observed by

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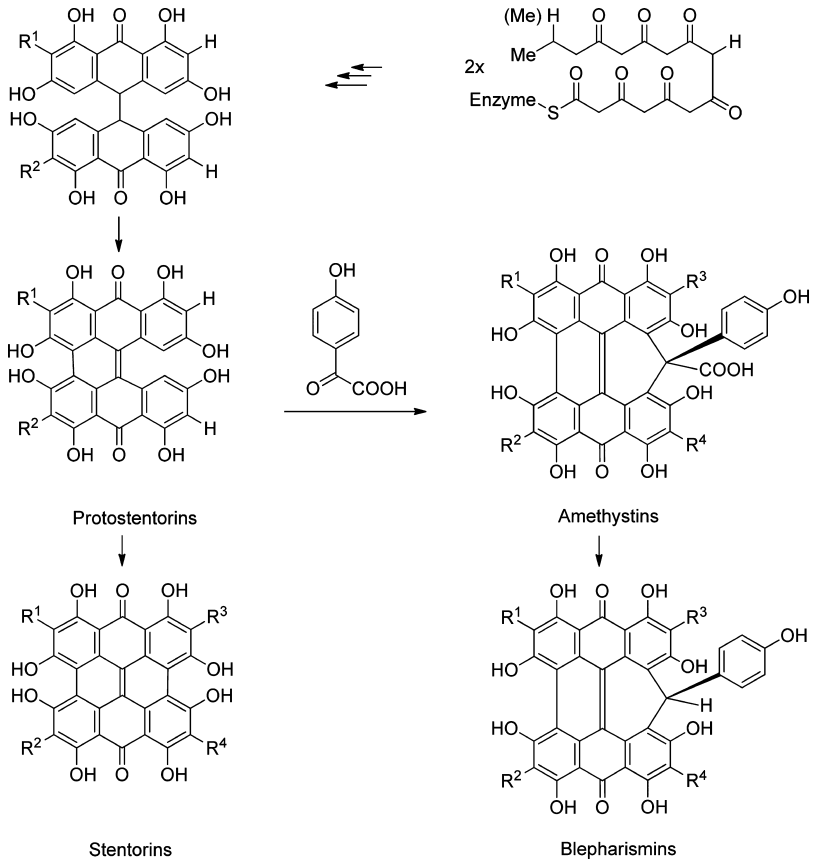
Scheme 1. Proposed Biosynthesis of the Ciliate Pigments Stentorin, Blepharismin, and Amethystin via the Hypothetical Intermediate Protostentorin[a]



[a]R^1, R^2 = Et, i-Pr; R^3, R^4 = H, Me.

HPLC/MS and separated by preparative HPLC. For **7** HRESIMS gave a molecular weight of 667.1624, indicating an elemental composition of $C_{42}H_{28}O_{10}$, which corresponds to loss of C_2O_2 compared to amethystin (**4**). The proton NMR spectrum shows asymmetry in the benzodianthrone system and low field signals for one of the C methyl groups (2.39 ppm) and for the C-18 protons of the hydroxyphenyl group. From that, stentorin-related structure **7** is derived, in which one of the bay hydroxy groups is replaced by the *p*-hydroxyphenyl moiety. For the second compound HRESIMS showed a molecular weight of 739.1473, corresponding to an elemental composition of $C_{42}H_{28}O_{13}$. This is one oxygen more than the educt, and consequently the compound was named oxyamethystin. Due to the lack of material, only proton NMR spectra could be measured, showing four conspicuous broad signals in the range 5.60 to 8.00 for the *p*-hydroxyphenyl group. From that, oxyamethystin is not structurally related to oxyblepharismin, which is characterized by a well-resolved AA′BB′ system at 6.45 and 6.65. Differing from oxyblepharismin, the hydroxyphenyl in oxyamethystin must be locked in an asymmetric position above the naphthodianthrone ring system exposed to strong shielding and deshielding effects. Its structure may be related to that of amethystin octaacetate (**5a**) and trimethyl ether (**5b**), which show similar, however less pronounced, shielding effects for the *p*-hydroxyphenyl protons.

The biosynthesis of the *Stentor* and *Blepharisma* pigments appears to be closely related to that of hypericin. Both are polyketides, which by dimerization of intermediate anthrones form symmetrical naphtho- and benzodianthrone systems. However, on closer inspection, profound differences turn up. Whereas emodin[33] and emodinanthrone,[34] the supposed biosynthetic intermediates to hypericin, dimerize selectively through the more activated dihydroxyphenyl moieties to protohypericin, such selectivity is not available for an analogous intermediate to stentorin (**1**), blepharismin (**2**), and amethystin (**4**) type compounds (Scheme 1). Both phenyl rings of a corresponding 13,13′-bisanthranol would be equally active in radical or oxidative coupling, resulting in isomeric mixtures with respect to the position of the ethyl or isopropyl groups. Such isomers have never been observed with **1**, **2**, and **4**. To achieve this strict selectivity in the dimerization, the corresponding enzyme must differentiate between ethyl/isopropyl and hydrogen in *both* building blocks to be coupled. After the second ring closure through C-11, and optionally methylation at C-3 and C-3′, the so far hypothetical protostentorins would be formed as a central branching point for further modifications. Whereas protostentorin undergoes another ring closure to give the stentorins, it is condensed with *p*-hydroxyphenylglyoxylic acid (or a biochemical equivalent) to give the amethystins in *S. amethystinus*. These, by decarboxylation in *B. japonicum*, eventually lead to the blepharismins. Interestingly, by HPLC/MS analysis of extracts from *B. japonicum* and *S. amethystinus* only small amounts of the corresponding stentorins, but no protostentorin, could be detected. No amethystin was detected in *B. japonicum*, and no blepharismin in *S. amethystinus*.

In conclusion, we have achieved the isolation of amethystin in a crystalline state, which is responsible for the red-violet coloration of *S. amethystinus*. Its benzodianthrone substructure was elucidated by spectroscopic measurements as a carboxy derivative of blepharismin (**2**) from *B. japonicum*. According to

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NMR measurements amethystin (**4**) exists in solution exclusively as an orthoester, whereas in the crystal and in the solid state the isomeric hydroxylactone form may be present to a moderate extent. To prove independently the orthoester and hydroxylactone structures, *O*-acetyl and *O*-methyl derivatives were prepared and characterized by spectroscopy. Reaction with base in the presence of oxygen produced oxyamethystin of unknown structure and a degradation product carrying a *p*-hydroxyphenyl residue at C-2.

S. amethystinus collected from rich blooms was found to contain high concentrations of amethystin (**4**), whereas sparingly occurring organisms appear uncolored and contain more than 10-fold less pigment. From that, it could be speculated that the very small amounts of pigments are localized in the cilia[28] responsible for photodetection, while larger amounts are concentrated in the granula during a bloom for defense.

Beyond that, a consistent biosynthetic scheme is proposed including the ciliate pigments, stentorin, blepharismin, and amethystin. As central intermediate, protostentorin is postulated in analogy with the biosynthesis of hypericin via protohypericin. The formation of protostentorin from two heptaketides by dimerization is a highly regioselective process, as only the smaller substituent H is found in the 3,3′-positions. Thus, the corresponding enzyme must provide an extended recognition site for both anthrone units. Apart from the search for early biosynthetic intermediates, genetic analysis of the biosynthetic machinery in the three species will give a clue to this mechanism and, moreover, explain the further species-specific modifications.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined with a Büchi 510 apparatus. UV spectra were recorded on a Shimadzu UV-2102 PC scanning spectrometer; IR spectra, with a Nicolet 20DXB FT-IR spectrometer. NMR spectra were recorded on a Bruker ARX 300 or ARX-400 spectrometer. EI and DCI mass spectra (reactant gas ammonia) were obtained on a Finnigan MAT 95 spectrometer, with high-resolution data acquired using peak matching (M/DM = 10 000). ESI mass spectra were obtained with a Q-Tof II from Micromass. HPLC/ESI/APCIMS: PE Sciex Api-2000 LC/MS. HRESIMS data were recorded on a Maxis spectrometer (Bruker Daltonics). Analytical RP HPLC was carried out on an Agilent 1100 HPLC system equipped with a UV diode-array detector. HPLC conditions: Nucleodur C-18, 5 μm, 2 × 125 mm column (Macherey-Nagel), solvent A: 5% MeCN in 5 mM of NH_4Ac buffer; solvent B: 95% MeCN, 5 mM of NH_4Ac buffer, pH 5.5; gradient 5:95 to 95:5 in 30 min. For analytical TLC aluminum sheets coated with silica gel Si 60 F_{254} and for preparative TLC precoated silica gel Si 60 F_{254} plates of 0.25 and 0.5 mm layer thickness (Merck) were used.

Isolation of Amethystin (4). (a) *Stentor amethystinus* and concomitant plankton were collected from the lake "Große Fuchskule" in Neubrandenburg, Germany (53°06′20.6″ N, 12°59′05.2″ E) in August 1995. The organisms were flushed with water from the plankton net (60 μm mesh size) and immediately extracted by the addition of four volumes of acetone, 1.92 L in total. From the absorption at 580 nm the presence of 10 mg of amethystin was estimated. The extract was concentrated *in vacuo* to give a dark yellow water phase and a brown precipitate, which were extracted with EtOAc. The organic layer was dried with Na_2SO_4 and evaportated to dryness to give 380 mg of extract. A second batch of organisms yielded 250 mg of extract. Separation of the combined extracts on Sephadex LH-20 (column 4 × 87 cm, MeOH) gave a dark red fraction of crude amethystin (31 mg). Final purification was achieved by preparative HPLC (Nucleosil RP18, column 2 × 25 cm, MeOH/5 mM NH_4Ac buffer 8:2), yielding 15 mg of **4** as a dark red, microcrystalline powder.

(b) *Stentor amethystinus* was collected from a small pond near Ostrach, Germany (47°55′14.6″ N, 9°23′56.0″ E) as described above in May 1996. The suspension of the collected organisms in pond water was filtered through a bed of Celite (8 × 30 cm). The column was washed with H_2O (2 L) and eluted with acetone (3 L) to yield a dark red aqueous acetone fraction, which was concentrated *in vacuo* at 30 °C. The dark-colored, oily residue was lyophilized. The residue was triturated with diethyl ether, and the suspension formed was diluted with petroleum ether, chilled, and filtered. This procedure was repeated four times to give 5.0 g of crude material, which was further purified and separated by HPLC. A 4.4 g portion of the crude material was separated in five batches on Lichroprep Si100, 15−25 μm silica gel (70 mL) with acetone as eluent. The dark red fraction was collected to give 1.55 g of crude **4**. Final purification was achieved by RP18 chromatography in two batches on HD-SIL 35−70 μm (column 83 × 9 cm, MeOH/10 mM NH_4OAc buffer, 9:1) to give 740 mg of amethystin (**4**).

Amethystin (4): dark red to black needles (EtOAc); slow decomposition above 200 °C without melting; UV (MeOH) λ_{max} 579 (4.87), 538 (4.73), 494 (4.70), 355 (sh) (4.80), 333 (4.98), 287 (5.07), 254 (5.22), 224 (5.31) nm; after addition of base: 580, 539, 491 427, 339, 287, 257, 225 nm; after addition of acid: 575, 539, 486, 403, 354, 330, 285, 252, 225 nm; IR (KBr) ν_{max} 1798 (weak to very weak), 1614, 1590, 1419 cm^{-1}; ^1H and ^{13}C NMR see Table 1; ESIMS (−) *m/z* 723; HRESIMS (+) *m/z* 725.1672 (calcd for $C_{42}H_{29}O_{12}$, 725.1659); anal. 67.92; H 4.07; O 28.00%, calcd for $C_{42}H_{30}O_{13}$, C 67.99; H 3.80; O 27.96%; TLC R_f = 0.48 (silica gel, EtOAc/MeOH/H_2O, 80:15:10), R_f = 0.33 (RP-18, MeOH/H_2O, 8:2); HPLC t_R = 6.15 min (Nucleosil C_{18}, 4 × 250 mm, MeOH/10 mM NH_4OAc buffer, 85:15, diode array detection).

(c) *Stentor amethystinus* was collected from a small pond at the Darmstadt University campus (49°52′9.1″ N, 8°40′51.5″ E) in the summer and fall of 2013 and separated from an excess of copepods by filtration through a nylon stocking. For analytical purposes a suspension of approximately 20 000 organisms was filtered through a bed of Celite and extracted as described above. HPLC/HRESIMS analysis (WatersXBridge C_{18}, 3,5 μm, 2.1 × 100 mm column, gradient MeOH/5 mM NH_4OAc buffer, 1:1, to 100% MeOH/5 mM NH_4OAc buffer in 20 min at 0.6 mL/min) revealed in addition to amethystin (66 μg, t_R = 13.5 min, 100%) three minor components with the chromophore of amethystin. They were identified as lower (t_R = 11.1 min, 1%) and higher (t_R = 14.9 min, 3%) homologues and as an isomer of amethystin (t_R = 12.4 min 5%). For ESIMS data see Supporting Information. A semipure sample of the isomer from the large-scale isolation showed the following: ^1H NMR (CD_3OD) δ 1.28 (t, *J* = 7.5 Hz, 3H), 1.50 (d, *J* = 7.5, 3H), 1.55 (d, *J* = 7.5, 3H), 2.50 (s, 3H), 2.95 (br q, 2H), 3.91 (m, 1H), 5.80 (br s, 1H), 5.97 (br s, 1H), 6.30 (br s, 1H), 6.46 (br s, 1H), 6.75 (s, 1H).

A more polar compound (t_R = 2.4 min 6%) by UV and molecular mass was identified as stentorin F (**1b**). It was isolated from the prerun of the above separation (b) by two consecutive RP HPLC runs, first with MeOH/50 mmol NH_4OAc buffer, 9:1, then MeCN/H_2O, 96:4, and 0.1% TFA to give 16 mg of stentorin F (**1b**): TLC R_f = 0.62 (EtOAc/MeOH/H_2O, 80:15:10); UV (MeOH) λ_{max} 602, 551. 463, 437 sh, 342, 329, 296, 242 sh, 225 nm; ^1H NMR (DMSO-d_6) δ 1.20 (t, *J* = 7.5, 23-H_3), 2.37 (br s, 21-H_3), 2.50 (q, *J* = 7.6 Hz, 22-H_2), 14.50 (br s, 4-OH, 8-OH); ESIMS (−) *m/z* 591 $[M - H]^-$ (100); HRESIMS (+) *m/z* 593.1438 $[M + H]^+$ (calcd for $C_{34}H_{25}O_{10}$, 593.1448).

Derivatization. *Amethystin Octaacetate (5a).* (a) Amethystin (4 mg) was dissolved in acetic anhydride (0.5 mL) and 13 μL of concentrated sulfuric acid and heated for 1 min to 90 °C. Some sodium acetate was added, and the solution evaporated to dryness. The residue was distributed between EtOAc and phosphate buffer pH 7. From the organic phase 1.1 mg of **5a** was isolated by preparative TLC (CH_2Cl_2/acetone, 95:5). (b) Amethystin (40 mg) was acetylated with acetic anhydride (1 mL) and pyridine (0.5 mL) at room temperature (rt). The reaction product was evaporated *in vacuo* at rt, and the residue was dissolved in 5 mL of toluene and evaporated again. This procedure was repeated four times. Then the residue was dissolved in 3 mL of toluene/CH_2Cl_2 (10:1) and filtered through a pad of kieselguhr, and the filtrate was evaporated to dryness. The yield

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of 40 mg of crude peracetate was separated by preparative TLC (CH_2Cl_2/ MeOH, 95:5) to give 11 mg of **5a** as a dark yellow solid: TLC R_f = 0.44 (CH_2Cl_2/acetone, 95:5); ^{1}H NMR ($CDCl_3$) δ 1.12 (t, J = 7.5, 3H), 1.17 (t, J = 7.5, 3H), 2.00 (s, 3H), 2.10 (s, 3H), 2.22 (s, 3H), 2,33 (s, 3H), 2.41 (s, 3H), 2.42 (s, 3H), 2.44 (s, 6H), 2.50 (s, 3H), 2.52 (s, 3H), 3.55−3.58 (m, 4H), 6.11 (br d, 1H), 6.41 (br d, 1H), 6.57 (br d, 1H), 6.68 (br d, 1H); EIMS m/z 1020 $[M - 40]^+$ (66), 978 (64), 936 (70), 894 (100), 852 (73), 810 (48), 768 (100); ESIMS (+) m/z 1083 $[M+ Na]^+$ (100).

*Amethystin Tri- and Tetramethyl Ether (**5b**, **6a**, **6b**).* A 50 mg amount of amethystin was added to 3 mL of diazomethane in diethyl ether. To solubilize the amethystin, some MeOH and EtOAc were added. After 10 min the solvents were evaporated and the residue was separated into two batches by HPLC (Nucleosil 100, 7 μm, column 2 × 25 cm, CH_2Cl_2/petroleum ether, 95:5, detection 254 nm). The center fraction (46 mg) was separated again to give pure **5b** (22 mg) as a dark red solid. A side fraction gave 3.4 mg of isomeric trimethyl ether **6a**, and another side fraction by preparative TLC (diethyl ether/ petroleum ether, 1:1) gave tetramethyl ether **6b** (~1 mg) and a pentamethyl derivative (~1 mg) of unknown structure.

***5b**:* TLC R_f = 0.42 (CH_2Cl_2/MeOH, 98:2); UV (MeOH) λ_{max} 565 (4.11), 531 (4.02), 486 (4.15), 416 (3.77), 367 (3.80), 282 (4.43), 246 (4.56) nm; IR (KBr) ν_{max} 1796, 1630, 1585 cm^{-1}; NMR spectroscopic data see Table S2, Supporting Information; DCIMS (NH_3) m/z 767 $[M + H]^+$ (100), 444 (60), 150 (80); HREIMS m/z 766.2029 (calcd for $C_{45}H_{34}O_{12}$, 766.2050), 645.1788 (calcd for $C_{38}H_{29}O_{10}$, 645.1761).

***6a**:* TLC R_f = 0.60 (CH_2Cl_2/MeOH, 98:2); UV (CH_3OH) λ_{max} 567, 531, 480, 363, 316 sh, 280 sh, 249, 226 nm; ^{1}H NMR ($CDCl_3$/ DMSO-d_6, 8:2) δ 0.93 (t, J = 7.5 Hz, 6H), 1.97 (s, 6H), 2.5−2.8 (m, 4H), 3.02 (s, 6H), 3.24 (s, 3H), 5.88 (s, 4H), 13.40 (s, 2H), 13.94 (s, 2H); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 8:2) δ 185.6, 164.3, 163.8, 161.4, 157.3, 155.8, 127.6, 125.8, 125.2, 124.7, 123.4, 123.0, 118.7, 117.4, 108.4, 107.7, 105.2, 60.2, 55.9, 52.1, 16.1, 12.8, 7.5; HRESIMS m/z 767.2115 $[M + H]^+$ (100) (calcd for $C_{45}H_{35}O_{12}$, 767.2128), 1533.4177 $[2M + H]^+$ (33) (calcd for $C_{90}H_{69}O_{24}$, 1533.4179).

***6b**:* TLC: R_f = 0.67 (diethyl ether/petroleum ether, 1:1); UV (CH_3OH) λ_{max} 566, 529, 483, 364, 317 sh, 286 sh, 251, 228 nm; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, J = 7.5 Hz, 6H), 2.40 (s, 6H), 3.0 (m, 4H), 3.40 (s, 6H), 3.50 (s, 3H), 3.68 (s, 3H), 6.36 and 6.41 (AA′BB′ system, 8 Hz, 4 H), OH signals were not observed; ESIMS (−) m/z 779 $[M - H]^-$ (100); HRESIMS (+) m/z 781.2281 $[M + H]^+$ (100) (calcd for $C_{46}H_{37}O_{12}$, 781.2280).

Amethystin pentamethyl ether: TLC R_f = 0.57 (diethyl ether/ petroleum ether, 1:1); UV (CH_3OH) λ_{max} 544, 479, 366, 318 sh, 282 sh, 250, 228 nm; ^{1}H NMR ($CDCl_3$, selection) δ 3.38 (s, 3H), 3.39 (s, 3H), 3.66 (s, 6H), 3.99 (s, 3H); HRESIMS (+) m/z 795.2443 $[M + H]^+$ (100) (calcd for $C_{47}H_{39}O_{12}$, 795.2442).

*Degradation of Amethystin Trimethyl Ether (**5b**) by Base.* A mixture of potassium hydroxide powder (50 mg) and **5b** (9 mg) was heated for a few minutes to form a black melt. H_2O (0.5 mL) was added and acidified with concentrated hydrochloric acid. The EtOAc extract was separated by TLC (EtOAc/MeOH/H_2O, 83:8:7) to give *p*-hydroxybenzoic acid (0.3 mg, 20%).

*Degradation of Amethystin (**4**) by Base.* Amethystin (10 mg) was dissolved in 3 N NaOH (1 mL), kept for 20 h at rt, acidified with acetic acid, and extracted with EtOAc. Two predominant compounds were detected by HPLC/MS with molecular masses of 668 (**7**, blue-violet) and 740 (red color, named oxymethystin). The compounds were separated by preparative RP18 HPLC (MeOH/H_2O, 9:1, and 1% acetic acid) to give **7** (4.5 mg), oxyamethystin (1.5 mg), and a mixture of both.

***7**:* TLC R_f = 0.52 (EtOAc/MeOH/H_2O, 80:15:10); UV (CH_3OH) λ_{max} 597, 555, 495, 457, 405, 332, 300, 244, 227 nm; ^{1}H NMR (CD_3OD) δ 1.22 (t, J = 7 Hz, 3H), 1.32 (br t, J = Hz, 3H), 2.08 (s, 3H), 2.39 (s, 3H), 3.0 (br m, 2H), 3.18 (br q, J = 7 Hz, 2H), 6.92 (br d, 2H), 7.80 (br d, 2H); HRESIMS (−) m/z 667.1624 $[M - H]^-$ (100) (calcd for $C_{40}H_{27}O_{10}$, 667.1604).

Oxyamethystin: TLC R_f = 0.40 (EtOAc/MeOH/H_2O, 80:15:10); UV (MeOH) λ_{max} 588, 545, 483, 447, 340, 327, 294, 248 sh, 224 nm; ^{1}H NMR (CD_3OD) δ 1.30 (m, 6H), 2.60 (br s, 6H), 3.10 (m, 4H), 5.58 (br s, 1H), 6.31 (br s, 1H), 7.12 (br s, 1H), 8.00 (br s, 1H); HRESIMS (−) m/z 739.1473 $[M - H]^-$ (100) (calcd for $C_{42}H_{27}O_{12}$, 739.1452).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The UV/vis spectrum of amethystin (**4**), analytical HPLC/UV/ HRESIMS of extracts (a) and (b) of *S. amethystinus*, and ^{1}H and ^{13}C NMR data and spectra of stentorin (**1b**), amethystin (**4**), amethystin octaacetate (**5a**), and trimethyl ether (**5b**) are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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