Aeruginascin, a Trimethylammonium Analogue of Psilocybin from the Hallucinogenic Mushroom *Inocybe aeruginascens*

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Dedicated to Dr. phil. II Dr. h.c.mult. Albert Hofmann in honour of his 100th birthday.

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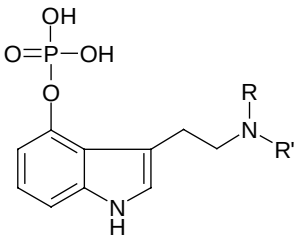
Supporting information is available online.

Abstract

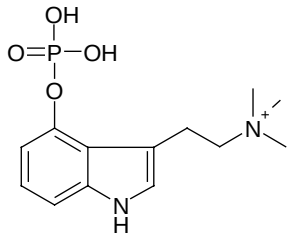
The hallucinogenic mushroom *Inocybe aeruginascens* contains several typical *Psilocybe* alkaloids including psilocybin. We have now elucidated the structure of a further indole derivative named aeruginascin as the quaternary ammonium compound *N*,*N*,*N*-trimethyl-4-phosphoryloxytryptamine. Aeruginascin is closely related to the frog skin toxin bufotenidine, and has been found exclusively in *Inocybe aeruginascens* so far.

Letter

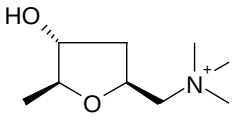
The hallucinogenic mushroom *Inocybe aeruginascens* (see supporting information) grows mostly at anthropogenic locations like parks and gardens, directly on sand or in short grass and is found widely distributed across central Europe [1]. Several unintentional intoxications have been reported and have resulted in characteristic hallucinogenic symptoms [2]. The hallucinogenic alkaloid psilocybin (**3**) [3] could be detected subsequently in this and in a few related *Inocybe* species [4-6].



1: R = R' = H (Norbaeocystin)
2: R = H, R' = Me (Baeocystin)
3: R = R' = Me (Psilocybin)



4 (Aeruginascin



5 (Muscarine)

Beside psilocybin (**3**) and the related alkaloid baeocystin (**2**), a so far unknown indole alkaloid named aeruginascin (**4**) has been observed in *Inocybe aeruginascens* extracts [6-8]. One of us (J. G.) has screened several related psilocybin-positive *Inocybe* species and many other hallucinogenic mushrooms [5-8], but aeruginascin (**4**) has never been detected in any other mushroom species so far.

Aeruginascin (**4**) is stable in dried mushrooms at room temperature for years, it is more polar

on silica gel than psilocybin (**3**) and exhibits the same colour reaction with Ehrlich's reagent as **3** and the demethyl analogues norbaeocystin (**1**) and baeocystin (**2**) [9]. However, the pinkish colour is stable and does not change to bluish violet on the TLC plate.

Fruiting bodies of *Inocybe aeruginascens* were extracted with polar solvents, and aeruginascin (**4**) was purified by repeated column chromatography on silica gel followed by size-exclusion chromatography on Sephadex G-10. The UV spectrum of the resulting pure compound closely matched those of **1**, **2** and **3**, pointing to the presence of a 4-hydroxyindole moiety as well [3,9]. In ^{1}H NMR experiments, a conspicuous sharp 9H singlet at δ = 3.2 gave evidence for the presence of a quaternary trimethylammonium group. ESI MS showed a $[M+H]^+$ molecular peak of *m/z* = 299.2, suggesting 4-phosphoryloxy-*N,N,N*-trimethyltryptamine (**4**) as a candidate structure. The exact mass was obtained by high-resolution mass spectrometry and clearly excluded a sulphate of similar molecular weight.

To differentiate between a substitution in 4- or 7-position of the indole ring, 4-phosphoryloxy-*N,N,N*-trimethyltryptamine (**4**) was synthesized by permethylation of **2** [10]. According to all analytical data, the compound thus obtained was identical with isolated aeruginascin (**4**) at controlled pH value. The methyl ester of aeruginascin (**4**) had been obtained previously as an intermediate during the Hofmann degradation of **3** [3].

Aeruginascin (**4**) shares the quaternary ammonium group with muscarine (**5**), a mushroom toxin mainly found in the genera *Inocybe* and *Clitocybe* that is absent in psilocybin positive *Inocybe* species [1,6-8]. It is tempting to speculate that the same methyltransferase that catalyzes the final methylation step in the biosynthesis of **5** in other *Inocybe* species may be responsible for the synthesis of **4** from **3** in *I. aeruginascens*.

The pharmacology and toxicology of aeruginascin (**4**) has not yet been tested. **4** can be assumed to undergo a rapid enzymatic dephosphorylation *in vivo* in animals, but due to the quaternary ammonium group it is unlikely to pass the blood-brain barrier, a requirement for centrally mediated hallucinogenic effects. Aeruginascin (**4**) is a close analogue of the frog skin toxins bufotenidine (N,N,N-trimethylserotonin, 5-HTQ) and its sulphuric acid ester [11]. Bufotenidine is a potent peripherally acting 5-HT_3 receptor agonist [12]. While the effects after ingestion of *I. aeruginascens* did not differ from those of other hallucinogenic mushrooms [2,6-8], larger doses of aeruginascin (**4**) might have dangerous peripheral consequences.

Materials and Methods

Fruiting bodies of *Inocybe aeruginascens* were collected in 1991 in Potsdam, Germany, and identified by one of us (J. G.). Voucher specimens have been deposited in the herbarium of the University of Leipzig (Germany). Powdered carpophores (9.5 g) were consecutively extracted with each 100 ml cyclohexane, ethyl acetate, ethanol, and 50 ml MeOH/H_2O/formic acid (80:20: 0.2). The latter extract tested positive for **3** and **4** on TLC with Ehrlich's reagent and was pre-separated on a short silica gel column (MeOH/H_2O/formic acid, 80:20:0.2). Positive fractions were further separated by column chromatography on silica gel with MeOH/H_2O/formic acid (80:20:0.2) and MeOH/H_2O/28% NH_3 (70:30:0.2) and on Sephadex G-10 (H_2O). Fractions with UV absorption at λ = 267 nm yielded 4.6 mg aeruginascin (**4**).

Aeruginascin (**4**): Colourless glassy hygroscopic material, R_f (**4**) / R_f (**3**) = 0.29/0.42 (*n*-BuOH/AcOH/H_2O, 2/1/1); for further systems see the supporting information; UV (H_2O): λ_{max} (log ε) = 219 (3.92), 267 (3.20), 282 (3.10, sh), 288 (3.00, sh) nm; ^{1}H NMR (7% formic acid in D_2O, 600 MHz): δ = 8.257 (s, *H*COOH, reference), 7.29 (1H, d, *J* = 8 Hz, 7-H), 7.25 (1H, s, 2-H), 7.18 (1H, dd, *J* = 2 × 8 Hz, 6-H), 7.06 (1H, d, *J* = 8 Hz, 5-H), 3.64 - 3.61 (2H, m, □-CH_2), 3.46 - 3.42 (2H, m, □-CH_2), 3.23 (9H, s, $N^+(CH_3)_3$); ^{13}C NMR (D_2O, 3% formic acid, 3% MeOH, 151 MHz): δ = 139.7 (C-7b), 124.9 (C-2), 123.5 (C-6), 119.4 (C-3b), 109.9 (C-3), 108.5 (C-5), 68.7 (α-CH_2), 54.1 [$N^+(CH_3)_3$], 21.3 (β-CH_2); (+)-ESI MS (H_2O/MeOH): *m/z* (%) 635 (7) $[2M+K]^+$, 619 (17) $[2M+Na]^+$, 597 (18) $[2M+H]^+$, 337 (12) $[M-H+K]^+$, 321 (52) $[M-H+Na]^+$, 299 (100) $[M]^+$, 240 (10) $[M-N(CH_3)_3+H]^+$, 219 (16) $[M-PO_3H]^+$, 160 (15) $[M-PO_3H-N(CH_3)_3+H]^+$; HR ESIMS *m/z* 299.115477 $[M]^+$ (calcd for $[C_{13}H_{20}N_2O_4P]^+$ 299.115520).

Acknowledgements

We thank Dietmar Schmidt (University of Tübingen, Germany) for measuring the HR mass spectrum of native aeruginascin, and the Deutsche Forschungsgemeinschaft for finan-

cial support (DFG grant La 414/8-2).

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Supporting Information

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Fig. 1: The psychoactive mushroom *Inocybe aeruginascens* (picture by courtesy of Gerhard Drewitz).

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Synthesis

Aeruginascin (**4**): Synthetic baeocystin (**2**) [1] (10 mg, 37.0 µmol) was dissolved in 250 µL of H_2O and mixed with 25 µL of methyl iodide (57 mg, 402 µmol) and 20 µL of diisopropyl-ethylamine (14.8 mg, 115 µmol) in a microtube. Methanol was added dropwise in order to get a homogenous solution. The mixture was kept at 50 °C for 60 min and then evaporated to dryness. The crude product was purified using the procedure described in the main part, starting with MeOH/H_2O/NH_3 column chromatography.

TLC Data

Developed TLC sheets were stained by immersion in Ehrlich's reagent (identical to Van Urk's reagent; 8% conc. hydrochloric acid and 1% *p*-dimethylaminobenzaldehyde (DMBA) in MeOH) and subsequent heating in a stream of hot air.

Further R_f values of aeruginascin (**4**) and psilocybin (**3**): R_f (**4**) / R_f (**3**) = 0.24/0.37 (*n*-BuOH/AcOH/H_2O, 24:10:10); 0.09/0.14 (*n*-PrOH/6% NH_3, 5:2); 0.30/0.46 (*n*-BuOH/AcOH/*i*-PrOH/H_2O, 8:2:3:5); 0.34/0.49 (*n*-BuOH/AcOH/*i*-PrOH/H_2O, 8:2:1:5); 0.21/0.32 (*n*-PrOH/ 28% NH_3, 5:3); 0.21/0.38 (*n*-PrOH/AcOH/H_2O, 10:3:3); 0.23/0.51 (MeOH/H_2O/28% NH_3, 70:30:0.2); 0.38/0.56 (MeOH/H_2O/formic acid, 80:20:0.2).

Table 1: R_f values of mushroom alkaloids and reference compounds. TLC on silica gel TLC sheets, run length around 60 mm. Compounds: aeruginascin (**4**) in an enriched *Inocybe aeruginascens* extract; synthetic aeruginascin (**4**) from baeocystin (**2**); psilocin in a crude extract of *Psilocybe azurescens*; psilocybin (**3**) in an enriched *Inocybe aeruginascens* extract; synthetic baeocystin (**2**); synthetic norbaeocystin (**1**); commercial samples of tryptophan, tryptamine, 5-hydroxytryptophan, and serotonin creatinine sulphate; putative 4-hydroxy-*N,N,N*-trimethyltryptamine (4-OH-TMT), 4-hydroxy-*N*-methyltryptamine (4-OH-NMT), and 4-hydroxytryptamine from crude aeruginascin (**4**), synthetic baeocystin (**2**), and synthetic norbaeocystin (**1**), respectively, by incubation with alkaline phosphatase. Although isolated and synthetic aeruginascin (**4**) gave slightly differing R_f values with these eluents, co-application of both samples resulted in a single spot in all three systems tested.

Compound	**Ehrlich's reagent color** (immediately)	**Ehrlich's reagent color** (after 48 h)	***n*-BuOH / AcOH / H_2O** (2 + 1 + 1) (n = 3, ±SEM)	***n*-BuOH / AcOH / H_2O** (24 + 10 + 10) (n = 3, ±SEM)	***n*-PrOH / NH_3 6%** (5 + 2)	***n*-BuOH / AcOH / *i*-PrOH / H_2O** (8 + 2 + 3 + 5)	***n*-BuOH / AcOH / *i*-PrOH / H_2O** (8 + 2 + 1 + 5)	***n*-PrOH / NH_3 28%** (5 + 3)	***n*-PrOH / AcOH / H_2O** (10 + 3 + 3) (n = 3, ±SEM)	**MeOH / H_2O / NH_3 28%** (70 + 30 + 0.2)	**MeOH / H_2O / formic acid** (80 + 20 + 0.2)
Aeruginascin (enriched extract)	purple	purple	0.30* (±0.02)	0.25* (±0.02)	0.08	0.31	0.35	0.20	0.25* (±0.03)	0.23	0.40
Aeruginascin (synthetic)	purple	purple	0.29* (±0.02)	0.24* (±0.01)	0.09	0.30	0.34	0.21	0.21* (±0.01)	0.23	0.38
Psilocin (crude extract)	violet	bluish violet	0.66 (±0.03)	0.62 (±0.04)	0.72	0.70	0.66	0.92	0.63 (±0.03)	0.32	0.71
Psilocybin (enriched extract)	purple	violet	0.42 (±0.03)	0.37 (±0.03)	0.14	0.46	0.49	0.32	0.38 (±0.03)	0.51	0.56
Baeocystin (synthetic)	purple	violet	0.49 (±0.03)	0.46 (±0.03)	0.12	0.55	0.54	0.22	0.51 (±0.03)	0.83	0.69
Norbaeocystin (synthetic)	purple	violet	0.60 (±0.02)	0.56 (±0.02)	0.13	0.64	0.60	0.18	0.64 (±0.03)	0.93	0.74
Tryptophan	violet	bluish green	0.75 (±0.03)	0.71 (±0.05)	0.62	0.77	0.72	0.69	0.82 (±0.03)	0.88	0.82
Tryptamine	purple, then violet	bluish green	0.74 (±0.03)	0.68 (±0.04)	0.66	0.75	0.69	0.78	0.80 (±0.02)	0.21	0.83
5-OH-Tryptophan	blue	blue	0.70 (±0.03)	0.66 (±0.05)	0.58	0.73	0.69	0.64	0.79 (±0.02)	0.87	0.84
Serotonin	blue	blue	0.77 (±0.06)	0.68 (±0.03)	0.60	0.74	0.69	0.72	0.80 (±0.02)	0.22	0.84
4-OH-TMT (from Aeruginascin)	gray	-	-	-	-	-	-	0.62	-	-	-
4-OH-NMT (from Baeocystin)	blue	-	-	-	-	-	-	0.67	-	-	-
4-OH-Tryptamine (from Norbaeocystin)	blue	-	-	0.78	-	-	-	0.68	0.70	-	-

UV spectra

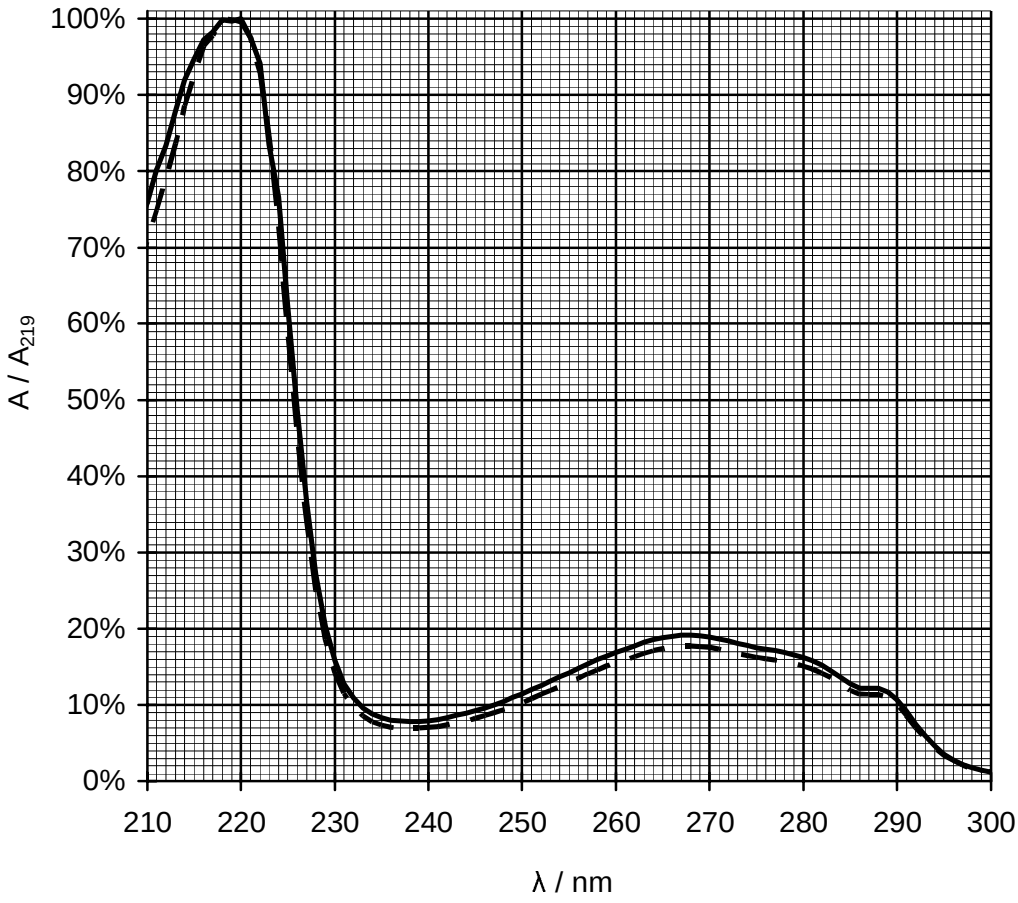

Fig. 2: UV spectra of isolated (continuous line) and synthetic (dashed line) aeruginascin (**4**). The absorption has been normalized to A_{219}.

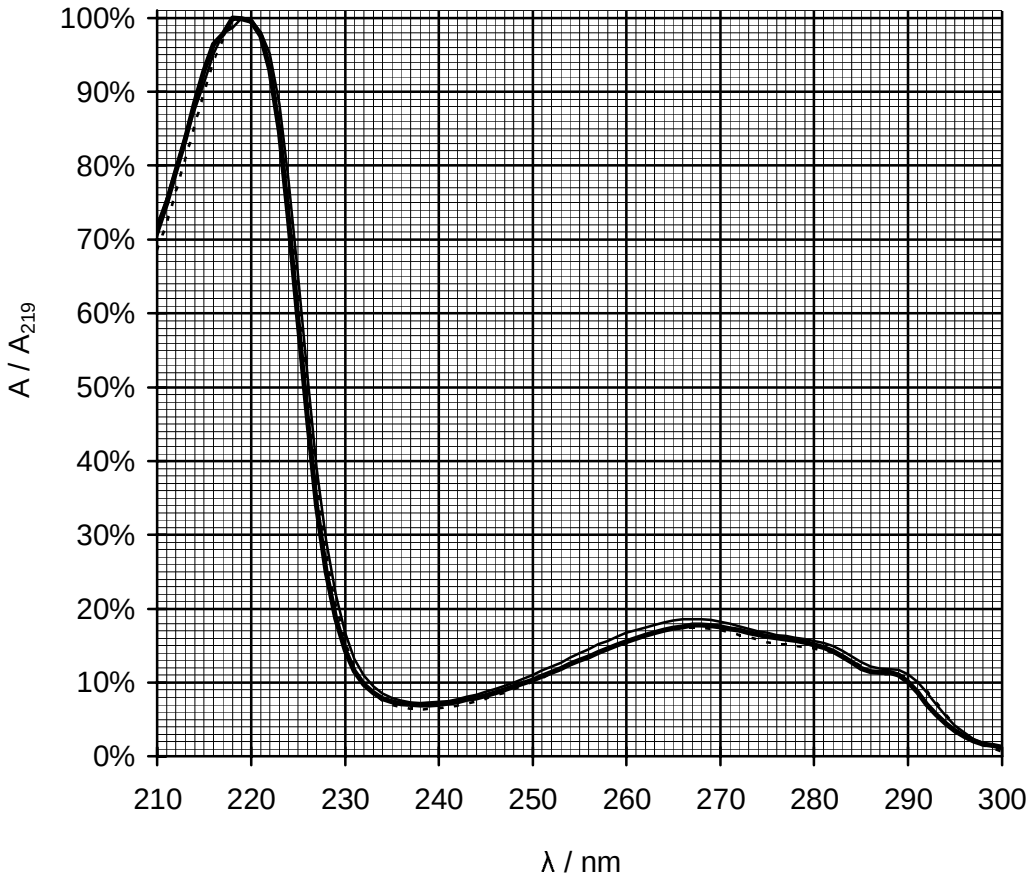

Fig. 3: UV spectra of synthetic aeruginascin (**4**) (bold line), baeocystin (**2**) (fine continuous line), and norbaeocystin (**1**) (dashed line). The absorption has been normalized to A_{219}.

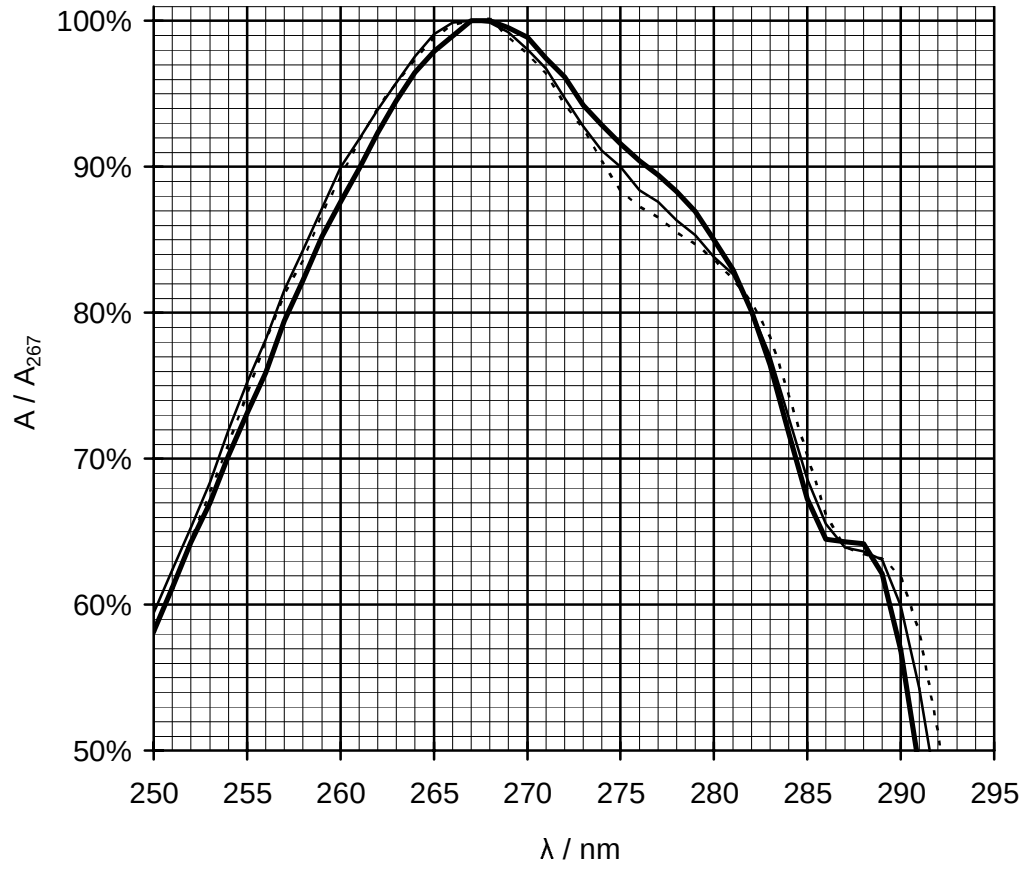

Fig. 4: Enlarged UV spectra of synthetic aeruginascin (**4**) (bold line), baeocystin (**2**) (fine continuous line), and norbaeocystin (**1**) (dashed line). The absorption has been normalized to A_{267}.

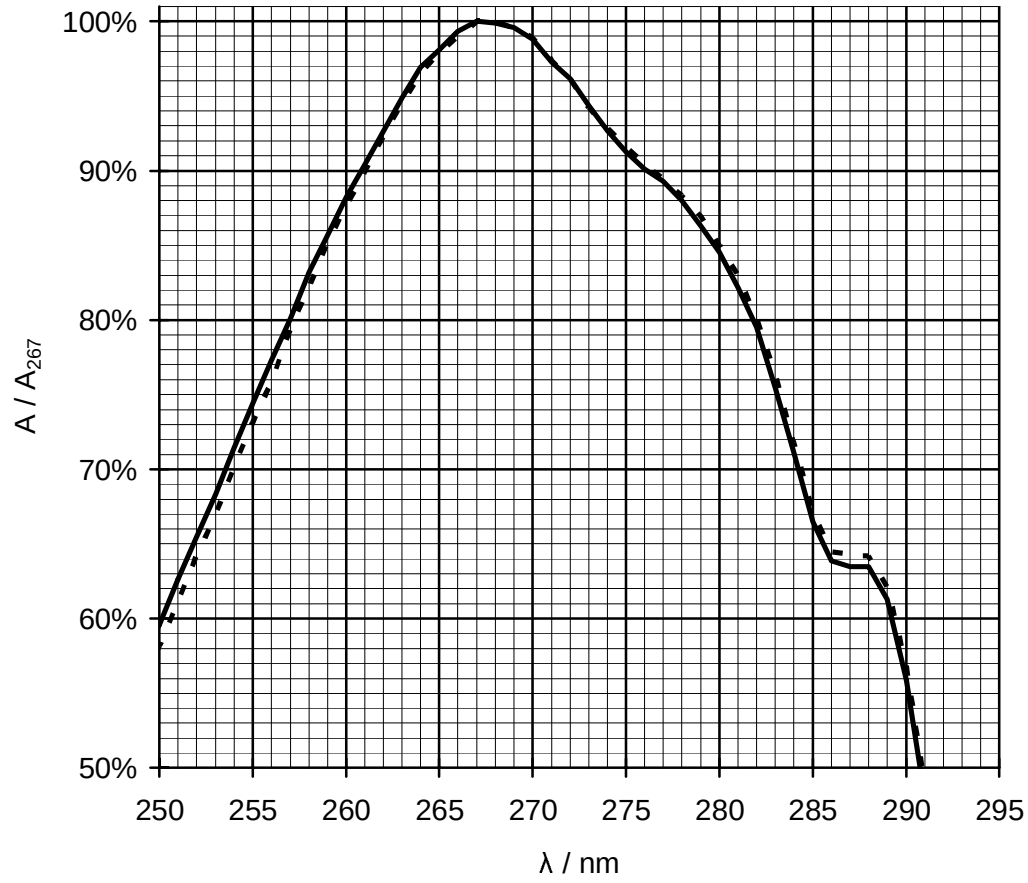

Fig. 5: Detailed view of the UV spectra of isolated (continuous line) and synthetic (dotted line) aeruginascin (**4**). The absorption has been normalized to A_{267}.

^{1}H NMR

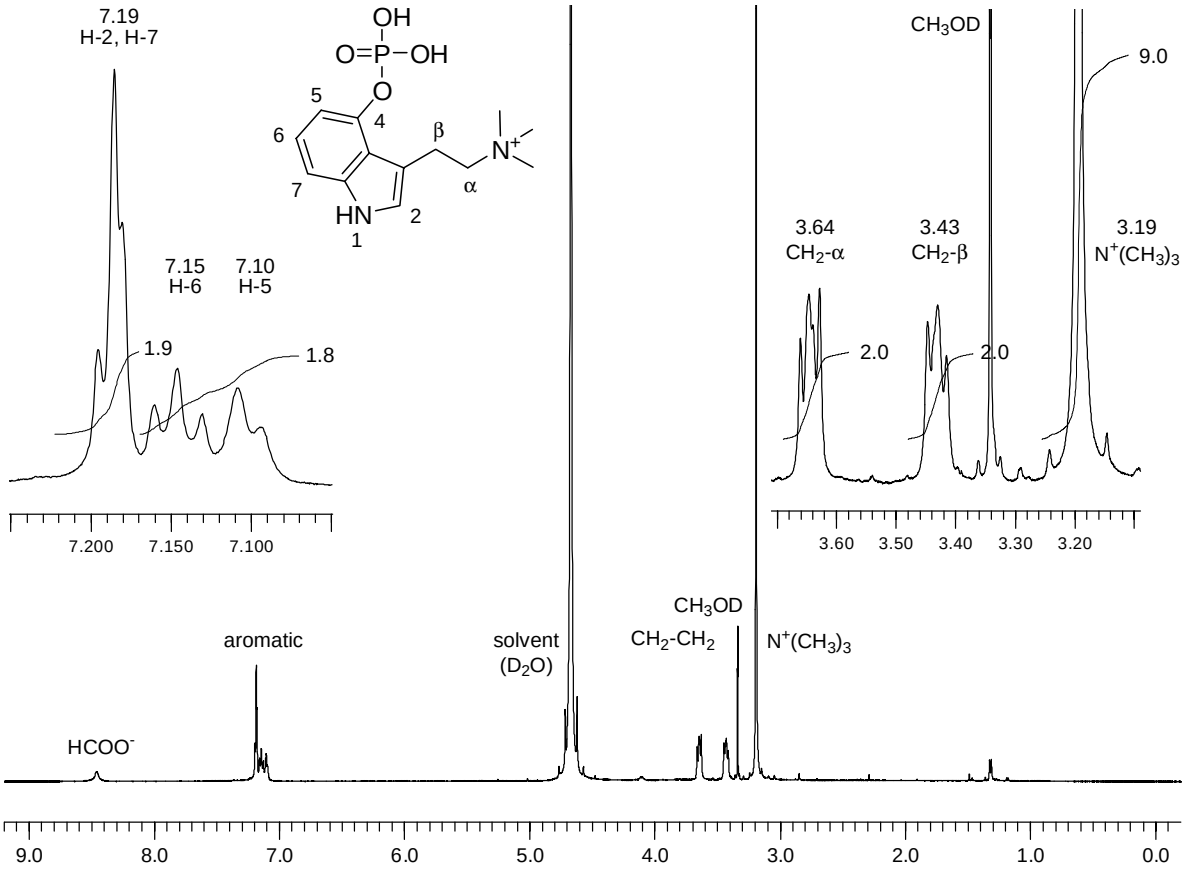

Fig. 6: ^{1}H NMR spectra of aeruginascin (**4**) under unbuffered conditions (500 MHz, D_2O). The chemical shifts of the aromatic protons were pH-sensitive and the signals could be separated under the acidic conditions.

^{1}H NMR of isolated aeruginascin (**4**) (600 MHz, D_2O / 7% formic acid): δ = 8.257 (s, HCOOH, reference), 7.29 (d, *J* = 8 Hz, 1 H, H-7), 7.25 (s, 1 H, H-2), 7.18 (dd, *J* = *J'* = 8 Hz, 1 H, H-6), 7.06 (d, *J* = 8 Hz, 1 H, H-5), 3.64 - 3.61 (m, 2 H, H_2-□), 3.46 - 3.42 (m, 2 H, H_2-β), 3.23 (s, 9 H, $N^+(CH_3)_3$).

^{1}H NMR synthetic aeruginascin (**4**) (600 MHz, D_2O / 7% formic acid): δ = 8.257 (s, HCOOH, reference), 7.29 (d, *J* = 8 Hz, 1 H, H-7), 7.24 (s, 1 H, H-2), 7.17 (dd, *J* = *J'* = 8 Hz, 1 H, H-6), 7.06 (d, *J* = 8 Hz, 1 H, H-5), 3.65 - 3.60 (m, 2 H, H_2-α), 3.45 - 3.40 (m, 2 H, H_2-□), 3.23 (s, 9 H, $N^+(CH_3)_3$).

^{1}H NMR of isolated aeruginascin (**4**) (500 MHz, D_2O): δ = 8.46 (s br, $HCOO^-$), 7.19 (s, 1 H, H-2), 7.19 (d, *J* = 7.5 Hz, 1 H, H-7), 7.15 (dd, *J* = 7.5 Hz, 7.5 Hz, 1 H, H-6), 7.10 (d, *J* = 7 Hz, 1 H, H-5), 3.64 (t, *J* = 8 Hz, 2 H, H_2-α), 3.43 (t, *J* = 8 Hz, 2 H, H_2-β), 3.19 (s, 9 H, $N^+(CH_3)_3$).

^{1}H NMR of synthetic aeruginascin (**4**) (500 MHz, D_2O): δ = 8.49 (s br, 1 H, $HCOO^-$), 7.25 (d, *J* = 8 Hz, 1 H, H-7), 7.17 (s, 1 H, H-2), 7.15 (dd, *J* = *J'* = 8 Hz, 1 H, H-6), 7.04 (d, *J* = 7.5 Hz, 1 H, H-5), 3.55 - 3.50 (m, 2 H, H_2-α), 3.37 - 3.32 (m, 2 H, H_2-β), 3.14 (s, 9 H, $N^+(CH_3)_3$).

^{1}H NMR of synthetic aeruginascin (**4**) (500 MHz, D_2O / 3% NEt_3): δ = 8.39 (s br, $HCOO^-$), 7.11 (s, 1 H, H-2), 7.10 - 7.07 (m, 3 H, H-5,6,7), 3.65 - 3.27 (m, 2 H, H_2-α), 3.42 - 3.37 (m, 2 H, H_2-β), 3.15 (s, 9 H, $N^+(CH_3)_3$).

^{13}C NMR

^{13}C-APT NMR of isolated aeruginascin (**4**) (126 MHz, D_2O, methanol): δ = 124.3 (CH-2), 123.7 (CH-6), 109.6 (CH-3), 107.3 (CH-5), 68.7 (CH_2-1'), 54.1 (3 CH_3-N^+), 21.1 (CH_2-2').

^{13}C NMR of isolated aeruginascin (**4**) (151 MHz, D_2O / 3% formic acid / 3% methanol): δ = 166.76 (CH-HCOOD), 165.29 (CH-HCOOH), 49.86 (CH_3-methanol, reference), 139.68 (C_q-7b), 124.91 (CH-2), 123.45 (CH-6), 119.49 (C_q-3b), 109.85 (CH-3), 108.53 (CH-5), 68.71 (CH_2-1'), 54.09 (3 CH_3-N^+), 21.31 (CH_2-2').

^{13}C NMR of synthetic aeruginascin (**4**) (151 MHz, D_2O / 3% formic acid / 3% methanol): δ = 166.68 (CH-HCOOD), 165.49 (CH-HCOOH), 49.89 (CH_3-methanol, reference), 146.84 (C_q-4), 139.55 (C_q-7b), 125.09 (CH-2), 123.49 (CH-6), 119.35 (C_q-3b), 109.93 (CH-3), 108.75 (CH-5), 68.52 (CH_2-1'), 54.09 (3 CH_3-N^+), 21.18 (CH_2-2').

^{31}P NMR

^{31}P NMR of isolated aeruginascin (**4**) (81 MHz, D_2O): δ = 0.7 (br s, R-OPO_3D_2).

^{31}P NMR of baeocystin (**4**) (81 MHz, D_2O): δ = 2.8 (s, R-OPO_3D_2).

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