

Isolation and Synthesis of Pulmonarin A and B,
Acetylcholinesterase Inhibitors from the Colonial Ascidian
Synoicum pulmonaria

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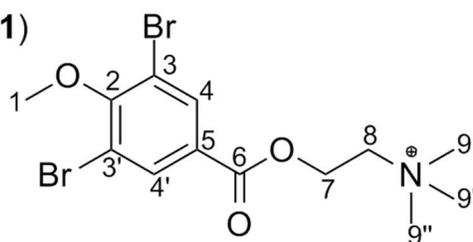
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ABSTRACT

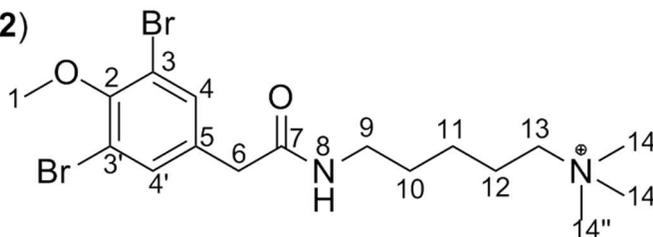
Pulmonarins A and B are two new dibrominated marine acetylcholinesterase inhibitors which were isolated and characterised from the sub-Arctic ascidian *Synoicum pulmonaria* collected off the Norwegian coast. The structures of natural pulmonarins A and B were tentatively elucidated by spectroscopic methods and later verified by comparison with synthetically prepared material. Both pulmonarins A and B displayed reversible, non-competitive acetylcholinesterase inhibition comparable to several known natural acetylcholinesterase inhibitors. Pulmonarin B was the strongest inhibitor with an inhibition constant (K_i) of 20 μM . In addition to reversible, non-competitive acetylcholinesterase inhibition, the compounds displayed weak antibacterial activity but no cytotoxicity or other investigated bioactivities.

Marine invertebrates and their associated bacterial symbionts represent organisms with great potential for the discovery of novel bioactive compounds and chemical scaffolds.¹⁻³ The sessile benthic lifestyle promotes the production of defensive secondary metabolites to provide an evolutionary advantage. To investigate their potential as sources for bioactive compounds, several hundred different Arctic marine invertebrates, algae and microorganisms have been collected during a systematic study of Arctic marine organisms along the Norwegian coast.⁴ The Arctic waters provide a significantly less studied biotope in comparison to warmer waters, however they are still rich in biodiversity.⁴⁻⁶ One such Arctic organism, the colonial ascidian *Synoicum pulmonaria* has been shown to contain a family of dibrominated bioactive compounds known as the synoxazolidinones.⁷⁻⁹ In the current paper, further studies of the extract from *S. pulmonaria* revealed additional novel compounds named pulmonarins A (**1**) and B (**2**).

Pulmonarin A (**1**)

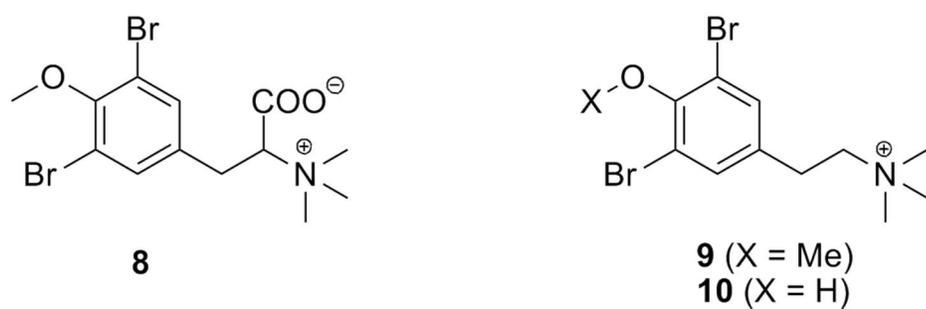
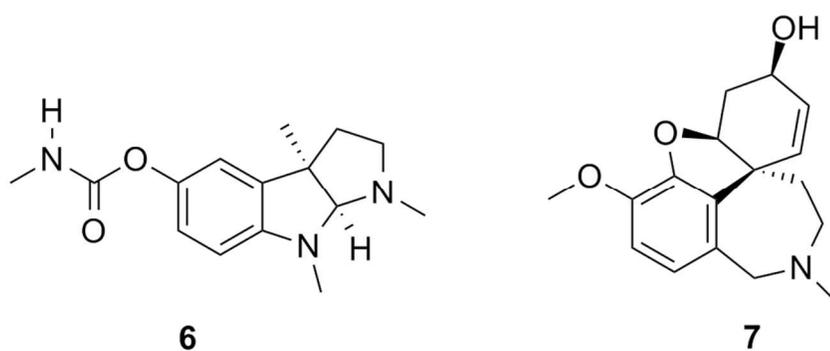
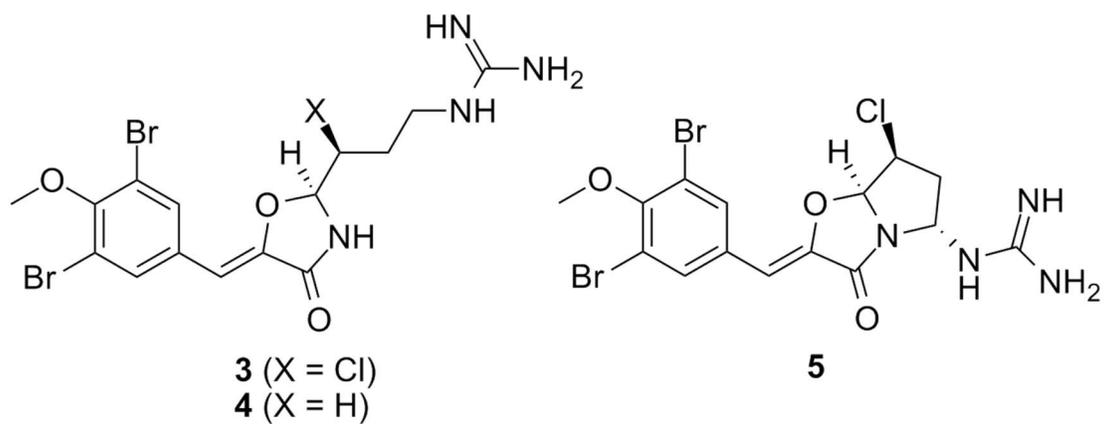


Pulmonarin B (**2**)



The pulmonarins are structurally related to the synoxazolidinones (**3-5**) which are heavily modified antimicrobial dipeptide derivatives displaying a rare 4-oxazolidinone core linking a dibrominated tyrosine derivative to an arginine/agmatine side chain. The pulmonarins do not

display the 4-oxazolidinone core or the guanidine group of the synoxazolidinones but instead they contain quaternary ammonium groups implying potential acetylcholinesterase (AChE) inhibiting properties. AChE inhibitors are a class of drugs used for the treatment of Alzheimer's disease, glaucoma, myasthenia gravis (an autoimmune disorder) and the recovery of neuromuscular block after surgery.^{10, 11} Some well-known AChE-inhibitors in clinical use today include physostigmine (**6**), a naturally occurring carbamate from the Calabar bean, used for the treatment of glaucoma.^{10, 12} Examples of other current AChE-inhibitor based drugs are galanthamine (**7**) and donepezil, both used in most countries for the treatment of Alzheimer's disease. Several toxins such as nerve agents, insecticides and antiparasitic chemicals used in agriculture and aquaculture also act by inhibiting AChE and consist mostly of organophosphorus compounds and carbamates.^{13, 14} At least three other marine natural products with similar structures to **1** and **2** have been reported in the literature. Compound **8**, with significant structural similarities to **1** was isolated by Fattorusso et al. from the Caribbean sponge *Pseudoceratina crassa* (later reclassified as *Aiolochoia crassa*) in 1994.¹⁵ Compound **8** was recently studied by Munoz and co-workers and shown to display weak antiparasitic properties.¹⁶ Compound **9** was isolated from another Caribbean sponge *Verongula sp.* but no biological activity was reported for the compound.¹⁷ A derivative of **9**, compound **10**, was isolated from the sponge *Verongia fistularis*.¹⁸



The current paper describes the isolation, structural characterisation and syntheses of **1** and **2** and the evaluation of their biological activities including AChE inhibition employing electric eel AChE.

RESULTS AND DISCUSSION

Step-wise fractionation of an aqueous extract of *S. pulmonaria* resulted in the isolation of two compounds. The aqueous extract was loaded on a solid phase extraction (SPE) cartridge and eluted with 40/60 CH₃CN/H₂O. The eluate was loaded on a semi-preparative RP-HPLC C₁₈-column. MS analysis revealed compounds with isotope patterns indicating dibrominated compounds. Compounds **1** and **2** were isolated as yellow gels with the molecular formula C₁₃H₁₈Br₂O₃N for **1** and C₁₇H₂₇Br₂O₂N₂ for **2**. Database searching suggested new compounds and their tentative structures were solved on the basis of a number of 1D and 2D NMR experiments and mass spectrometric analyses. Spectroscopic similarities with the synoxazolidinones were rapidly established.⁷ Table 1 lists proton and carbon NMR shift values and both gCOSY and gHMBC correlations of natural **1** and **2**.

Table 1. 1D and 2D NMR Data (600/175 MHz DMSO-*d*₆) of Natural **1** and **2**.

no	δ_C , type ^a	δ_H (mult, <i>J</i> in Hz)	gCOSY	gHMBC
Pulmonarin A				
1	61.2, CH ₃	3.84, s		2
2	158.2, C			
3/3'	118.5, C			
4/4'	133.9, CH	8.15, s		2, 3/3', 4/4', 6
5	*			
6	163.2, C			
7	59.9, CH ₂	4.66, m	8	
8	64.2, CH ₂	3.78, t (4.5)	7	9
9/9'/9''	53.3, CH ₃	3.14, s		8
Pulmonarin B				
1	60.9, CH ₃	3.77, s		2
2	151.9, C			
3/3'	117.0, C			
4/4'	133.7, CH	7.53, s		2, 3/3', 4/4'
5	136.1, C			
6	41.0, CH ₂	3.38, s		4/4', 5, 7
7	169.3, C			
8	NH	8.19, t (5.5)	9	
9	38.9, CH ₂	3.05, m	8, 10	
10	29.0, CH ₂	1.45, m	9, 11	
11	23.8, CH ₂	1.25, m	10, 12	
12	22.3, CH ₂	1.66, m	11, 13	
13	65.2, CH ₂	3.24, m	12	
14/14'/14''	52.2, CH ₃	3.02, s		13, 14

^a Carbon shifts obtained from gHSQC and gHMBC experiments. * Not observed

An initial inspection of the 2D NMR data revealed similar motifs in the aromatic region as those found in the synoxazolidinones.⁷ The carbon shifts (Table 1) used for the structure elucidation were obtained from the gHSQC experiment, while the quaternary carbon signals are derived from gHMBC data by comparison with the structurally related synoxazolidinones isolated from the same organism. The tentative structure of **1** was solved based on the following correlations: The gHMBC correlation from the H-1 methoxy protons to C-2 indicated the position of the

methoxy group on the substituted aromatic ring; the shift value of C-3/3' (δ_C 118.5) was indicative of two bromine atoms at these positions⁷; and protons H-4/4' showed a gHMBC correlation to the ester carbonyl C-6. Furthermore, a gCOSY correlation was observed between the two adjacent methylene protons H-7 and H-8 and the high chemical shift of H-7 (δ_H 4.66) placed it adjacent to the ester oxygen. The gHMBC correlation from protons H-9/9'/9'' to C-8 supported the terminal trimethylated quaternary ammonium. The shift values of C-9/9'/9'' (δ_C 53.3) and H-9/9'/9'' (δ_H 3.14) are consistent with literature values.¹⁹⁻²¹ The assignment of the terminal trimethylated ammonium was also supported by a loss of 59 mass units from the parent ion as indicated by a prominent peak at m/z 334 in the mass spectrum of **1**.

Compound **2** displayed signals in the aromatic area of the ¹H and ¹³C NMR spectra that were nearly identical to **1**, suggesting a similar sub-structure. The chemical shift of H-4/4' is somewhat lower than that of the corresponding protons found in both **1** and the synoxazolidinones due to the methylene at C-6. The H-6 methylene protons displayed a high shift (δ_H 3.38) and showed gHMBC correlations to C-4/4', C-5 and the amide carbonyl C-7 placing it between the amide and the aromatic ring. The chemical shifts for H-6, C-6 and C-7 correlated well with a similar synthetic methoxy phenyl acetamide derivative of orthidine F.²² C-9 was placed next to the amide nitrogen based on gCOSY correlations between amide proton H-8 and H-9. This spin system further involved H-10 to H-13 generating a saturated five carbon segment between the amide and terminal trimethyl ammonium which was further verified by the gHMBC correlations from protons H-14/14'/14'' to C-13 in analogy to **1**. This spin system matched well with the chemical shifts of the analogous spin system reported for clavatadine D,

isolated from the Australian sponge *Suberea clavata*.²³ Figure 1 shows key gHMBC and gCOSY correlations of natural **1** and **2**.

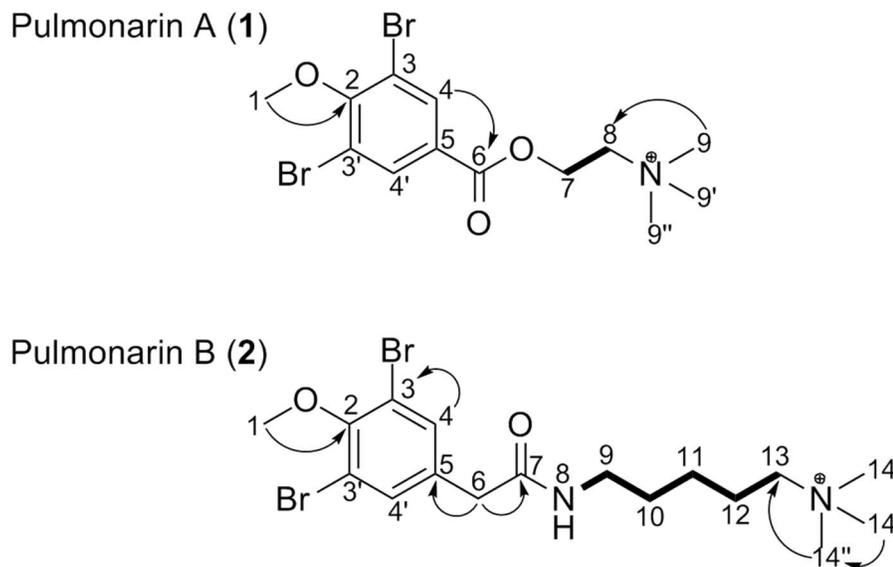
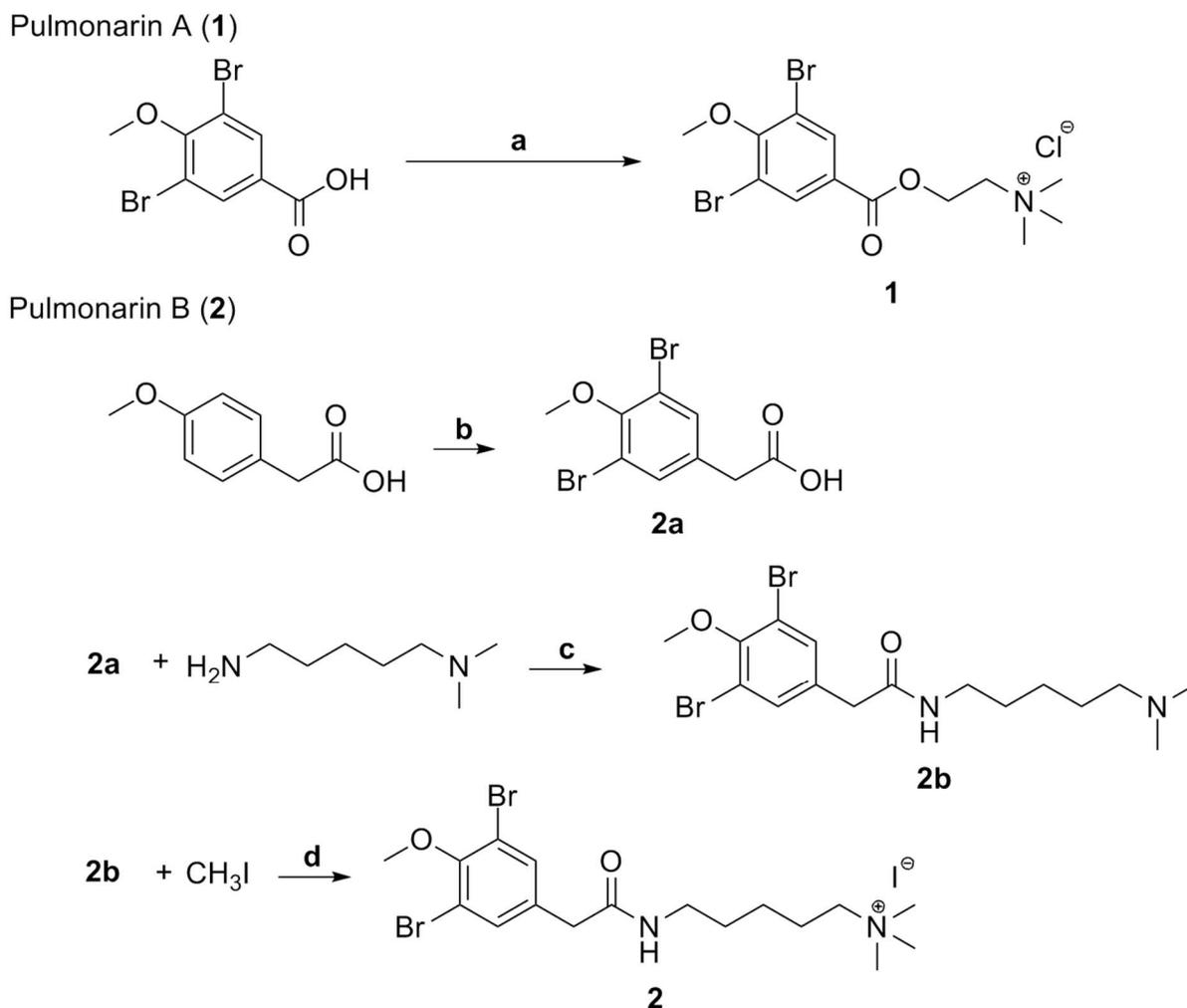


Figure 1. Key gHMBC (H→C) and gCOSY (—) correlations of **2**.

Because the purification led to sample loss, and the incomplete NMR data of the compounds, total synthesis was required to generate sufficient material for structural verification and biological testing. Compound **1** was prepared in one step. DCC was used as coupling agent to react 3,5-dibromo-4-methoxybenzoic acid and choline chloride in the presence of a catalytic amount of DMAP in CH₂Cl₂. The reaction was slow and sufficient amounts of **1** could only be obtained after an extended reaction time followed by HPLC purification. Several different solvent systems were evaluated, but only CH₂Cl₂ generated **1** due to solubility issues. Compound **2** was synthesised in three steps from 4-methoxyphenylacetic acid which was initially dibrominated with Br₂/FeBr₃ to produce 3,5-(dibromo-4-methoxyphenyl)acetic acid (**2a**) according to Weller et al.²⁴ The bromination reaction was followed with MS and a complete

conversion to the desired dibrominated product required additional $\text{Br}_2/\text{FeBr}_3$ after 24 h. Compound **2a** was then coupled with 5-(dimethylamino)amylamine using HBTU as coupling agent to generate 2-(3,5-dibromo-4-methoxyphenyl)-*N*-(5-(dimethylamino)pentyl)acetamide (**2b**). *N*-methylation of **2b** with CH_3I completed the synthesis of **2**. The synthetic routes to **1** and **2** are displayed in Scheme 1.



Scheme 1. Synthesis of **1** and **2**. Reagents, conditions, isolated yield (a) Choline chloride, DMAP, DCC, CH_2Cl_2 , rt, 6 days, 4% (HPLC yield); (b) $\text{Br}_2/\text{FeBr}_3$ ²⁴, CHCl_3 , rt, 48h, 21%; (c) HBTU, DIPEA, DMF, rt, 16h, not isolated; (d) K_2CO_3 , CH_2Cl_2 ; 16 h, 13% (HPLC yield).

Both ^1H and ^{13}C NMR shifts of the natural and synthetic compounds are analogous, thus validating their proposed structures (Table S3). This is further supported by comparing the 2D-NMR data.

The para-methoxy dibrominated phenyl group moiety is a common structural motif among marine secondary metabolites, and particularly those that are tyrosine derivatives.²⁵ The isolated compounds bear structural resemblances with not only the synoxazolidinones,^{7, 8} and **8**, **9** and **10**^{15, 17, 18} but also with ianthelline,²⁶ and subereamine B²⁷ amongst others.

As both pulmonarins are terminally trimethylated ammonium compounds they were evaluated as AChE inhibitors in addition to the standard screens routinely performed during our screening process.⁴ Electric eel AChE, sharing a high degree of sequence homology and having a 3D structure very similar to human AchE,²⁸ was employed as a model vertebrate AChE system and the well-established method of Ellman was employed to study the inhibition kinetics.^{29, 30} Synthetic **1** induced 50% inhibition of electric eel AChE at 42 $\mu\text{g}/\text{mL}$ (105 μM) in the presence of acetylthiocholine chloride as a substrate (Figure 2). The inhibitory constant (K_i) was determined from the Dixon plot³¹ and revealed **1** to be a reversible, non-competitive inhibitor with a K_i of 36 $\mu\text{g}/\text{mL}$ (90 μM) (Figure 3). Compound **2** showed a stronger reversible, non-competitive inhibition and 50% inhibition was obtained at 16 $\mu\text{g}/\text{mL}$ (35 μM) with a K_i of 9 $\mu\text{g}/\text{mL}$ (20 μM) (Figures 2 and 3).

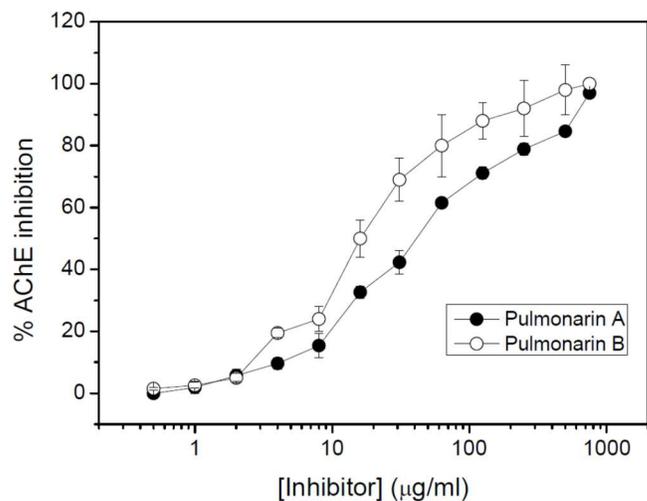


Figure 2. Inhibition of vertebrate AChE by **1** and **2**. 50% inhibition was observed at 42 and 16 µg/mL, (105 µM and 36 µM) respectively.

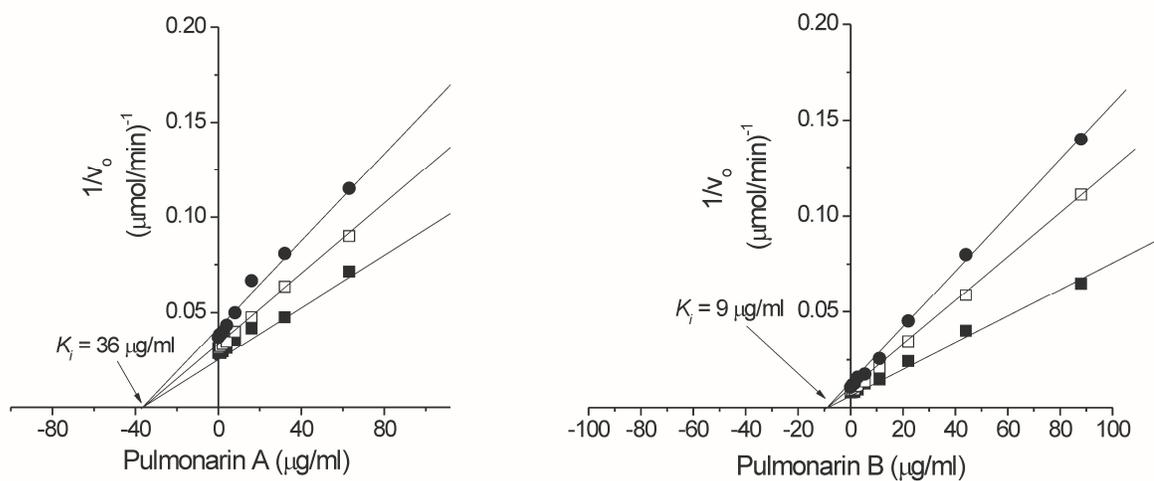


Figure 3. Determination of the type of inhibition and the inhibition constant K_i for **1** (left graph) and **2** (right graph) by Dixon plot analysis. The concentrations of the substrate acetylthiocholine were 0.25 (●), 0.5 (□), and 1 mM (■). K_i was determined to 36 µg/mL (90 µM) for **1** and to 9 µg/mL (20 µM) for **2**.

A K_i of 9 $\mu\text{g/mL}$ (20 μM) for **2** indicates moderate binding affinities for vertebrate AChE, similar to that of the FDA-approved drug galanthamine.³² The affinity of **1** for AChE is four times weaker. AChE inhibitors with significantly varying binding affinities are used in approved medications. Physostigmine is a strong AChE inhibitor with an IC_{50} value of approximately 30 nM³³ while galanthamine, used to combat Alzheimer's disease, displays a moderate K_i of 2-20 μM against AChE from different vertebrate species.³² The K_i values obtained here are therefore within the pharmaceutically interesting regime. For competitive inhibitors of AChE, studies based on molecular modelling and quantitative structure-activity relationship (QSAR) analysis have shown that a degree of hydrophobicity and the presence of an ionisable nitrogen are prerequisites for the inhibitor-enzyme interactions.¹⁰ Considering this, it was expected that the more hydrophobic **1**, as determined by HPLC retention times, would be more active. As the observed AChE inhibition contradicts this, it is clear that further investigations are required for a mechanistic explanation.

As an allosteric inhibitor of AChE, the presence of a quaternary nitrogen in both compounds suggests binding through electrostatic interactions at the peripheral anionic site (PAS) of the enzyme, which is located on the surface of the protein at the entrance of the active site cleft. This site is implicated in a number of non-classical functions such as amyloid deposition, cell adhesion and neurite outgrowth.³⁴ The PAS is thus a potential target in rational drug design for the development of novel and improved inhibitors and therapeutics for the treatment of neural cancers, nerve regeneration and neurodegenerative disorders such as Alzheimer's disease.^{11, 34} QSAR studies of **1** and **2** could potentially contribute to future development of new drug candidates in various therapeutic fields.

We have previously reported that the 40/60 (v/v) CH₃CN/H₂O SPE fraction of *S. pulmonaria* exhibited antibacterial activity against a number of bacteria, showing an activity of 160 µg/ml against *Corynebacterium glutamicum* and 80 µg/ml against *Staphylococcus aureus*.³⁵ These compounds were therefore also included in the extensive screening performed at MabCent to investigate other potential bioactivities.⁴ A lack of activity at concentrations above 100 µg/mL classified the compounds as “inactive” in all assays apart from the antibacterial screen. Compound **1** displayed only very mild activity against one bacterial strain, *C. glutamicum*, at a concentration of 500 µg/mL indicating a possible synergistic effect in the active fraction. Compound **2** was slightly more active against *C. glutamicum* displaying a minimal inhibitory concentration of 250 µg/mL. No activities were seen in the other bacterial screens in our panel (test strains used were *S. aureus* (ATTC 9144), *E. coli* (ATCC 25922), *P. aeruginosa* (ATTC 27853), and *C. glutamicum* (ATTC 13032)) (data not included). As well as testing negative against other bacteria, both **1** and **2** also tested negative for cytotoxic activities against three adherent cancer cell lines (A2058 melanoma, HT29 colon carcinoma and MCF7 breast carcinoma cells) and against adherent, non-malignant lung fibroblasts (MRC-5 cells) (data not included). They were also inactive in an anti-inflammatory assay targeted against TNF- α and in a biofilm formation inhibition assay using *S. epidermidis* (ATCC 35984) (data not included). Finally **1** and **2** were shown to be inactive as cellular antioxidants (data not included). Compounds **1** and **2** thus appear to possess a highly specific action as reversible non-competitive AChE inhibitors displaying no apparent cellular toxicity. As reported for the synoxazolidinones (**3-5**), it was not possible to determine whether symbiotic microorganisms are responsible for the biosynthesis of **1** and **2** which is a common case for marine natural products.¹ Based on the key structural differences between the synoxazolidinones and the compounds in the present study it

seems unlikely that they share a common biosynthetic route in the organism apart from being tyrosine derivatives. Compound **1** can be considered as a dibrominated version of *p*-hydroxybenzoylcholine which has been isolated from the seeds of *Sinapis albae* and it was proposed to originate biosynthetically from a *p*-hydroxy benzyl alcohol derivative.^{36, 37} An analogous biosynthetic route towards **1** is therefore plausible. The proposed biosynthesis of **2** is similar and is suggested to be derived from amide formation between methylated 2-(4-hydroxy-3,5-dibromophenyl)acetic acid and ascophylline, both previously described in extracts of marine organisms^{38, 39}.

CONCLUSIONS

Two new marine bioactive compounds have been isolated and characterised from *S. pulmonaria*. Curtailed by the lack of the natural compounds, total synthesis provided enough material for structure verification and biological testing. Both compounds turned out to be nontoxic, reversible, non-competitive inhibitors of AChE. The isolation of **1** and **2** yet again demonstrates the chemical diversity of the cold-water marine organism *S. pulmonaria* and the potential of the species for the discovery of new structural motifs for further lead compound development in a number of therapeutic areas.

EXPERIMENTAL SECTION

General Experimental Procedures

UV spectrum was determined on a Lambda 25 spectrometer from Perkin Elmer Instruments.

Infrared spectra were recorded on an Avatar 320 FT-IR spectrometer from Nicolet. 1D and 2D

NMR spectra of natural products were recorded on a Varian VNMRS 600 MHz spectrometer in DMSO-*d*₆. Carbon resonances were derived from gHSQC and gHMBC experiments. NMR spectra of synthetic compounds were recorded on a Varian 7000e 400 MHz spectrometer in DMSO-*d*₆. Chemical shifts were referenced to the residual solvent peaks, δ_{H} 2.50 and δ_{C} 39.5. Mass spectra were acquired on a LTQ Orbitrap XL Hybrid Fourier Transform mass spectrometer from Thermo Scientific and the Thermo Scientific Accela HPLC-LTQ Ion Trap-Orbitrap Discovery system. HPLC of synthetic compounds was performed on a 1200 series instrument with a binary pump and a photodiode array detector from Agilent Technologies. All starting materials were purchased from Sigma-Aldrich apart from 5-(dimethylamino)amylamine which was provided by Matrix Scientific. Chemicals were used without further purification except for methyl iodide which was distilled and stored over Cu (s) prior to use. Electric eel AChE (6.25 U/mL) was provided by Sigma-Aldrich.

Extraction and Purification of Pulmonarins A and B

Specimens of *Synoicum pulmonaria* (Ellis and Solander, 1786) were collected off the coast of Tromsø in northern Norway and identified by Professor Bjørn Gulliksen (Department of Arctic and Marine Biology, University of Tromsø, Tromsø, Norway). The organisms are stored at the Norwegian College of Fishery Science, University of Tromsø, Norway. Specimens (80 g, wet weight) of the organism were pooled, lyophilised and extracted with 10 volumes (v/w) of 60/40 CH₃CN/H₂O containing 0.1% TFA, at 4 °C. The supernatant was removed after 24 h and the extraction procedure was repeated. The combined supernatants were then placed in a -20 °C freezer for 2 h, resulting in phase separation between a CH₃CN rich organic phase and an aqueous phase. The aqueous phase was loaded on a C₁₈ solid phase extraction (SPE) cartridge

and eluted with 10, 40 and 80% CH₃CN. The 40% CH₃CN SPE eluate was subsequently loaded on a semi-prep HPLC (column; Waters, Sunfire C18, 250 × 10 mm) with photodiode array (PDA) detection. Compounds **1** (3.0 mg) and **2** (3.9 mg) were eluted after 41.9 min and 38.4, min respectively, using a gradient of 0% to 40% CH₃CN/0.05% TFA in H₂O.

Pulmonarin A (1). Yellow gel; UV (MeOH) λ_{\max} (log ϵ) 221 nm (4.4); IR ν_{\max} 3036, 1684, 1474, 1200, 1123 cm⁻¹; ¹H and ¹³C NMR data, see Table 1. HRESIMS m/z 393.9650 [M]⁺ (calcd for C₁₃H₁₈⁷⁹Br₂O₃N, 393.9654).

Pulmonarin B (2). Yellow gel. UV (MeOH) λ_{\max} (log ϵ) 220 nm (4.5); IR ν_{\max} 3036, 1688, 1473, 1200, 1125 cm⁻¹; ¹H and ¹³C NMR data, see Table 1. HREIMS m/z 449.0439 for [M]⁺ (calcd for C₁₇H₂₇O₂N₂Br₂, 449.0440).

Synthesis

2-((3,5-dibromo-4-methoxybenzoyl)oxy)-*N,N,N*-trimethylethanaminium chloride (1)

DCC (1.12 g, 3 equiv., 5.40 mmol), 1.68 g of 3,5-dibromo-4-methoxybenzoic acid (1.5 equiv., 5.40 mmol) and 0.16 g of DMAP (0.2 equiv., 1.28 mmol) were added to 2-hydroxy-*N,N,N*-trimethylethanaminium chloride (0.67 g, 4.80 mmol) in 10 mL of CH₂Cl₂. The mixture was stirred at room temperature (rt) for 6 days. A white solid was obtained which was extracted with H₂O (3 × 20 mL), filtered and dried. An aliquot of the dried filtrate (713 mg) was dissolved in 5% CH₃CN to a concentration of 25 mg/mL and purified by HPLC using a gradient of 5-100% CH₃CN over 45 min, employing a Waters Sunfire Prep C-18 column (10 μ m, 90 Å, 10 × 250

mm) until 70 mg of the sample was collected for biological activity screening. The isolated compound (4% yield) was a yellow viscous gel. IR ν_{\max} 3036, 1684, 1474, 1200, 1123 cm^{-1} ; ^1H NMR and ^{13}C NMR, see table S3 in Supporting information. HRESIMS m/z 393.9650 $[\text{M}]^+$ (calcd for $\text{C}_{13}\text{H}_{18}^{79}\text{Br}_2\text{O}_3\text{N}$, 393.9654).

3,5-(dibromo-4-methoxyphenyl)acetic acid (2a)

2a was prepared in 21% yield according to the method described by Weller et al.²⁴ Spectral data matched those previously reported.

2-(3,5-dibromo-4-methoxyphenyl)-*N*-(5-(dimethylamino)pentyl)acetamide (2b)

0.25 mL 5-(dimethylamino)amylamine (1.61 mmol, 1.05 equiv.) was added to a stirred solution of **2a** (494.0 mg, 1.53 mmol) and HBTU (580 mg, 1.53 mmol, 1.0 equiv.) in 5 mL DMF and 1.06 mL DIPEA (6.12 mmol, 4 equiv.). The reaction mixture was stirred overnight at rt before being diluted with 20 mL EtOAc, washed with 2×25 mL 10% citric acid, 25 mL 10% NaHCO_3 and 25 mL sat. brine. The EtOAc extract was dried over Na_2SO_4 , filtered and concentrated *in vacuo* to yield a yellow oil. No further purification was performed. ^1H NMR (400 MHz, CDCl_3): δ = 7.36 (s, 2H), 3.79 (s, 3H), 3.34 (s, 2H), 3.14 (q, J = 6.0 Hz, 2H), 2.28 (m, 2H), 2.24 (s, 6H), 1.47 (m, 2H), 1.40 (m, 2H), 1.24 (m, 2H) ppm; ^{13}C NMR (100 MHz, CDCl_3): δ = 170.1 (C), 153.0 (C), 134.2 (C), 133.5 (CH), 118.2 (C), 60.7 (CH_3), 59.1 (CH_2), 45.1 (CH_3), 41.8 (CH_2), 39.7 (CH_2), 29.1 (CH_2), 26.8 (CH_2), 24.3 (CH_2) ppm. HRESIMS m/z 435.0279 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{16}\text{H}_{25}^{79}\text{Br}_2\text{O}_2\text{N}_2$, 435.0283).

5-(2-(3,5-dibromo-4-methoxyphenyl)acetamido)-*N,N,N*-trimethylpentan-1-aminium iodide
(2)

380 mg of **2b** (0.87 mmol) was dissolved in 5 mL CH₂Cl₂ and 5 mL 1 M K₂CO₃ solution was added. The resulting mixture was stirred at room temperature for 15 min before 0.41 mL of methyl iodide (8.70 mmol, 10 equiv.) was added and the reaction stirred overnight. The pH of the reaction was adjusted to 7 with 2 M HCl. All volatiles were evaporated and the crude solid material (537 mg) was dissolved in CH₂Cl₂. Silica was added and the crude material was adsorbed onto the silica under vacuum. Silica column purification was performed using a mixture of HOAc:H₂O:MeOH:EtOAc (3:2:3:3) and EtOAc 1:9 as eluent. A final purification of the isolated 237 mg of **2** with semi-preparative reversed-phase HPLC was necessary. The isolated **2** was dissolved in 5% CH₃CN to a concentration of 25 mg/mL and HPLC purification was performed employing a Waters Sunfire Prep C-18 column (10 μm, 90 Å, 10 × 250 mm) using a gradient of 16-40% CH₃CN in H₂O over 40 min until 50 mg (13% yield) of **2** was collected. IR ν_{\max} 3019, 2400, 1600, 1422, 1218 cm⁻¹; UV (MeOH) λ_{\max} 220 nm; ¹H NMR and ¹³C NMR, see table S3 in Supporting information. HRESIMS m/z 449.0439 [M]⁺ (calcd for C₁₇H₂₇⁷⁹Br₂O₂N₂, 449.0440).

Acetylcholinesterase Inhibition Assay

AChE activity was measured by Ellman's method^{29, 30} using acetylthiocholine chloride (0.25, 0.5 or 1 mM) as a substrate in 100 mM potassium phosphate buffer pH 7.4 at 25 °C, and electric eel AChE as a source of enzyme (final concentration in the test 0.0075 U/mL). Hydrolysis of acetylthiocholine chloride was followed on a Kinetic Microplate Reader (Dynex Technologies)

at 405 nm. Stock solutions (2 mg/mL) of **1** and **2** were prepared in deionised H₂O and DMSO, respectively. AChE inhibition by **1** and **2**, progressively diluted in H₂O, was monitored for 5 min. All readings were corrected for their appropriate blanks and a run with only acetylthiocholine chloride served as assay positive control. In the case of **2**, the blank reactions without the inhibitor were run in the presence of the appropriate dilution of DMSO. Every measurement was repeated at least three times.

Antibacterial Assay

Test strains used were *Staphylococcus aureus* (ATTC 9144), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATTC 27853), and *Corynebacterium glutamicum* (ATTC 13032). All isolates were grown at 37 °C in Mueller Hinton Broth (MHB; Difco Laboratories). Bacterial growth was continuously monitored with an Envision plate reader (Perkin Elmer). The test was performed in 96-well Nun microtitre plates, in which 50 µL of test fractions dissolved in H₂O were incubated with 50 µL of a suspension of an actively growing (log phase) culture of bacteria diluted to a starting concentration of approximately 5×10^5 cells per well. The antimicrobial peptide, cecropin B (25 µM), was used as a positive control. The minimum inhibitory concentration (MIC) was defined as the minimum concentration resulting in no change in optical density after incubation for 24 h at 37 °C. Compounds were tested at concentrations ranging from 8-500 µg/mL.

ASSOCIATED CONTENT

Supporting Information. 1D and 2D NMR and HREIMS data of both natural and synthetic **1** and **2**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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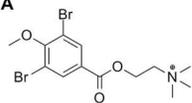
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TOC Graphic



Pulmonarin A



Pulmonarin B

