Improved Isolation Procedure for Azaspiracids from Shellfish, Structural Elucidation of Azaspiracid-6 and Stability studies

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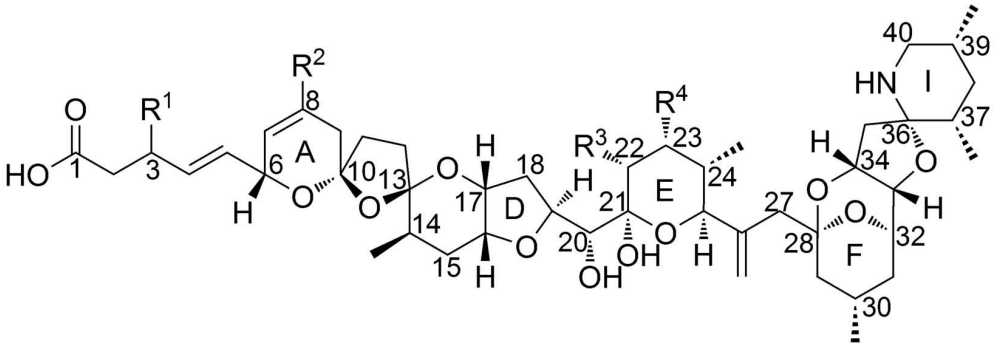

	R^1	R^2	R^3	R^4	$[M+H]^+$ *m/z*
Azaspiracid-1	H	H	CH_3	H	842.5
Azaspiracid-2	H	CH_3	CH_3	H	856.5
Azaspiracid-3	H	H	H	H	828.5
Azaspiracid-4	OH	H	H	H	844.5
Azaspiracid-5	H	H	H	OH	844.5
Azaspiracid-6	H	CH_3	H	H	842.5
Azaspiracid-7	OH	H	CH_3	H	858.5
Azaspiracid-8	H	H	CH_3	OH	858.5
Azaspiracid-9	OH	CH_3	H	H	858.5
Azaspiracid-10	H	CH_3	H	OH	858.5
Azaspiracid-11	OH	CH_3	CH_3	H	872.5
Azaspiracid-12	H	CH_3	CH_3	OH	872.5
Azaspiracid-13	OH	H	H	OH	860.5
Azaspiracid-14	OH	H	CH_3	OH	874.5
Azaspiracid-15	OH	CH_3	H	OH	874.5
Azaspiracid-16	OH	CH_3	CH_3	OH	888.5
Azaspiracid-17	H	H	CO_2H	H	872.5
Azaspiracid-19	H	CH_3	CO_2H	H	886.5
Azaspiracid-21	OH	H	CO_2H	H	888.5
Azaspiracid-23	OH	CH_3	CO_2H	H	902.5

Figure 1: Structures of AZAs with substitution points for analogues. Note that only AZA1–6 have structures unambiguously established by NMR spectroscopy, while the remaining structures are tentative, base on MS fragmentations, biosynthetic and metabolic considerations, and analogy with known analogues.
139x167mm (300 x 300 DPI)

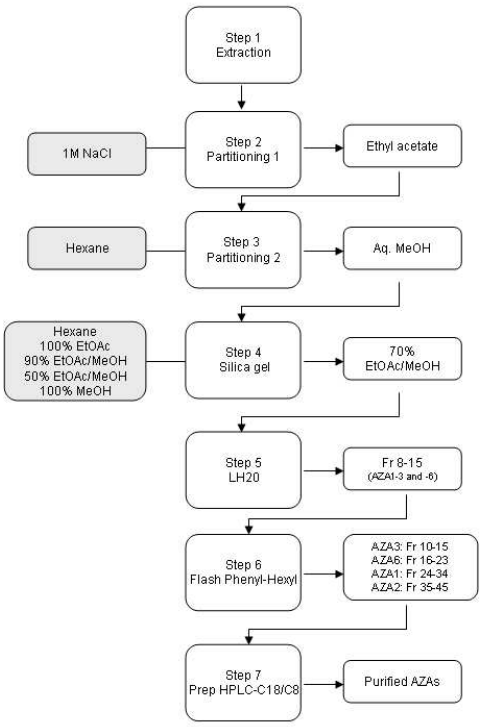

Figure 2: Schematic diagram of AZA isolation procedure.
254x190mm (96 x 96 DPI)

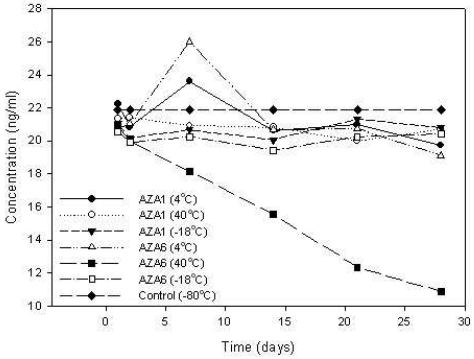

Figure 3: Short-term stability of AZA1 and AZA6 stored in methanol at −18°C, 4°C, and 40°C.
254x190mm (96 x 96 DPI)

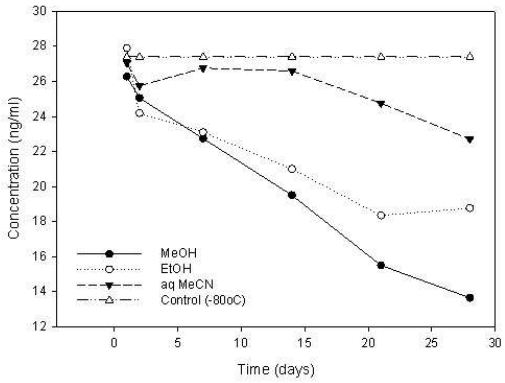

Figure 4: Short-term stability of AZA6 stored at 40°C in methanol, ethanol and 20% aqueous acetonitrile.

254x190mm (96 x 96 DPI)

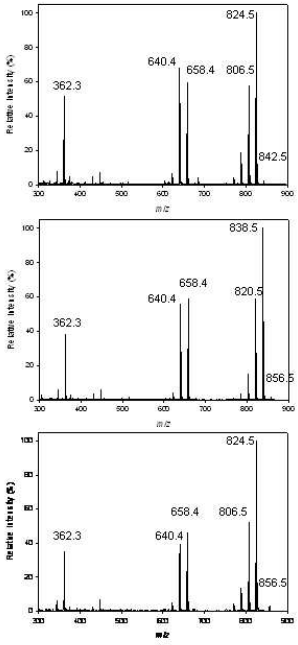

Figure 5: Mass spectra of a) AZA6, b) AZA6 methyl ester and c) AZA6 methyl ketal.
254x190mm (96 x 96 DPI)

Improved Isolation Procedure for Azaspiracids from Shellfish, Structural Elucidation of Azaspiracid-6 and Stability studies

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Abstract

Azaspiracids (AZAs) are a group of lipophilic polyether toxins produced by the small dinoflagellate *Azadinium spinosum*. They may accumulate in shellfish and can result in illnesses when consumed by humans. Research into analytical methods, chemistry, metabolism and toxicology of AZAs has been severely constrained by the scarcity of high-purity AZAs. Consequently, since their discovery in 1995, considerable efforts have been made to develop methods for isolation of AZAs in sufficient amounts and purities for toxicological studies, in addition to the preparation of reference materials. A 7-step procedure was improved for the isolation of AZA1–3, increasing recoveries two-fold compared to previous methods and leading to isolation of sufficiently purified AZA6 for structural determination by NMR spectroscopy. The procedure, which involved a series of partitioning and column chromatography steps, was performed on 500 g of *Mytilus edulis* hepatopancreas tissue containing ~ 14 mg of AZA1. Overall yields of AZA1 (52%), AZA2 (43%), AZA3 (43%) and AZA6 (38%) were good, and purities were confirmed by NMR spectroscopy. The structure of AZA6 was determined by 1- and 2-dimensional NMR spectroscopy and mass spectrometry. The stability of AZA6 relative to AZA1 was also assessed in three solvents in a short-term study that demonstrated greatest stability in aqueous acetonitrile.

KEYWORDS: azaspiracid, stability, NMR, mass spectrometry, purification.

INTRODUCTION

Azaspiracids (AZAs) were discovered after 8 people in the Netherlands became ill in 1995 after consuming mussels harvested off the west coast of Ireland (*1*). Contaminated mussels from this incident were sent to Tohoku University in Japan, where the primary causative agents (AZA1–3) were isolated and characterized (*2, 3*). The illness caused by the consumption of AZAs was named azaspiracid shellfish poisoning (AZP), and severe acute symptoms include nausea, vomiting, diarrhea, and stomach cramps (*4*). The AZA group now includes more than 20 analogues that are either produced by phytoplankton, through biotransformation in shellfish, or as by-products formed as a result of storage of the toxin (*5, 6*). However, only AZA1–3 are currently regulated by the European Union (*7*) . The other analogues had initially been found at lower concentrations and were therefore not deemed to be significant, but little is known about these additional analogues and to date only AZA1–5 have been isolated and fully characterized.

The Irish national biotoxin monitoring program was set up in 2001 and since that time the detection of AZAs in shellfish samples have resulted in significant shellfish farm closures (*8*). AZAs have since been found in other European countries, Morocco, Eastern North America, Japan and more recently Chile (*9-13*). The EU has set maximum levels of 160 μg/kg of toxins from the AZA group (defined as the sum of AZA1–3, corrected for their estimated toxic equivalence factors) for shellfish to be placed on the market (*7*). Until recently, the mouse bioassay (MBA) was the EU reference method for the detection of marine biotoxins in shellfish. However, there were problems with this method in terms of sensitivity, accuracy, false positives and ethics (*14*). Although the current regulatory limit for AZAs may be detected by both MBA or LC-MS/MS methods, the MBA is not capable of detecting lower levels and the non-specific character of the assay has prevented its effective use in routine monitoring (*15, 16*). The MBA has now been replaced with liquid

chromatography coupled to mass spectrometry (LC-MS/MS) as the reference method for the detection of lipophilic marine biotoxins in shellfish (*7*).

Considerable efforts were made to try to identify the biological source of AZAs, and in 2002 James *et al.* reported *Protoperidinium crassipes* as the causative organism (*17*). However, this species was not found to produce AZAs in culture (Tillmann and Krock, unpublished data). Furthermore, analysis of picked cells of *P. crassipes* in Norway showed no presence of AZAs (*18*). As *P. crassipes* is a heterotrophic dinoflagellate, it is possible that it might feed on AZA-producing phytoplankton. In 2007, during an oceanographic survey in the North Sea, a small (5 µm in width) photosynthetic thecate dinoflagellate was identified (subsequently named *Azadinium spinosum*) that was abundant in water samples that also contained AZAs by LC-MS/MS. *A. spinosum* was subsequently found to produce AZA1 and AZA2 in culture *(19, 20)*. It is believed that most of the other AZA analogues are produced as a result of metabolic processes in shellfish or as a result of storage (*6, 21*). This belief was corroborated by a study in which an Irish strain of *A. spinosum* was fed directly to shellfish resulting in the formation of the analogues AZA3, AZA6, AZA17 and AZA19 (*22*).

A number of toxicological studies have been performed showing AZAs to be teratogenic in fish (*23*), damaging to the gastrointestinal tract in mice (*24, 25*), and potential lung-tumor promoters (*26*). However, more detailed toxicological studies need to be performed on as many AZA analogues as is possible in order to establish more accurate regulatory limits and to identify all analogues that are relevant for public health protection. A recent study, investigating an increase in AZA3 concentration in shellfish tissue upon heating, showed that AZA3 is produced as a result of decarboxylation of AZA17 (*6*), which in turn is a metabolic product of AZA1 (*27*). The same phenomenon was observed for AZA6 (i.e. decarboxylation of AZA19, which similarly appears to be a metabolic product of AZA2) (*6*). AZA2 was found to be the predominant toxin detected in Portugal, Morocco, Japan, and in scallops in Chile (*10, 12, 28, 29*), so it would not be surprising if

71 the ratio of AZA6 to AZA3 was higher in samples from these countries than in profiles observed in
72 European shellfish.
73
74 Isolation of AZAs has been reported previously (*3, 30-33*), however in three of these studies the
75 purity was not assessed by NMR (*30-32*) and in some cases the recoveries were ambiguously
76 described. In this paper we describe the isolation of AZA1–3 and AZA6 from shellfish using a
77 modified procedure with improved recoveries and purities. This enabled the confirmation, by NMR
78 spectroscopy, of the structure for AZA6 (Figure 1) that had previously been proposed based on MS
79 fragmentation studies and analogy with AZA3. We also assess the relative stabilities of AZA1 and
80 AZA6 in three solvents.

MATERIALS AND METHODS

Chemicals. All solvents (pestican grade) were purchased from Labscan (Dublin, Ireland). Sodium chloride (99+%), triethylamine (99%), ammonium acetate (97+%), ammonium formate (reagent grade), formic acid (>98%), and silica gel (10-40 µm, type H) were purchased from Sigma Aldrich (Steinheim, Germany). Sephadex LH-20 was from GE Healthcare (Uppsala, Sweden), LiChroprep RP C8 (25-40 µm) was from Merck (Darmstadt, Germany), Luna Phenyl-Hexyl (15 µm) was from Phenomenex (Cheshire, UK), and methanol-d_3 (CD_3OH, 99.5%) was from Cambridge Isotope Laboratories (MA, USA). AZA1–3 certified reference materials (CRMs) were obtained from the NRC, Certified Reference Material Program (Halifax, NS, Canada).

Assessment of extraction and clean up efficiency from freeze dried and wet tissue. Three 10 g (W1) hepatopancreas (HP) samples were freeze-dried and extracted three times (Ultra turrax, IKA-Werke T25 at 11,000 rpm) for 1 min with ethanol (15 mL) in parallel with three wet samples. Extracts were centrifuged (3,950 x g) for 5 min and the supernatant decanted into 20 mL volumetric flasks which were brought to volume with ethanol. Prior to analysis by LC-MS/MS (Method A) the samples were filtered (Whatman, 0.2 µm, cellulose acetate filter). The clean-up efficiency ((W1-W2)/W1 x 100) was assessed by combining the relevant extracts, evaporating off the solvent *in vacuo* and determining the weight of the remaining residue (W2).

Comparison of flash chromatography stationary phases. Two stationary phases (LiChroprep RP-8 and Luna Phenyl-Hexyl) were assessed for separation, clean-up and recovery efficiencies. Each stationary phase (packed in a 19.9 × 2 cm column) was loaded with 200 µg of residue in acetonitrile–water (6:4, plus 0.1% triethylamine), which had been brought through the first 5 steps of the isolation procedure, and eluted with acetonitrile–water (3:7, plus 0.1% triethylamine) at 4 mL/min. Fractions

containing AZAs, as determined by flow injection analysis with mass spectrometry detection (FIA-MS/MS, Method C), were combined and analyzed by LC-MS/MS (Method A).

Isolation from shellfish. Cooked whole-mussel tissue (2.5 kg) from *M. edulis* collected in 2005 from Bruckless, Donegal, Ireland, was dissected to yield 500 g of HP, which was homogenized with a Waring blender and freeze-dried (final weight 130 g). The freeze-dried HP was extracted with ethanol (5 × 500 mL) using a Waring blender. The extracts were combined, evaporated *in vacuo*, and partitioned between ethyl acetate (150 mL) and aqueous NaCl (1 M, 50 mL). The ethyl acetate fraction was evaporated to dryness *in vacuo* and the oily residue was partitioned between hexane (200 mL) and methanol-water (9:1, 200 mL). The methanolic fraction was evaporated to dryness in vacuo, dissolved in ethyl acetate (20 mL), and ~ 4 g of silica gel was added. The sample was then carefully evaporated to dryness in vacuo, mixed to a fine powder and loaded onto a silica gel (55 g) column (19.5 × 5 cm). Vacuum assisted elution was performed successively with hexane, ethyl acetate, ethyl acetate–methanol (9:1), (7:3), (1:1), and methanol (300 mL of each, all containing 0.1% acetic acid except for hexane). The 7:3 ethyl acetate–methanol fraction which FIA-MS/MS (Method C) showed to contain the AZAs, was evaporated *in vacuo*, loaded in MeOH onto a Sephadex LH-20 column (150 × 1.5 cm, packed in MeOH) and eluted by gravity (~ 1 mL/min) with methanol. The first 20 min of eluate was collected separately, with 3-min fractions collected thereafter. Fractions containing AZAs (fractions 8-15), as determined by FIA-MS/MS, were combined, evaporated to dryness *in vacuo*, and the sample loaded in acetonitrile-water (6:4, plus 0.1% triethylamine) onto a column packed with Phenyl-Hexyl (19.9 × 2 cm). The sample was eluted with acetonitrile–water (3:7, plus 0.1% triethylamine) at 4 mL/min, and 5 mL fractions were collected. Appropriate fractions were combined (AZA3, fractions 10–15; AZA6, fractions 16–23; AZA1, fractions 24–34 and AZA2, fractions 35–45) based on FIA-MS/MS analysis.

Final purification of AZA1 was achieved by semi-preparative LC (Agilent 1200) with photodiode array (PDA) detection (210 nm) using a Luna C8 (5 µm, 250 × 10 mm, Phenomenex) column eluted

with acetonitrile–water (1:1, plus 2 mM ammonium acetate) at 4 mL/min. The column temperature was 30 °C. AZA2, AZA3 and AZA6 were purified using the same system as for AZA1, but with a narrower-bore column (Cosmosil C18, 5µm, 250 × 4.6 mm, Nacalai tesque) eluted with acetonitrile–water (1:1, plus 2 mM ammonium acetate) at 1 mL/min. Purified AZAs were recovered by diluting the fractions with water (to 20% acetonitrile), loading on to solid-phase extraction (SPE) cartridges (Oasis HLB, 200 mg), washing with methanol–water (1:9, 10 mL) to remove the buffer and eluting with methanol–water (9:1, 20 mL).

Purified samples were tested for phthalates (Method E) which, if present, were removed by partitioning the sample in methanol-water (4:1, 20 mL) with 20 mL of hexane. Removal of solvent by evaporation *in vacuo* afforded purified AZAs as white solids.

Mass Spectrometry. Two LC-MS/MS systems were used in positive ion mode, both of which were equipped with a z-spray ESI source.

Method A. Recoveries were determined by quantitative analysis of fractions on a Waters 2695 LC coupled to a Micromass triple-stage quadrupole (TSQ) Ultima operated in multiple reaction monitoring (MRM) mode, with the following transitions: AZA1 *m/z* 842.5→654.4 and 842.5→672.4, AZA2 856.5→654.4 and 856.5→672.4, AZA3 828.5→640.4 and 828.5→658.4, AZA6 842.5→640.5 and 842.5→658.4. The cone voltage was 60 V and the collision voltage was 40 V, the cone and desolvation gas flows were set at 100 and 800 L/h, respectively, and the source temperature was 150 °C.

Binary gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid) in a minor modification to the method of Quilliam et al. (*34*). Chromatography was performed with a Hypersil BDS C8 column (50 × 2.1 mm, 3 µm, with a 10 × 2.1 mm guard column of the same stationary phase) (Thermo Scientific). The gradient was from 30% B, to 90% B over 8 min at 0.25 mL/min, held for 5 min, then held at 100% B at 0.4 mL/min for 5 min, and returned to the initial conditions

and held for 4 min to equilibrate the system. The injection volume was 5 µL and the column and sample temperatures were 25 °C and 6 °C, respectively.

Method B. Purity was initially assessed on a Micromass time-of-flight (QTof) Ultima coupled to a Waters 2795 LC by running MS scans (*m*/*z* 100–1000) using the same chromatographic conditions as above. Identification of other contaminant AZA analogues was also determined by performing product ion scans, where the precursor ions were selected and then fragmented, for all the known AZA analogues (Table 1).

Method C. Qualitative analysis of fractions for AZAs was performed by FIA-MS/MS using a Micromass QTof Ultima coupled to a Waters 2795 LC. Samples (2 µL) were injected, using the 2795 autosampler, directly (no column) into the mass spectrometer monitoring for the precursor ions.

LC-PDA purity analysis.

Method D. A concentrated sample (~ 500 µg/mL) was injected (1 µL) onto the semi preparative system (Shimadzu 10AVp) with photodiode array (PDA) detection (210 nm) using a Cosmosil C18 column, 5 µm, 250 × 4.6 mm eluted with acetonitrile–water (1:1, plus 2 mM ammonium acetate) at 1 mL/min. The column temperature was 30 °C.

Method E. An additional method employed to detect any strongly retained compounds (e.g. phthalates) used an analytical LC system (Shimadzu LC 10AVp) with PDA detection at 210 nm. The sample collected after the SPE step was injected (5 µL) onto a Vydac C18, column (10 µm, 250 × 4.6 mm, Grace) and eluted with methanol–water (9:1) at 1 mL/min, maintaining the column temperature at 30 °C.

NMR Spectroscopy. NMR-purity was assessed by ^{1}H NMR using a Bruker DRX-500 spectrometer. The structure of AZA6 was determined by analysis of ^{1}H, COSY, TOCSY, NOESY, ROESY, HSQC and HMBC spectra using a Bruker Avance III 700 spectrometer fitted with a 1.7 mm proton-detect

184 micro-cryoprobe. Approximately 50 μg of AZA6 was dissolved in 30 μL CD_3OH, and proton-
185 detected spectra were acquired with pre-saturation of the OH peak The TOCSY spectrum was
186 recorded using an MLEV sequence with a 120 ms mixing time. The ROESY spectrum was acquired
187 with a spin-lock pulse of 200 ms and a spin-lock field of approximately 3 kHz. Two HMBC spectra
188 were recorded, optimized for long-range couplings of 8.33 Hz and 5.56 Hz (60 ms and 90 ms
189 evolution times, respectively). All samples were tuned and matched to 50 Ω resistive impedance.
190 Chemical shifts were referenced to internal C*H*D_2OH (3.31 ppm) or *C*D_3OH (49.15 ppm).
191
192 **Stability studies.** A side-fraction from the final step in the isolation procedure, containing both
193 AZA6 and AZA1, was used to assess stability. Aliquots of the fraction were evaporated under a
194 stream of N_2 and taken up in three solvents (methanol, ethanol, and 4:1 acetonitrile-water) and stored
195 in flame-sealed ampoules (under nitrogen) at −18 °C, 4 °C and 40 °C for a 4-week period. Samples
196 were ampouled in triplicate for each of the temperature and time points. The study was performed
197 isochronously, and samples were analyzed simultaneously by LC-MS/MS (Method A) with
198 specimens stored at −80 °C used as the control.
199
200 **Methylation with diazomethane**
201 To identify whether the degradation products formed during the stability study were methyl esters or
202 other methyl derivatives, AZA6 methyl-ester was synthesized. A purified sample (~ 60 ng) of AZA6
203 was added to the outside tube of an Aldrich diazomethane generator with System 45 connection, and
204 1 mL MeOH and 1.5 mL Et_2O were added. Diazomethane was generated in the inner tube of the
205 apparatus and allowed to react *in situ* with the extract. After reacting for 45 min at 0 °C with
206 occasional swirling, the extract was transferred to a glass vial, evaporated to dryness under a stream
207 of N_2, and the residue dissolved in MeOH (1 mL) for LC-MS/MS analysis (Method B).
208
209

Cleavage with sodium periodate

Aliquots (50 µL) of 0.2 M solution of sodium periodate were added to 50 µL of purified AZA6 and AZA3 (~ 80 ng/mL in methanol) in insert vials, vortex mixed for 20 s and analysed after ca. 2 hr by LC-MS/MS (Method B).

RESULTS AND DISCUSSION

Extraction and partitioning (steps 1–3). An exhaustive trial extraction was performed on 130 g of freeze-dried HP sample resulting in a 95% clean up (Table 1). Small scale tests with methanol and ethanol as extraction solvents showed that both solvents were equivalent in terms of extraction efficiency. Ethanol was chosen as the extraction solvent primarily to minimize the formation of side products, which can be significant when methanol is used as extractant (*5*), and also because it is a less toxic solvent.

Freeze-drying of shellfish prior to extraction has been successfully employed previously in the isolation of pinnatoxins from Australian oysters (*35*). This has many advantages, including avoiding the necessity of using water-miscible extraction solvents, complete control of extractant composition, and low water content in the extract (thus avoiding difficulties during evaporation and potentially toxin stability problems). The effect of freeze-drying the mussel HP prior to extraction of AZAs was therefore explored. Higher extraction efficiencies were achieved for the freeze-dried samples after the first and second extractions with 12% and 2% more AZAs being extracted respectively. No difference was observed in clean up efficiency (94.2% for both freeze-dried and wet tissues), but the extracts from the freeze-dried samples evaporated more quickly with little or no foaming in the subsequent vacuum-evaporation step. The two subsequent liquid–liquid partitioning steps resulted in only minor losses of toxin (~ 95% recovery) with an overall clean up efficiency of 67% (Table 1).

Silica gel (step 4). The sample was eluted from the silica gel column with step gradients of hexane, ethyl acetate, ethyl acetate–methanol and methanol. AZAs eluted in 7:3 ethyl acetate–methanol, with only small losses of toxin. The ethyl acetate–methanol mixtures contained 0.1% acetic acid. Previous studies have shown that AZAs are unstable in acidic environments, but that shellfish tissue appears to have a protective effect (*36*). As the sample at this stage of the isolation was still quite crude, and there appeared to be no degradation of the AZAs during small-scale trials, it was deemed to be safe

to use acetic acid in the eluent at this point of the procedure. Attempts to replace the acetic acid with 0.1% triethylamine were unsuccessful, with the toxins eluting over three of the mobile-phase compositions, thereby reducing the clean-up efficiency significantly. Of all the steps in the procedure, silica gel chromatography (step 4) gave the greatest efficiency in terms of clean up (93%) and recovery (~ 95%) (Table 1).

Sephadex LH-20 chromatography (step 5). AZAs eluted together after ca. 64 min and were collected in 11 fractions. The clean-up efficiency of 66% was achieved with a recovery of 85%.

Phenyl-Hexyl flash chromatography (step 6). Acidic mobile phases have previously been used for reverse-phase flash chromatographic purification (*33*), but bring with them an inherent risk of acid-promoted degradation of AZAs during storage or evaporation. We found the use of triethylamine to be a safer alternative, with the toxins being stable whilst stored in the freezer as a dry sample (after evaporation of the mobile phase containing 0.1% triethylamine) for at least one month (data not shown).

Both the RP-8 and Phenyl-Hexyl stationary phases performed similarly in terms of clean-up efficiency and recovery however, with respect to resolution, the Phenyl-Hexyl proved to be much more efficient at separating the AZA analogues than the RP-8 stationary phase (Table 2). Separation of the AZA analogues at this stage in the procedure improved recoveries and purities in the final semi-preparative LC step (step 7), so the Phenyl-Hexyl stationary phase was chosen as the stationary phase for flash chromatography. This step resulted in a clean-up of 64% (assessed after the RP-8 vs Phenyl-Hexyl experiment, see Materials and Methods section) and a recovery of ~ 90% (Table 1).

Prep HPLC (step 7). An acidic mobile phase was used for semi-preparative LC purification in preliminary studies, but AZAs were very unstable when evaporated to dryness from the acidic eluent

(unpublished information). Therefore, a neutral mobile phase was chosen to prevent AZA degradation. Acceptable chromatography was obtained for AZA1 and AZA2 using the neutral mobile phase, but broad, fronting peaks were observed for AZA3 and AZA6. Similar chromatography for AZA3 was also observed using alkaline conditions on an analytical scale (*37*). This is presumably related to the fact that both AZA3 and AZA6 lack a methyl group at the R^3 position (Figure 1) which somehow affects their chromatographic behavior. All fractions were collected based on UV detection at 210 nm to minimize contamination with non-AZA analytes.

Most (80%) of the AZA6 from the flash chromatography (step 6) was recovered in the AZA6 fraction, and 20% came from the AZA3 fraction. The recovery of AZA6 from the semi-preparative LC (61%) was slightly less than for the other AZA analogues (all ~ 85%), probably because co-eluting compounds necessitated significant heart cutting.

SPE recovery of AZAs from eluent. Fractions from the semi-preparative LC purification were diluted with water and recovered on SPE cartridges in order to remove any buffer remaining in the sample, but also to reduce the water content in, and volume of, the AZA fractions prior to evaporation, and as an additional final clean-up step to remove trace contaminants introduced via the LC eluents. This SPE recovery resulted in very little loss of toxin, with recoveries of >95% being achieved, and greatly facilitated evaporation of the purified AZA-fractions to dryness.

Overall recoveries. 7.3 mg of AZA1 was purified along with 1.6 mg of AZA2, 2.0 mg of AZA3 and 300 µg of AZA6. Overall recoveries (steps 1–7) were 52% for AZA1, 43% for AZA2 and -3, and 38% for AZA6, and represent a two-fold increase in recovery compared to previous isolations carried out as part of the ASTOX project (*33, 38*). Furthermore, the improved procedure is significantly easier to perform and less labor intensive.

292 **Purity testing by MS, UV and NMR**. The purity of the samples was first determined by mass
293 spectrometry. An LC-MS scan was performed in the range *m*/*z* 100–1000, followed by LC-MS/MS
294 analysis for all the known AZA analogues as well as for any additional masses picked up in the MS
295 scan (Method B). The sample was also analyzed using the LC-PDA semi-preparative method
296 (Method D) to ensure that no additional peaks were observed in the UV trace. To determine whether
297 strongly retained compounds, such as phthalates, were present in the sample, isocratic LC-PDA was
298 performed (Method E). Previous NMR analysis had shown the presence of a phthalate in some
299 fractions which was detectable by LC-PDA (λ_{max} 205, 225 and 275 nm). This contaminant was
300 conveniently removed by partitioning with hexane. Once samples were deemed to be sufficiently
301 pure (LC-MS/MS and LC-PDA), they were prepared for NMR spectroscopy. The ^{1}H NMR spectra
302 of AZA1–3 were compared to published NMR data and found to be essentially identical, and
303 examination of the spectra indicated purities of >95%.
304
305 **AZA6 structural elucidation by NMR spectroscopy.** NMR data for AZA6 have not been
306 published, and its proposed structure was based only on MS/MS fragmentation and on analogy with
307 the structure of AZA3. AZA6 was therefore subjected to a more thorough series of 1- and 2D NMR
308 experiments to verify its presumed structure.
309 NMR analysis confirmed the previously postulated structure of AZA6, a methyl group at the R^2
310 position (C-8) and a methylene at C-22 (R^3 = H), see Figure 1 and Table 4. Structural elucidation of
311 AZA-6 was done using 1- and 2-dimensional homonuclear ^{1}H and heteronuclear ^{1}H{^{13}C} NMR
312 spectroscopy to assign the ^{1}H and ^{13}C resonances, the chemical shifts of which were then compared
313 with published data for AZA-1, -2 and -3 (*2, 3*). One-dimensional ^{1}H NMR and edited HSQC spectra
314 showed that AZA6 had 6 methyl, 16 methylene, and 17 methine groups. Chemical shifts for eight
315 quaternary carbons were ascertained from HMBC correlations (2.20,2.31/181.6, C-1;
316 5.71,5.32/72.29, C-6; 1.67,1.96/130.8, C-8; 1.93,0.91/106.9, C-10; 1.93,0.91/110.9, C-13; 2.13/98.1,
317 C-21; 3.93/146.6, C-26; 2.14/97.6, C-28; and 0.83/95.6, C-36). Chemical shifts reported in Table 4

318 are from the HSQC (for ^{1}H and protonated ^{13}C atoms) and HMBC (for quaternary carbon atoms)
319 spectra.
320
321 Analysis of the COSY and TOCSY spectra led to the identification of 9 spin-systems based on
322 protons and methyl groups attached to C-2–C-7; 8-CH_3; C-9; C-11–C-12; C-14–C-20; C-22–C-25;
323 26=CH_2; C-27; C-29–C-35; and C-37–C-40. The following HMBC correlations defined the
324 connections of the spin systems: C-6 to H-7; C-7 to 8- CH_3; C-8 to 8- CH_3 and H-9a,b; C-9 to 8-
325 CH_3; C-10 to H-9; C-10 to H-11b; C-13 to H-12b; C-13 to H-14; C-13 to 14- CH_3; C-21 to H-22a,b;
326 C-25 to 26=CH_2; 26=*C*H2 to H-27b; C-26 to H-27a,b; C-28 to H-27a,b; C-38 to 37-CH_3; and C-36
327 to H-40b. Periodate treatment of AZA6 yielded the same C-20–C-21-cleavage product as was
328 obtained by treatment of AZA3, thereby establishing the presence of a 20,21-diol in AZA6 and a link
329 between the C-14–C-20 and C-22–C-25 spin-systems.
330
331 The presence of a resonance at 1.67 ppm (8-Me) was consistent with the vinylic methyl group such
332 as present in AZA2. The olefinic resonance at 5.32 ppm (H-7) showed more complex coupling than
333 could be accounted for by its original assignment as H-9. When the ^{1}H spectrum was observed with
334 resolution enhancement (Gaussian window function, LB = −2.0 Hz, GB = 0.25) the resonance at
335 5.32 ppm (8-Me) showed splitting into a multiplet ($J \approx 1.4$ Hz) implying coupling to more than 3
336 protons. In addition there was a weak COSY correlation from 5.32 ppm (H-7) to 4.70 ppm (H-6) and
337 an HMBC correlation from C-6 (72.3 ppm) to 5.32 ppm (H-7). This leads to the assignment of this
338 vinylic proton resonance (5.32 ppm) to H-7 and it defines the double bond as between C-7 and C-8
339 in AZA6, consistent with the structural revision of AZA1 by Nicolaou et al. (*39*). A detailed analysis
340 of NMR data for AZA1 and AZA2 (C. O. Miles, A. L. Wilkins, F. Rise and J. Kilcoyne,
341 unpublished) gave essentially identical results, so the original assignments (*2, 3*) for AZA1–3 for C-
342 7–C-9 and their attached protons and methyl groups are revised accordingly in Table 4.
343

Analysis of the TOCSY spectrum of AZA6 corresponding to the C-22 to C-25 spin system indicated that there was only one methyl group, and an additional methylene group, in ring-E compared to AZA1 and AZA2. This, along with COSY correlations, led to the conclusion that there is no methyl at C-22 of AZA6, analogous to AZA3.

ROESY NMR data (Supporting Information) confirmed that the relative stereochemistry of AZA6 was the same as that published for AZA1 (*39*). ROESY correlations were observed between H-30 and H-34, H-32 and H-33, and H-3 and H-34, consistent with the stereochemistry around rings F, G and H having H-32, H-33 and H-34 as equatorial, equatorial, and axial, respectively, with the 30-Me equatorial. In addition, ROESY correlations between the 37-Me and both H-33 and H-35a place the NH in ring-I on the β-face of ring-H. ROESY correlations between the 14-Me and both H-6 and H-11b support C-12 being axial to ring-C and the absence of a correlation between H-14 and H-16, were consistent with 14-Me being equatorial, and confirms the stereochemistry in this section of AZA6 as being that assigned to AZA1–3 by Nicolaou et al. (*39-41*). The ROESY correlation between H-16 and H-17, and H-16 and H18b, supports the cis-fusion of the 5-membered ring-D to ring-C. All the NMR data is thus consistent with the structure shown for AZA6 in Figure 1, as is the MS/MS fragmentation reported previously and used to propose the original tentative structure for this compound (*42*). The periodate cleavage established that AZA6 had the same structure and relative stereochemistry as for AZA3 in the C21–C-40 moiety. Furthermore, AZA6 is a metabolite produced by oxidative decarboxylation of the 22-Me group of AZA2 in shellfish (*6*), so it must have the same absolute stereochemistry as AZA2.

AZA6 stability. The stability of AZA6 was compared with that of AZA1. Figure 3 shows that AZA6 is significantly less stable ($p<0.05$, Student's t-test) than AZA1 when stored in methanol at 40 °C. These results parallel the observations of Perez et al. (*33*), who showed that AZA3 was less stable than AZA1 under these conditions and confirms the results of McCarron et al. (*43*) showing that

AZA6 exhibited similar instability to AZA3 in tissue CRM extracts. AZA6, like AZA3, but unlike AZA1 and AZA2, has no methyl group on the C-22 position. The mechanism responsible for this reduced stability is as yet unclear.

In this study the stability of AZA6 was determined in three solvents. Figure 4 shows that AZA6 is significantly more stable ($p<0.05$, Student's t-test) in 4:1 acetonitrile-water than in methanol or ethanol. The appearance of additional LC-MS/MS peaks at *m/z* 856.5 (Supplementary Information) and 870.5 after storage in methanol and ethanol, respectively, indicated that these solvents were reacting with AZA6 to produce methyl and ethyl derivatives. Methylation may occur at the C1 to produce the methyl ester or, alternatively, at C21 to produce the methyl ketal. A purified sample of AZA6 was reacted with diazomethane to produce AZA6 methyl ester. The semi-synthetic methyl ester differed from the derivative observed during the stability study in both LC-MS retention time (Supplementary Information) and fragmentation pattern. The mass spectrum of the methyl ester showed a loss of 18 amu (*m/z* 838.5) from the parent ion, while the derivative showed a loss of 32 amu (*m/z* 824.5) from the parent ion, suggesting that the AZA6 is being methylated at the C21 position to form a methyl ketal during storage in methanol (Figure 5). The methyl ester of AZA6 also shows a different retention time to that of the methyl ketal with the methyl ester being retained longer on the column. These results confirm observations in by Jauffrais et al. (submitted to Analytical and Bioanalytical Chemistry) which showed the formation of AZA1 and AZA2 methyl ketals in *A. spinosum* methanolic extracts.

In summary, a method was optimized for the isolation of AZAs from highly contaminated *M. edulis* HP. A seven-step procedure involving extraction, two partitioning, and four chromatography steps was employed. The method was adapted to limit degradation of sample by replacing acidic mobile phases with slightly basic and neutral mobile phases in two of the chromatography steps. Improved separation of the AZAs during the penultimate step (flash chromatography; step 6) was achieved by

using a Phenyl-Hexyl stationary phase, leading to a more efficient final clean up step by semi-preparative LC. Overall recoveries of ~ 40–50% were achieved for AZA1–3 and AZA6. Sufficient AZA6 was isolated for structural elucidation by NMR which confirmed the previously postulated structure (Figure 1). A short-term stability study showed that AZA6 is significantly more stable in aqueous acetonitrile than in methanol (the usual storage solvent) at 40 °C. The isolated AZAs are of sufficient purity for toxicological research and for the preparation of analytical standards.

ABBREVIATIONS USED

AZA, azaspiracid; EU, European Union; FIA, flow-injection analysis; HP, hepatopancreas; LC, liquid chromatography; MBA, mouse bioassay; MS/MS, tandem mass spectrometry.

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Supporting information available:

Structures of AZA6 showing COSY and HMBC correlations, as well as selected ROESY correlations, and a Molfile (AZA6.mol) showing the 3-dimensional structure of AZA6. LC-MS/MS chromatograms (QTof) of AZA1 and AZA6 samples in methanol after treatment with diazomethane and storage at 40 °C for 4 weeks are also shown, together with LC-MS/MS chromatograms (QTof) of AZA6 and AZA3 before and after treatment with periodate. This material is available free of charge via the Internet at http://pubs.acs.org.

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Captions for Figures

Figure 1. Structures of AZAs with substitution points for analogues. Note that only AZA1–6 have structures unambiguously established by NMR spectroscopy, while the remaining structures are tentative, based on MS fragmentations, biosynthetic and metabolic considerations, and analogy with known analogues.

Figure 2. Schematic diagram of AZA isolation procedure.

Figure 3. Short-term stability of AZA1 and AZA6 stored in methanol at −18 °C, 4 °C, and 40 °C.

Figure 4. Short-term stability of AZA6 stored at 40 °C in methanol, ethanol and 20% aqueous acetonitrile.

Figure 5. Mass spectra of a) AZA6, b) AZA6 methyl ester and c) AZA6 methyl ketal.

Table 1. Batch summary table for purification of AZA1-3 and AZA6.

Step No	Step	AZA1 (mg)	AZA2 (mg)	AZA3 (mg)	AZA6 (mg)	Weight (g)
	Subsampling (HP)	14.1	4.0	4.8	0.78	505.0
1	1st crude extract	14.0	3.9	4.7	0.77	26.9
2	1st partitioning	13.3	3.7	4.4	0.73	23.9
3	2nd partitioning	12.6	3.5	4.2	0.69	8.9
4	Silica gel	11.9	3.3	4.0	0.65	0.6
5	LH20	10.1	2.8	3.4	0.55	0.2
6	Flash (Phenyl-Hexyl)*	9.2	2.5	2.4	0.49	-
7	Prep HPLC (C8/C18)	7.3	1.7	2.0	0.30	-
	% Recovery	**52**	**43**	**43**	**38**	
	% Purity	**>95**	**>95**	**>95**	**>95**	

* AZA1-3 and AZA6 were separated from each other in this step

Table 2. Resolution efficiencies of LiChroprep RP-8 and Luna Phenyl-Hexyl stationary phases. Note: The resolution expresses the extent of separation between compounds. The higher the resolution of the chromatogram, the better separation of the analytes by the column.

Stationary phase	AZA1/2 resolution	AZA3/6 resolution	AZA1/6 resolution
LiChroprep RP-8	0.6	1.1	0.5
Phenyl-Hexyl	1.1	1.2	2.1

Table 3. Isolation optimisation experiments performed.

Step	Optimisation performed	Method improved with	Reason(s)
1st (Extraction)	MeOH vs EtOH	EtOH	- no difference in extraction recoveries - less toxic - formation of esters is limited
1st (Extraction)	Freeze-dried vs Wet	Freeze-dried	- no difference in extraction recoveries - less solvent required - less foaming - faster evaporation
4th (Silica gel)	0.1% TEA vs 0.1% HOAc	0.1% HOAc	- higher recoveries - sample matrix protects AZAs from acidic degradation
6th (Flash)	0.1% TEA vs 0.1% HOAc	0.1% TEA	- higher recoveries - sample stable
6th (Flash)	RP-8 vs Phenyl-Hexyl	Phenyl-Hexyl	- significantly improved resolution
7th (Prep HPLC)	0.1% HOAc vs 2 mM NH_4OAc	2 mM NH_4OAc	- pure AZAs more stable at neutral pH

Table 4. NMR assignments for AZA6 (in CD_3OH) and AZA1–3 (in CD_3OD)[a,b]

Atom	AZA6		AZA1[a]		AZA2[b]		AZA3[b]	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	181.6		180.3		177.8		177.8	
2	38.2	2.20, 2.20	37.4	2.31, 2.31	35.6	2.34, 2.34	35.4	2.37, 2.37
3	34.5	2.31, 2.31	30.3	2.33, 2.33	29.5	2.31, 2.31	29.4	2.33, 2.33
4	130.7	5.71	133.8	5.74	132.8	5.68	133.0	5.73
5	133.2	5.39	131.8	5.46	132.1	5.42	132.4	5.47
6	72.3	4.70	73.2	4.81	73.3	4.72	73.1	4.81
7[c]	122.7	5.32	130.1	5.65	123.6	5.32	130.0	5.63
8	130.8		124.1	5.76	132.8		124.2	5.75
8-Me	22.64	1.67			23.8	1.67		
9[c]	40.05	1.94, 2.39	36.5	2.15, 2.49	41.1	1.97, 2.42	36.5	2.13, 2.48
10	106.9		107.9		108.3		108.0	
11	32.9	1.62, 2.29	33.9	1.68, 2.33	34.0	1.65, 2.33	34.0	1.66, 2.34
12	37.1	1.93, 2.14	38.3	1.97, 2.16	38.3	1.96, 2.16	38.3	1.96, 2.15
13	110.9		112.1		112.1		112.1	
14	30.5	1.98	31.7	2.02	31.7	2.00	31.7	2.02
14-Me	16.3	0.91	17.4	0.94	17.4	0.93	17.3	0.95
15	32.3	1.71, 1.79	33.4	1.77, 1.85	33.4	1.73, 1.83	33.4	1.75, 1.84
16	77.5	3.89	79.1	3.89	79	3.87	79.0	3.91
17	72.7	4.13	74.2	4.25	74.2	4.20	74.0	4.23
18	38.1	1.98, 2.04	37.8	2.00, 2.01	37.7	1.98, 1.98	38.2	1.98, 1.98
19	79.3	4.39	79.9	4.44	79.9	4.42	80.3	4.43
20	80.0	3.26	77.6	3.94	77.6	3.93	80.6	3.63

21	98.1		101.1		101.0		98.7	
22	39.0	2.13, 2.13	37.6	2.09	37.6	2.07	33.4	1.55, 2.07
22-Me			17.2	0.91	17.2	0.89		
23	29.0	1.56, 1.56	38.9	1.44, 1.44	39.0	1.43, 1.43	30.1	1.61, 1.61
24	39.0	1.30	43.1	1.35	43.1	1.33	42.3	1.28
24-Me	17.8	0.79	18.8	0.84	18.9	0.83	18.9	0.86
25	80.1	3.93	80.4	4.00	80.4	3.97	80.7	4.08
26	146.6		149.1		149.1		149.2	
26=CH_2	115.5	5.10, 5.19	117.2	5.18, 5.36	118.1	5.17, 5.35	118	5.18, 5.35
27	48.1	2.14, 2.33	50.4	2.26, 2.43	50.1	2.24, 2.42	50.2	2.26, 2.43
28	97.6		99.5		99.5		99.5	
29	43.9	1.30, 1.96	44.9	1.37, 2.05	44.9	1.36, 2.03	44.9	1.37, 2.05
30	26.3	2.23	27.2	2.23	27.2	2.22	27.2	2.24
30-Me	23.5	0.90	24.3	0.96	24.1	0.93	24.3	0.96
31	35.4	1.45, 1.75	36.1	1.54, 1.84	36.1	1.51, 1.82	36.1	1.53, 1.83
32	72.3	4.21	73.6	4.38	73.6	4.35	73.6	4.37
33	78.9	3.68	82.3	4.08	82.4	4.06	82.4	4.07
34	75.3	4.76	75.6	5.02	75.6	5.00	75.6	5.03
35	42.8	1.86, 2.36	42.5	2.50, 2.64	42.4	2.49, 2.62	42.3	2.54, 2.64
36	95.6		97.4		97.4		97.4	
37	31.5	1.63	36.4	1.99	36.5	1.97	36.5	1.99
37-Me	15.8	0.83	16.2	0.98	16.2	0.97	16.2	0.98
38	39.8	1.10, 1.51	38.4	1.31, 1.70	38.4	1.29, 1.68	38.3	1.31, 1.68
39	37.6	1.71	30.2	1.89	30.2	1.86	30.1	1.90
39-Me	19.2	0.82	19.3	0.95	19.3	0.94	19.3	0.95

40	47.5	2.46	46.9	2.84, 2.91	46.9	2.83, 2.91	46.9	2.84, 2.92

[a]Data from Satake et al.(*3*)

[b]Data from Ofuji et al.(*2*)

[c]Published assignments for positions 7 and 9 of AZA1–3 (*2, 3*) are interchanged as a consequence of the revised position of the olefin in ring-A (*39*)