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

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Supporting Information

ABSTRACT: Azaspiracids 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 azaspiracids has been severely constrained by the scarcity of high-purity azaspiracids. Consequently, since their discovery in 1995, considerable efforts have been made to develop methods for the isolation of azaspiracids in sufficient amounts and purities for toxicological studies, in addition to the preparation of standard reference materials. A seven-step procedure was improved for the isolation of azaspiracids-1−3 (**1**, **2**, and **3**) increasing recoveries 2-fold as compared to previous methods and leading to isolation of sufficiently purified azaspiracid-6 (**6**) 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 **1**. Overall yields of **1** (52%), **2** (43%), **3** (43%), and **6** (38%) were good, and purities were confirmed by NMR spectroscopy. The structure of **6** was determined by one- and two-dimensional NMR spectroscopy and mass spectrometry. The stability of **6** relative to **1** was also assessed in three solvents in a short-term study that demonstrated the greatest stability in aqueous acetonitrile.

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

INTRODUCTION

Azaspiracids were discovered after eight 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 **1**, **2**, and **3** were isolated and characterized.[2,3] The illness caused by the consumption of azaspiracids was named azaspiracid shellfish poisoning (AZP), and severe acute symptoms include nausea, vomiting, diarrhea, and stomach cramps.[4] The azaspiracid group now includes more than 20 analogues that are either produced by phytoplankton, through biotransformation in shellfish, or as a byproduct formed as a result of storage of the toxin.[5,6] However, only **1**−**3** are currently regulated by the European Union (EU).[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 **1**−**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 azaspiracids in shellfish samples has 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 azaspiracid group (defined as the sum of **1**−**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 liquid chromatography coupled to mass spectrometry (LC-MS/MS) methods, the MBA is not capable of detecting lower levels, and the nonspecific character of the assay has prevented its effective use in routine monitoring.[15,16] The MBA has now been replaced with 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 azaspiracids, and in 2002, James et al.[17] reported *Protoperidinium crassipes* as the causative organism. However, this species was not found to produce azaspiracids in culture (Tillmann and Krock, unpublished data). Furthermore, analysis of picked cells of *P. crassipes* in Norway showed no presence of azaspiracids.[18] As *P. crassipes* is a heterotrophic dinoflagellate, it is possible that it might feed on azaspiracid-producing phytoplankton. In 2007, during an oceanographic survey in the North Sea, a small (5 μm in width)

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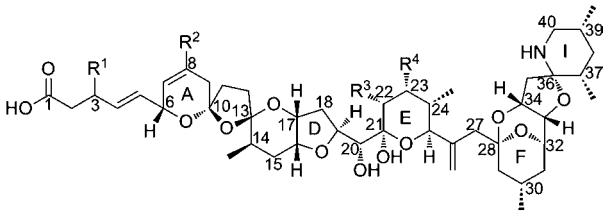
photosynthetic thecate dinoflagellate was identified (subsequently named *Azadinium spinosum*) that was abundant in water samples that were also shown by LC-MS/MS to contain azaspiracids. *A. spinosum* was subsequently found to produce **1** and **2** in culture.[19,20] It is believed that most of the other azaspiracid 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 **3**, **6**, **17**, and **19**.[22]

A number of toxicological studies have been performed showing azaspiracids to be teratogenic to 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 azaspiracid analogues as is possible 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 **3** concentration in shellfish tissue upon heating, showed that **3** is produced as a result of decarboxylation of **17**,[6] which in turn is a metabolic product of **1**.[27] The same phenomenon was observed for **6** (i.e., decarboxylation of **19**, which similarly appears to be a metabolic product of **2**).[6] Azaspiracid-2 (**2**) was found to be the predominant toxin detected in Portugal, Morocco, and Japan and in scallops in Chile,[10,12,28,29] so it would not be surprising if the ratio of **6** to **3** was higher in samples from these countries than in profiles observed in European shellfish.

The isolation of azaspiracids has been reported previously;[3,30−33] however, in three of these studies, the purity was not assessed by NMR.[30−32] In this study, we investigated the isolation of **1**−**3** and **6** from shellfish using a modified procedure with improved recoveries and purities. This enabled the confirmation, by NMR spectroscopy, of the structure for **6** (Figure 1) that had previously been proposed based on MS fragmentation studies and analogy with **3**. We also assessed the relative stabilities of **1** and **6** in three solvents.



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

Figure 1. Structures of azaspiracids with substitution points for analogues. Note that only **1**−**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.

MATERIALS AND METHODS

Chemicals. All solvents were purchased from Labscan (Dublin, Ireland). Sodium chloride, triethylamine, ammonium acetate, ammonium formate, formic acid, and silica gel were purchased from Sigma Aldrich (Steinheim, Germany). Sephadex LH-20 was from GE Healthcare (Uppsala, Sweden), and LiChroprep RP C8 was from Merck (Darmstadt, Germany). Luna phenyl-hexyl was from Phenomenex (Cheshire, United Kingdom), and methanol-d_3 (CD_3OH) was from Cambridge Isotope Laboratories (MA). Azaspiracids-1−3 (**1**, **2**, and **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 samples were freeze-dried and extracted three times (Ultra turrax, IKA-Werke T25 at 11000 rpm) for 1 min with ethanol (15 mL) in parallel with three wet samples. Extracts were centrifuged (3950 *g*) for 5 min, and the supernatant was 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 cleanup efficiency [(W1 − W2)/W1 × 100] was assessed by combining the relevant extracts, evaporating off the solvent *in vacuo*, and determining the weight of the remaining residue (W2).

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 hepatopancreas, which was homogenized with a Waring blender and freeze-dried (final weight, 130 g). The freeze-dried hepatopancreas 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* and dissolved in ethyl acetate (20 mL), and ∼4 g of silica gel (10−40 μm, type H) 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 cm × 5 cm). Vacuum-assisted elution was performed successively with hexane, ethyl acetate, ethyl acetate−methanol (9:1, 7:3, and 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 flow-injection analysis (FIA)-MS/MS (method C) showed to contain the azaspiracids, was evaporated *in vacuo*, loaded in MeOH onto a Sephadex LH-20 column (150 cm × 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 azaspiracids (fractions 8−15), as determined by FIA-MS/MS, were combined and evaporated to dryness *in vacuo*, and the sample was loaded in acetonitrile−water (6:4, plus 0.1% triethylamine) onto a column packed with phenyl-hexyl (19.9 cm × 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 (**3**, fractions 10−15; **6**, fractions 16−23; **1**, fractions 24−34; and **2**, fractions 35−45) based on FIA-MS/MS analysis.

Final purification of **1** was achieved by semipreparative LC (Agilent 1200) with photodiode array (PDA) detection (210 nm). The column used was a 250 mm × 10 mm i.d., 5 μm, Luna C8 (Phenomenex) eluted with acetonitrile−water (1:1, plus 2 mM ammonium acetate) at 4 mL/min. The column temperature was 30 °C. Azaspiracid-2 (**2**), **3**, and **6** were purified using the similar conditions as for **1** but with a narrower-bore column; 250 mm × 4.6 mm i.d., 5 μm, Cosmosil C18 (Nacalai tesque) eluted with acetonitrile−water (1:1, plus 2 mM ammonium acetate) at 1 mL/min. Purified azaspiracids were recovered

Table 1. Batch Summary Table for Purification of 1−3 and 6

		mg				
step no.	step	1	2	3	6	weight (g)
	subsampling	14.1	4.0	4.8	0.78	505.0
1	first crude extract	14.0	3.9	4.7	0.77	26.9
2	first partitioning	13.3	3.7	4.4	0.73	23.9
3	second 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)[a]	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	

[a]Compounds **1**−**3** and **6** were separated from each other in this step.

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 azaspiracids as white solids.

Comparison of Flash Chromatography Stationary Phases. Two stationary phases (LiChroprep RP-8, 25−40 μm and Luna phenyl-hexyl, 15 μm) were assessed for separation, cleanup, and recovery efficiencies. Each stationary phase (packed in a 19.9 cm × 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 five steps of the isolation procedure and eluted with acetonitrile−water (3:7, plus 0.1% triethylamine) at 4 mL/min. Fractions containing azaspiracids, as determined by FIA-MS/MS (method C), were combined and analyzed by LC-MS/MS (method A).

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: **1**, *m*/*z* 842.5 → 654.4 and 842.5 → 672.4; **2**, 856.5 → 654.4 and 856.5 → 672.4; **3**, 828.5 → 640.4 and 828.5 → 658.4; and **6**, 842.5 → 640.5 and 842.5 → 658.4. The cone voltage was 60 V, 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 consisting 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] The column used was a 50 mm × 2.1 mm i.d., 3 μm, Hypersil BDS C8 with a 10 mm × 2.1 mm guard column of the same material (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 and 6 °C, respectively.

Method B. The 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 azaspiracid analogues was also determined by performing product ion scans, where the precursor ions were selected and then fragmented, for all of the known azaspiracid analogues (Table 1).

Method C. Qualitative analysis of fractions for azaspiracids 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 semipreparative system (Shimadzu 10AVp) with photodiode array (PDA) detection (210 nm). The column used was a 250 mm × 4.6 mm i.d., 5 μm, Cosmosil C18 (Nacalai tesque) 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). The column used was a 250 mm × 4.6 mm i.d., 10 μm, Vydac C18 (Grace) 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 **6** was determined by analysis of ^{1}H, correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), rotating frame Overhauser effect spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra using a Bruker Avance III 700 spectrometer fitted with a 1.7 mm proton-detect microcryoprobe. Approximately 50 μg of **6** was dissolved in 30 μL of CD_3OH, and proton-detected spectra were acquired with presaturation of the OH peak. The TOCSY spectrum was recorded using an MLEV sequence with a 120 ms mixing time. The ROESY spectrum was acquired with a spin-lock pulse of 200 ms and a spin-lock field of approximately 3 kHz. Two HMBC spectra were recorded and optimized for long-range couplings of 8.33 and 5.56 Hz (60 and 90 ms evolution times, respectively). All samples were tuned and matched to 50 Ω resistive impedance. Chemical shifts were referenced to internal CHD_2OH (3.31 ppm) or CD_3OH (49.15 ppm).

Stability Studies. A side fraction from the final step in the isolation procedure, containing both **6** and **1**, was used to assess stability. Aliquots of the fraction were evaporated under a stream of N_2 and taken up in three solvents (methanol, ethanol, and 4:1 acetonitrile−water) and stored in flame-sealed ampoules (under nitrogen) at −18, 4, and 40 °C for a 4 week period. Samples were ampouled in triplicate for each of the temperature and time points. The study was performed isochronously, and samples were analyzed by LC-MS/MS (method A) with specimens stored at −80 °C used as the control.

Methylation with Diazomethane. To identify whether the degradation products formed during the stability study were methyl esters or other methyl derivatives, **6** methyl-ester was synthesized. A purified sample (∼60 ng) of **6** was added to the outside tube of an Aldrich diazomethane generator with System 45 connection, and 1 mL of MeOH and 1.5 mL of Et_2O were added. Diazomethane was generated in the inner tube of the apparatus and allowed to react in situ with the extract. After it was reacted for 45 min at 0 °C with

occasional swirling, the extract was transferred to a glass vial and evaporated to dryness under a stream of N_2, and the residue was dissolved in MeOH (1 mL) for LC-MS/MS analysis (method B).

Cleavage with Sodium Periodate. Aliquots (50 μL) of 0.2 M solution of sodium periodate were added to 50 μL of purified **6** and **3** (~80 ng/mL in methanol) in insert vials, vortex mixed for 20 s, and analyzed after ca. 2 h 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 hepatopancreas sample resulting in a 95% clean up (Table 1). The use of ethanol as an extraction solvent for the purification of azaspiracids has previously been reported.[33] 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 an extractant.[5]

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 hepatopancreas prior to extraction of azaspiracids was therefore explored. Higher extraction efficiencies were achieved for the freeze-dried samples after the first and second extractions with 12 and 2% more azaspiracids 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. Azaspiracids 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 azaspiracids 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 azaspiracids 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 cleanup efficiency significantly. Of all of 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). Azaspiracids eluted together after ca. 64 min and were collected in 11 fractions. The cleanup 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 azaspiracids during storage or evaporation. We found the use of triethylamine to be a safer alternative, with the toxins being stable while stored in the freezer as a dry sample (after evaporation of the mobile phase containing 0.1% triethylamine) for at least 1 month (data not shown).

Both the RP-8 and the phenyl-hexyl stationary phases performed similarly in terms of cleanup efficiency and recovery; however, with respect to resolution, the phenyl-hexyl proved to be much more efficient at separating the azaspiracid analogues than the RP-8 stationary phase. Separation of the azaspiracid analogues at this stage in the procedure improved recoveries and purities in the final semipreparative LC step (step 7), so the phenyl-hexyl stationary phase was chosen as the stationary phase for flash chromatography. This step resulted in a cleanup of 64% (assessed after the RP-8 vs phenyl-hexyl experiment) and a recovery of ~90% (Table 1).

Prep HPLC (Step 7). An acidic mobile phase was used for semipreparative LC purification in preliminary studies, but azaspiracids were very unstable when evaporated to dryness from the acidic eluent (unpublished information), confirming the results of Alfonso et al.[36] Therefore, a neutral mobile phase was chosen to prevent azaspiracid degradation. Acceptable chromatography was obtained for **1** and **2** using the neutral mobile phase, but broad, fronting peaks were observed for **3** and **6**. Similar chromatography for **3** was also observed using alkaline conditions on an analytical scale.[37] This is presumably related to the fact that both **3** and **6** 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-azaspiracid analytes.

Most (80%) of the **6** from the flash chromatography (step 6) was recovered in the **6** fraction, and 20% came from the **3** fraction. The recovery of **6** from the semipreparative LC (61%) was slightly less than for the other azaspiracid analogues (all ~85%), probably because co-eluting compounds necessitated significant heart cutting.

SPE Recovery of Azaspiracids from Eluent. Fractions from the semipreparative LC purification were diluted with water and recovered on SPE cartridges to remove any buffer remaining in the sample but also to reduce the water content in, and volume of, the azaspiracid fractions prior to evaporation and as an additional final cleanup 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 azaspiracid fractions to dryness.

Overall Recoveries. A 7.3 mg amount of **1** was purified along with 1.6 mg of **2**, 2.0 mg of **3**, and 300 μg of **6**. Overall recoveries (steps 1−7) were 52% for **1**, 43% for **2** and **3**, and 38% for **6** and represent a 2-fold increase in recovery as 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.

Purity Testing by MS, UV, and NMR. The purity of the samples was first determined by mass spectrometry. A LC-MS scan was performed in the range m/z 100−1000, followed by LC-MS/MS analysis for all of the known azaspiracid analogues as well as for any additional masses picked up in the MS scan (method B). The sample was also analyzed using the LC-PDA semipreparative method (method D) to ensure that no additional peaks were observed in the UV trace. To determine

Table 2. NMR Assignments for 6 (in CD_3OH) and 1−3 (in CD_3OD)[a,b]

	6		1[a]		2[b]		3[b]	
atom	^{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	37.6	1.71	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	31.5	1.63	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 **1**−**3**[2,3] are interchanged as a consequence of the revised position of the olefin in ring A.[39]

whether strongly retained compounds, such as phthalates, were present in the sample, isocratic LC-PDA was performed (method E). Previous NMR analysis had shown the presence of a phthalate in some fractions, which was detectable by LC-PDA (λ_{max} 205, 225, and 275 nm). This contaminant was conveniently removed by partitioning with hexane. Once samples were deemed to be sufficiently pure (LC-MS/MS and LC-PDA), they were prepared for NMR spectroscopy. The ^{1}H NMR spectra of **1**−**3** were compared to published NMR data and found to be essentially identical, and examination of the spectra indicated purities of >95%.

Azaspiracid-6 Structural Elucidation by NMR Spectroscopy. NMR data for **6** have not been published, and its proposed structure was based only on MS/MS fragmentation and on analogy with the structure of **3**. Azaspiracid-6 (**6**) was

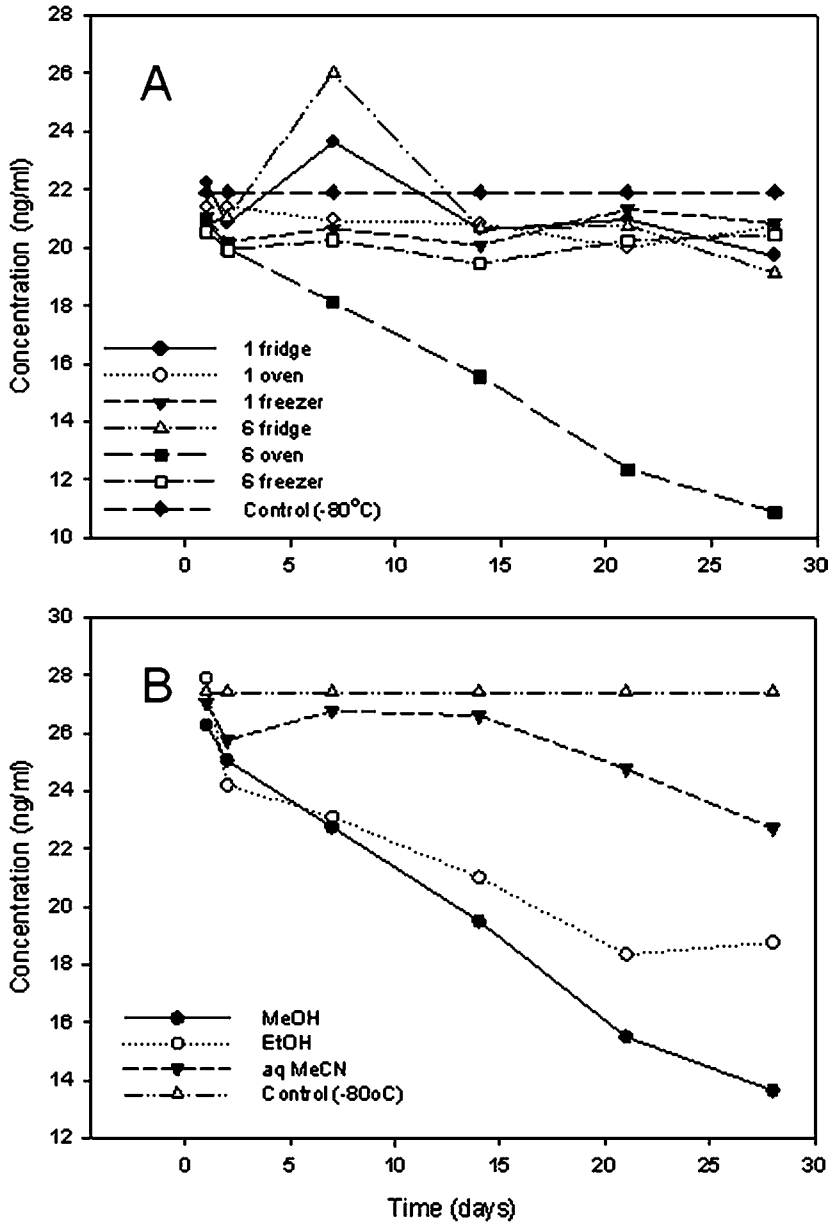


Figure 2. (A) Stability of **1** and **6** stored in methanol at −18, 4, and 40 °C and (B) stability of **6** stored at 40 °C in methanol, ethanol, and 20% aqueous acetonitrile.

therefore subjected to a more thorough series of 1D and 2D NMR experiments to verify its presumed structure.

NMR analysis confirmed the previously postulated structure of **6**, a methyl group at the R^2 position (C-8) and a methylene at C-22 (R^3 = H) (Figure 1 and Table 2). Structural elucidation of **6** was done using 1D and 2D homonuclear ^{1}H and heteronuclear ^{1}H{^{13}C} NMR spectroscopy to assign the ^{1}H and ^{13}C resonances, the chemical shifts of which were then compared with published data for **1**−**3**.[2,3] One-dimensional ^{1}H NMR and edited HSQC spectra showed that **6** had 6 methyl, 16 methylene, and 17 methine groups. Chemical shifts for eight quaternary carbons were ascertained from HMBC correlations (2.20, 2.31/181.6, C-1; 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, 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 2 are from the HSQC (for ^{1}H and protonated ^{13}C atoms) and HMBC (for quaternary carbon atoms) spectra.

Analysis of the COSY and TOCSY spectra led to the identification of nine spin systems based on 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, 26-CH_2, C-27, C-29−C-35, and C-37−C-40. The following HMBC correlations defined the 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-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; C-25 to 26-CH_2; 26-CH_2 to H-27b; C-26 to H-27a,b; C-28 to H-27a,b; C-38 to 37-CH_3; and C-36 to H-40b. Periodate treatment of **6** yielded the same C-20−C-21-cleavage product as was obtained by treatment of **3**, thereby establishing the presence of a 20,21-diol in **6** and a link between the C-14−C-20 and the C-22−C-25 spin systems.

The presence of a resonance at 1.67 ppm (8-Me) was consistent with the vinylic methyl group such as present in **2**. The olefinic resonance at 5.32 ppm (H-7) showed more complex coupling than could be accounted for by its original

assignment as H-9. When the ^{1}H spectrum was observed with resolution enhancement (Gaussian window function, LB = −2.0 Hz, GB = 0.25) the resonance at 5.32 ppm (8-Me) showed splitting into a multiplet ($J \approx 1.4$ Hz), implying coupling to more than three protons. In addition, there was a weak COSY correlation from 5.32 (H-7) to 4.70 ppm (H-6) and an HMBC correlation from C-6 (72.3 ppm) to 5.32 ppm (H-7). This leads to the assignment of this vinylic proton resonance (5.32 ppm) to H-7, and it defines the double bond as between C-7 and C-8 in **6**, consistent with the structural revision of **1** by Nicolaou et al.[39] A detailed analysis of NMR data for **1** and **2** (unpublished results) gave essentially identical results, so the original assignments[2,3] for **1**−**3** for C-7−C-9 and their attached protons and methyl groups are revised accordingly (Table 2).

Analysis of the TOCSY spectrum of **6** 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 as compared to **1** and **2**. This, along with COSY correlations, led to the conclusion that there is no methyl at C-22 of **6**, analogous to **3**.

ROESY NMR data confirmed that the relative stereochemistry of **6** was the same as that published for **1**.[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 was consistent with 14-Me being equatorial and confirms the stereochemistry in this section of **6** as being that assigned to **1**−**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 five-membered ring D to ring C. All of the NMR data are thus consistent with the structure shown for **6** 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 **6** had the same structure and relative stereochemistry as for **3** in the C-21−C-40 moiety. Furthermore, **6** is a metabolite produced by oxidative decarboxylation of the 22-Me group of **2** in shellfish,[6] so it must have the same absolute stereochemistry as **2**.

Azaspiracid-6 Stability. The stability of **6** was compared with that of **1**. Figure 2 shows that **6** is significantly less stable ($p < 0.05$, Student's t test) than **1** when stored in methanol at 40 °C. These results parallel the observations of Perez et al.,[33] who showed that **3** was less stable than **1** under these conditions, and confirms the results of McCarron et al.,[43] showing that **6** exhibited similar instability to **3** in tissue CRM extracts. Compound **6**, like **3**, but unlike **1** and **2**, 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 **6** was determined in three solvents. Figure 2 shows that **6** 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 and 870.5 after storage in methanol and ethanol, respectively, indicated that these solvents were reacting with **6** to produce methyl and ethyl derivatives.

The formation of azaspiracid methyl esters after storage in methanol has previously been reported; however, little evidence was provided to suggest that these compounds were in fact methyl esters.[5] Methylation may occur at the C-1 to produce the methyl ester or, alternatively, at C-21 to produce the methyl ketal. A purified sample of **6** was reacted with diazomethane to produce **6** methyl ester. The semi-synthetic methyl ester differed from the derivative observed during the stability study in both LC-MS/MS retention time 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 **6** is being methylated at the C-21 position to form a methyl ketal during storage in methanol (Figure 3). The methyl ester of **6** also shows a different retention time to that of the methyl ketal, with the methyl ester being retained longer on the column. Furthermore, when the sample containing the methyl

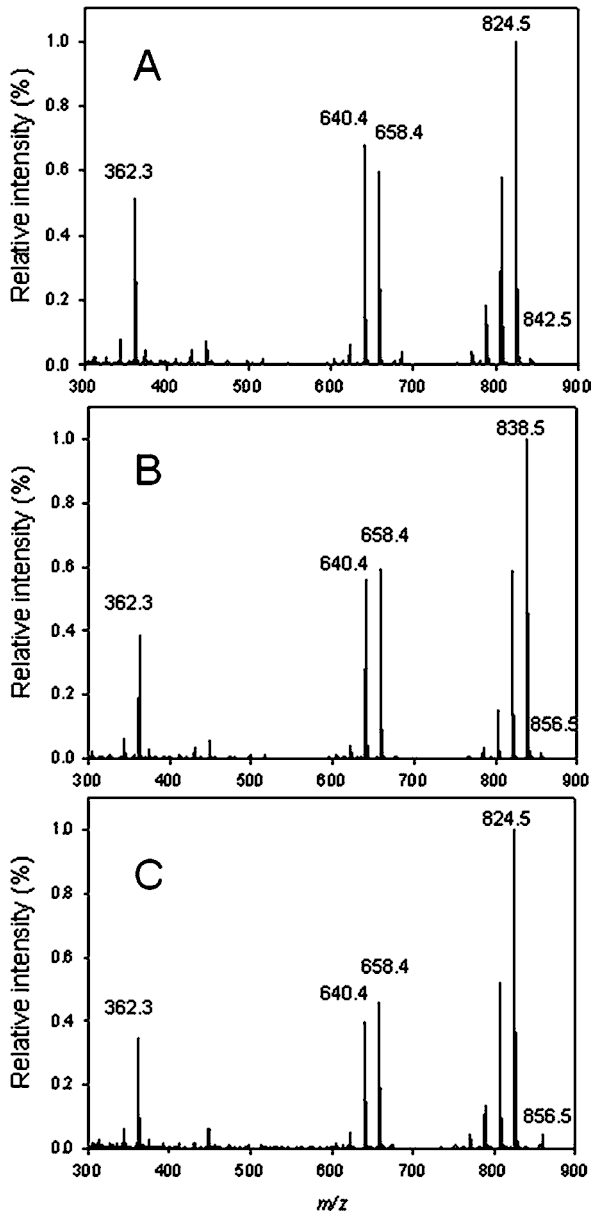


Figure 3. Mass spectra of (A) **6**, (B) **6** methyl ester, and (C) **6** methyl ketal.

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derivative was treated with sodium periodate, the compound remained intact, consistent with the proposition that the **6** derivative is methylated at the 21-position (i.e., **6** 21-methyl ketal). These results support observations reported by Jauffrais et al.,[44] which showed the formation of **1** and **2** methyl ketals in *A. spinosum* methanolic extracts.

In summary, a method was optimized for the isolation of azaspiracids from highly contaminated *M. edulis* hepatopancreas. 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 azaspiracids 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 semipreparative LC. Overall recoveries of ∼40−50% were achieved for **1**−**3** and **6**. Sufficient **6** was isolated for structural elucidation by NMR, which confirmed the previously postulated structure (Figure 1). A short-term stability study showed that **6** is significantly more stable in aqueous acetonitrile than in methanol (the usual storage solvent) at 40 °C. The isolated azaspiracids are of sufficient purity for toxicological research and for the preparation of analytical standards.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectrum of **6**; structures of **6** showing COSY and HMBC correlations, as well as selected ROESY correlations, and a Molfile (**6**.mol) showing the three-dimensional structure of **6**; and LC-MS/MS chromatograms (QTof) of **1** and **6** samples in methanol after treatment with diazomethane, after storage at 40 °C for 4 weeks and after treatment with periodate, together with LC-MS/MS chromatograms (QTof) of **6** and **3** 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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Notes

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■ ABBREVIATIONS USED

EU, European Union; FIA, flow-injection analysis; MBA, mouse bioassay

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