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Formation of Azaspiracids-3, -4, -6, and -9 via Decarboxylation of Carboxyazaspiracid Metabolites from Shellfish

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The azaspiracid (AZA) class of phycotoxins has been responsible for extended closures of shellfisheries in various locations around Europe, where levels of AZA1−3 are regulated in shellfish. Since their discovery in 1995, AZAs have been the focus of much research, resulting in the discovery of numerous analogues. During studies of procedures for processing of AZA-contaminated mussels (*Mytilus edulis*), an unusual phenomenon was observed involving AZA3. In uncooked tissues, AZA3 levels would increase significantly when heated for short periods of time in the absence of water loss. A similar increase in AZA3 concentrations occurred during storage of shellfish tissue reference materials for several months at temperatures as low as 4 °C. Concentrations of AZA1 and AZA2 did not change during these experiments. Several possible explanations were investigated, including an AZA3-specific matrix effect upon heating of tissues, release of AZA3 from the matrix, and formation of AZA3 from a precursor. Preliminary experiments indicated that toxin conversion was responsible, and more detailed studies focused on this possibility. LC−MS analysis of heating trials, deuterium labeling experiments, and kinetic studies demonstrated that a carboxylated AZA analogue, AZA17, undergoes rapid decarboxylation when heated to produce AZA3. Heat-induced decarboxylation of AZA19, AZA21, and AZA23 to form AZA6, AZA4, and AZA9, respectively, was also demonstrated. This finding is of great significance in terms of procedures used in the processing of shellfish for regulatory analysis, and it exemplifies the role that chemical analysis can play in understanding the contribution of metabolic processes to the toxin profiles observed in shellfish samples.

KEYWORDS: Azaspiracid; shellfish; metabolism; LC−MS; stability; cooking; mussel; decarboxylation

INTRODUCTION

The accumulation in bivalve shellfish of toxins originating from marine phytoplankton has serious implications for human health. Numerous classes of toxins have been identified which, after consumption of contaminated tissues, induce a variety of symptoms in humans including nausea, abdominal cramps, diarrhea, memory loss, and in extreme cases paralysis and even death (*1*).

In November 1995, mussels cultivated in Killary Harbour, on the west coast of Ireland, were implicated in the poisoning of at least eight people in The Netherlands (*2*). Symptoms resembling those of diarrhetic shellfish poisoning (DSP) were reported by those affected, including nausea, vomiting, stomach cramps, and severe diarrhea. However, when mussels from the location were analyzed for the presence of the DSP toxins okadaic acid and dinophysistoxins, only very low concentrations were present. A new toxic compound was soon identified as the causative agent and provisionally named Killary toxin-3 (KT3) in recognition of the location where the mussels originated (*3*). Following elucidation of the structure, it was renamed azaspiracid-1 (AZA1) (*4*), although its structure was subsequently revised on the basis of synthetic studies (*5*) (**Figure 1**). Subsequently, 8-methyl (AZA2) and 22-desmethyl (AZA3) analogues (*6*) of AZA1 were isolated and their structures determined by NMR analysis. Two hydroxylated analogues (AZA4 and AZA5) of AZA3 were also isolated and identified by NMR (*7*). Six additional AZA analogues were detected and their structures proposed on the basis of mass spectrometric evidence (*8, 9*) together with the known MS characteristics of AZAs (*10*). A recent LC−MS study has identified a number of

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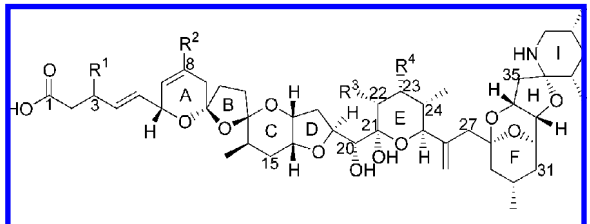

	R^1	R^2	R^3	R^4	$[M+H]^+$ *m/z*
Azaspiracid-1	H	H	CH_3	H	842.5
Azaspiracid-2	H	CH_3	CH_3	H	856.5
Azaspiracid-3	H	H	H	H	828.5
Azaspiracid-4	OH	H	H	H	844.5
Azaspiracid-5	H	H	H	OH	844.5
Azaspiracid-6	H	CH_3	H	H	842.5
Azaspiracid-7	OH	H	CH_3	H	858.5
Azaspiracid-8	H	H	CH_3	OH	858.5
Azaspiracid-9	OH	CH_3	H	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 and *m/z* for the $[M + H]^+$ ions of azaspiracids detected in shellfish. Structures for AZA1−5 are firmly established from NMR and synthesis (*4*−*7*), whereas the remaining structures have been proposed on the basis of mass spectrometric and biosynthetic considerations (*8, 9, 11*).

additional AZA analogues and provided further information about the MS fragmentation of the AZAs (*11*). The defined and proposed structures of all AZA analogues which have been detected in shellfish are shown in **Figure 1**.

Although AZAs were initially reported in Ireland (*2*), they have since been detected in various European locations (*12*) and more recently outside of Europe (*13*). After consumption of shellfish contaminated with AZAs, sickness occurs between 3 and 18 h, with full recovery after 2−5 days (*2*). Human exposure data are limited, however. A number of studies have been carried out examining the in vivo toxicity of AZAs (*14, 15*). The toxicological mode of action for AZAs is not yet known, but it is different from those of okadaic acid and the dinophysistoxins as AZA1 does not inhibit protein phosphatase 2A (*16*). Within the European Union, the maximum allowable level of AZAs in shellfish is 160 μg/kg AZA equivalents (*17*). This regulation only includes AZA1, -2, and -3, as only these analogues have been found in shellfish at concentrations sufficient to pose a risk to human health and also due to the limited toxicological information available for the other AZAs. Toxic equivalence factors of 1.8 and 1.4 have been reported for AZA2 and -3, respectively, relative to AZA1 (*6*). AZA4 and AZA5 were 2.5 and 5 times less toxic (*7*) than AZA1, respectively, while in vivo toxicity data for other AZA analogues have not been published.

The influence of cooking on AZA concentrations in mussels has been examined, and considerable increases in concentrations after steaming of fresh samples have been reported (*12*). This change was attributed to water loss during steaming, with the AZAs concentrating by a factor of ca. 2-fold in the cooked tissue as a result. However, stability studies as part of developmental work on the production of reference materials for a variety of marine toxins revealed an unusual phenomenon involving AZA3. A laboratory reference material (LRM) had been prepared from fresh mussel tissues contaminated with AZAs and was bottled in hermetically sealed polypropylene tubes. Upon storage of these LRMs at temperatures ≥4 °C for periods of up to 8 months, significant increases in the concentration of AZA3, but not of AZA1 or AZA2, were observed (*18*). A similar phenomenon was also observed during studies of the fate of individual AZAs during cooking (*19*).

Protoperidinium crassipes was reported to be the progenitor of these toxins in Ireland (*20*), but subsequent studies have not supported this proposal. There was no correlation between the very high levels of AZAs measured in Irish mussels during 2005 and the presence of the *P. crassipes* in phytoplankton samples (*21*). James et al. (*20*) noted that *P. crassipes* was predatory and that it was possible that AZAs were produced by a consumed species, and Miles et al. (*22*) found evidence of transfer of DSP toxins from prey (*Dinophysis*) but no AZAs in several *Protoperidinium* species. Rundberget et al. (*23*) observed only AZA1 and -2 in passive adsorption disks in waters where mussels were contaminated with AZA1−3 and -6 and speculated that AZA3 and -6 might be products of metabolism in shellfish. The subsequent isolation from the North Sea of an alga that produces AZA1 and AZA2, but not AZA3 (*24*), is consistent with this suggestion.

The aim of this study was to explain increased AZA3 concentrations upon heating of fresh mussel tissues. This was achieved through a combination of mass spectrometric, isotopic labeling, and chemical degradation studies which, when taken together with metabolic and biosynthetic considerations, provide valuable new information about the AZA group of shellfish toxins.

MATERIALS AND METHODS

Standards and Chemicals. The AZA calibrants used were dilutions of AZA1 isolated during 2001 from mussels (*Mytilus edulis*) originating in Ireland (Killary Harbour in 1996 and Bantry Bay in 2000) under the supervision of M. Satake (Tohoku University, Sendai, Japan). For the standard-addition experiment, a solution purified as part of the ASTOX project (*25*) containing AZA1 and -3 in methanol was used. Methanol and acetonitrile were obtained as pestiscan grade solvents from Labscan (Dublin, Ireland). A reverse osmosis purification system (Barnstead International, Dubuque, IA) supplied water for the mobile phase. Formic acid, ammonium formate, and deuterated methanol (CH_3OD; >99.5 atom % D) were from Sigma-Aldrich (St. Louis, MO).

Tissue Samples. Mussels (*M. edulis*) containing AZAs were obtained from Bruckless, Donegal Bay, on the northwest coast of Ireland during August 2005. The sample arrived in the laboratory within 24 h of being removed from the water. Whole flesh was removed from the raw mussels, homogenized, and stored frozen (−20 °C). This homogenate was used for the initial study demonstrating the increase of AZA3 upon cooking. From a separate portion of the raw Bruckless sample, hepatopancreas (HP) was dissected and homogenized to produce a more concentrated extract for the ultrafiltration and kinetics experiments and for gel permeation chromatography.

Two uncooked mussel samples, received as part of the Irish routine monitoring program for lipophilic toxins, were also utilized. Upon receipt, the whole flesh of each was removed, homogenized, and stored frozen (−20 °C). The first sample, from Clew Bay, was received in June 2007. It had AZA concentrations below the limit of detection by LC−MS and was used for the standard-addition experiment. The second sample was harvested in October 2007 from Bantry Bay on the southwest coast of Ireland. It contained AZAs above the regulatory level and was used for initial investigation of the toxin conversion hypothesis.

Heat Treatment of Fresh Mussel Homogenate. Aliquots (2 g, $n = 18$) of whole tissue homogenate from Bruckless were transferred to centrifuge tubes and capped to prevent water loss. Three replicate aliquots were heated for 10 min in a water bath (Grant Ltd., Cambridge,

U.K.) at temperatures of 50, 60, 70, 80, or 90 °C. The tubes were then cooled by immersion in cold water for 5 min. Control samples were maintained at ambient temperature. The contents of each tube were extracted with 2 × 9 mL of methanol, and the supernatants from the two extractions were combined and made up to 25 mL with methanol in a volumetric flask as described previously (*18*). Aliquots of the extracts were filtered through a 0.2 μm Schleicher & Schuell filter (Whatman, Maidstone, U.K.) into HPLC vials for LC−MS analysis (method A).

Ultrafiltration Trial. Homogenized HP (2 g) from the Bruckless mussels was extracted with 8 mL of methanol using an Ultraturrax (IKA-Werke, Staufen, Germany) at 11 000 rpm for 1 min. The sample was then centrifuged for 5 min at 3950*g* and 15 °C. The extract was filtered as before, and an aliquot (1 mL) was made up to volume with deionized water in a 5 mL volumetric flask to give a sample that was compatible with the ultrafiltration membrane. Microcon YM-3 ultrafiltration cartridges were used which had a regenerated cellulose membrane (0.32 cm^2 surface area) with a nominal molecular weight limit of 3000 (Millipore, Bedford, MA). An aliquot (450 μL) of the diluted extract was applied to a centrifugal ultrafiltration cartridge and centrifuged at 10500*g*. The filtrate was analyzed by LC−MS directly (method A) and again after heating at 70 °C for 20 min. The filter residue was recovered by inverting the filter and back-flushing with methanol and also analyzed by LC−MS (method A).

Standard-Addition Experiment. Mussel homogenate from Clew Bay was thawed, and a portion (ca. 15 g) was heated in a water bath at 90 °C for 15 min in a closed centrifuge tube to mimic the effect of cooking but without the loss of water from the tissue. Raw and heated mussel samples were extracted using the procedure described above. Aliquots (0.5 mL) of extract from the raw or cooked shellfish, or of methanol (for the controls), were added to HPLC vials ($n = 7$ for each treatment).

The AZA stock solution contained 14.31 μg/mL AZA1 and 7.34 μg/mL AZA3. A working solution was prepared by 5-fold dilution of the stock, which was further diluted with a Hamilton Microlab diluter (AGB Scientific, Dublin, Ireland) to produce seven dilutions (16−1468 ng/mL AZA3) in methanol for spiking in the standard-addition experiment. Aliquots of the various working dilutions were added to the vials using the diluter, generating a series of methanolic extracts of raw and cooked mussel, and methanol controls, each containing AZA3 at seven concentrations in the range 0.8−50.3 ng/mL. Each sample was analyzed by LC−MS (method A) in triplicate.

Investigation of Toxin Conversion with a Fresh Mussel Sample. Fresh mussel homogenate from Bantry Bay was thawed, and an aliquot (2 g) was placed in a 50 mL centrifuge tube, the tube capped, and the homogenate heated in a water bath at 90 °C for 10 min. This sample and a 2 g aliquot of unheated tissue were extracted using the procedure described above. Filtered aliquots of both extracts were analyzed using LC−MS method B.

Kinetic Analysis. The rates of change in the concentrations of the carboxylated AZAs (AZA17 and AZA19) and of AZA3 and -6 were measured. HP from the Bruckless mussels was extracted with methanol as described above (2 g with 2 × 9 mL, made up to 25 mL). Aliquots (500 μL) were dispensed into HPLC vials. Triplicate aliquots of the extract were heated at 70 °C for varying lengths of time (2, 5, 10, 20, 30, and 60 min), then rapidly chilled, and stored at −20 °C until LC−MS analysis.

Fractionation of the Raw HP Extract by Gel Permeation Chromatography. HP (20 g) was dissected from the fresh mussels from Bruckless and extracted with methanol (2 × 20 mL) with an Ultraturrax. The extracts were combined and partitioned against an equal volume of hexane. The methanolic layer was concentrated without heating under a stream of nitrogen, the residual water was removed with a freeze-drier (Labonco, Kansas City, MO), and the residue was dissolved in 3 mL of methanol.

The sample was loaded onto a column of Sephadex LH20 (Amersham Biosciences, Buckinghamshire, U.K.) with a stationary bed size of 850 mm × 15 mm and eluted with methanol. Fifty 3 mL fractions were collected and stored at −20 °C until analysis. An aliquot of each fraction was heated to 90 °C for 10 min. Unheated and heated aliquots of each fraction were screened by flow-injection analysis (FIA) on the Q-ToF mass spectrometer for the presence of AZA3 (*m*/*z* 828.5) and AZA1 (*m*/*z* 842.5) to identify fractions that contained components responsible for increased AZA3 levels upon heating. Fractions of interest were transferred to vials and stored at −20 °C until LC−MS analysis.

Deuterium Incorporation. Fraction 17 from the gel permeation column, which showed a marked increase in AZA3 and -6 when heated, was selected for a deuterium incorporation experiment. A methanolic extract of an in-house LRM prepared from cooked mussels contaminated with AZAs was used as a control.

For both fraction 17 and the LRM extract, two 500 μL aliquots were transferred to HPLC vials and evaporated under N_2 without the use of heat. The residues were dissolved in 500 μL of CH_3OD and the vials capped. One aliquot of each of fraction 17 and the LRM extract in CH_3OD were heated in a water bath at 70 °C for 10 min. The heated and nonheated aliquots of fraction 17 and the LRM extract were evaporated under N_2 without the use of heat. The residues were then redissolved in 500 μL of methanol and analyzed by LC−MS using method B for AZA1, -2, -3, -6, -17, and -19 and for deuterated AZA1, -2, -3, and -6 (*m*/*z* 843.5, 857.5, 829.5, and 843.5, respectively) and method C for AZA4, -9, -21, and -23 and for deuterated AZA4 (*m*/*z* 845.5) and AZA9 (*m*/*z* 859.5).

Treatment of the Gel Permeation Chromatography Fraction with Periodate. Aliquots (50 μL) of fraction 17 that had been heated in CH_3OH and CH_3OD (above) were oxidized by adding 25 μL of 0.2 M $NaIO_4$. The samples were analyzed immediately by LC−MS using method A, but including additional ion traces at *m*/*z* 434.4 (for the AZA oxidation product) and *m*/*z* 435.4 (for the monodeuterated AZA oxidation product).

LC−MS Analysis. A Waters 2795 HPLC instrument coupled to a Micromass Q-ToF mass spectrometer, equipped with a Z-spray electrospray ionization source, was used in positive ion mode. The Q-ToF instrument was used in ToF-MS/MS mode with a capillary voltage of 3.2 kV, a cone voltage of 100 V, a source temperature of 130 °C, a desolvation temperature of 350 °C, and a collision energy of 50 eV. A binary mobile phase was used, with (A) water and (B) 95% aqueous acetonitrile, each containing 2 mM ammonium formate and 50 mM formic acid. Method A: A 30 mm × 2.1 mm i.d., 3 μm, ACE-C18 column (Advanced Chromatography Technologies, Aberdeen, Scotland) was eluted isocratically with 60% B for 7 min. Retention times for AZA3, -1, and -2 were ca. 2.8, 4.0, and 5.1 min, respectively. The following molecular ions ($[M + H]^+$) were selected as parent ions for Q-ToF analysis: AZA3, *m*/*z* 828.5; AZA1, *m*/*z* 842.5; AZA2, *m*/*z* 856.5. Fragment ions were scanned over the *m*/*z* range from 100 to 900. Method B: A linear gradient was run on a 50 mm × 2 mm i.d., 3 μm, BDS-Hypersil C8 column (Thermo Fisher Scientific, Loughborough, U.K.) with a 10 mm × 2 mm, 3 μm, guard column from 30% to 90% B over 8 min, held for 2.5 min, decreased to 30% B over 0.5 min, and equilibrated for 3 min until the next run. The following protonated molecules were selected as parent ions for Q-ToF analysis ($[M + H]^+$): AZA17, *m*/*z* 872.5; AZA19, *m*/*z* 886.5; AZA3, *m*/*z* 828.5; AZA1 and -6, *m*/*z* 842.5; AZA2, *m*/*z* 856.5. Fragment ions were scanned over the *m*/*z* range from 100 to 900. These AZA analogues eluted from 8.5 to 13.0 min. This method is validated at the Marine Institute for analysis of azaspiracids, okadaic acid, and dinophysistoxins in shellfish tissues. Method C: A linear gradient adapted from that described previously for the analysis of all reported AZA analogues (*11*) was run on a 50 mm × 2 mm i.d., 3 μm, BDS-Hypersil C8 column (Thermo Fisher Scientific, Loughborough, U.K.) with a 10 mm × 2 mm, 3 μm, guard column from 30% to 100% B over 20 min, decreased to 30% B over 1.0 min, and equilibrated for 4 min until the next run. The following protonated molecules were selected as parent ions for Q-ToF analysis ($[M + H]^+$): AZA4, *m*/*z* 844.5; AZA9, *m*/*z* 858.5; AZA21, *m*/*z* 888.5; AZA23, *m*/*z* 902.5. Fragment ions were scanned over the *m*/*z* range from 100 to 900. The collision energy and cone voltage were set at 50 and 40 eV, respectively.

RESULTS AND DISCUSSION

Increases in AZA3 levels have been observed in fresh mussels during heating and cooking (*18*, *19*), but earlier experiments

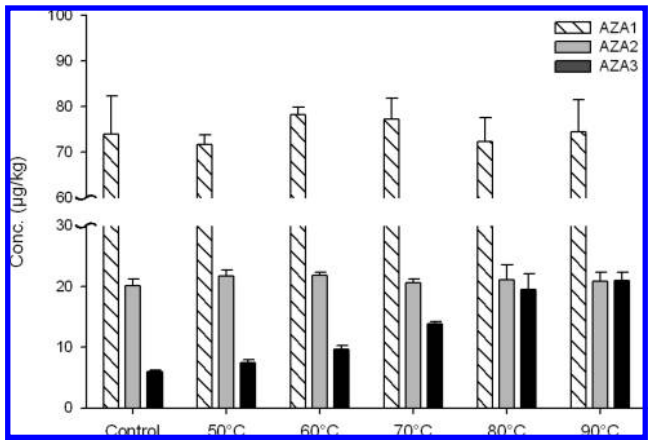

Figure 2. Levels of AZA1, -2, and -3 extracted from aliquots ($n = 3$) of uncooked whole mussel homogenate from Bruckless heated for 10 min at increasing temperatures. Error bars shown represent ±1 SD.

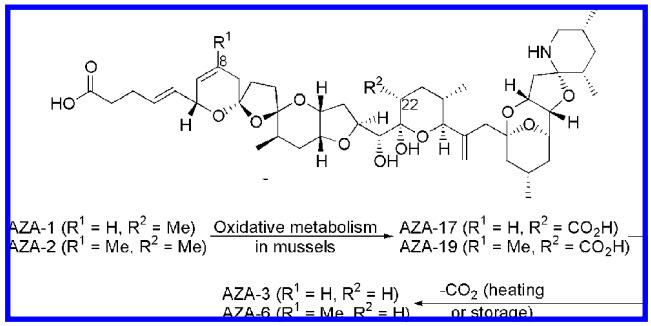

Figure 3. Proposed oxidative metabolism of AZA1 and -2 in shellfish to form 22-carboxylated metabolites (AZA17 and -19), which undergo decarboxylation when heated to form AZA3 and -6, respectively.

were confounded by loss of water from the tissues during cooking which effectively increased the tissue concentration of all AZA toxins. Therefore, the influence of heating fresh mussels in sealed containers, to prevent water loss, on the levels of AZAs was studied. AZA1, -2, and -3 concentrations were measured (LC−MS method A) in raw mussel tissues before and after heating at a range of temperatures for 10 min (**Figure 2**). The AZA3 concentration increased from 7 μg/kg in the control samples (unheated) to 21 μg/kg when heated at 90 °C, whereas the concentrations of AZA1 and -2 were unaffected. The increases of concentration with temperature were all statistically significant ($P < 0.05$), with the exception of the change between 80 and 90 °C ($P > 0.05$). This experiment clearly demonstrated that levels of AZA3 measured in fresh mussels increased upon heating and that this was not related to water loss. In this experiment only the influence of temperature was examined, and not heating duration. However, in the feasibility study for the preparation of reference materials, increased concentrations of AZA3 were both time- and temperature-dependent, with the increase in AZA3 concentration and its subsequent degradation occurring more rapidly at higher temperatures (*18*). In the same study, AZA3 levels were stable over an 8 month period when the tissues were stored at −20 °C. Possible explanations for this phenomenon include release of AZA3 bound to tissue components as a result of heating, reduction of matrix effects or ion suppression in the LC−MS analysis of AZA3 due to heating of tissues, or conversion of AZA analogues to AZA3 during heating.

Ultrafiltration Trial. A recent study demonstrated that AZAs are weakly bound to proteins in mussel tissues (*26*). An ultrafiltration experiment was therefore carried out to examine the possibility that AZA3 was bound to components of the matrix, such as proteins. The methanol extract of raw mussel HP tissue containing AZAs was passed through a filter with a nominal molecular weight cutoff of 3000. Molecules with molecular weights significantly below 3000, such as AZAs, should pass through such a filter, whereas protein-bound AZAs should be retained. LC−MS analysis of the filtrate showed a greater than 3-fold increase in AZA3 concentration after heating, indicating that the AZA3 precursor in raw tissues had a molecular weight of <3000. The concentrations of AZA1 and -2 in the filtrate did not change with heat treatment. This trial showed that AZA3 was not released from the matrix and that binding of AZAs to shellfish protein did not contribute significantly to the increase in the levels of AZA3 after heating. Therefore, the possible role of matrix effects on the LC−MS analysis was investigated.

Standard-Addition Experiment. Matrix effects are a common problem in the LC−MS analysis of biological extracts, and studies have demonstrated their ability to influence shellfish toxin analyses (*27*−*29*). A possible explanation for the apparent increase in the concentration of AZA3 was that heating caused a reduction in the LC−MS matrix effects for AZA3. In the heat treatment experiment, the AZA3 concentration increased approximately 3-fold upon heating at 90 °C for 10 min. That matrix effects were responsible for this observation was considered unlikely because the measured concentrations of the closely related analogues AZA1 and -2 were unaffected by heating, and furthermore, the LC−MS matrix effects for AZA1 in raw and cooked shellfish tissues were very similar (*30*). However, different instrumentation was used in the previous study on matrix effects (*30*), and differing ionization efficiencies for the regulated AZA analogues have been reported (*31*).

To find out whether LC−MS analysis of AZA3 was influenced by matrix effects to a different extent than that of AZA1, and to rule out the possibility that matrix effects were responsible for the measured increases in AZA3 concentrations, a standard-addition experiment was performed. The slopes for AZA3 were very similar in methanol and in the cooked and raw extracts (average slope 0.983 ± 0.004). This showed that there was no ion suppression or enhancement during analysis of AZA3 in matrixes from cooked or uncooked shellfish using method A (isocratic elution), and similar findings were obtained for AZA1 (average slope 0.93 ± 0.02). Matrix effects were observed during analysis of the shellfish tissue extracts with a triple-quadruple LC−MS system, but the suppression was similar for both the cooked and raw tissues for both AZA1 and AZA3, confirming the findings of Fux et al. (*30*). Thus, the increase in AZA3 concentration upon heating was not due to matrix effects.

Toxin Conversion. In view of the above results, the most likely explanation for the observed increase in AZA3 concentrations upon heating was the presence of a thermally labile AZA3 precursor in the shellfish and their extracts. Until recently, only 11 AZA analogues had been reported (*4*, *6*−*9*), differing in the position (or number) of their methyl groups (AZA1, -2, -3, or -6) or containing an additional hydroxyl group (AZA4, -5, or -7−11). There was nothing in their structures suggesting the possibility of conversion to AZA3 upon heating, especially under the mild conditions in which the phenomenon was sometimes observed. The possibility of carboxylic acid derivatives of AZAs had been suggested on the basis of MS data, but no structures were proposed (*8*). Recent work demonstrated the existence of carboxylated AZAs (AZA17, -19, -21, and -23), although the location of the carboxylic acid group could not be definitively

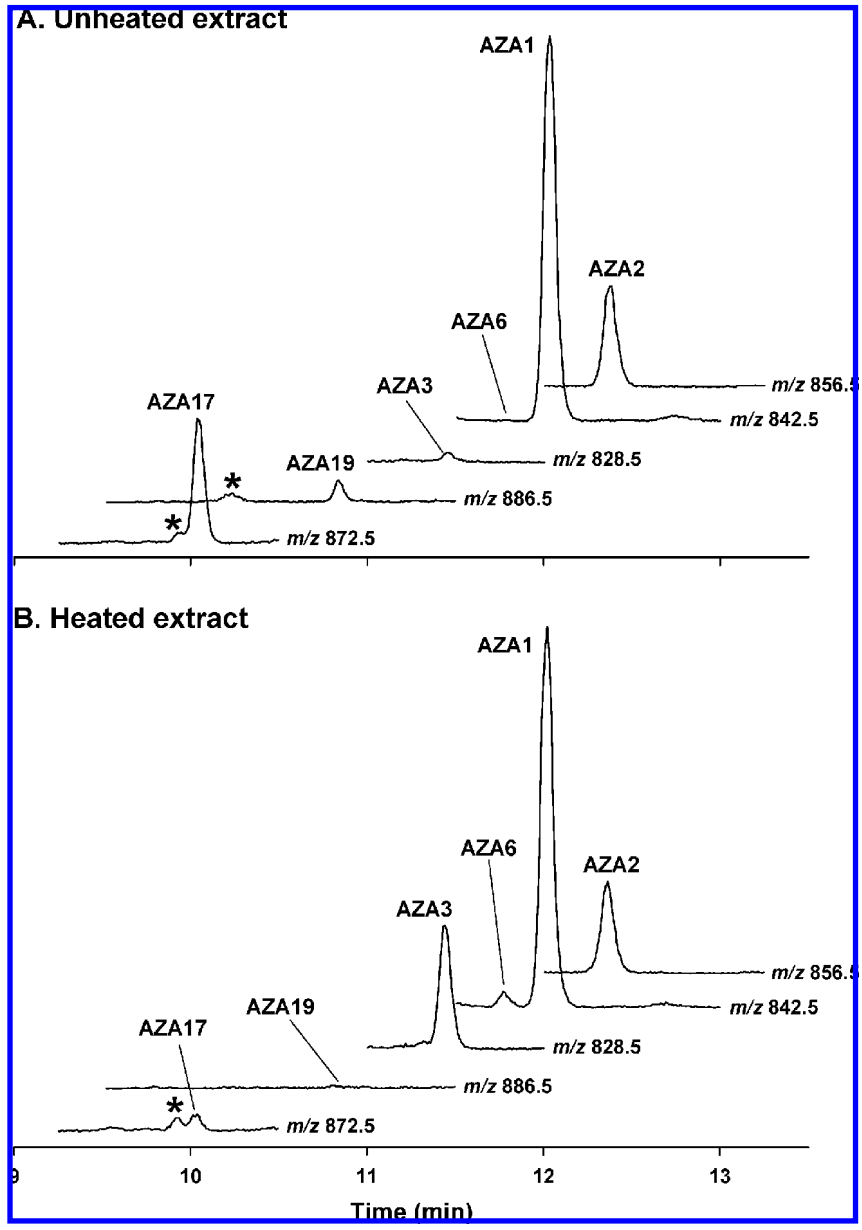

Figure 4. LC−MS (method B) chromatograms of (A) unheated and (B) heated aliquots of fresh mussel homogenate from Bantry Bay. Traces show peaks for AZA17 and -19 in the unheated sample, with subsequent absence in the heated sample. AZA3 and -6 peaks appear after heating. The mass spectra of the peaks marked with an asterisk did not correspond to azaspiracids.

assigned (*11*). In terms of the increased AZA3 concentrations upon heating of fresh tissues, these carboxylated analogues were of interest due to the possibility that they might undergo thermal decarboxylation. All AZAs contain a −COOH group at C1. The carboxylated AZAs have a second −COOH, and we postulated that the carboxylated analogue AZA17 might eliminate the additional −COOH group as CO_2, upon heating, to form AZA3. Rundberget et al. (*23*) recently suggested that AZA3 and -6 are products of metabolic conversion in mussels, because only AZA1 and -2 were detectable by LC−MS in water samples while the mussels collected from the same site contained high levels of AZA1, -2, -3, and -6. On the basis of these considerations, we hypothesized that AZA17 is 22-carboxy-AZA3, produced by oxidative metabolism of the 22-methyl of AZA1 in the mussel, and that heating eliminates CO_2 from the 22-COOH to generate AZA3 (**Figure 3**). This hypothesis also predicts that the levels of AZA6 will also increase through an equivalent series of transformations via AZA19. Provided that AZA17 and -19 decarboxylate under mild conditions to produce AZA3 and -6, this hypothesis would provide an explanation for the absence of AZA3 and -6 in seawater (*23*) as well as for the increase in AZA3 upon heating fresh tissues.

To investigate whether AZA17 is decarboxylated by heating to form AZA3, separate aliquots of the fresh mussel sample from Bantry Bay were extracted before and after heating at 90 °C in a closed container for 10 min. LC−MS analyses were run for AZA1, -2, and -3, as in the normal routine method, but additional MS traces were run for AZA17 and AZA19 (method B). The chromatograms (**Figure 4**) showed a significant peak for AZA17 in the raw sample, which was greatly reduced upon heating. Conversely, AZA3 was virtually absent in the raw sample, but was a significant component in the heated sample. As in the initial heating trial, levels of AZA1 and -2 were not affected by heating. This supported the idea of toxin conversion during the heating process and in particular that the increase in AZA3 was due to decarboxylation of AZA17. **Figure 4** also shows an increase in AZA6 in the heated sample and a concomitant decrease in AZA19, supporting a similar origin

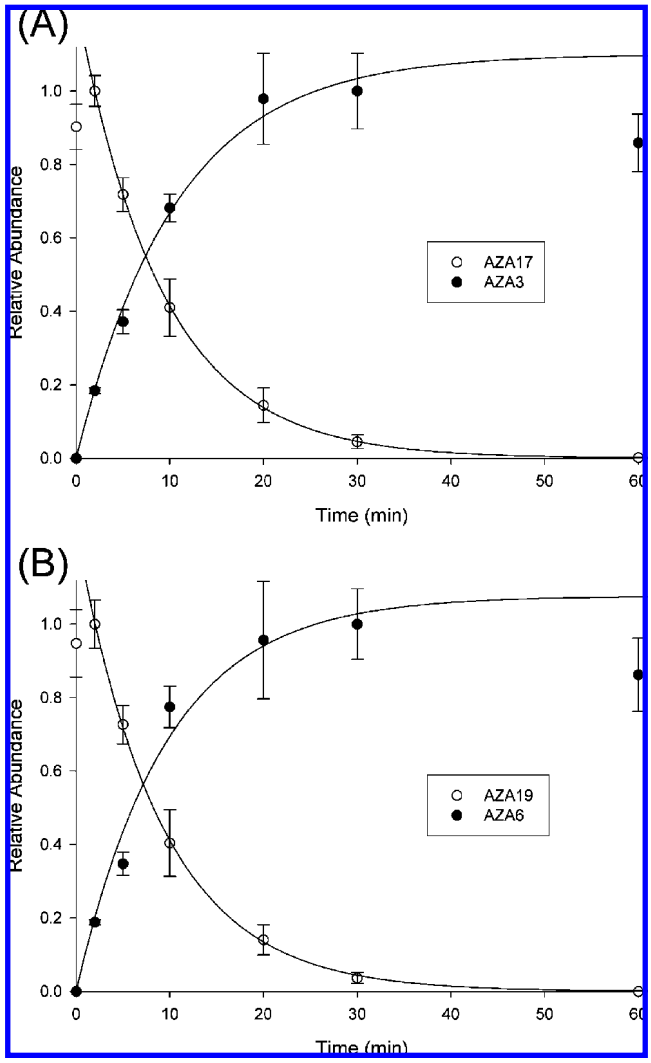

Figure 5. Change in concentration with time of (A) AZA17 and AZA3 and (B) AZA19 and AZA6 in Bruckless mussel HP extracts upon heating at 70 °C. Error bars represent ±1 SD. The analysis was carried out using LC−MS method B. Exponential curves were fitted to 0−30 min for AZA3 and -6 due to degradation with prolonged heating and to 2−30 min for AZA17 and -9.

for AZA6. AZA6 has been observed as a significant component in Norwegian mussels (*23*).

The concentrations of AZA3, -6, -17, and -19 in a raw mussel HP extract were followed over time during heating. Kinetic analysis was carried out to determine the rates of formation of AZA3 and -6 and the rates of degradation of AZA17 and -19 (**Figure 5**). The maximum levels of AZA3 and -6 were reached at ca. 30 min, with degradation as a result of prolonged heating being observed after that. The first-order half-lives for the reactions were the same within experimental error ($t_{1/2}$ = 6.3 ± 0.1 (AZA17), 7.4 ± 0.8 (AZA3), 6.2 ± 0.1 (AZA19), and 6.7 ± 1.2 (AZA6) min). This was consistent with direct interconversion of the toxins. Due to differences in the absolute values of the peak areas for carboxy/decarboxylated pairs, attributable to differences in response in the LC−MS, data in **Figure 5** are plotted as normalized values. The carboxy-AZAs appeared to have higher response factors relative to the decarboxylated toxins, by approximately 1.7-fold, in these analyses.

As the ultrafiltration and kinetics experiments had shown that the AZA3 precursor was methanol-extractable, a concentrated methanolic extract of fresh mussel tissues was fractionated by gel permeation chromatography to provide a sample for more detailed studies of the decarboxylation reaction. FIA−mass spectrometry (MS) of fractions from the column revealed several fractions in which the AZA3 levels increased significantly upon heating. Because a similar phenomenon was anticipated for AZA6, a fraction (fraction 17) was chosen for study which also produced a significant quantity of AZA6 upon heating. **Figure 6** shows the chromatograms of this fraction analyzed by LC−MS before and after heating (parts A and C, respectively, of **Figure 6**). Before heating, this sample was dominated by AZA17, together with moderate levels of AZA19 and almost no AZA3 or -6. After heating, the fraction was dominated by AZA3, with moderate levels of AZA6, and only traces of AZA17 and -19 remained. Again, the levels of AZA1 and -2 were unaffected. It should be noted that, as this fraction was from a preparative chromatographic preparation, the AZA profile was different from that typical of naturally contaminated mussel samples from Ireland (**Figure 4**). As a result, the level of AZA6 in this fraction after heating was almost equivalent to that of AZA1, making it useful for further studies.

To test the decarboxylation hypothesis, fraction 17 was heated in CH_3OD to determine whether deuterium was specifically incorporated into AZA3 and -6 during decarboxylation of AZA-17 and -19. Mass spectra (**Figure 7**) were obtained for AZA3 and -6 in the unheated fraction 17 (**Figure 7**A,B), an LRM (containing AZA3 and -6 but no AZA17 or -19) heated with CH_3OD, and fraction 17 heated in CH_3OD (**Figure 7**C,D). All samples were evaporated to dryness without heating and redissolved in CH_3OH to remove exchangeable deuterium before analysis. LC−MS analysis showed that AZA3 (**Figure 7**C) and AZA6 (**Figure 7**D) were monodeuterated when fraction 17 (containing AZA17 and -19) was heated with CH_3OD, although no incorporation of deuterium was observed for AZA1 and -2. Furthermore, no deuterium was incorporated into AZA3 or -6 in fraction 17 without the application of heat (**Figure 7**A,B), nor was deuterium incorporated into AZA3 or -6 when these compounds were heated in CD_3OD.

Examination of the mass spectrum of the monodeuterated AZA3 (**Figure 7**C) showed that the characteristic fragment ions for AZA3 had all clearly increased by a mass of 1 Da down as far as the fragmentation in the E ring (*m*/*z* 362). This, together with the presence of *m*/*z* 449 and 431 ions, showed that the D atom was located on C20, C22, or C23. Analogous results were obtained for AZA6 (**Figure 7**D). This confirms that AZA3 and -6 are directly derived through decarboxylation of AZA17 and -19, respectively, and locates the position of the second carboxyl group in AZA17 and -19 at C20, C22, or C23.

Samples of fraction 17 that had been heated in CH_3OH and in CH_3OD were oxidized with sodium periodate, which cleaves the vicinal diol moiety between C20 and C21 to give a lactone containing only C21−C40 and appended functional groups. LC−MS analysis of the oxidized samples showed that the sample heated in methanol had the expected molecular isotope pattern for the periodate reaction products of AZA6 and AZA3, with the *m*/*z* 434.4 ion being dominant. The *m*/*z* 435.4 ion was dominant in the isotope cluster for the sample heated in CH_3OD, showing that the deuterium atom cannot be located at C20, leaving only C22 and C23 as possible locations. However, no AZAs have yet been identified with an additional carbon atom attached at C23, whereas the most abundant AZAs (AZA1 and -2) both contain a methyl group at C22, making the latter a much more likely location for the additional carboxy group. These results are therefore in complete agreement with the proposal for formation of AZA3 and -6 shown in **Figure 3**.

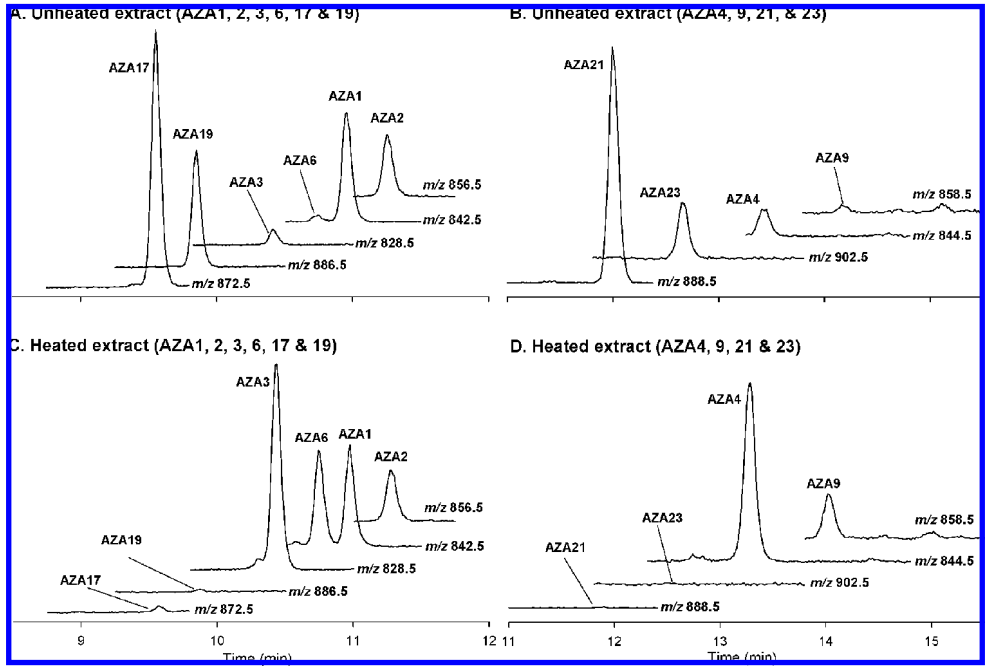

Figure 6. LC−MS (method B) chromatograms of (A) unheated and (C) heated aliquots of fraction 17 from gel filtration of the raw HP extract. The traces show peaks for AZA17 and -19 in the unheated sample, with subsequent absence in the heated sample, with a corresponding increase in AZA3 and AZA6 upon heating. Also shown are LC−MS analyses (method C) of fraction 17 (B) before and (D) after heating, showing reduction in AZA21 and -23 levels and an increase in AZA4 and AZA9 levels.

Although the present study can only show that the additional carboxyl groups of AZA17 and AZA19 are located on either C22 or C23, strong circumstantial evidence suggests that they are located at C22 with the same stereochemistry as the 22-methyl of AZA1 and AZA2. NMR analysis of isolated and purified AZA17 and -19 would be required to confirm this proposal.

Metabolism and Bioconversion of AZAs. In addition to the carboxy-AZAs, Rehmann et al. (*11*) also reported a set of hydroxylated carboxy-AZAs. As with the carboxy-AZAs, only what were apparently hydroxylated analogues of carboxy-AZA3 and -6 were detected (AZA21 and -23, respectively). Loss of the additional carboxyl group from these analogues would result in the formation of AZA4 (3-hydroxy-AZA3) and AZA9 (3-hydroxy-AZA6). LC−MS analysis of aliquots of fraction 17 using methodologies described above similarly demonstrated the conversion of AZA21 and -23 into AZA4 and -9, respectively (**Figure 6**), and one deuterium atom was also incorporated at C22 or C23 in AZA4 and AZA9 after the fraction was heated in CH_3OD.

To date, 32 different AZAs have been detected in samples or have been proposed to exist, and this number increases when a number of minor epimers and rearrangement isomers that have also been observed are taken into consideration (*11*, *32*). Research using passive sampling devices, through comparison of the toxin profiles in the water column with those in shellfish tissues, suggests that mainly AZA1 and -2 are produced by the primary causative organism. AZA3 has been detected infrequently and only at much lower concentrations (*33*) or not at all (*23*) in some field studies. Indeed, the AZA-producing organism recently isolated from the North Sea produces only AZA1 and -2, as well as a hitherto unknown isomer of AZA2, in culture (*24*). This indicates that, apart from AZA1 and -2, the remaining known AZA analogues may be products of bioconversion. The studies reported here show that AZA3 and AZA6 are formed by decarboxylation of AZA17 and -19, respectively. This implies that the carboxy-AZAs are metabolites of AZA1 and -2 formed by oxidation of the 22-methyl group (**Figure 3**). This in turn would explain why some of the proposed carboxy (AZA18 and -20) or hydroxycarboxy (AZA22 and -24) analogues of AZA1 or -2, respectively, were not detected in the previous study (*11*), as these would require insertion of a carboxyl group into the AZA skeleton rather than oxidation of an existing methyl group. Therefore, it appears that the AZAs which have been detected are formed as a result of hydroxylation at the 3- or 23-position and/or oxidation of the 22-methyl group of the parent toxins (AZA1 and -2) to generate AZA7, -8, -11, -12, -15, -16, -17, -19, -21, and -23 and that decarboxylation of AZA17, -19, -21, and -23 affords AZA3, -6, -4, and -9, respectively. Presumably the 22-desmethyl-23-hydroxy-AZAs (AZA5, -10, -13, and -14) are also formed via a similar process, although 22-carboxy-23-hydroxy-AZAs may be too sterically demanding to be present as intermediates in shellfish in significant quantities. The proposed metabolic grid, consisting of oxidation of AZA1 and -2 at C3, C23, and 22-methyl, together with the observed nonenzymatic decarboxylation at C22, is thus capable of accounting for all the currently identified AZAs in shellfish.

Since their initial discovery, AZAs have been found in many European countries, with the typical profile in raw mussels being dominated by AZA1, with lesser amounts of AZA2 and with only small amounts of AZA3. Recent reports of AZAs in southern Europe (*34*) and in northwest Africa (*13*) have shown a different profile. In AZA-contaminated mussels from these regions, AZA2 is dominant followed by AZA1 with only traces of AZA3, if detected at all. This may indicate different strains of the AZA-producing organism in varying geographical regions. From the findings of the work reported here, it would be expected that the level of AZA6 would increase to a greater degree than that of AZA3 upon heating mussel tissues in regions where AZA2 was more abundant than AZA1, provided that the shellfish species in these areas are capable of oxidizing the 22-methyl group.

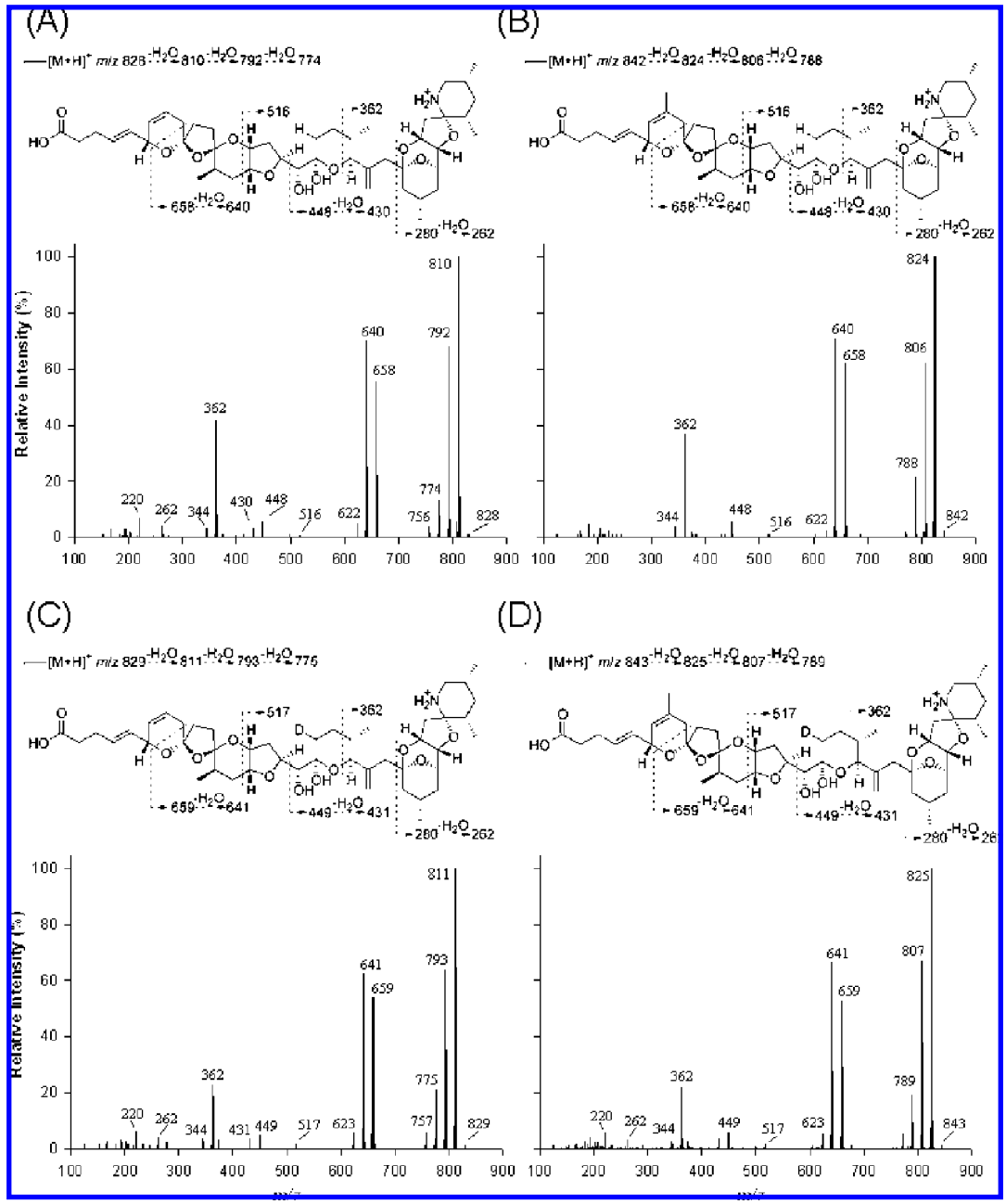

Figure 7. Q-ToF-MS/MS spectra of (A) AZA3 and (B) -6 from fraction 17 (control, unheated in CH_3OD) and (C) AZA3 and (D) -6 from fraction 17 heated in CH_3OD. The insets show the proposed MS/MS fragmentations of (A) AZA3 and (B) -6 and (C) AZA3 and (D) -6 with a deuterium incorporated at C22.

This study explains why AZA3 is frequently below detection limits in contaminated shellfish received as part of the Irish routine monitoring program (Hess, unpublished observation), because the regulatory analytical procedure prescribes analysis of raw shellfish tissues. The fact that detailed research on this phenomenon with AZAs in mussels has not been previously reported is explained by the predominant use of cooked or processed samples for research purposes, resulting in the typical AZA1, -2, and -3 profiles. A similar mechanism for the interconversion of analogues has not been described for any other group of marine toxins. A recent study of a fresh water cyanobacterium identified a novel anatoxin derivative, 11-carboxyanatoxin-a, and found that sample preparation procedures could result in enzymatic decarboxylation to form anatoxin-a (*35*).

It was noted that the novel AZA analogues in the cooked samples used for their identification comprised less than 5% of the total AZAs present (*11*). However, in raw samples the contribution of these analogues would be significantly greater, in particular for the carboxy- and hydroxycarboxy-AZAs, with the contribution from the decarboxylation products being reduced in parallel. Therefore, further research into the toxicity of these carboxylated analogues could be relevant to shellfish species that are consumed raw.

The aim of this work was to explain the changes in the levels of AZA3 in fresh mussel tissues upon heating. After exclusion of some initial hypotheses, toxin conversion via decarboxylation was shown to be responsible for increased levels not only of AZA3, but also of AZA6, -4, and -9. Decarboxylation was demonstrated to be thermally accelerated, with rates being both temperature- and time-dependent, and a previous study (*18*) showed that at least one of the carboxy-AZAs (AZA17) was stable for up to eight months at −20 °C as AZA3 levels only increased at temperatures of 4, 20, and 37 °C. Although

LC−MS/MS was utilized to monitor the experiments, NMR of purified compounds is required for definitive structural assignment. With the sample sizes used, it would not have been possible to obtain sufficient quantities of pure compounds in this study, but possible approaches for isolation of some of the novel AZAs from raw tissues were demonstrated. Availability of the toxins in their pure form for toxicological studies would also give insight into how these bioconversions influence the overall toxicity of AZA-contaminated tissues, as well as the structure−activity relationships within the AZAs.

This work has relevance in terms of regulations applied in the control of AZA levels in shellfish, as well as to the methodology used for handling and processing the samples received as part of toxin monitoring programs. Additionally, it provides information key to the understanding of AZA analogue formation and highlights how chemical studies can produce valuable information on the complex metabolic processes that marine bioactives undergo in shellfish.

ABBREVIATIONS USED

AZA, azaspiracid; DSP, diarrhetic shellfish poisoning; HP, hepatopancreas; FIA, flow injection analysis; Q-ToF, quadrupole time-of-flight (hybrid mass spectrometer); LRM, laboratory reference material.

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Supporting Information Available: Analysis of samples from the standard-addition experiment using a Micromass Q-ToF mass spectrometer, analysis of samples from the standard-addition experiment using a Micromass Quattro Ultima (triple-quadrupole) mass spectrometer, Q-ToF MS/MS spectra of AZA3 and -6 from the deuterium incorporation experiment including control mass spectra, mass spectra of AZA periodate oxidation products from fraction 17 heated in CH_3OH or CH_3OD, and mass spectra of AZA4 and AZA9 monodeuterated at C22 (or possibly C23) following decarboxylation of AZA21 and AZA23, respectively, in CH_3OD. This material is available free of charge via the Internet at http://pubs.acs.org.

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