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Antibodies with Broad Specificity to Azaspiracids by Use of Synthetic Haptens

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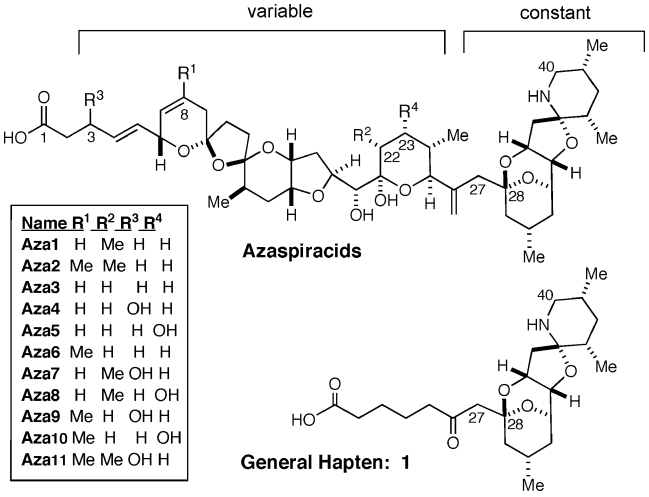
The azaspiracids (Figure 1) are natural products that accumulate in shellfish and have severe acute and suspected chronic toxicological effects on humans.[1] The seasonal recurrence of azaspiracid contamination in European cultured shellfish coupled with the potential for global distribution of toxin-harboring strains of the dinoflagellate *Protoperidinium crassipes*[2] has spurred worldwide surveillance programs.[3] Existing methods for detecting and quantifying azaspiracids have been restricted to mass spectrometry and mouse toxicity assays.[4] The development of general, sensitive, portable, and quantitative assays is urgently needed. Attempts to use azaspiracids isolated from environmental samples as haptens for antibody development that can be translated to an ELISA format for the detection of the natural products are underway.[5] Meanwhile, herein we report the successful detection of natural azaspiracids by antibodies raised against a synthetic hapten representing the common C28−C40 domain of the azaspiracids.

Initially reported in 1998,[6] the azaspiracids were shrouded in structural ambiguity. Neither the relative stereochemistry among the discrete cyclic domains nor the absolute configuration of any was fully established until recently.[7,8] We hypothesized that antibodies raised against two enantiomeric forms of a synthetic C28−C40 azaspiracid hapten could correlate the absolute configuration of this domain between synthetic and natural products via enantiospecific recognition. This immuno-stereochemical determination strategy would utilize the C26−C40 domain that is invariant among the azaspiracids. An antibody that universally cross-reacts with the azaspiracid natural products via their common C28−C40 domain would serve important roles in development of matrices for immunoaffinity purification and sensitive, quantitative detection via ELISA.[9]

In 2001, enantioselective total syntheses of dextrarotatory enantiomers of *N*-protected forms of the C26−C40 domain of the azaspiracids were reported,[10a,b] whereas a C26−C40 degradation product of azaspiracid 1 (AZA1) was reported to be levarotatory.[11] Consequently, a levarotatory derivative of the C26−C40 domain was initially targeted for hapten development. The complete stereochemistry of AZA1 has since been established via correlation of natural product degradation fragments with synthetic products, culminating in a total synthesis by Nicolaou and co-workers in 2004.[7]

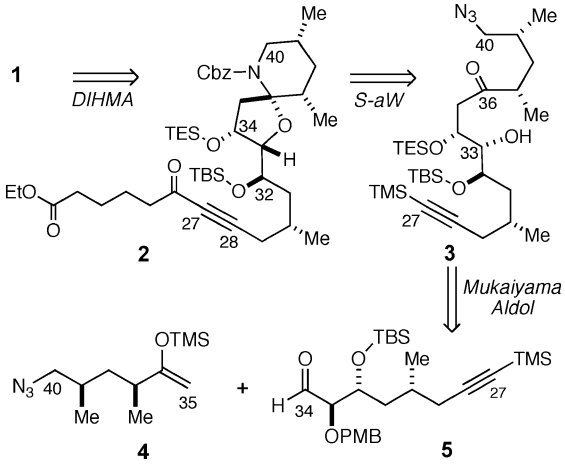
Our original synthesis of the C26−C40 domain relied upon a stepwise Lewis acid-induced spiroaminal formation followed by a double intramolecular hetero-Michael addition (DIHMA) to assemble the bridged F−G rings.[10] We have since developed more efficient entries.[12] DIHMA disconnection of ketal **1** (Scheme 1) yields ynone **2**, the spiroaminal of which would arise from a



Name	R^1	R^2	R^3	R^4
Aza1	H	Me	H	H
Aza2	Me	Me	H	H
Aza3	H	H	H	H
Aza4	H	H	OH	H
Aza5	H	H	H	OH
Aza6	Me	H	H	H
Aza7	H	Me	OH	H
Aza8	H	Me	H	OH
Aza9	Me	H	OH	H
Aza10	Me	H	H	OH
Aza11	Me	Me	OH	H

Figure 1. Structures of the azaspiracids with variable functionality R^1−R^4 at C3−C24 and the hapten **1**.

Scheme 1. Retrosynthesis of **1**



Staudinger reduction/aza-Wittig[13] (S-aW) initiated closure from azido-hydroxy-ketone **3**. A chelation-controlled Mukaiyama aldol reaction[14] of silyl enol ether **4** and aldehyde **5** would install the requisite stereochemistry and functionality in **3**.

The synthesis of **1** began with addition of the alkyl lithium derived from C27−C31 iodide **6** to the C32−C34 aldehyde **7** to generate a diastereomeric mixture of C32 alcohols (Scheme 2). Subsequent oxidation−reduction enhanced the desired (32*R*)-configuration, and a silylation−desilylation sequence provided primary alcohol **9**. Oxidation of **9** gave aldehyde **5**. Early incorporation of the C33 *p*-methoxy benzyl ether emergent in **5** facilitated an α-chelated Mukaiyama aldol reaction[14] with methyl

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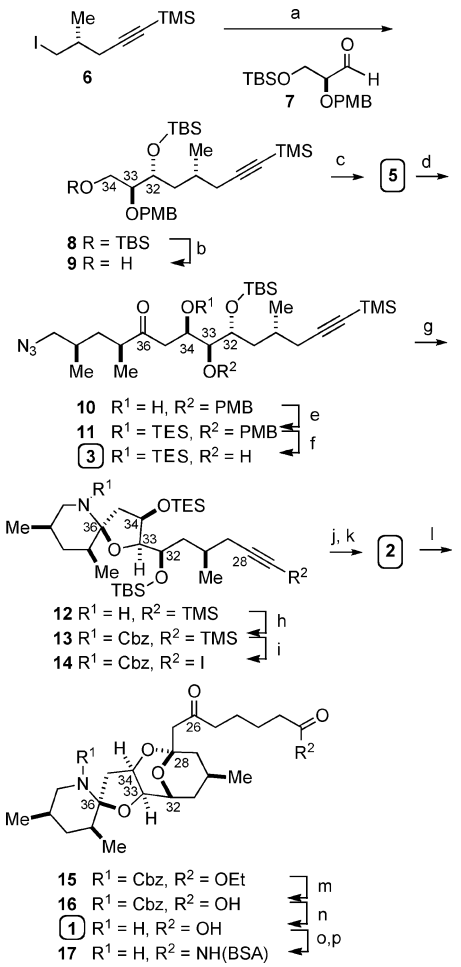
Scheme 2. Synthesis and Protein Conjugation of **1**[a]



[a] (a) (i) *t*-BuLi, pentanes/Et_2O, −78 °C then **7**, 90%, (ii) SO_3·Pyr, *i*-Pr_2NEt, DMSO, CH_2Cl_2, 0 °C, 70%, (iii) LiI, Et_2O, −40 °C, then $LiAlH_4$, −100 °C, 100%, (iv) TBSCl, DMAP, CH_2Cl_2, 96%; (b) HF·Pyr, Pyr, THF, 69%; (c) SO_3·Pyr, *i*-Pr_2NEt, DMSO, CH_2Cl_2, 0 °C, 100%; (d) **4**, $MgBr_2$·OEt_2, CH_2Cl_2, −78 °C to −25 °C, 12 h, 79%; (e) TESOTf, 2,6-lutidine, CH_2Cl_2, −10 °C, 92%; (f) DDQ, *t*-BuOH, H_2O, CH_2Cl_2, 5 min, 93%; (g) Et_3P, PhH, 1.5 h, ∼100%; (h) CbzCl, K_2CO_3, 4 Å MS, CH_2Cl_2, 14 h, 73%; (i) CF_3COOAg, NIS, acetone, 10 min, 95%; (j) ethyl 6-oxo-hexanoate, $CrCl_2$, $NiCl_2$ (0.2%), THF, 12 h, 70%; (k) MnO_2, K_2CO_3, CH_2Cl_2, 85%; (l) TBAF, THF, 12 h, 76%; (m) LiOH, *t*-BuOH, THF, H_2O, 12 h, 90%; (n) H_2, Pd/$CaCO_3$ (5%), EtOAc, 2 h, 80%; (o) *N*-hydroxysuccinimide, DIPCDI, DMF; (p) cationized bovine serum albumin (cBSA).

ketone derivative **4** to yield C33,34 syn-aldol **10**. Thereafter, the C34 hydroxyl of **10** was silylated in parallel with the C32 group in anticipation of F−G ring closure. Intervening spiroaminal formation was preceded by liberation of the C33 hydroxyl group from **11**. The resultant azido-keto-alcohol **3** was poised to cascade into sprioaminal **12** via a one-pot Staudinger reduction/intramolecular aza-Wittig reaction[13]−imine capture sequence. This was accomplished by treating **3** with Et_3P in benzene under anhydrous conditions to provide spiroaminal **12** and its C36 epimer in 75% combined yield and ca. 3:1 ratio, respectively. The thermodynamically favored (36*S*)-configuration in **12** was established via ^1H NMR spectroscopy.[10a] Conversion of the amine and alkyne of **12** into carbamate and iodo alkyne functionalities, respectively, provided iodide **14**. This was coupled with ethyl 6-oxo-hexaoate under NHK conditions,[15] and the resultant propargyl alcohol was oxidized to ynone **2**. Fluoride-initiated bis-conjugate addition of the liberated C32 and C34 oxygens upon the C28 Michael acceptor gave ketal **15**. The resultant (28*S*)-configuration of ketal **15** is geometrically predisposed in ynone (32*R*,34*R*)-**2**. Deprotection of the *C*- and *N*-termini provided carboxylic acid **1**, which was then conjugated to cBSA[16] and ovalbumin (OVA) to yield conjugates **17** and OVA−**1**, respectively.

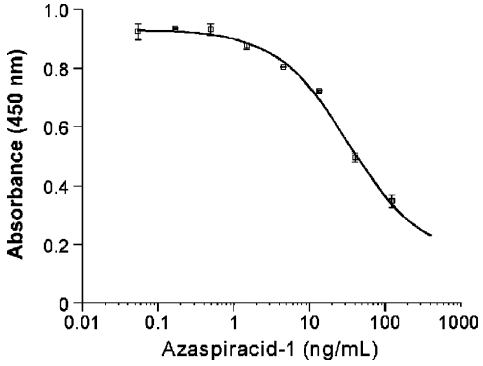


Figure 2. ELISA inhibition curve for antibodies derived from hapten **1** with natural azaspiracid 1.

Sheep were repeatedly immunized with cBSA conjugate **17** and the antisera harvested.[9b] ELISA plates were coated with OVA−**1** and blocked with polyvinylpyrrolidone. Competitive ELISA analyses were performed by adding appropriate amounts of antiserum and analyte to the ELISA wells. The bound antiserum was detected using a commercial anti-sheep−HRP conjugate, and the absorbance was measured after reaction with a colorimetric substrate.[9b] ELISA analyses showed that the antibodies in the antiserum recognized and bound to OVA−**1** in the absence of AZA1 and that this binding was inhibited by addition of **1**. Addition of AZA1 also caused concentration-dependent inhibition of antibody binding (Figure 2), indicating that the antibodies produced against **1** recognized AZA1. A partially purified extract containing a ca. 2:2:2:1 mixture of AZA1, AZA2, AZA3, and AZA6 was available from Norwegian mussels, and its AZA content was determined by LC-MS[17] against the AZA1 standard. Addition of this mixture caused a degree of inhibition of antibody binding consistent with its total AZA content, rather than just its content of AZA1. This result suggests that the antibodies also have a similar affinity for AZA2, AZA3, and AZA6 as they do for AZA1 and that such antibodies are suitable for analysis of AZAs in shellfish samples, although assay optimization will be required.

The broad recognition of the antibodies was further confirmed by immunoaffinity chromatography. The IgG fraction from the antiserum was purified using HiTrap Protein G HP columns (Amersham Biosciences, A.B.) and coupled to cyanogen bromide-activated Sepharose 4B (A.B.) according to the manufacturer's instructions. The mixture of AZA1, AZA2, AZA3, and AZA6 was applied together with a standard of yessotoxin (YTX)[18] to a column containing the immobilized anti-AZA IgG. The column was washed with buffer and eluted with methanol. LC-MS analysis of the fractions showed the YTX was present in the buffer wash and was not retained on the immunoaffinity column, but that AZA1, AZA2, AZA3, and AZA6 were retained on the column with buffer and eluted by the methanol. This shows that the antibodies produced against hapten **1** did not bind to the structurally dissimilar polyether algal toxin YTX, but did bind to a wide array of naturally occurring AZA analogues. In addition to demonstrating the broad specificity of the antibodies for AZA congeners, the immunoaffinity columns have considerable potential for concentrating and purifying shellfish extracts prior to LC-MS analysis and could be useful for identifying novel AZA metabolites.

The absolute configurational assignment of C28−C40 of the azaspiracids made initially via chiroptical correlation of synthetic

products[10] with a C26−C40 AZA1 degradation product[11] is corroborated by the recognition of natural AZA1 by the levarotatory hapten-derived antibody [(−)-AZAAb]. Further corroboration derives from fragment[19] and total synthesis.[7] The complete array of naturally occurring azaspiracids (Figure 1) has not yet been assayed against (−)-AZAAb. It is anticipated, however, that minor structural variations within the C1−C26 region will not substantially affect molecular recognition between the natural products and antibodies raised against the common F−I ring terminal domain. Preliminary studies with the azaspiracids mixture are consistent with this proposal. Optimization of the C26-linker structure in subsequent haptens is expected to enhance derived antibody sensitivity toward the natural toxins.

The results from this and an earlier study[9a] highlight the advantages of applying synthetic organic chemistry to produce carefully designed haptens containing conserved substructures of complex toxin molecules. Such haptens can be used as immunogens to generate antibodies that bind a whole family of toxin molecules. Additional advantages of this approach are that it is possible to generate antibodies to natural toxins whose availability is severely limited, as in the present study, and this approach may also be useful where the whole toxin molecule is unstable in vitro or in vivo.

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Supporting Information Available: Preparation procedures and characterization data for compounds **1**−**16**.

References

(1) (a) Twiner, M. J.; Hess, P.; Bottein, Dechraoui, M.-Y.; McMahon, T.; Samons, M. S.; Satake, M.; Yasumoto, T.; Ramsdell, J. S.; Doucette, G. J. *Toxicon* **2005**, *45*, 891−900. (b) Colman, J. R.; Twiner, M. J.; Hess, P.; McMahon, T.; Satake, M.; Yasumoto, T.; Doucette, G. J.; Ramsdell, J. S. *Toxicon* **2005**, *45*, 881−890. (c) Magdalena, A. B.; Lehane, M.; Moroney, C.; Furey, A.; James, K. J. *Food Addit. Contam.* **2003**, *20*, 154−160. (d) Roman, Y.; Alfonso, A.; Louzao, M. C.; de la Rosa, L. A.; Leira, F.; Vieites, J. M.; Vieytes, M. R.; Ofuji, K.; Satake, M.; Yasumoto, T.; Botana, L. M. *Cell. Signalling* **2002**, *14*, 703−716. (e) James, K. J.; Lehane, M.; Moroney, C.; Fernandez-Puente, P.; Satake, M.; Yasumoto, T.; Furey, A. *Food Addit. Contam.* **2002,** *19*, 555−561. (f) Ito, E.; Satake, M. *Recent Adv. Mar. Biotechnol.* **2002**, *7*, 31−39. (g) Ito, E.; Satake, M.; Ofuji, K.; Higashi, M.; Harigaya, K.; McMahon, T.; Yasumoto, T. *Toxicon* **2001**, *40*, 193−203. (h) Ito, E.; Satake, M.; Ofuji, K.; Kurita, N.; McMahon, T.; James, K.; Yasumoto, T. *Toxicon* **2000,** *38*, 917−930.

(2) James, K. J.; Moroney, C.; Roden, C.; Satake, M.; Yasumoto, T.; Lehane, M.; Furey, A. *Toxicon* **2003**, *41*, 145−151.

(3) Hungerford, J. M. *J. AOAC Int.* **2005**, *88*, 299−313.

(4) (a) Lehane, M.; Saez, M. J. F.; Magdalena, A. B.; Canas, I. R.; Sierra, M. D.; Hamilton, B.; Furey, A.; James, K. J. *J. Chromatogr., A* **2004**, *1024*, 63−70. (b) Hamilton, B.; Diaz Sierra, M.; Lehane, M.; Furey, A.; James, K. J. *Spectroscopy (Amsterdam, Netherlands*) **2004**, *18*, 355−362. (c) James, K. J.; Sierra, M. D.; Lehane, M.; Brana, Magdalena, A.; Furey, A. *Toxicon* **2003**, *41*, 277−283. (d) Moroney, C.; Lehane, M.; Brana-Magdalena, A.; Furey, A.; James, K. J. *J. Chromatogr., A* **2002**, *963*, 353−361. (e) Lehane, M.; Brana-Magdalena, A.; Moroney, C.; Furey, A.; James, K. J. *J. Chromatogr., A* **2002,** *950*, 139−147. (f) Furey, A.; Brana-Magdalena, A.; Lehane, M.; Moroney, C.; James, K. J.; Satake, M.; Yasumoto, T. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 238−242. (g) Flanagan, A. F.; Callanan, K. R.; Donlon, J.; Palmer, R.; Forde, A.; Kane, M. *Toxicon* **2001**, *39*, 1021−1027. (h) Draisci, R.; Palleschi, L.; Ferretti, E.; Furey, A.; James, K. J.; Satake, M.; Yasumoto, T. *J. Chromatogr., A* **2000**, *871*, 13−21.

(5) McEvoy, J.; McMahon, T.; Yakkundi, S.; Hess, P.; Moran, Siobhan, M. In *Proceedings of the 3rd Irish Marine Biotoxin Workshop*, Galway, Nov 14, 2002; Food Safety Authority of Ireland: Dublin, 2002; pp 37−42.

(6) Satake, M.; Ofuji, K.; Naoki, H.; James, K. J.; Furey, A.; McMahon, T.; Silke, J.; Yasumoto, T. *J. Am. Chem. Soc.* **1998**, *120*, 9967−9968.

(7) Nicolaou, K. C.; Koftis, T. V.; Vyskocil, S.; Petrovic, G.; Ling, T.; Yamada, Y. M. A.; Tang, W.; Frederick, M. O. *Angew. Chem., Int. Ed.* **2004**, *43*, 4318−4324.

(8) Geisler, L. K.; Nguyen, S.; Forsyth, C. J. *Org. Lett.* **2004**, *6*, 4159−4162.

(9) (a) Fischer, W. J.; Garthwaite, I.; Miles, C. O.; Ross, K. M.; Aggen, J. B.; Chamberlin, A. R.; Towers, N. R.; Dietrich, D. R. *Environ. Sci. Technol.* **2001**, *35*, 4849−4856. (b) Briggs, L. R.; Miles, C. O.; Fitzgerald, J. M.; Ross, K. M.; Garthwaite, I.; Towers, N. R. *J. Agric. Food Chem.* **2004**, *52*, 5836−5842.

(10) (a) Forsyth, C. J.; Hao, J.; Aiguade, J. *Angew. Chem., Int. Ed.* **2001**, *40*, 3663−3667. (b) Nicolaou, K. C.; Pihko, P. M.; Diedrichs, N.; Zou, N.; Bernal, F. *Angew. Chem., Int. Ed.* **2001**, *40*, 1262−1265. (c) Sasaki, M.; Iwamuro, Y.; Nemoto, J.; Oikawa, M. *Tetrahedron Lett.* **2003**, *44*, 6199. (d) Zhou, X.-T.; Lu, L.; Furkert, D. P.; Wells, C. E.; Carter, R. G. *Angew. Chem., Int. Ed.* Published Online: Oct 17, 2006; DOI: 10.1002/anie.200603353.

(11) Ofuji, K.; Satake, M.; Oshima, K.; Naoki, H.; Yasumoto, T. In *43rd Symposium on the Chemistry of Natural Products*; Symposium papers; Symposium on the Chemistry of Natural Products: Osaka, Japan, 2001; pp 353−358. The structures of synthetic ($[\alpha]_D^{20}$ +4.6 (*c* 0.22, CH_2Cl_2); $[\alpha]_D^{23}$ +5 (*c* 0.26, $CHCl_3$))[10a,b] and naturally derived ($[\alpha]_D^{25}$ −59 (*c* 0.016, MeOH)) products differed: the synthetic products bore *N*-carbamoyl and C27 alkenes, whereas in the degradation product the nitrogen was unfunctionalized and C26 was oxidized to a carboxylic acid.

(12) Nguyen, S.; Xu, J.; Forsyth, C. J. *Tetrahedron* **2006**, *62*, 5338−5346.

(13) (a) Eguchi, E.; Matsushita, Y.; Yamashita, *Org. Prep. Proc.* **1992**, *24*, 209. (b) Molina, P.; Vilaplana, M. J. *Synthesis* **1994**, 1197−1218.

(14) Reetz, M. T. *Angew. Chem., Int. Ed.* **1984**, *23*, 556−569.

(15) Fürstner, A. *Chem. Rev.* **1999**, *99*, 991−1045.

(16) Hermansen, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1995.

(17) Aasen, J.; Torgersen, T.; Aune, T. Application of an improved method for detection of lipophilic marine algal toxins (OA/DTXs, PTXs, YTXs, and AZAs) with LC/MS. In *Fourth International Conference on Molluscan Shellfish Safety*; Villalba, A., Reguera, B., Romalde, J. L., Beiras, R., Eds.; Xunta de Galicia and IOC of UNESCO: Santiago de Compostella, Spain, 2002; pp 49−55.

(18) Miles, C. O.; Wilkins, A. L.; Hawkes, A. D.; Selwood, A.; Jensen, D. J.; Aasen, J.; Munday, R.; Samdal, I. A.; Briggs, L. R.; Beuzenberg, V.; MacKenzie, A. L. *Toxicon* **2004**, *44*, 325−336.

(19) Nicolaou, K. C.; Koftis, T. V.; Vyskocil, S.; Petrovic, G.; Tang, W.; Frederick, M. O.; Chen, D. Y.-K.; Li, Y.; Ling, T.; Yamada, Y. M. A. *J. Am. Chem. Soc.* **2006**, *128*, 2859−2872.

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