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A TOTAL SYNTHESIS DETECTIVE STORY

Saga of marine toxin's structure determination and synthesis has many twists and turns

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Unless cast-iron evidence for the structure of a natural product is available, the total synthesis of a compound isolated from natural sources can be a molecular gamble, observes Joe B. Sweeney. A reader in chemistry at the University of Reading, in England, Sweeney carries out research on the synthesis of biologically important molecules.

"The risk of major pitfalls awaiting practitioners of the art of total synthesis is almost unavoidable, especially where only very small amounts of a very potent natural material are available," he says.

In the case of azaspiracid-1, a shellfish toxin, the gamble initially failed. This is the story of that failure, and what happened next.

The story starts in November 1995 with an incident of human food poisoning in the Netherlands following the consumption of blue mussels, *Mytilus edulis*, that had been harvested in Killary Harbour, Ireland. The symptoms--nausea, vomiting, severe diarrhea, and stomach cramps--were similar to diarrhetic shellfish poisoning (DSP). Levels of DSP toxins in the mussels, however, were found to be very low, so the involvement of a new marine toxin was suspected.

SEA SLEUTHS Postdocs Taotao Ling (from left), Wenjun Tang, Goran Petrovic, Theocharis V. Koftis, and Stepan Vyskocil, and Ph.D. student Michael Frederick at Scripps Research Institute and UC San Diego looked for clues to revise the structure of azaspiracid-1.
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In 1998, chemistry professors Takeshi Yasumoto and Masayuki Satake and their coworkers at Tohoku University, Sendai, Japan, reported the isolation of a 2-mg sample of azaspiracid from the contaminated mussels and identified the compound, a colorless amorphous solid, as the major causative agent of the Netherlands outbreak [*J. Am. Chem. Soc.*, **120**, 9967 (1998)].

The following year, Yasumoto and coworkers isolated the compound and two new analogs from mussels collected at Aran Island (Arranmore), Ireland. The analogs, named azaspiracid-2 and azaspiracid-3, were slightly more potent than the original compound, azaspiracid-1, but were present in much lower concentrations. The levels of DSP toxins in the mussels were again insignificant. The team therefore named the new toxic syndrome azaspiracid poisoning.

NICOLAOU
PHOTO COURTESY OF K. C. NICOLAOU

Azaspiracids are neurotoxins produced by marine microalgae in the shellfish food chain. Last year, a report revealed that the toxic mussels are widely distributed at certain times of the year along the western coastal region of Ireland and that azaspiracids accumulate in other bivalve mollusks, including oysters, scallops, clams, and cockles [*Environ. Sci. Technol.*, **37**, 3078 (2003)]. The azaspiracids have also been identified in shellfish from France and Spain [*Toxicon*, **42**, 105 (2003)]. Both studies were carried out by Kevin J. James, a principal investigator at Ireland's Cork Institute of Technology, and coworkers.

In their 1998 paper, Yasumoto and coworkers proposed the structure of azaspiracid-1 on the basis of nuclear magnetic resonance and mass spectrometric analyses of their small sample of the toxin. The proposed structure has two four-ring systems, ABCD and FGHI, bridged by an E-ring system, and 20 stereogenic centers. However, the group was unable to elucidate fully the relative stereochemistry between the ABCDE (C1-27) and FGHI (C28-40) domains and the absolute stereochemistry of the structure.

IN VIEW OF the health and environmental hazards of the toxin and to clarify its structure, several research groups around the world embarked on its total synthesis following the report of its structure.

"The total synthesis became urgent since it was apparent that the substance was badly needed as a standard for analytical purposes," explains K. C. Nicolaou, chemistry professor at Scripps Research Institute and the University of California, San Diego, who led one of the groups. "The substance was also needed to develop antibodies against it in order to develop bioassays for testing mussels and other seafoods for its presence and quantity before sending the seafood to market."

The Nicolaou group synthesized the two possible diastereoisomers of the structure reported by the Japanese team, but neither matched the natural substance chromatographically or spectroscopically.

"At that point, the real odyssey that turned into a fascinating detective story began," Nicolaou says. "After synthesizing the obvious diastereoisomers and proving them to be wrong, we began a rational approach that relied on subtle clues and chemical synthesis efforts."

The structure and nuclear magnetic resonance spectral data of lissoketal, a marine natural product isolated from sea grass blades in shallow waters around Palau in the Pacific Ocean, yielded the first clue. Although the lissoketal molecule is much smaller than azaspiracid-1, it contains a structural domain that is similar to the AB region of the acid. The remarkable resemblance of the NMR spectra of the two compounds led the Nicolaou team to conclude that the double bond in the A ring of azaspiracid-1 is located at the C7-8 position and not at C8-9 as originally reported.

"We synthesized the new structure corresponding to the ABCD ring system," Nicolaou says, but that, too, proved to be wrong.

His team then collaborated with Japan's Satake group members, who by now possessed only a minute amount of natural azaspiracid-1. By chemical means, the researchers split the molecule into its ABCD and EFGHI components for comparison with their synthetic counterparts.

"These comparisons of degradatively derived materials with synthetic materials allowed us to locate the structural discrepancies in the ABCD region while telling us what the EFGHI domain was precisely," Nicolaou says.

Nicolaou and coworkers then focused their attention on determining the true structure of the ABCD region. However, this four-ring fragment has seven stereogenic centers and therefore 2^7 or 128 possible structures--too many for synthesis. The team used computer and manual modeling studies to narrow the field down to two candidates.

The key difference between the two relates to a double-spiroketal bridge that spans the A, B, and C rings. The bridge has two carbon atoms, known as spirocenters: one at C10 that the A and B rings have in common, and one at C13 that the B and C rings have in common.

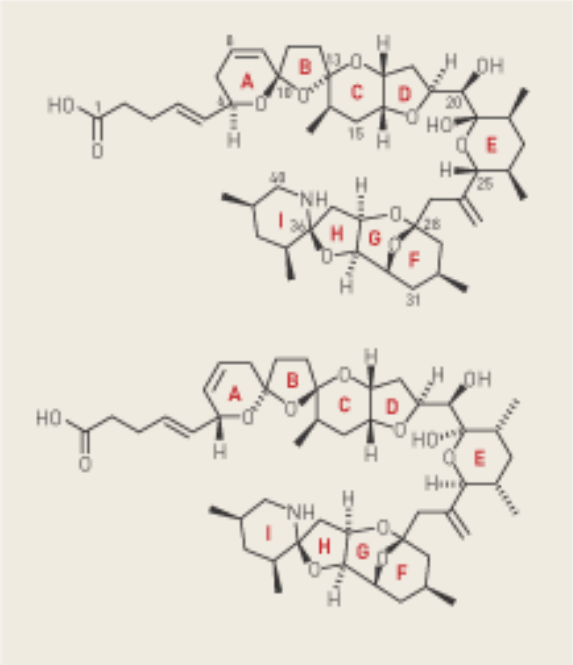
The team initially attempted to synthesize the candidate with the same ABC double-spiroketal bridge architecture as that in the structure proposed in 1998 but with the opposite stereochemistry of the methyl group situated on ring C. The synthetic material proved to be less stable in acidic conditions than the material obtained by degradation of the natural product. The chemists were once again on the wrong trail.

The team then synthesized the second candidate, in which the double-spiroketal bridge is inverted. The product was more stable, and NMR spectroscopy revealed that it matched the ABCD compound obtained from natural azaspiracid-1.

"Due to the tiny amounts of the natural product available, there was not enough degradation product to measure accurately its optical rotation, and thus the absolute stereochemistry of the ABCD ring system remained in question," Nicolaou says. "Luckily, the optical rotation of the FGHI part of the molecule was barely measurable, so we could determine the absolute stereochemistry of that region."

The Nicolaou team synthesized both enantiomers of the second candidate for the ABCD ring segment (C1-20). The chemists then used a convergent strategy for the total synthesis. They combined one of the enantiomers of the ABCD segment with the E segment (C21-27) and the FGHI segment (C28-40) through the use of two well-known coupling reactions: dithiane coupling and palladium-catalyzed cross-coupling.

"The first enantiomer of the ABCD system to be incorporated into the new structure of azaspiracid-1 turned out to be the wrong one, leading to the wrong diastereoisomer of the natural product," Nicolaou observes. "Murphy's law had once again prevailed. It was only after the incorporation of the second enantiomer into the final structure that we were led, by total synthesis, to the true structure of azaspiracid-1.



SPOT THE DIFFERENCE The incorrect (top) and correct structures of azaspiracid-1 both have three spirocenters (at C10, 13, and 36); a carboxylic acid chain attached to ring A; and an unusual azaspiro ring structure (I ring), after which the compound is named.

"Rarely before have we experienced such a drama in a quest for a natural product in my group," he continues. "This is not to criticize structural chemists whose pioneering contributions keep us all so busy. It shows that, because of the stunning advances in spectroscopic techniques, including mass spectrometry and X-ray crystallography, isolation chemists are able to make daring proposals of highly complex natural products working with only minute amounts of material."

THE TOTAL synthesis of azaspiracid-1, starting from l-malic acid, involved 96 steps. The longest linear sequence in the synthesis is 50 steps with an overall yield in this sequence of 0.00017%. The structural revision and total synthesis are described in two papers published this month [*Angew. Chem. Int. Ed.*, **43**, 4312 and 4318 (2004)].

"As a synthetic chemist, it can be painfully frustrating to discover that you have been chasing 'phantom molecules' and not the true natural product structure," comments Ian Paterson, chemistry professor at the University of Cambridge. "The azaspiracid saga represents an intriguing detective story as well as a classic adventure in complex target molecule synthesis and structural elucidation, relying throughout on the combination of chemical insight; flexible synthetic planning; and, above all, sheer perseverance."

Sweeney remarks that the work is deeply impressive. "It is a great demonstration of the power of modern stereoselective, target-oriented synthesis, especially when reinforced by

cutting-edge analytical methods," he says. "However, even with the efficiency of modern synthetic methodology, there can oftentimes be little substitute for sheer weight of manpower. This Herculean task could not have been completed without the provision of a large and highly skilled team of coworkers."

As a result of this Herculean effort, Nicolaou and coworkers have now synthesized about 10 mg of synthetic azaspiracid-1.

"The synthesis is significant because, until now, the only available source of the toxin has been contaminated shellfish, which are in limited supply and give very low yields," says Marian Kane, manager of the National Diagnostics Centre, National University of Ireland, Galway.

KANE'S INTEREST in azaspiracids started in the late 1990s when she became involved in Ireland's national shellfish monitoring program.

"At that time, the mouse bioassay was the only method for the detection of azaspiracid contamination in shellfish," she explains. "We started to explore alternative assay procedures and succeeded in developing a cell-culture-based cytotoxicity assay which could distinguish azaspiracids from the DSP class of toxins. The cell assay was equivalent in sensitivity to the mouse assay."

The European Union, however, has set limits on permitted levels of azaspiracids in shellfish that are below the detection limits of either of the cell and mouse assays. Screening for the presence of azaspiracids has therefore recently employed more sensitive liquid chromatography and mass spectrometry assays.

"Antibody-based assays could also provide the sensitivity required and would be much more convenient than current methods," Kane says. "To date, the very limited availability of purified azaspiracids has hindered the development of the specific antibodies for azaspiracids which are needed for immunoassay development."

The maximum contamination level of DSP-type toxins permitted by EU legislation reflects the sensitivity limit of the animal test and not necessarily the minimum concentration of toxin that could induce severe poisoning in humans, notes Reinhard Tiebach, head of the National Reference Laboratory for the Control of Marine Biotoxins, Berlin.

"Alternative methods of analysis for the quantitative determination of marine biotoxins are allowed only if they are validated according to an internationally accepted protocol," he explains. "The required validation of any alternative method of analysis has to be performed using certified reference standards or certified reference materials with a known content of toxic agent. With the synthesis of azaspiracid-1, we now have at hand a chemical compound that allows the validation of analytical methods for a group of marine biotoxins and consequently the replacement of the animal test by a chemical method of analysis."

Nicolaou's team is now in the process of making more azaspiracid-1.

"Whether this will be enough to satisfy the demand, I do not know because I have no feeling

for how much is needed," Nicolaou says. "We are, in the meantime, planning to optimize our route to cut down the number of steps and increase the overall yield."

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