Biosynthesis Hot Paper

Deutsche Ausgabe: DOI: 10.1002/ange.201602971
Internationale Ausgabe: DOI: 10.1002/anie.201602971

Cyclic Guanidine Compounds from Toxic Newts Support the Hypothesis that Tetrodotoxin is Derived from a Monoterpene

*Yuta Kudo, Takeshi Yasumoto, Dietrich Mebs, Yuko Cho, Keiichi Konoki, and Mari Yotsu-Yamashita**

Abstract: *The biosynthesis of tetrodotoxin (TTX), a potent neurotoxin consisting of a 2,4-dioxaadamantane skeleton and a guanidine moiety, is an unsolved problem in natural product chemistry. Recently, the first C5–C10 directly bonded TTX analogue, 4,9-anhydro-10-hemiketal-5-deoxyTTX, was obtained from toxic newts and its carbon skeleton suggested a possible monoterpene origin. On the basis of this hypothesis, screening of predicted biosynthetic intermediates of TTX was performed using two MS-guided methods. Herein, five novel cyclic guanidine compounds from toxic newts are reported which commonly contain a cis-fused bicyclic structure including a six-membered cyclic guanidine. These structures could be biosynthetically derived from geranyl guanidine through oxidation, cyclization, and/or isomerization steps. LC–MS analysis confirmed the widespread distribution of the five novel compounds in toxic newt species. These results support the hypothesis that TTX is derived from a monoterpene.*

Tetrodotoxin (TTX; **1**),[1] a potent and selective blocker of voltage-gated sodium ion channels,[2] causes fatal pufferfish poisoning. A recent study also advised caution with regards to low concentrations of TTX contamination in bivalve mollusks from some areas of Europe.[3] This molecule is still attracting the interest of pharmacologists and synthetic chemists.[4] TTX is distributed among various marine (such as pufferfish and snails)[5] and terrestrial (newts, frogs, and toads)[6] animals. In spite of the high interest in this unique toxin, its biosynthetic pathway is still unknown. Although toxic marine animals are considered to accumulate TTX produced by several species of bacteria,[7] bacterial gene clusters corresponding to TTX biosynthesis have not been elucidated thus far. In several studies, problems involving the isolation and cultivation of TTX-producing bacteria have been reported.[8] The debate on the internal or external origin of TTX in toxic terrestrial animals still continues,[9] but recently, no significant production of TTX or of its analogues have been observed in captive-reared newts.[10] Feeding experiments performed in toxic newts by Shimizu and Kobayashi[11] resulted in no incorporation of potential TTX precursors. Although biosynthetic pathways of some natural toxins that contain guanidine group(s) have been elucidated,[12] the actual biosynthesis of TTX is still a matter of speculation.

In an attempt to obtain clues for the biosynthesis of TTX, we[13] and Kotaki and Shimizu[14] identified several TTX analogues and predicted arginine and a C5 unit (isoprene or sugar) as precursors. However, we recently discovered the first C5–C10 directly bonded TTX analogue, named 4,9-anhydro-10-hemiketal-5-deoxyTTX (**2**), which is widely distributed among toxic newt species.[15] Based on its chemical structure (**2**), which consists of a guanidine and a C10 unit, we hypothesized that a monoterpene (geranyl pyrophosphate) is a precursor of TTX in terrestrial animals (Figure 1).[15]

On the basis of this proposed pathway, we performed mass spectrometry (MS)-guided screening to find possible biosynthetic intermediates of TTX at an early stage, assuming that geranyl guanidine is a key intermediate (geranyl guanidine is a linear monoterpene with a guanidine group). In this study, we predicted various guanidine compounds which could be derived from geranyl guanidine by oxidation and searched for them in toxic newts. Herein, we present five novel cyclic guanidine compounds that could be related to the synthesis of TTX.

The proposed TTX intermediates with molecular formulae $C_{11}H_{[x]}N_3O_{[y]}$ (Figure 1) were comprehensively screened using hydrophilic interaction chromatography (HILIC)[13c,16] and reverse-phase (RP) chromatography high-resolution LC–MS methods. As a result, five peaks corresponding to possibly novel compounds (**3**–**7**) were detected in the Japanese swordtail newt, *Cynops ensicauda popei* (see Figure S1 in the Supporting Information; for the molecular formulae of **3**–**7**, see page S23 in the Supporting Information).

Cep-210 (**3**) and Cep-212 (**4**; see Figure 2 for molecular structures of **3** and **4**) were obtained from both methanol and MeOH/H_2O (1:1, v/v) whole body extracts (415 g, 70 specimens) except for the viscera of *C. e. popei*. The extract was purified on reverse-phase columns and on a weak cation-exchange column. Eventually, **3** (69 nmol; estimated by ^{1}H NMR spectroscopy) and **4** (130 nmol) were isolated. To obtain compounds **5**–**7**, *C. e. popei* and Japanese fire-bellied newts, *C. pyrrhogaster*, were extracted with 0.2 M acetic acid at 100 °C, and then purified on an activated charcoal column, on weak cation-exchange columns, and a HILIC column. For **7**, additional purification was performed on a reverse-phase column. Finally, Cep-242 (**5**, 2.2 μmol; estimated by

[*] Dr. Y. Kudo, Dr. Y. Cho, Prof. Dr. K. Konoki, Prof. Dr. M. Yotsu-Yamashita
Graduate School of Agricultural Science, Tohoku University
1-1 Tsutsumidori-Amamiyamachi, Aoba-ku
Sendai, Miyagi, 981-8555 (Japan)
E-mail: myama@biochem.tohoku.ac.jp
Prof. Dr. T. Yasumoto
Tama Laboratory, Japan Food Research Laboratories
6-11-10 Nagayama, Tama-shi, Tokyo, 206-0025 (Japan)
Prof. Dr. D. Mebs
Institute of Legal Medicine, University of Frankfurt
Kennedyallee 104, 60596 Frankfurt (Germany)

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201602971.

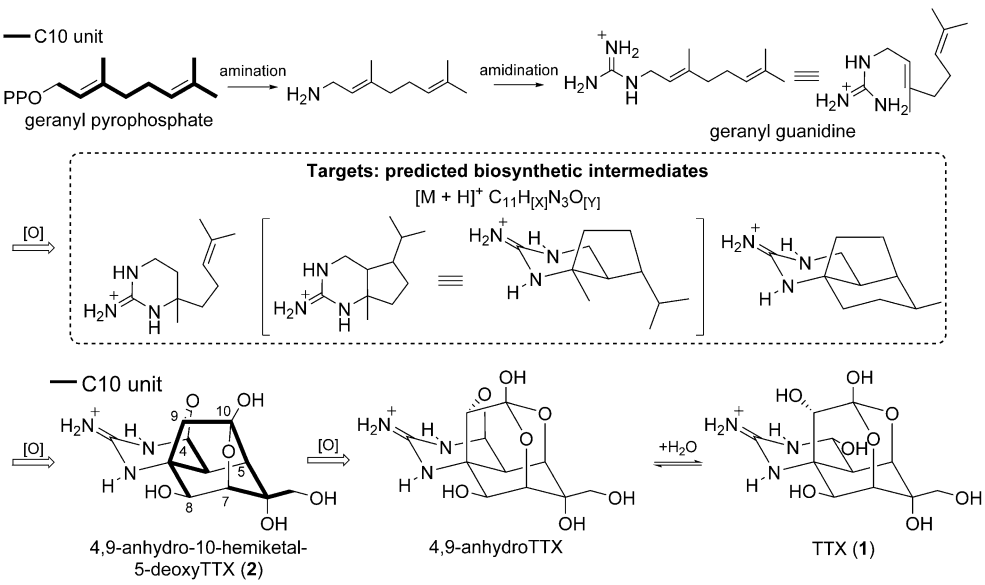

Figure 1. Proposed biosynthetic pathway of TTX based on the structure of **2**,[15] and the predicted intermediates as the target molecules in this study.

^{1}H NMR), Cep-240 (**6**, 0.37 μmol; by ^{1}H NMR) and Cpy-240 (**7**, 0.46 μmol; by ^{1}H NMR) were obtained (see Figure 3 for molecular structures of **5**–**7**).

The NMR spectra of compounds **3** and **4** were measured in CD_3OD. The signals for all protons, except for those which undergo exchange with deuterium atoms in **3**, were assigned (Table S1).

The planar structure of **3** was identified as a bicyclic compound consisting of a six-membered cyclic guanidine and a five-membered carbon ring based on COSY and HMBC correlations (Figure 2). The stereochemistry of **3** was elucidated by NOESY 1D experiments. The mutual NOEs between H4a and the three protons at the C8 position enabled us to deduce that **3** is a *cis*-fused bicyclic compound. The geometry of the C5–C6 alkene was confirmed to be in the *Z* configuration through the appearance of positive NOEs between the protons at the C10 and C11 positions (Figure 2).

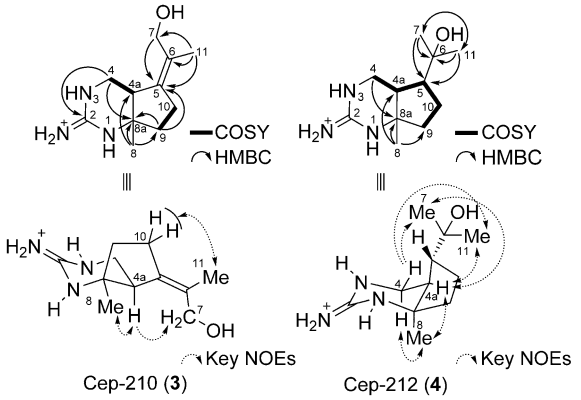

Figure 2. The planar structures of **3** and **4** with 2D NMR correlations and their proposed stereostructures with key NOEs. The atom numbering scheme is based on that used for **1**.

Compound **4** was also elucidated as containing a carbon backbone similar to **3** on the basis of various 2D NMR experiments (see Table S1; Figure 2). Although a HMBC correlation between C2/H4 was not detected in **4**, the existence of a guanidine moiety in **4** was assumed from the molecular formula and the chemical shifts for the C4 and C8a centers in **4**, which were similar to those of C4 and C8a centers in **3**. The relative stereochemistry of **4** was assigned on the basis of NOEs as shown in Figure 2.

The NMR spectra of **5**–**7** were measured in CD_3COOD/D_2O (4:96 v/v). Compound **5** was observed as a mixture of two components, which were assigned as Cep-242 (**5a**) and Cep-242-hemiketal (**5b**; by ^{1}H NMR spectroscopy, the relative ratio of **5a**:**5b** was estimated to be 8:3). Both structures were elucidated as *cis*-fused bicyclic compounds with a six-membered cyclic guanidine and a seven-membered carbon ring by NMR analysis (Table S2, Figure 3; see also Figure S10). Compound **5b** was identified as a C6-*O*-C9 hemiketal of **5a** based on the chemical shifts of the C6 and C9 centers in **5b**, and the equilibrium between **5a** and **5b** was indicated by ^{1}H NMR spectroscopy. The correlation between the resonance signal for the N1 center and the three protons at the C8 position in the ^{15}N-HMBC spectrum clearly revealed the direct bonding of N1 to C8a in **5a** and **5b**. The positive NOEs that were detected between H4a and H_3-8 demonstrated their *cis*-configuration at C4a–C8a in **5a** and **5b**. Although it is difficult to determine the stereochemistry of C5 and C6 centers in **5a**, the relative stereostructure of **5b** was suggested by the key NOEs as shown Figure 3. The stereochemistry of **5a** should be identical to that of **5b** since all NOE data from **5a** showed no conflicts with the proposed structure. Compounds **6** and **7** were elucidated as **5b**-type compounds as shown Figure 3 (Tables S3, S4). To confirm their structures, the reduction of **7** to **5** by $NaBH_4$ in 0.5 M acetic acid was performed (Figure S3).

The identification of **3**–**7** in this study supports the hypothesis that TTX is derived from a monoterpene, because these compounds are predicted to be derived from geranyl guanidine through the routes shown in Figure 4 (see also Figure S5).

Compounds **3** and **4** have a common skeleton structure that is equivalent to the C7–C8 cleaved structure of **2**. The transformation from geranyl guanidine to **3** and **4** could be initiated by epoxidation of the dimethyl-substituted olefin, followed by sequential cyclization, which was likely induced by the acid-catalyzed ring opening of this epoxide (Fig-

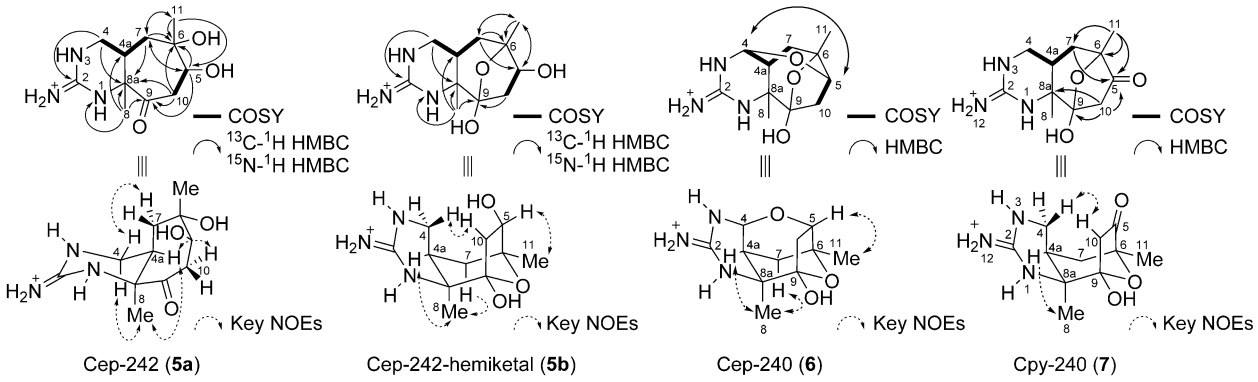

Figure 3. The planar structures of **5a**, **5b**, **6**, and **7** with 2D NMR correlations and their proposed stereostructures with key NOEs. Atom numbering scheme is based on that used for **1**.

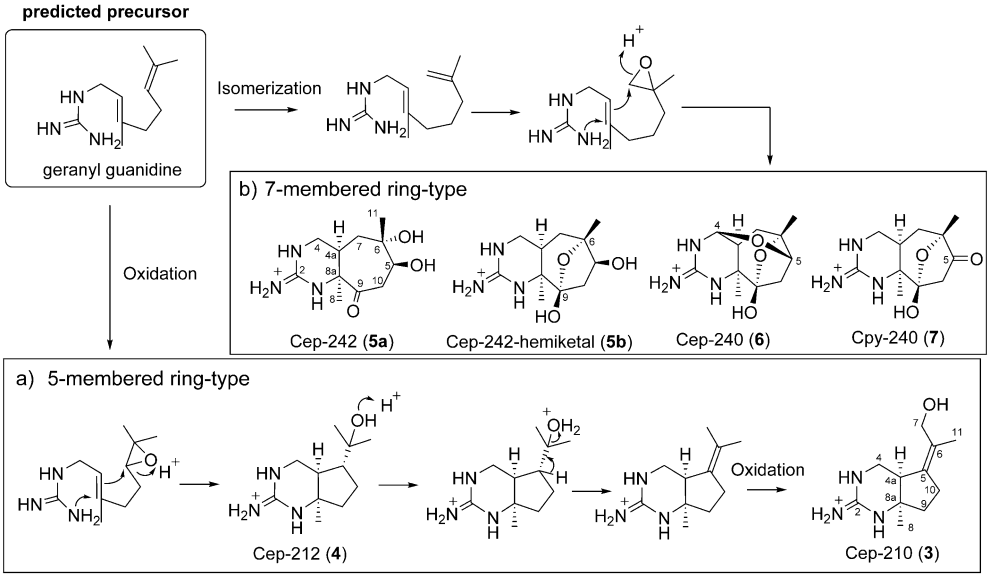

Figure 4. Proposed biosynthetic pathways to the cyclic guanidine compounds from geranyl guanidine: a) **3** and **4**, b) **5–7**.

ure 4a). These reactions could form the bicyclic structure of **3** and **4**, which is consistent with Baldwin's rules.[17] We have not obtained related compounds to predict the mechanism underlying the formation of the C7–C8 bond in **3** and **4** to build the tricyclic skeleton of **2**. However, oxidation at the C7 and C8 positions may be necessary to form a bond between them, because all natural TTX analogues found so far possess oxygen atoms at both C7 and C8 centers. Likewise, the structures of compounds **5–7** could be derived from geranyl guanidine through isomerization of the olefin followed by epoxidation and sequential cyclization to form N1–C8a and C4a–C7 bonds (Figure 4b). However, compounds **5–7** may be shunt products because the formation of **2** or **1** from **5–7**, consisting of a seven-membered ring, is not conclusively predictable.

The distribution of **1** and **3–7** was examined in 22 specimens of six newt species from the USA, Japan, and China using two LC–MS methods (Figure 5; Table S5). Compounds **3–7** were found in almost all species tested.

The statistical analysis of concentrations indicated that significant correlations exist ($P < 0.01$, Spearman's rank correlation coefficient test) between **3** and TTX ($r_s = 0.72$; Figure S6) and between **4** and TTX ($r_s = 0.66$; Figure S7), respectively (see the Supporting Information for **5–7**, pages S20—S22, Figures S8, S9). In contrast, **3–7** were not detected in the captive-reared nontoxic newt *C. pyrrhogaster* (Table S5).[10] Similarly, in weakly toxic newts, specifically *Taricha granulosa* from South Alaska ([TTX] = $< 1\ \mu g\,g^{-1}$), only **3** was identified at the limit of quantitation level (Table S5). Interestingly, compounds **2–7** have not been detected to date in marine organisms such as pufferfish, snails, or others. Differences in the distribution of some TTX analogues have also been observed between marine and terrestrial animals (see Figure S18). Unlike TTX, some other guanidine toxins such as anatoxin-a(s),[12a] cylindrospermopsin,[12b] and saxitoxin[12c] are all reportedly biosynthesized from amino acids in microorganisms. However, the guanidine alkaloids, nitensidines[18a] and plumbagines[18b] isolated from plants, and siphonodictidine[18c] isolated from marine sponges, are predicted to be derived from monoterpenes or sesquiterpenes because of their structures. The enzymes that catalyze such *N*-heterocyclizations might be unusual terpene cyclases.[19]

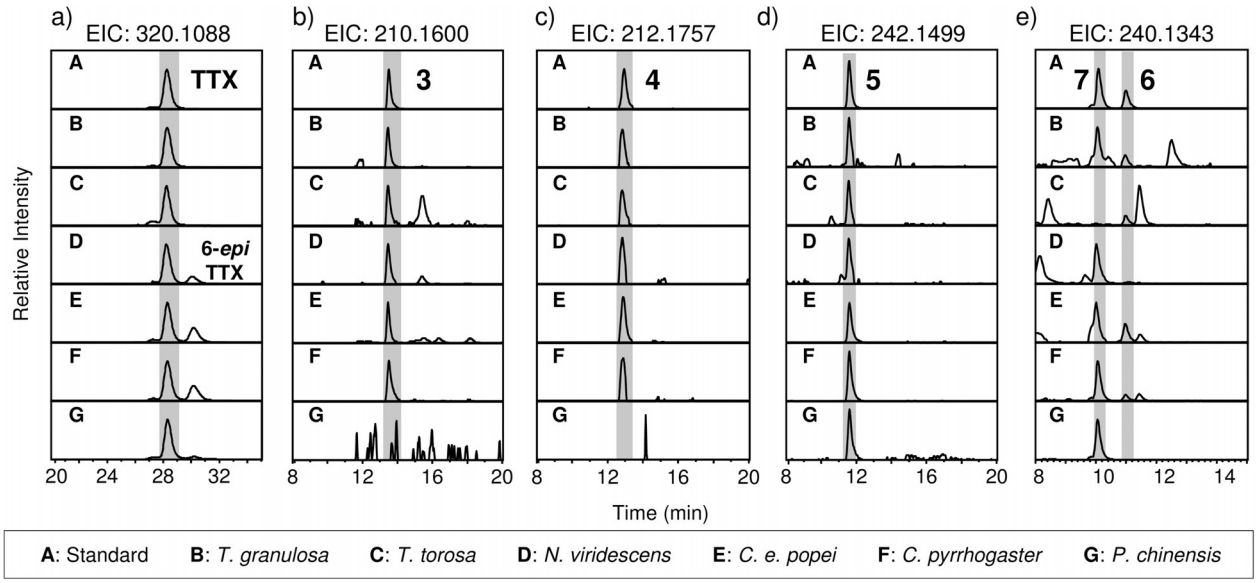

Figure 5. Extracted ion chromatograms (EICs) of a, d, e) hydrophilic interaction chromatography LC–MS and b, c) reverse-phase LC–MS for TTX (**1**) and **3**–**7** from six species of toxic newts.

Acknowledgements

This work was funded by the JSPS (no. LS012) and the KAKENHI (no. 26292057) to M.Y.-Y. Y.K. is a research fellow of the JSPS (DC1; no. 25-5534).

Keywords: biosynthesis · mass spectrometry · natural products · structure elucidation · tetrodotoxin

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 8728–8731
Angew. Chem. **2016**, *128*, 8870–8873

[1] a) K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, K. Sakai, C. Tamura, O. Amakasu, *Chem. Pharm. Bull.* **1964**, *12*, 1357–1374; b) R. B. Woodward, *Pure Appl. Chem.* **1964**, *9*, 49–74; c) T. Goto, Y. Kishi, S. Takahashi, Y. Hirata, *Tetrahedron* **1965**, *21*, 2059–2088.

[2] a) T. Narahashi, J. W. Moore, W. R. Scott, *J. Gen. Physiol.* **1964**, *47*, 965–974; b) C. Y. Kao, *Pharm. Rev.* **1966**, *18*, 997–1049.

[3] a) A. D. Turner, A. Powell, A. Schofield, D. N. Lees, C. Baker-Austin, *Euro. Surveill.* **2015**, *20*(2), pii = 21009; b) A. Vlamis, et al., *Toxins* **2015**, *7*, 1779–1807.

[4] a) N. Ohyabu, T. Nishikawa, M. Isobe, *J. Am. Chem. Soc.* **2003**, *125*, 8798–8805; b) A. Hinman, J. Du Bois, *J. Am. Chem. Soc.* **2003**, *125*, 11510–11511; c) T. Nishikawa, M. Isobe, *Chem. Rec.* **2013**, *13*, 286–302.

[5] a) T. Yasumoto, H. Nagai, D. Yasumura, T. Michishita, A. Endo, M. Yotsu, Y. Kotaki, *Ann. N. Y. Acad. Sci.* **1986**, *479*, 44–51; b) T. Noguchi, O. Arakawa, *Mar. Drugs* **2008**, *6*, 220–242.

[6] a) H. D. Buchwald, L. Durham, H. G. Fischer, R. Harada, H. S. Mosher, C. Y. Kao, F. A. Fuhrman, *Science* **1964**, *143*, 474–475; b) Y. H. Kim, G. B. Brown, H. S. Mosher, F. A. Fuhrman, *Science* **1975**, *189*, 151–152.

[7] a) T. Yasumoto, D. Yasumura, M. Yotsu, T. Michishita, A. Endo, Y. Kotaki, *Agric. Biol. Chem.* **1986**, *50*, 793–795; b) T. Noguchi, J. Jeon, O. Arakawa, H. Sugita, Y. Deguchi, Y. Shida, K. Hashimoto, *J. Biochem.* **1986**, *99*, 311–314.

[8] L. R. Salvitti, S. A. Wood, P. McNabb, S. C. Cary, *Toxins* **2015**, *7*, 255–273.

[9] C. T. Hanifin, *Mar. Drugs* **2010**, *8*, 577–593.

[10] Y. Kudo, C. Chiba, K. Konoki, Y. Cho, M. Yotsu-Yamashita, *Toxicon* **2015**, *101*, 101–105.

[11] Y. Shimizu, M. Kobayashi, *Chem. Pharm. Bull.* **1983**, *31*, 3625–3631.

[12] a) B. S. Moore, I, Ohtani, C. B. de Koning, R. E. Moore, W. W. Carmichael, *Tetrahedron Lett.* **1992**, *33*, 6595–6598; b) T. K. Mihali, R. Kellmann, J. Muenchhoff, K. D. Barrow, B. A. Neilan, *Appl. Environ. Microbiol.* **2008**, *74*, 716–722; c) R. Kellmann, T. K. Mihali, Y. J. Jeon, R. Pickford, F. Pomati, B. A. Neilan, *Appl. Environ. Microbiol.* **2008**, *74*, 4044–4053.

[13] a) T. Yasumoto, M. Yotsu, M. Murata, H. Naoki, *J. Am. Chem. Soc.* **1988**, *110*, 2344–2345; b) Y. Kudo, T. Yasumoto, K. Konoki, Y. Cho, M. Yotsu-Yamashita, *Mar. Drugs* **2012**, *10*, 655–667; c) M. Yotsu-Yamashita, Y. Abe, Y. Kudo, R. Ritson-Williams, V. J. Paul, K. Konoki, Y. Cho, M. Adachi, T. Imazu, T. Nishikawa, M. Isobe, *Mar. Drugs* **2013**, *11*, 2799–2813; d) T. Yasumoto, M. Yotsu-Yamashita, *J. Toxicol.* **1996**, *15*, 81–90; e) M. Yotsu-Yamashita, *J. Toxicol. Toxin. Rev.* **2001**, *20*, 51–66.

[14] Y. Kotaki, Y. Shimizu, *J. Am. Chem. Soc.* **1993**, *115*, 827–830.

[15] Y. Kudo, Y. Yamashita, D. Mebs, Y. Cho, K. Konoki, T. Yasumoto, M. Yotsu-Yamashita, *Angew. Chem. Int. Ed.* **2014**, *53*, 14546–14549; *Angew. Chem.* **2014**, *126*, 14774–14777.

[16] M. Yotsu-Yamashita, J. H. Jang, Y. Cho, K. Konoki, *Forensic Toxicol.* **2011**, *29*, 61–64.

[17] J. E. Baldwin, *J. Chem. Soc. Chem. Commun.* **1976**, 734–736 (DOI: 10.1039/C39760000734).

[18] a) L. O. Regasini, et al., *J. Nat. Prod.* **2009**, *72*, 473–476; b) H. J. Cong, S. W. Zhang, Y. Shen, Y. Zheng, Y. J. Huang, W. Q. Wang, Y. Leng, L. J. Xuan, *J. Nat. Prod.* **2013**, *76*, 1351–1357; c) B. Sullivan, D. J. Faulkner, L. Webb, *Science* **1983**, *221*, 1175–1176.

[19] M. Baunach, J. Franke, C. Hertweck, *Angew. Chem. Int. Ed.* **2015**, *54*, 2604–2626; *Angew. Chem.* **2015**, *127*, 2640–2664.

Received: March 31, 2016
Published online: June 1, 2016

