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Synthesis and identification of proposed biosynthetic intermediates of saxitoxin in the cyanobacterium *Anabaena circinalis* (TA04) and the dinoflagellate *Alexandrium tamarense* (Axat-2)†

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Here, we describe the synthesis of the genetically predicted biosynthetic intermediates of the neurotoxin saxitoxin (STX) (1), 2, 6 and 7, and identification of 2 and 6 in toxin-producing microorganisms. This is the first chemical evidence supporting the genetically predicted biosynthetic route toward 1.

Saxitoxin (STX) (**1**)[1] is a well-known guanidine neurotoxin, one of the agents in the paralytic shellfish toxins (PSTs) and the most potent and selective voltage-gated sodium channel blocker.[2] Its high potency and characteristic structure have attracted synthetic chemists.[3] PSTs are responsible for human food poisoning and cause serious damage to the production of shellfish worldwide.[1] These toxins are produced by several species of freshwater cyanobacteria[4] and marine dinoflagellates.[5] The biosynthetic pathway that produces STX was first proposed by Shimizu *et al*.[6] based on the results of feeding experiments using stable isotope labelled compounds with the toxin-producing cyanobacterium. In the proposed route (Fig. 1a) by Shimizu *et al*.,[6] an acetate group was introduced to the α-carbon of L-arginine through a Claisen-condensation, releasing CO_2 to form the bi-cyclic guanidino-keto-amine (i); this structure was converted to the bis-guanidino-ketone (ii), and a methyl group was transferred from *S*-adenosyl methionine to form the C-13 of STX. Recently, the STX biosynthetic gene cluster (*sxt*) for a cyanobacterium has been reported by Kellmann *et al*.;[7] they proposed a different route from that proposed by Shimizu *et al*., as shown in Fig. 1b.[7] In the route of Kellmann *et al*., Intermediate-A' (Int-A') (**2**) was predicted to be synthesized from L-arginine, which accepts the propionyl group from the propionyl-acyl carrier protein and releases CO_2.

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†Electric supplementary information (ESI) available: Detailed experimental procedures, characterization data and ^1H and ^{13}C NMR data.

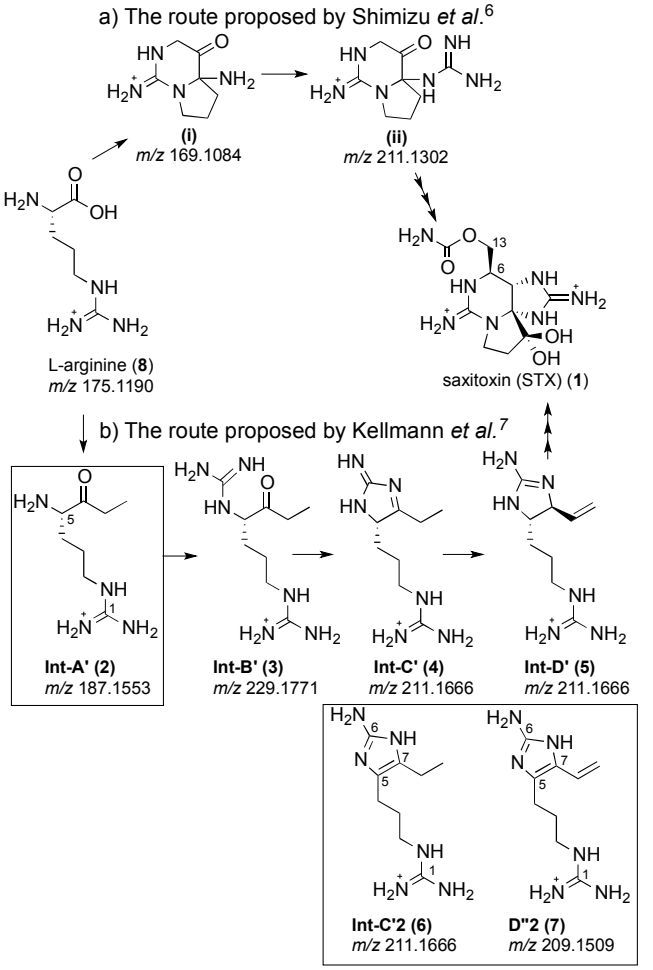


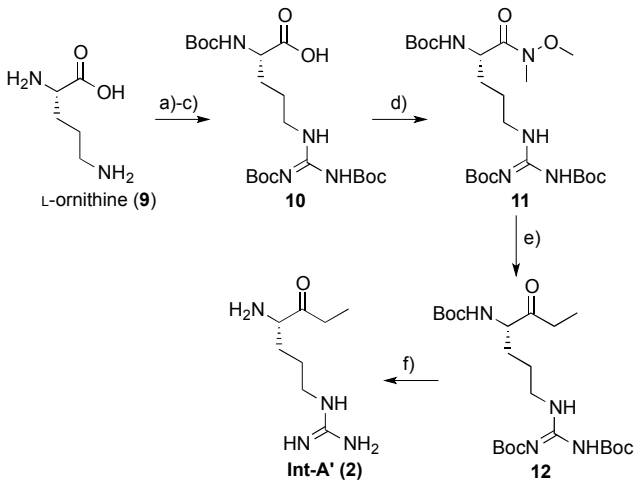
Fig.1 Proposed biosynthetic routes (partial) toward saxitoxin (STX)(**1**) by Shimizu *et al*.[6] (a) and Kellman *et al*.[7] (b), and the synthesized compounds in this study (**2**,**6**,**7** in the boxes).

Then, **2** should form a bis-guanidino-ketone, Int-B' (**3**), which is then cyclized to form Int-C' (**4**) and then Int-D' (**5**) and, finally, STX after several steps.

These reactions were predicted to be catalyzed by the enzymes (SxtA-U) coded by *sxtA-U* genes.[7] The corresponding genes were also found in dinoflagellate strains *Alexandrium* by Stüken *et al*.[8] However, each enzymatic reaction and the corresponding intermediates have not been sufficiently supported by the chemical and analytical data. We planned to determine which route is more plausible (a or b) in Fig. 1 by analysing the predicted intermediates during early STX biosynthesis.

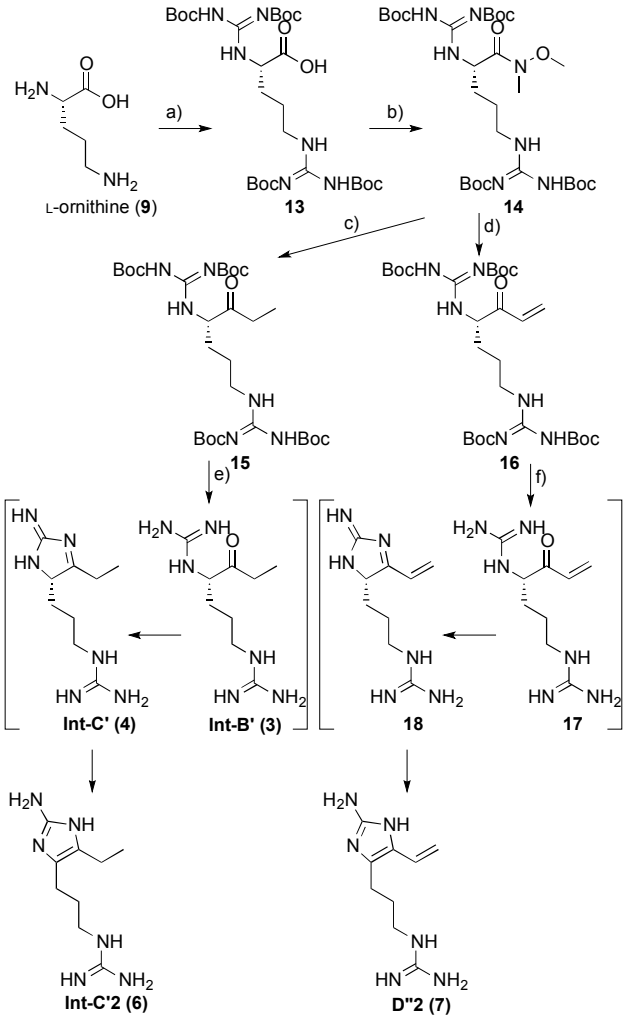
In this report, we describe the synthesis of **2** and two related compounds named Int-C'2 (**6**) and D"2 (**7**), which have structures similar to those of **4** and **5**, respectively. We synthesized **7** instead of **5** because the peak suspected to be characteristic of **5** was not detected in the data for the toxic microorganisms when using LC-MS, but two unidentified peaks suggesting the loss of 2H from **5** were detected at *m/z* 209.1509 (Fig. 2) during early studies. We preferred to examine whether **7** would give such a peak. Synthesized **2**, **6** and **7** were used as standards for the high-resolution LC-MS analysis used to identify the compounds of interest in the cells of the toxin-producing cyanobacteria and dinoflagellate strains.

For the synthesis of **2**, tris-*tert*-butoxycarbonyl (Boc) group protected L-arginine (**10**) derived from L-ornithine (**9**)[9] (37% yield, for three steps) (Scheme 1) was converted to the corresponding Weinreb amide **11** (95% yield), followed by the reaction with ethylmagnesium bromide to ethyl ketone **12** in 30% yield. Then, all three Boc groups of **12** were deprotected with trifluoroacetic acid (TFA) to produce **2** in 92% yield.



Scheme 1 Reagents and conditions: a) $CuCO_3$, H_2O. b) *N,N'*-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (1.0 eq), DIEA, formamide, 1,4-dioxane. C) Boc_2O, EDTA2Na, $NaHCO_3$, H_2O, acetone, 37% three steps. d) *N,O*-dimethylhydroxylamine hydrochloride salt, EDC, HOBt, NMM (N-methylmorpholine), CH_2Cl_2, 95%. e) EtMgBr, THF, 30%. f) TFA, CH_2Cl_2, 92%.

Next, we attempted to synthesize the second predicted intermediate, **3** (Scheme 2). L-Ornithine (**9**) reacted with *N,N'*-bis(Boc)-1H-pyrazole-1-carboxamidine (2 equiv.) to produce four Boc groups protected bis-guanidine carboxylic acid **13** in 55% yield. After conversion of carboxylic acid **13** into Weinreb amide **14** (71% yield), the amide reacted with ethylmagnesium bromide at 40-50°C to produce ethyl ketone **15** in 40% yield. Deprotection of all Boc groups in **15** was conducted by treatment with TFA to produce mono-guanidine **6** (named as Int-C'2) in 93% yield. In this reaction, the α-guanidino ketone moiety in **15** was cyclized into 2-amino-imidazole, and both **3** and **4** predicted by Kellmann *et al*.[7] were not observed by NMR and MS analysis. We assumed that the cyclization of the α-guanidino ketone moiety in **15** occurred under acidic dehydrative conditions; therefore, synthesis of **3** was also attempted using benzyloxycarbonyl (Cbz) groups instead of Boc groups as the protecting groups by the similar reaction route (Scheme S1†). However, the deprotection of Cbz groups in the corresponding ketone (Cbz protected **15**) under hydrogenolysis conditions using hydrogen in the presence of 10% Pd/C in ethyl acetate-MeOH (2:1, v/v) yielded **6** in 63% together with a structurally unidentified byproduct, but no **3** and **4** were further obtained (see ESI†). Pearlman's catalyst was also attempted for this reaction, but the result was almost same (see ESI†). These results strongly suggest that **3** spontaneously cyclize to form a cyclic imine **4** which is easily isomerized to **6** even under the neutral and/or non-dehydrative conditions.



Scheme 2 Reagents and conditions: a) *N,N'*-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (2.04 eq), DIEA, formamide, 1,4-dioxane, 55%. b) *N,O*-dimethylhydroxylamine hydrochloride salt, EDC, HOBt, NMM, CH_2Cl_2, 71%. c) EtMgBr, THF, 40-50°C, 40%. d) VinylMgBr, THF, 40-50°C, 29%. e) TFA, CH_2Cl_2, 79%. f) TFA, CH_2Cl_2, 80%.

Furthermore, a vinyl substituted imidazole bearing guanidine compound **7** (named as D"2) was also synthesized by the similar synthetic route of **6** from **14** via **16** (Scheme 2). Thus, Weinreb amide **14** reacted with vinylmagnesium bromide at 40-50°C to form vinyl ketone **16** (29% yield) followed by the deprotection of all four Boc groups of **16** by TFA that produced **7** (80% yield). Similarly to the case of **6**, ketone **17** and isomer **18** were not observed in the product mixtures in the deprotection process.

The presence of **2**, **6** and **7** in the toxic (TA04)[10] and non-toxic (NIES-1645)[11] (for reference) strains of the cyanobacterium *Anabaena circinalis* and in the toxic (Axat-2)[12] and non-toxic (UAT-014-009)[12] strains of the dinoflagellate *Alexandrium tamarense* were examined using high-resolution LC-MS (UPLC-Q-TOF-MS). HILIC (hydrophilic interaction chromatography) mode was used for the LC separation. The extracted ion chromatograms (EICs) of the extracts from the toxic strains (Fig. 2) of *A. circinalis* and *A. tamarense* were determined, with the $[M+H]^+$ ion detected at *m/z* 175.1190±0.01 for arginine, *m/z* 187.1553±0.01 for **2**, *m/z* 211.1666±0.01 for **6** and *m/z* 209.1509±0.01 for **7**. Peaks for the authentic arginine, **2**, **6** and **7** were detected at 9.1, 4.8, 4.2 and 4.2 min, respectively (Fig. 2A). On the EICs of the cell extract of the *A. circinalis* (TA04) toxic strain (Fig. 2B) and the *A. tamarense* (Axat-2) toxic strain (Fig. 2C), peaks corresponding to arginine, **2** and **6** were detected at almost the same retention times as the peaks for the authentic compounds; no other major peaks were detected under these conditions. Identification of **2** and **6** in the cells was also confirmed by spiking the authentic compounds into the sample solutions (Fig. S1†). Interestingly, although the peak corresponding to **7** was not observed on the EICs for both toxic strains, two unidentified peaks at different retention times (5.2 and 6.3 min) were observed, suggesting the presence of at least two unidentified compounds with the same molecular formula as that of **7**. The structures of these compounds will be identified during future work.

For the EIC $[M+H]^+$ data of (i) and (ii) (*m/z* 169.1084, 211.1302) predicted by Shimizu *et al*.[6] (Fig. 1a) no major peak was detected. For the EIC data of Int-B' (**3**) (*m/z* 229.1771) predicted by Kellmann *et al*.[7] (Fig. 1b) only a slight peak was detected without using authentic standard of **3**. Kellmann *et al*.[7] proposed that two enzymes (SxtB/C) are implicated in the conversion from **3** to **4**. The reason **3** was not detected as a major peak in the cells might be explained by the quick conversion to **6** via **4** based on the results of above chemical reactions. On the EICs of the extracts from the non-toxic strains, no peaks except that for arginine were detected, supporting the prediction that **4** and **6** are biosynthetic intermediates of **1**.

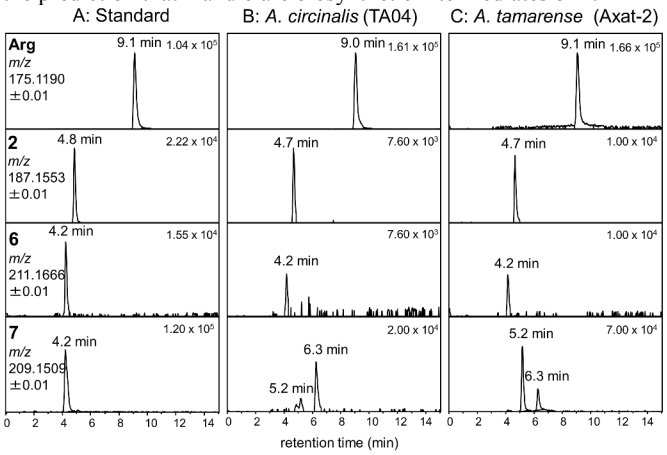


Fig. 2 High resolution LC-MS extracted ion chromatograms (EICs) of the authentic compounds (A), the toxic strains *A. circinalis* (TA04) (B) and *A. tamarense* (Axat-2) (C). Liquid chromatography was performed on a 2.1 i.d. × 150 mm (130Å, 1.7 µm) ACQUITY UPLC BEH Amide column. The mobile phase A was 200 mM $HCOONH_4$/200 mM HCOOH/water (2.5:2.5:95, v/v, pH 3.9), and the mobile phase B was 200 mM $HCOONH_4$/200 mM HCOOH/water/CH_3CN (2.5:2.5:1.5:95, v/v). A gradient elution program was applied as follows: 0-3 min 85% B, 3-11 min 85%-70% B,11-13 min 70% B, 13-20 min 85% B. The flow rate was 0.3 mL/min. The oven temperature was 40°C. The liquid chromatography system was connected to a Q-TOF mass spectrometer, MicrOTOFQII equipped with an ESI source. The amounts of the authentic standards of arginine, **2**, **6** and **7** were 50, 10, and 10 pmol (in 5 µL), respectively.

Compounds **2** and **6** in the toxic strains were further studied using high-resolution LC-MS/MS. The MS/MS spectra of these compounds are shown in Fig. 3. The precursor ions were *m/z* 175.12 for arginine, *m/z* 187.20 for Int-A' (**2**) and *m/z* 211.20 for Int-C'2 (**6**). Overall, the MS/MS spectra of **2** and **6** in the extracts from *A. circinalis* (TA04) (Fig. 3B) and *A. tamarense* (Axat-2) (Fig. 3C) showed almost identical fragmentation patterns compared to the authentic standards (Fig. 3A), supporting the identification of these compounds.

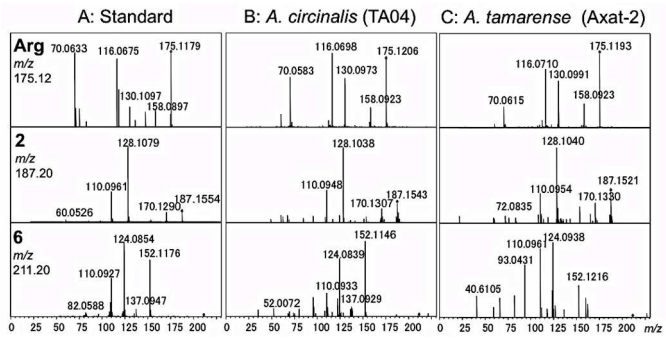
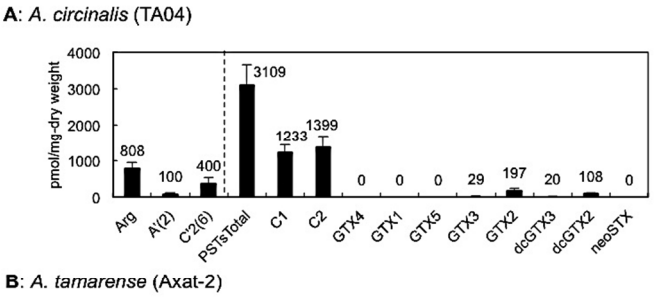
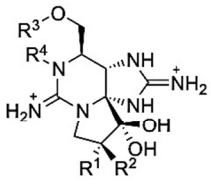


Fig. 3 High-resolution LC-MS/MS spectra of arginine, **2** and **6**. A: authentic standard, B: *Anabaena circinalis* (TA04), C: *Alexandrium tamarense* (Axat-2). The LC-MS conditions were the same as those used for Fig. 2. The MS/MS spectra were measured in MRM mode. The precursor ions were *m/z* 175.12 width 2 Da for arginine, *m/z* 187.20 width 2 Da for **2** and *m/z* 211.20 width 2 Da for **6**. The sweeping collision energy was 40-160 eV. The amounts of the authentic standards of arginine, **2**, **6** and **7** were 5 ng.





PST	R^1	R^2	R^3	R^4
neoSTX	H	H	$CONH_2$	OH
GTX1	OSO_3^-	H	$CONH_2$	OH
GTX2	OSO_3^-	H	$CONH_2$	H
GTX3	H	OSO_3^-	$CONH_2$	H
GTX4	H	OSO_3^-	$CONH_2$	OH
GTX5	H	H	$CONHSO_3^-$	H
C1	OSO_3^-	H	$CONHSO_3^-$	H
C2	H	OSO_3^-	$CONHSO_3^-$	H
dcGTX2	OSO_3^-	H	H	H
dcGTX3	H	OSO_3^-	H	H

Fig. 4 The contents of arginine, **2**, **6** and PSTs in *A. circinalis* (TA04) (A) and *A. tamarense* (Axat-2) (B). Arginine, **2** and **6** were quantified using LC-MS/MS (MRM), while the PSTs were quantified using LC-FLD[13].

This study is the first to achieve a complete identification of the biosynthetic intermediates of STX in the microorganisms and strongly supports the genetically predicted route described by Kellmann *et al*. (Fig. 1b),[7] because **6** should be derived from predicted intermediates **3** and **4** due to their chemical stability. In the cells it may be possible that biosynthesis would proceed via **3** and **4** catalyzed by enzymes as predicted.[7] In order to investigate the significance of **6** in biosynthesis, we are planning feeding study with isotope labelled **6**.

The contents of arginine, **2** and **6** in the cells of *A. circinalis* (TA04) and *A. tamarense* (Axat-2) were quantified using LC-MS/MS with a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode (see ESI†) and compared with the major PSTs quantified using LC-fluorescent detection (LC-FLD)[13]. As shown in Fig. 4, the arginine, **2** and **6** contents were 808±150, 100±13 and 400±130 pmol/mg-dry weight (mean, s.d., n=3), respectively, in *A. circinalis* (TA04), accounting for 26, 3.2 and 13% (mol/mol, average) of the total PST content (3.1 nmol/mg-dry weight), respectively. In *A. tamarense* (Axat-2), the arginine, **2** and **6** contents were 41.1±0.6, 4.4±0.2 and 5.5±2.2 fmol/cell (mean, s.d., n=3), respectively, accounting for 52, 5.5 and 6.9% (mol/mol, average) of the total PST content (79.6 fmol/cell), respectively. The contents of **2** and **6** appeared to be much less than the total PST content, but their levels were similar to those of the minor PSTs constituents. Although STX (**1**) was not detected in both of these microorganisms, the analogues of **1**, dcGTX2 and dcGTX3, or only dcGTX3, were detected. DcGTX2 and dcGTX3 were previously predicted to be biosynthesized via the same route as that of **1** in the cyanobacterium *Lyngbya wollei*.[14] Therefore, what is accumulated in these microorganisms in this study could be precursors of **1**. We will confirm it by feeding experiment during our future work.

Conclusions

In conclusion, we synthesized a genetically predicted biosynthetic intermediate of saxitoxin, Int-A' (**2**) and two related compounds (Int-C'2 (**6**) and D"2 (**7**)) and completely identified and quantified **2** and **6** in the toxin-producing cyanobacterium *Anabaena circinalis* and the dinoflagellate *Alexandrium tamarense* using the synthesized compounds. The results strongly support the biosynthetic route proposed by Kellmann et al. Int-C'2 (**6**) might be spontaneously derived from the predicted intermediate Int-B' (**3**) via Int-C' (**4**). Not all of these intermediates were detected in the non-toxic strains of these microorganisms. The synthesis and identification of the other biosynthetic intermediates are in progress.

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