

# Elucidation and Total Synthesis of the Correct Structures of Tridecapeptides Yaku'amides A and B. Synthesis-Driven Stereochemical Reassignment of Four Amino Acid Residues

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# **Supporting Information**

**ABSTRACT:** Yaku'amides A (1) and B (2) possess four  $\alpha,\beta$ dehydroamino acid residues in their linear tridecapeptide sequence and differ in their residue-3 (Gly for 1 and Ala for 2). The highly unsaturated peptide structure, characteristic cytotoxicity profile, and extreme scarcity from natural sources motivated us to launch synthetic studies of 1 and 2. Here, we report the total synthesis of the originally proposed structure of yaku'amide B (2a) by applying the route to 1a, which was previously established in our group. However, this accomplishment only proved that 2a and natural 2 were structurally different and prompted investigations directed toward determining the true structure of 2. Extensive Marfey's analyses of minute



amounts of natural 2 and its degradation products presented us the possible stereoisomers, all of which were synthetically prepared for chromatographic comparison with the authentic fragments of 2. Based on this detective work, we proposed a corrected structure for yaku'amide B (2c), in which the orders of residues-7 and -8 and residues-11 and -12 are reversed. Finally, the total synthesis of 2c led to confirmation of its structural identity. Moreover, the revised structure of yaku'amide A (1c) was constructed by switching Ala-3 to Gly-3 and was found to be chromatographically matched with the re-isolated natural 1. The present work demonstrated the high reliability and sensitivity of the MS- and LC-based structural analyses and the indispensable role of chemical synthesis in structural elucidation of scarce natural products.

# INTRODUCTION

Yaku'amides A (1, Figure 1) and B (2) were isolated from a deep-sea sponge Ceratopsion sp. collected at Yakushinsone in the East China Sea as minute components (1, 1.3 mg; 2, 0.3 mg).<sup>1,2</sup> Matsunaga and co-workers disclosed their unprecedented tridecapeptide structures in 2010. The linearly assembled 13 amino acid residues contain one  $\alpha_{,\beta}$ -dehydrovaline ( $\Delta$ Val, residue-13), three  $\alpha_{,\beta}$ -dehydroisoleucine ( $\Delta$ Ile, residues-2, -4, and -9), and seven other nonproteinogenic amino acids (residues-1, -5, -6, -7, -8, -10, and -12) and are capped with an N-terminal acyl group (NTA) and C-terminal amine (CTA). The structural difference between 1 and 2 resides in their residues-3 (Gly for 1 and Ala for 2). The geometries of the double bonds of the three  $\Delta$ Ile-2, -4, and -9 were determined on the basis of NOESY data, and the absolute configurations of the component amino acids and CTA were established by Marfey's analysis. Taking these data together, Matsunaga proposed the stereostructures of 1 and 2 to be 1a/ 1b and 2a/2b, respectively. Only the C4-stereochemistry of NTA was left undefined.

Natural peptides **1** and **2** were reported to exhibit exceptionally potent cytotoxicity toward P388 murine leukemia cells ( $IC_{50} = 8.5$  and 2.4 nM, respectively). Moreover, examination of the growth-inhibitory activity of **1** against a panel of 39 human cancer cell lines (JFCR39)<sup>3</sup> revealed its distinct profile from those of 38 anticancer drugs. Therefore, **1** is likely to exert its cytotoxicity through a potentially novel mode of action.<sup>4</sup> Further evaluation of the biological activities of these potential anticancer agents **1** and **2** has been impeded because minute amounts of the isolated yaku'amides existed after 2010. As compounds **1** and **2** were utilized for the structural and biological studies, none of **1** and approximately 0.1 mg of **2** remained.

To supply sufficient amounts of 1 and 2, their chemical constructions are the sole realistic solution. Motivated by their unique structures and bioactivities, we started the synthetic studies of yaku'amides, and accomplished the total synthesis of 1a and its C4-epimer 1b in 2013 (Figure 1c).<sup>5-7</sup> The  $\alpha,\beta$ -

Received: May 29, 2015

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**Figure 1.** (a) The originally proposed structures of yaku'amide A (1a/1b) and B (2a/2b). (b) The revised structures of yaku'amides A (1c) and B (2c). The corrected structures are highlighted in yellow. (c) Structures of compounds 3–7 used for total synthesis of 1a/1b and 2a/2b. (d) Cumediated enamide formation used in this study. Boc = *tert*-butoxycarbonyl;  $\Delta IIe = \alpha_{,\beta}$ -dehydroisoleucine;  $\Delta Val = \alpha_{,\beta}$ -dehydrovaline; OHIle = hydroxyisoleucine; OHVal = hydroxyvaline.

unsaturated amino acid residues of fragments 4, 5, and 7 were constructed in a geometrically selective fashion by employing the powerful Cu-mediated cross-coupling reactions (e.g.,  $A + B \rightarrow C$ , Figure 1d).<sup>8</sup> Stepwise assembly of the four synthetic fragments 3a/3b, 4, 5, and 7 then resulted in the entire structure of 1a/1b. Since the <sup>1</sup>H and <sup>13</sup>C NMR spectra of natural 1 matched those of 1a in comparison to 1b, we concluded that natural 1 possessed the C4S-stereochemistry of 1a.

Herein, we report the total syntheses of the C4-epimers of the originally proposed structures of yaku'amides B (2a and 2b, Figure 1a) by application of the route to 1a and 1b. Chromatographic comparison between natural 2 and 2a/2b validated that 2a/2b did not represent the true structure of the natural substance. This recognition prompted investigations directed at determining the true structure of natural 2. The structural information was gathered through degradative studies of the minute amounts of natural 2 available and synthetic preparation of the possible structures of the degraded partial structures. Combination of the degradation and synthesis allowed us to revise the structure of natural 2 to be 2c (Figure 1b), which was chemically constructed to confirm its structural authenticity. These studies further led to correction of the structure of natural yaku'amide A. Thus, the revised structure 1c was synthesized and was shown to be chromatographically identical with natural 1, which was re-isolated in this study. Below we present the details of these synthetic and analytical efforts.

#### RESULTS AND DISCUSSION

Total Syntheses of the Two Possible Proposed Structures of Yaku'amide B. To validate the unestablished C4-stereochemisty at NTA, we initially planned to synthesize the two C4-isomers of the proposed structures of yaku'amide B (2a and 2b, Figure 1). The fragments that were used in the total synthesis of 1a and 1b would be directly utilized except for 5.5 Thus, the requisite fragment 6 was first prepared prior to assembly of 2a/2b (Scheme 1). When Boc-L-Ala-NH<sub>2</sub> (8) and ethyl (E)-2-iodo-3-methylpent-2-enoate (9) were treated with catalytic CuI, N,N'-dimethylethylenediamine, and Cs2CO3 in dioxane at 70 °C, the hindered C–N bond was stereoselectively formed to afford dipeptide 10 with the E-didehydroisoleucine moiety.9,10 Saponification of 10 afforded carboxylic acid 11, which was transformed into the requisite fragment 6 in three steps: (i) allyl ester formation, (ii) Boc introduction at the enamide nitrogen atom, (iii) Pd-catalyzed removal of the allyl group at the C-terminus.<sup>11,12</sup>

Total syntheses of **2a** and **2b** were accomplished by elongation from nonapeptide 7 by the stepwise condensation with fragments **6**, **4**, and **3a/3b** (Scheme 2). Two cycles of TFA-promoted Boc removal and PyBOP<sup>13</sup>/HOAt<sup>14</sup>-mediated amidation were performed on 7 using dipeptides **6** and **4**. As a result, tridecapeptide **13** was obtained in 51% yield in 4 steps ( $7 \rightarrow 12 \rightarrow 13$ ). Next, treatment of **13** with TFA liberated the N-terminal amine, which was conjugated with NTA fragments **3a** and **3b** by the action of COMU<sup>15</sup> and 2,4,6-collidine to deliver the two C4-epimers **2a** and **2b**, respectively. Hence the two





possible structures of the originally proposed structures of yaku'amide B (2a and 2b) were chemically constructed.

However, neither of the final products 2a and 2b matched the natural yaku'amide B (2) by the chromatographic analysis. Specifically, injection of 2a/2b and 2 to ultrahigh-performance liquid chromatography (UHPLC) afforded peaks of significantly different retention times (Figure 2a,b). Co-injection reinforced the distinct nature of these peaks (Figure 2c), clarifying that 2a or 2b did not represent the true structure of natural yaku'amide B. This recognition set in motion a next wave of investigations directed at determining the true structure of yaku'amide B.



Figure 2. UHPLC charts of natural 2 and synthetic 2a/2b. (a) Synthetic 2a/2b. (b) Authentic 2. (c) Co-injection of 2 and 2a/2b. Column: Accucore phenyl hexyl 2.1 × 150 mm; column oven: 45 °C; flow rate: 0.25 mL/min; eluent: 35% 1-PrOH/H<sub>2</sub>O containing 1% AcOH; detection: 254 nm.

Re-elucidation of the Planar Structure of Natural 2. Since the proposed structure could contain one or more errors in unknown positions, the planar structure of the natural yaku'amide B (2) was first re-evaluated. The sequence of the amino acid residues, NTA, and CTA was verified by combination of the MS and NMR data. As shown in Figure 3, the in-source MS fragmentation of 2 enabled the highly sensitive peptide sequencing of 2 by detection of the diagnostic MS fragments, corroborating the sequence from residue-2 to residue-10. Although connectivities of NTA/residue-1 and residue-11/-12/-13/CTA were not established by the MS experiments, the reported interresidue HMBC correlations (red arrows) clearly indicated the correct order of these components.<sup>1</sup> On the other hand, the isolation paper unambiguously assigned the double-bond geometries of the three  $\Delta$ Ile moieties of natural vaku'amide A (1) based on the NOESY correlations (blue arrows). <sup>1</sup>H and <sup>13</sup>C NMR chemical

Scheme 2. Total Synthesis of the Proposed Structures of Yaku'amide B  $(2a/2b)^a$ 



"TFA = trifluoroacetic acid; PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; HOAt = 1-hydroxy-7-azabenzotriazole; COMU = (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate.

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Figure 3. Confirmation of the planar structure of natural yaku'amide B (2).

Scheme 3. Degradation of Natural Yaku'amide B (2) and Marfey's Analyses of Total and Partial Hydrosate



shifts corresponding to  $\Delta$ Ile-2,  $\Delta$ Ile-4, and  $\Delta$ Ile-9 are almost identical among 1, natural 2, and synthetic 2a/2b, indicating that the stereochemistry of these tetra-substituted olefins is the same. Taken together, these data confirmed that the planar structure of natural 2 contained no mis-assignments.

Marfey's Analysis of Natural 2 and its Degradation Product 15. Having confirmed the planar structure of 2, we decided to re-establish the absolute configurations of all the 13 stereocenters within the entire structure of 2. In doing so, degradative studies of natural 2 were executed to collect the stereochemical information by Marfey's analyses.<sup>16</sup> This turned out to be an extremely challenging task, because a maximum of 0.1 mg of 2 could be utilized. First, natural 2 (0.04 mg in total) was subjected to aqueous 6 M HCl solution at 110 °C for 12 h to induce complete hydrolysis, leading to a mixture of the component amino acids (Scheme 3). The resulting amino acids were treated with 1-fluoro-2,4-dinitrophenyl-5-alanine amide (FDAA), and then the derivatives were analyzed by the LC-MS method. To realize highly sensitive analysis using a trace amount of the hydrolysate, the following experimental conditions were designed. Namely, the amino acids were derivatized by both enantiomers of FDAA to cross-check the information, and the online MS detection of the analyte was employed to acquire the structural data in real-time.<sup>17</sup> In addition, a naphthylethyl-conjugated silica gel column

(COSMOSIL  $\pi$ -NAP) was used instead of the standard ODS column to attain the highly resolved HPLC peaks. In fact, the four FDAA-derivatives of L-Ile/L-allo-Ile and D-Ile/D-allo-Ile were found to be separable only upon use of COSMOSIL  $\pi$ -NAP, presumably due to its stronger attractive interaction with the dinitrophenyl moieties (Figure 4). Consequently, the L- and D-FDAA-derivatives of  $(2R_3R)-/(2S_3R)-/(2R_3S)-/(2S_3S)-$ hydroxyisoleucine (OHIle), L-/D-Ala, L-/D-Val, L-Ile/L-allo-Ile/D-Ile/D-allo-Ile, L-/D-hydroxyvaline (OHVal), and (R)-/(S)-CTA were used as the standard samples, and the obtained retention times of these compounds were compared with the L- and D-FDAA-treated hydrolysate of natural 2 (Marfey's analysis



**Figure 4.** UHPLC charts of the L-FDAA derivatives of L-Ile, L-*allo*-Ile, D-Ile, and D-*allo*-Ile. FDAA = 1-fluoro-2,4-dinitrophenyl-5-alanine amide. Column: COSMOSIL 2.5  $\pi$ -NAP 2.0 × 100 mm; column oven: 40 °C; flow rate: 0.4 mL/min; eluent: linear gradient from 10% to 50% MeCN/H<sub>2</sub>O containing 0.05% TFA over 32 min; detection: 340 nm.

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A). The experiments revealed the structures of residues-1 and -6 to be (2S,3R)-OHIle and D-allo-Ile, respectively, thus establishing four of the stereocenters of **2**. Furthermore, the L/D ratios of the three component amino acids were determined as follows: Ala (L/D = 1:1), Val (L/D = 1:2), and OHVal (L/D = 1:1). On the other hand, the FDAA-derivatives of CTA were not detected because of its instability toward the highly acidic conditions.

When another 0.01 mg of **2** was treated with aqueous 1 M HCl solution at 70 °C for 36 h, the three fragments **14**, **15**, and **16** were produced (Scheme 3). Interestingly, this controlled hydrolysis transformed the enamide moieties of  $\Delta$ Ile to the N-terminal dicarbonyl structures of **14** and **15** (1:1 stereoisomers at their branched carbons). Therefore, **15** was utilized for the optimized Marfey's analysis. After complete hydrolysis of **15** with 6 M HCl, the lysate was functionalized with L-FDAA to identify the presence of D-Ala at residue-10 (Marfey's analysis B). Considering that **2** possesses a 1:1 ratio of L- and D-Ala, the structure of residue-3 was determined to be L-Ala at this stage. Therefore, the degradative studies indicated that the originally assigned absolute stereochemistry of residues-1, -3, -6, and -10 should be correct [(2*S*, 3*R*)-OHIle-1, L-Ala-3, D-*allo*-Ile-6, D-Ala-10, respectively].

Syntheses of Eight Possible Isomers of 16. The structural ambiguities in 2 resided in the seven absolute configurations of NTA, residues-5, -7, -8, -11, and -12, and CTA. Since fragment 16 generated by the partial hydrolysis contained residues-11 and -12 and CTA (Scheme 3), we planned to determine the three unknown stereocenters of 16 by chromatographic comparison with the eight possible isomers of synthetic 16a-h (Scheme 4). Among them, compound 16a corresponded to the proposed structure and was already prepared via 21a.<sup>5</sup>

A divergent synthesis of other seven isomers 16b-h is summarized in Scheme 4.<sup>18</sup> Four isomers of 19 were prepared utilizing the Cu-catalyzed coupling reaction of vinyl iodide 17a/ 17b and primary amide 18a/18b. TFA-treatment of 19 removed the Boc group, and the resulting amine reacted with Boc-L- or D-Val-OH in the presence of COMU to generate the seven isomers of 20. Next, N<sub>a</sub>-deprotection of 20 and COMUpromoted amidation with Boc-D-Ala-OH gave rise to 21b-h. It is of note that use of COMU in these two amidations was important to suppress the C<sub>a</sub>-epimerization of the sterically hindered amino acids. Finally, the obtained 21b-h were converted to the requisite amines 16b-h.

The synthesized eight possible isomers 16a-h were then chromatographically compared with authentic 16 by LC-MS experiments. After extensive screening of the HPLC conditions, 16a-h was found to exhibit eight separate peaks as shown in Figure 5a. The retention time of authentic 16 matched that of synthetic 16d, but not those of the other seven stereoisomers, by separate injection (Figure 5b) as well as by coinjection (Figure 5c). Consequently, the absolute structures of residues-11, and -12 and CTA were determined to be D-Val, L-Val, and (S)-CTA, respectively. As Marfey's analysis of the total hydrolysate shed light on the existence of one L-Val and two D-Val in 2 (*vide supra*), residue-5 was fixed to be D-Val. Through these experiments, four of the seven stereocenters were resolved.

**Syntheses of Two Possible Isomers of 14.** Marfey's analysis A specified the presence of one L- and one D-OHVal within the peptide sequence of **2** (Scheme 3). Accordingly, correct placement of L- and D-OHVal to residues-7 and -8 was





**Figure 5.** LC-MS charts of authentic **16** and synthetic **16a**–**h**. (a) Mixture of **16a**–**h**. (b) Authentic **16**. (c) Co-injection of **16** and **16a**–**h**. Column: COSMOSIL 2.5  $\pi$ -NAP 2.0 × 100 mm; column oven: 30 °C; flow rate: 0.3 mL/min; eluent: linear gradient from 10% to 15% MeCN/H<sub>2</sub>O containing 0.05% TFA over 18 min; detection: m/z = 497.

time (min)

the next issue for the structural determination. Thus, two possible isomers 14a and 14b with these residues were planned to be prepared and analyzed by UHPLC along with the degradatively obtained 14. Compound 21d, which was prepared according to Scheme 4, was elongated to 14a and 14b in 10 steps (Scheme 5). Treatment of 21d with TFA liberated the amine, and condensation of the amine with enantiomeric dipeptides 22a<sup>5</sup> and 22b by using PyBOP and HOAt furnished hexapeptides 23a and 23b, respectively. Three rounds of TFA-promoted N<sub> $\alpha$ </sub>-deprotection and COMUmediated amidation transformed 23a and 23b into nonapeptides 25a and 25b, respectively, when 24a/24b<sup>5</sup> (24a for 25a, 24b for 25b), Boc-D-allo-Ile, and Boc-D-Val were sequentially used. Finally, TFA treatment of 25a and 25b,

# Scheme 5. Syntheses of Two Possible Isomers of 14



followed by amidation with acid **26** by the action of PyBOP/ HOAt, gave rise to ketones **14a** and **14b**.

Figure 6a-c depicts the UHPLC charts of the two synthetic isomers 14a and 14b, authentic 14, and their combination,



Figure 6. UHPLC charts of authentic 14 and synthetic 14a/14b. (a) Synthetic 14a/14b. (b) Authentic 14. (c) Co-injection of 14 and 14a/14b. Column: Accucore phenyl hexyl  $2.1 \times 150$  mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 40% MeCN/H<sub>2</sub>O containing 0.05% TFA; detection: 226 nm.

respectively. Each compound gave two peaks, because they were 1:1 diastereomeric mixtures at the methyl group of the N-terminal acyl portion. Nevertheless, it was clear that the retention time of **14b** was identical to **14**. Hence, residues-7 and -8 were established to be D-OHVal and L-OHVal, respectively.

The series of degradative and synthetic efforts described herein permitted us to unambiguously determine the entire stereostructure of natural yaku'amide B except for the NTA moiety. Most importantly, the revised structure 2c/2d differs from the originally proposed structure 2a/2b in the orders of residues-7 and -8 and residues-11 and -12 (Scheme 6). Therefore, L-OHVal-7, D-OHVal-8, L-Val-11, and D-Val-12 of 2a/2b were corrected to be D-OHVal-7, L-OHVal-8, D-Val-11, and L-Val-12 of 2c/2d, respectively.

Total Synthesis of the Two Possible Revised Structures of Yaku'amide B. The possible structures of natural yaku'amide B (2) were narrowed down to the two C4epimers 2c and 2d. Determination of the absolute stereochemistry of the last remaining C4-stereocenter of NTA necessitated the total synthesis of 2c and 2d (Scheme 6). Similar to the route to 2a and 2b in Scheme 2, the stepwise  $N_{a}$ deprotection/amidation from nonapeptide 25b delivered the target molecules in six transformations. Nonapeptide 25b was deprotected and coupled with acid 6 by the action of PyBOP and HOAt to yield the undecapeptide, which underwent in situ deprotection by TFA to deliver amine 27.19 Condensation of 27 with 4 in turn delivered tridecapeptide 29. Lastly, TFA induced the detachment of the Boc group of 29 to afford the amine, which was separately coupled with enantiomeric carboxylic acids 3a and 3b using COMU and 2,4,6-collidine to provide 2c and 2d, respectively.

When the UHPLC experiments of natural 2 and the two synthetic isomers 2c and 2d were conducted (Figure 7), the retention time of 2c with the C4S-stereochemistry was found to be identical to natural 2, thus defining the entire stereostructure of natural yaku'amide B to be that shown in Scheme 6. Additionally, the <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts of synthetic 2c were assigned based on HMBC and HSQC correlations and were in accordance with those of natural 2. Considering the NMR spectra of 2c changed depending on the conditions in the NMR tube (e.g., concentration, temperature, and purity), the <sup>1</sup>H NMR spectra of the mixture of natural 2 and synthesized 2c were also obtained for further confirmation of their structural identity.







Figure 7. UHPLC charts of natural 2 and synthetic 2c/2d. (a) Synthetic 2c/2d. (b) Authentic 2. (c) Co-injection of 2 and 2c/2d. Column: Accucore C18 2.1 × 150 mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 70% MeCN/H<sub>2</sub>O containing 0.05% TFA; detection: 226 nm.

**Re-isolation, Structural Revision, and Total Synthesis** of Yaku'amide A. Our previous study reported the total synthesis of the originally determined structure of yaku'amide A C4S-1a and C4*R*-1b and permitted the assignment of the C4Sstereochemistry of NTA based on the similarities of the NMR spectra of 1a with those of natural 1.<sup>5</sup> However, the aforementioned structural revision of yaku'amide B (2) jeopardized the credibility of the original structure of natural 1 and thus forced us into defining the correct stereostructure of 1. Since variable NMR spectra of peptide natural products often complicate structural determination,<sup>20</sup> our NMR-based comparison of 1 and 1a could have led to the incorrect conclusion.

Chromatographic comparison has been demonstrated to be a powerful method for the structural assignment of large peptides. No sample of authentic 1 remained, and thus reisolation of authentic 1 was required to apply the chromatographic method. In this situation, we focused our attention on the remaining side-fraction in the original isolation procedures.<sup>1</sup> After optimizing the purification protocol, <0.1 mg of natural **1** was isolated from the side fraction, which was then used for the UHPLC experiments. As clearly shown in Figure 8, retention times of re-isolated **1** and previously synthesized **1a**/**1b** were completely different, suggesting assignment errors in the proposed structure of yaku'amide A.



Figure 8. UHPLC charts of re-isolated 1 and synthetic 1a/1b. (a) Synthetic 1a/1b. (b) Authentic 1. (c) Co-injection of 1 and 1a/1b. Column: Accucore phenyl hexyl 2.1 × 150 mm; column oven: 45 °C; flow rate: 0.25 mL/min; eluent: 30% 1-PrOH/H<sub>2</sub>O containing 1% AcOH; detection: 254 nm.

The above results led us to synthesize 1c, which shares all the structure of the revised structure of yaku'amide B except residue-3 (Gly for 1c and L-Ala for 2c). Scheme 6 shows the total synthesis of 1c along with its C4-epimer 1d. The common intermediate 25b underwent chain elongation through repeating the N<sub> $\alpha$ </sub>-deprotection and amidation by using 5, 4, and 3a/3b to yield 1c and 1d (25b  $\rightarrow$  28  $\rightarrow$  30  $\rightarrow$  1c/1d).

The retention time of C4S-epimer 1c was indeed identical to natural 1 (Figure 9), while 1 and 1d exhibited distinct chromatographic behavior. Therefore, the absolute structure of yaku'amide A should be revised from 1a to 1c.

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Figure 9. UHPLC charts of re-isolated 1 and synthetic 1c/1d. (a) Synthetic 1c/1d. (b) Authentic 1. (c) Co-injection of 1 and 1c/1d. Column: Accucore C18 2.1 × 150 mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 65% MeCN/H<sub>2</sub>O containing 0.05% TFA; detection: 226 nm.

**Cytotoxicity of the Revised Structures of Yaku'amides A and B.** A cytotoxicity assay of the revised structures 1c and 2c against P388 mouse leukemia cells was performed using the XTT method.<sup>21</sup> Yaku'amides A (1c) and B (2c) both displayed extremely potent cytotoxicity (1c: 0.88 nM and 2c: 0.51 nM), corroborating the authenticity of our structural revisions.

#### CONCLUSION

In summary, the structural revision and total synthesis of yaku'amides A and B were achieved by judicious combination of natural product degradation, MS and chromatographic analyses, and chemical synthesis.<sup>22</sup> The total synthesis of the proposed structure of yaku'amide B (2a) proved its mistaken structural identity. The structural information on 2 was then acquired by applying the highly sensitive Marfey's analysis of the minute amounts of natural 2 and its fragments. The potential substructures suggested by these degradative studies were chemically synthesized and chromatographically evaluated to obtain the two possible revised structures 2c and 2d. Finally, the total synthesis defined the correct structure of yaku'amide B to be 2c. Moreover, re-isolation of natural 1, total synthesis of 1c, and their chromatographic comparison indicated 1c as the revised structure of yaku'amide A. Both of the revised structures possess the inverse configurations at the  $C_{\alpha}$ -positions of the four amino acid residues (residues-7, -8, -11, and -12) of the original structures and S-configuration at C4 of NTA. These studies demonstrated the efficiency and robustness of our convergent strategy for assembly of the various yaku'amide stereoisomers and the high reliability and sensitivity of the MSand LC-based structural analyses. Moreover, the present work showed once again the indispensable role of chemical synthesis in structural elucidation of scarce, but highly valuable, natural products. Establishment of the true structures of yaku'amides provides a starting point for systematic synthesis of analogues, detailed structure-activity relationship studies, and elucidation of the unknown mode of action of these potent cytotoxins. Such investigations are currently underway in our laboratory.

## ASSOCIATED CONTENT

# **Supporting Information**

Characterization data for all new compounds and experimental procedures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/ jacs.5b05550.

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#### Notes

The authors declare no competing financial interest.

#### ACKNOWLEDGMENTS

This research was financially supported by the Funding Program for a Grant-in-Aid for Scientific Research (A) to M.I.

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