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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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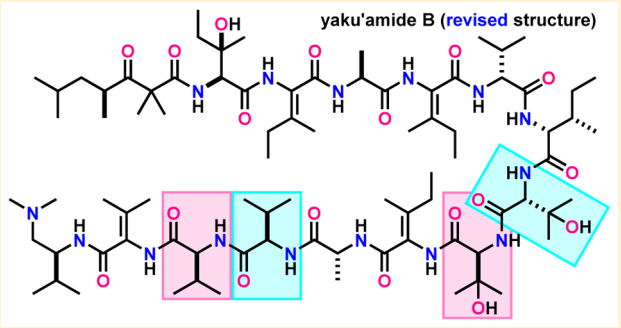
Supporting Information

ABSTRACT: Yaku'amides A (**1**) and B (**2**) possess four α,β-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).[1,2] Matsunaga and co-workers disclosed their unprecedented tridecapeptide structures in 2010. The linearly assembled 13 amino acid residues contain one α,β-dehydrovaline (ΔVal, residue-13), three α,β-dehydroisoleucine (ΔIle, 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 ΔIle-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)[3] 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.[4] 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).[5−7] The α,β-

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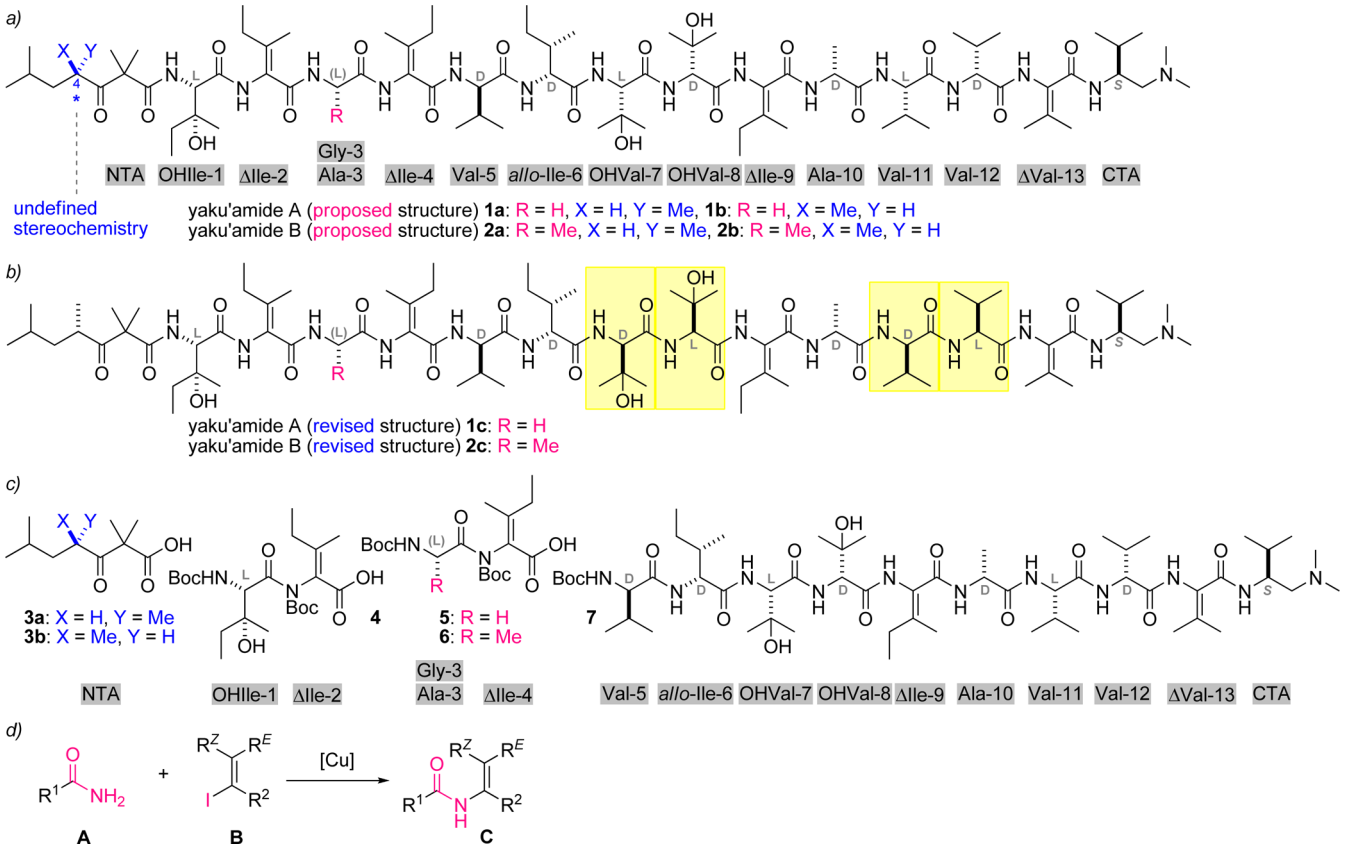

Figure 1. (a) The originally proposed structures of yaku'amide A (**1a**/**1b**) and B (**2a**/**2b**). (b) The revised structures of yaku'amides A (**1c**) and B (**2c**). The corrected structures are highlighted in yellow. (c) Structures of compounds **3**–**7** used for total synthesis of **1a**/**1b** and **2a**/**2b**. (d) Cu-mediated enamide formation used in this study. Boc = *tert*-butoxycarbonyl; ΔIle = *α*,*β*-dehydroisoleucine; ΔVal = *α*,*β*-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** → **C**, Figure 1d).[8] Stepwise assembly of the four synthetic fragments **3a**/**3b**, **4**, **5**, and **7** then resulted in the entire structure of **1a**/**1b**. Since the ^{1}H and ^{13}C NMR spectra of natural **1** matched those of **1a** in comparison to **1b**, we concluded that natural **1** possessed the C4*S*-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_2 (**8**) and ethyl (*E*)-2-iodo-3-methylpent-2-enoate (**9**) were treated with catalytic CuI, *N*,*N*′-dimethylethylenediamine, and Cs_2CO_3 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.[11,12]

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[13]/HOAt[14]-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** → **12** → **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[15] and 2,4,6-collidine to deliver the two C4-epimers **2a** and **2b**, respectively. Hence the two

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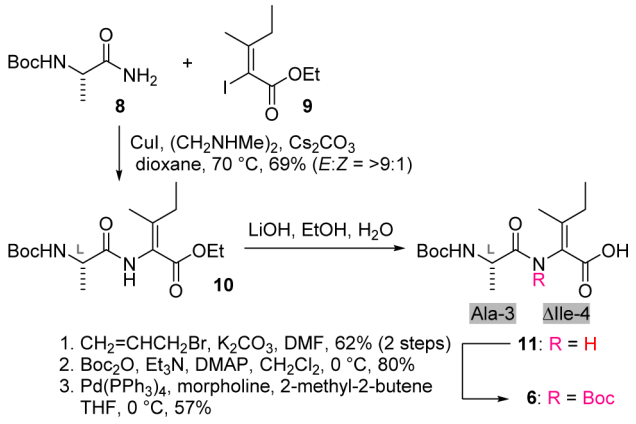
Scheme 1. Synthesis of Dipeptide 6[a]



[a]DMF = *N*,*N*-dimethylformamide; DMAP = *N*,*N*-dimethyl-4-aminopyridine; THF = tetrahydrofuran.

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.

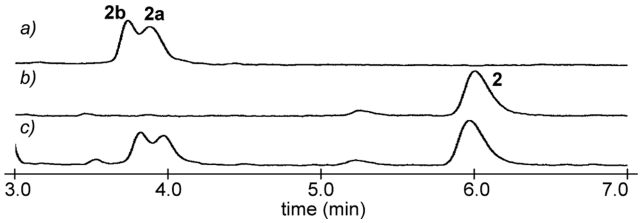
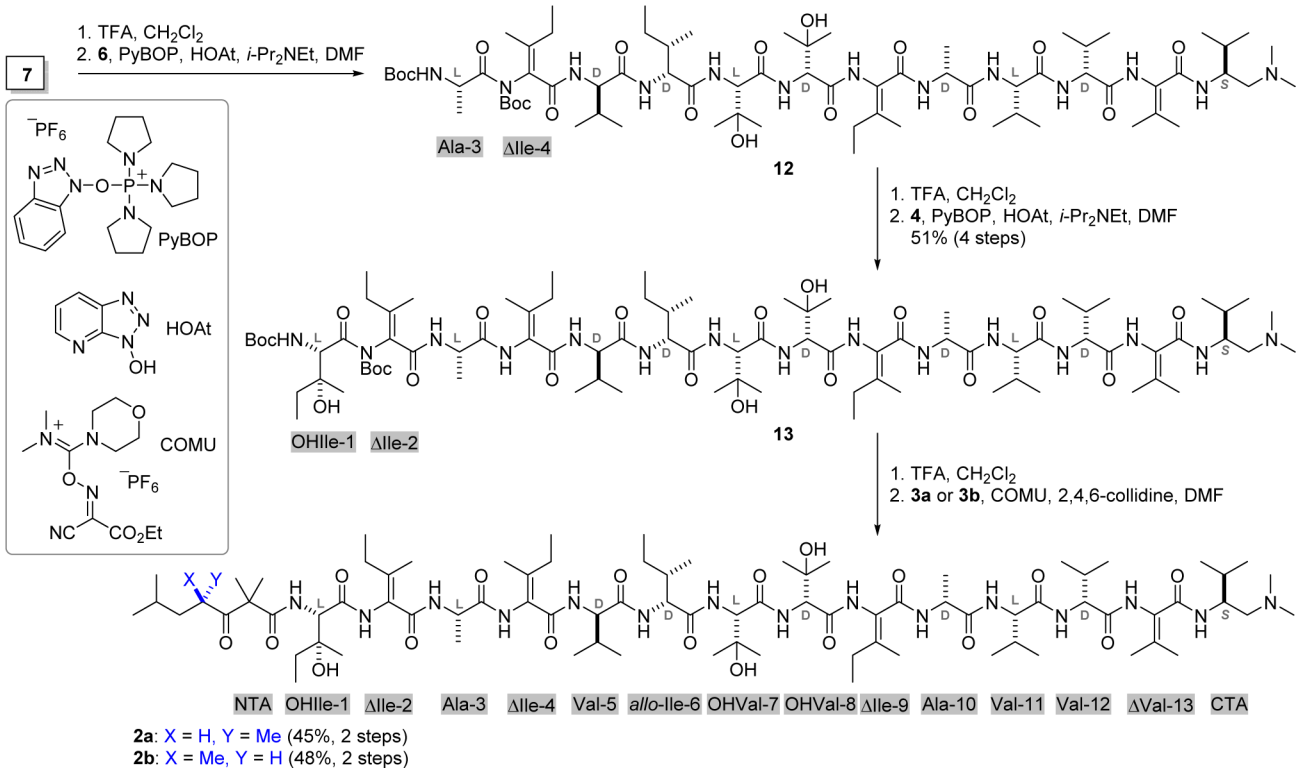


Figure 2. UHPLC charts of natural **2** and synthetic **2a**/**2b**. (a) Synthetic **2a**/**2b**. (b) Authentic **2**. (c) Co-injection of **2** and **2a**/**2b**. Column: Accucore phenyl hexyl 2.1 × 150 mm; column oven: 45 °C; flow rate: 0.25 mL/min; eluent: 35% 1-PrOH/H_2O containing 1% AcOH; detection: 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.[1] On the other hand, the isolation paper unambiguously assigned the double-bond geometries of the three ΔIle moieties of natural yaku'amide A (**1**) based on the NOESY correlations (blue arrows). ^1H and ^{13}C NMR chemical

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



[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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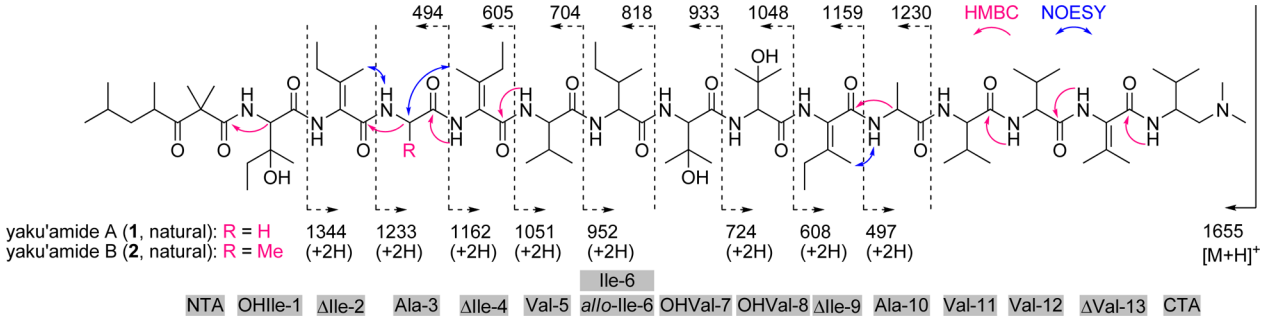
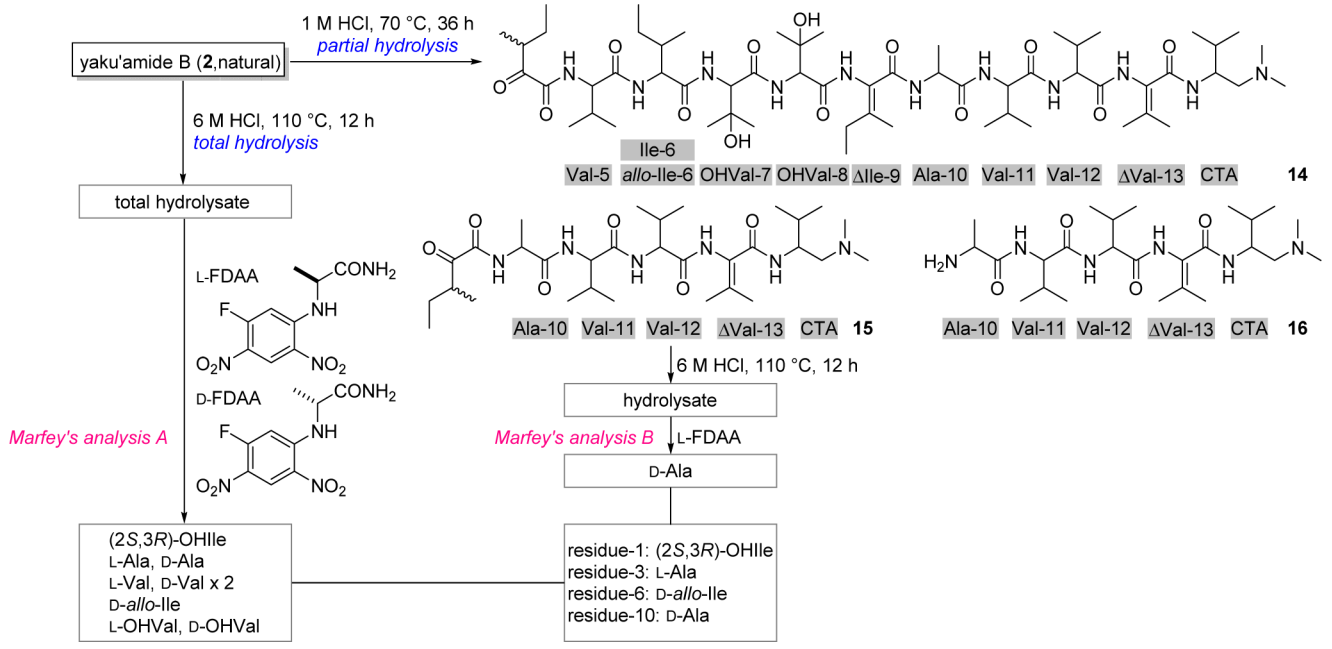


Figure 3. Confirmation of the planar structure of natural yaku'amide B (**2**).

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



shifts corresponding to ΔIle-2, ΔIle-4, and ΔIle-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.[16] 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.[17] In addition, a naphthylethyl-conjugated silica gel column (COSMOSIL π-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 π-NAP, presumably due to its stronger attractive interaction with the dinitrophenyl moieties (Figure 4). Consequently, the L- and D-FDAA-derivatives of (2*R*,3*R*)-/(2*S*,3*R*)-/(2*R*,3*S*)-/(2*S*,3*S*)-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

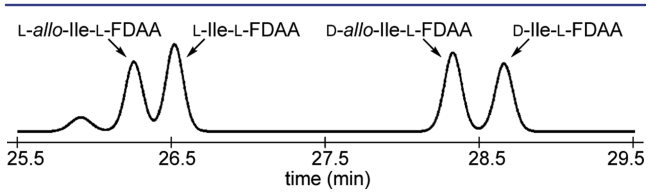


Figure 4. UHPLC charts of the L-FDAA derivatives of L-Ile, L-*allo*-Ile, D-Ile, and D-*allo*-Ile. FDAA = 1-fluoro-2,4-dinitrophenyl-5-alanine amide. Column: COSMOSIL 2.5 π-NAP 2.0 × 100 mm; column oven: 40 °C; flow rate: 0.4 mL/min; eluent: linear gradient from 10% to 50% MeCN/H_2O containing 0.05% TFA over 32 min; detection: 340 nm.

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A). The experiments revealed the structures of residues-1 and -6 to be (2*S*,3*R*)-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 ΔIle 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].

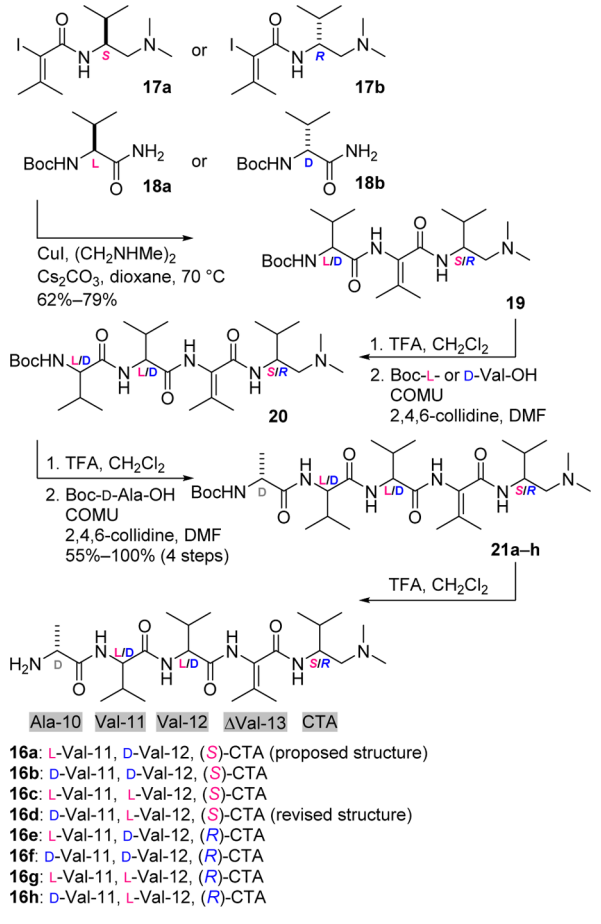
Syntheses of Eight Possible Isomers of 16. The structural ambiguities in **2** resided in the seven absolute configurations of NTA, residues-5, -7, -8, -11, and -12, and CTA. Since fragment **16** generated by the partial hydrolysis contained residues-11 and -12 and CTA (Scheme 3), we planned to determine 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**.[5]

A divergent synthesis of other seven isomers **16b–h** is summarized in Scheme 4.[18] 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_α-deprotection of **20** and COMU-promoted 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_α-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

Scheme 4. Syntheses of Eight Possible Isomers of 16



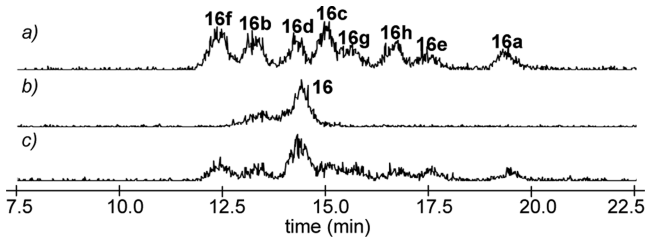


Figure 5. LC-MS charts of authentic **16** and synthetic **16a–h**. (a) Mixture of **16a–h**. (b) Authentic **16**. (c) Co-injection of **16** and **16a–h**. Column: COSMOSIL 2.5 π-NAP 2.0 × 100 mm; column oven: 30 °C; flow rate: 0.3 mL/min; eluent: linear gradient from 10% to 15% MeCN/H_2O containing 0.05% TFA over 18 min; detection: m/z = 497.

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**[5] and **22b** by using PyBOP and HOAt furnished hexapeptides **23a** and **23b**, respectively. Three rounds of TFA-promoted N_α-deprotection and COMU-mediated amidation transformed **23a** and **23b** into non-apeptides **25a** and **25b**, respectively, when **24a**/**24b**[5] (**24a** for **25a**, **24b** for **25b**), Boc-D-*allo*-Ile, and Boc-D-Val were sequentially used. Finally, TFA treatment of **25a** and **25b**,

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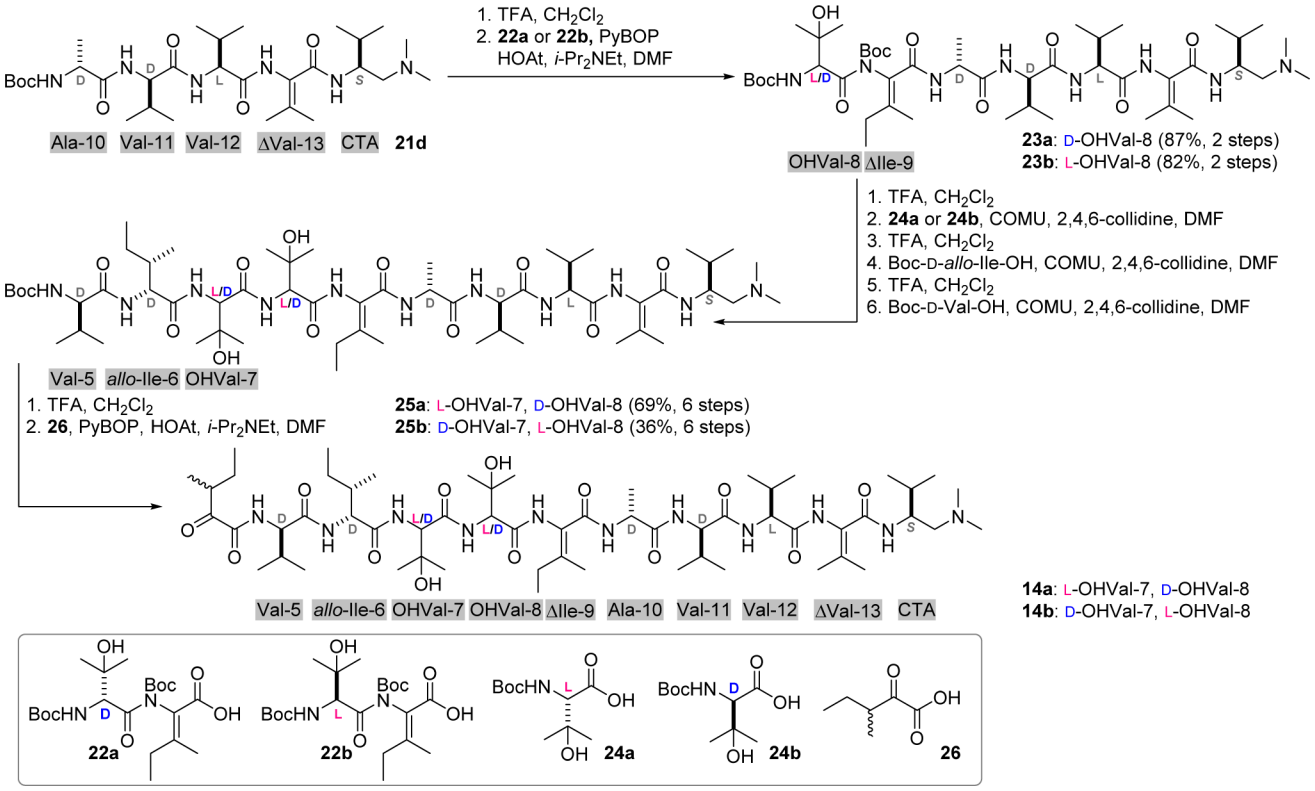
Scheme 5. Syntheses of Two Possible Isomers of 14



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,

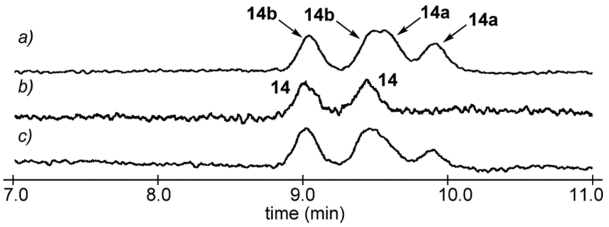


Figure 6. UHPLC charts of authentic **14** and synthetic **14a**/**14b**. (a) Synthetic **14a**/**14b**. (b) Authentic **14**. (c) Co-injection of **14** and **14a**/**14b**. Column: Accucore phenyl hexyl 2.1 × 150 mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 40% MeCN/H_2O containing 0.05% 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 C4-epimers **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_α-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 C4*S*-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 ^1H and ^{13}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 ^1H NMR spectra of the mixture of natural **2** and synthesized **2c** were also obtained for further confirmation of their structural identity.

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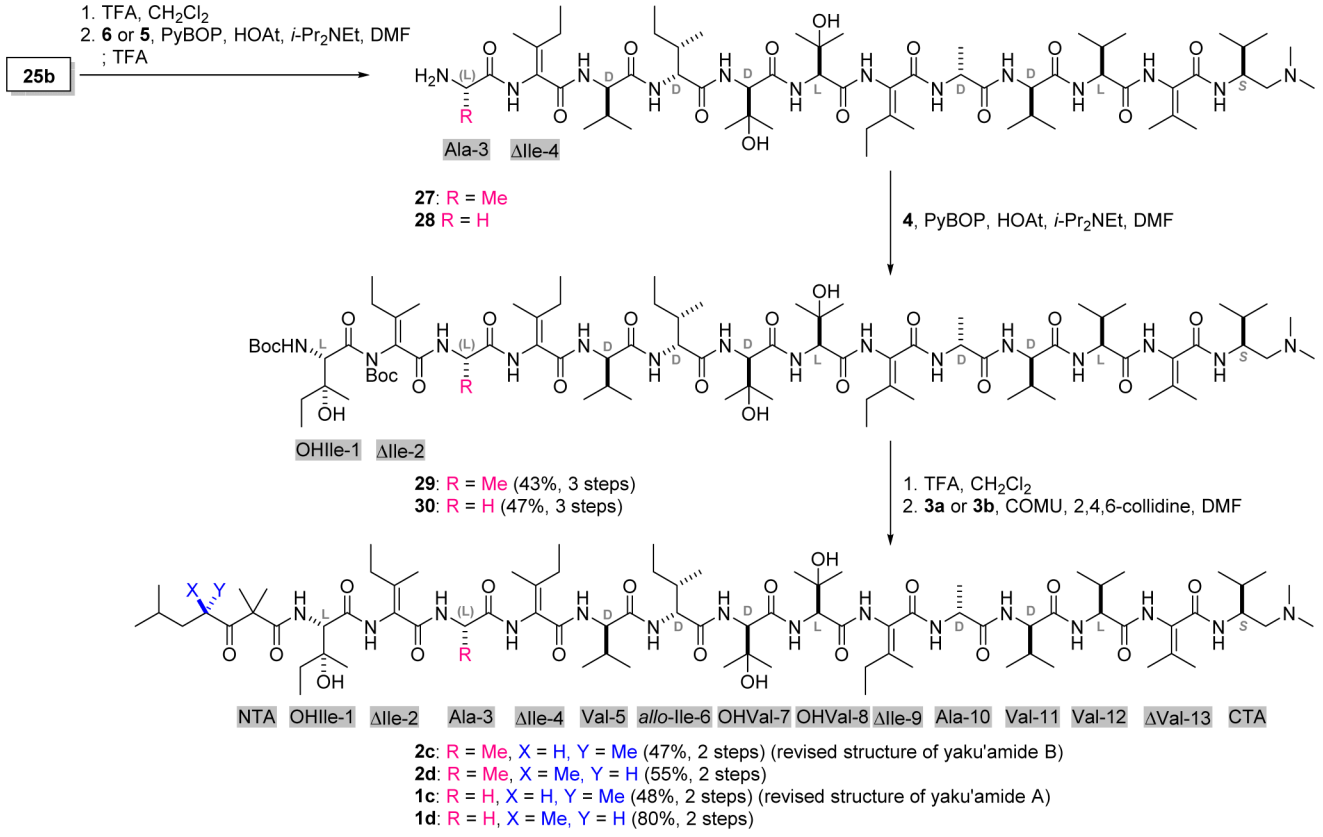
Scheme 6. Total Synthesis of the Revised Structures of Yaku'amide B (2c), A (1c), and their C4-Epimers (2d, 1d)



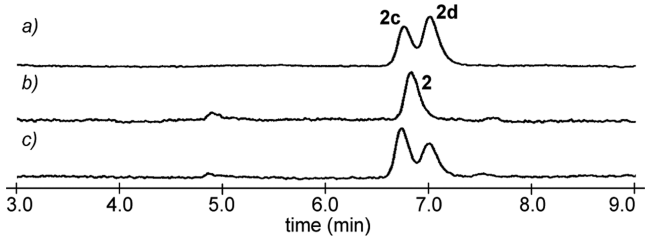


Figure 7. UHPLC charts of natural **2** and synthetic **2c**/**2d**. (a) Synthetic **2c**/**2d**. (b) Authentic **2**. (c) Co-injection of **2** and **2c**/**2d**. Column: Accucore C18 2.1 × 150 mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 70% MeCN/H_2O containing 0.05% TFA; detection: 226 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 C4*S*-**1a** and C4*R*-**1b** and permitted the assignment of the C4*S*-stereochemistry of NTA based on the similarities of the NMR spectra of **1a** with those of natural **1**.[5] 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,[20] 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 re-isolation 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.[1]

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.

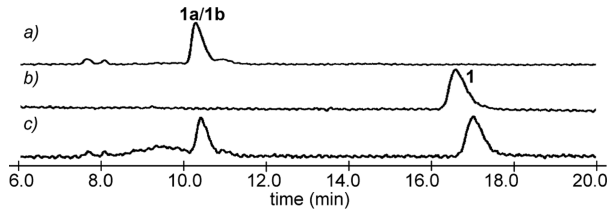


Figure 8. UHPLC charts of re-isolated **1** and synthetic **1a**/**1b**. (a) Synthetic **1a**/**1b**. (b) Authentic **1**. (c) Co-injection of **1** and **1a**/**1b**. Column: Accucore phenyl hexyl 2.1 × 150 mm; column oven: 45 °C; flow rate: 0.25 mL/min; eluent: 30% 1-PrOH/H_2O containing 1% AcOH; detection: 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_α-deprotection and amidation by using **5**, **4**, and **3a**/**3b** to yield **1c** and **1d** (**25b** → **28** → **30** → **1c**/**1d**).

The retention time of C4*S*-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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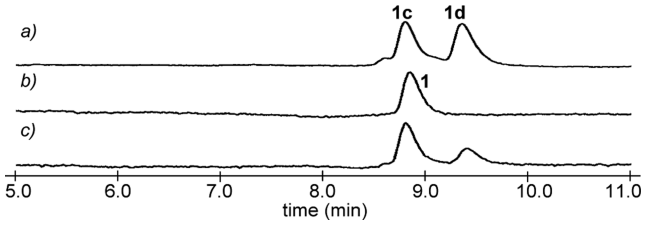

Figure 9. UHPLC charts of re-isolated **1** and synthetic **1c**/**1d**. (a) Synthetic **1c**/**1d**. (b) Authentic **1**. (c) Co-injection of **1** and **1c**/**1d**. Column: Accucore C18 2.1 × 150 mm; column oven: 40 °C; flow rate: 0.5 mL/min; eluent: 65% MeCN/H_2O containing 0.05% TFA; detection: 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.[21] 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.[22] 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_α-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 MS- and 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.

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REFERENCES

(1) Ueoka, R.; Ise, Y.; Ohtsuka, S.; Okada, S.; Yamori, T.; Matsunaga, S. *J. Am. Chem. Soc.* **2010**, *132*, 17692.

(2) For selected reviews of marine natural products, see: (a) Yasumoto, T.; Murata, M. *Chem. Rev.* **1993**, *93*, 1897. (b) Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793. (c) Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2162. (d) Blunt, J. W.; Copp, B. R.; Keyzers, R. A.; Munro, M. H. G.; Prinsep, M. R. *Nat. Prod. Rep.* **2015**, *32*, 116.

(3) (a) Yamori, T. *Cancer Chemother. Pharmacol.* **2003**, *52*, S74. (b) Yaguchi, S.; Fukui, Y.; Koshimizu, I.; Yoshimi, H.; Matsuno, T.; Gouda, H.; Hirono, S.; Yamazaki, K.; Yamori, T. *J. Natl. Cancer Inst.* **2006**, *98*, 545. (c) Yamori, T.; Matsunaga, A.; Sato, S.; Yamazaki, K.; Komi, A.; Ishizu, K.; Mita, I.; Edatsugi, H.; Matsuba, Y.; Takezawa, K.; Nakanishi, O.; Kohno, H.; Nakajima, Y.; Komatsu, H.; Andoh, T.; Tsuruo, T. *Cancer Res.* **1999**, *59*, 4042.

(4) For recent reviews on application of natural products to drug discovery, see: (a) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461. (b) Cragg, G. M.; Grothaus, P. G.; Newman, D. J. *Chem. Rev.* **2009**, *109*, 3012. (c) Mishra, B. B.; Tiwari, V. K. *Eur. J. Med. Chem.* **2011**, *46*, 4769. (d) Butler, M. S.; Robertson, A. A. B.; Cooper, M. A. *Nat. Prod. Rep.* **2014**, *31*, 1612.

(5) Kuranaga, T.; Sesoko, Y.; Sakata, K.; Maeda, N.; Hayata, A.; Inoue, M. *J. Am. Chem. Soc.* **2013**, *135*, 5467.

(6) For synthetic studies on yaku'amides from other groups, see: (a) Ma, Z.; Naylor, B. C.; Loertscher, B. M.; Hafen, D. D.; Li, J. M.; Castle, S. L. *J. Org. Chem.* **2012**, *77*, 1208. (b) Saavedra, C. J.; Boto, A.; Hernández, R. *Org. Lett.* **2012**, *14*, 3788. (c) Ma, Z.; Jiang, J.; Luo, S.; Cai, Y.; Cardon, J. M.; Kay, B. M.; Ess, D. H.; Castle, S. L. *Org. Lett.* **2014**, *16*, 4044.

(7) For recent accounts on total synthesis and biological evaluation of peptidic natural products from our laboratory, see: polytheonamide B (a) Itoh, H.; Inoue, M. *Acc. Chem. Res.* **2013**, *46*, 1567. antillatoxin (b) Inoue, M. *Proc. Jpn. Acad., Ser. B* **2014**, *90*, 56.

(8) For reviews on Cu-mediated enamide formation, see: (a) Evano, G.; Blanchard, N.; Toumi, M. *Chem. Rev.* **2008**, *108*, 3054. (b) Ma, D.; Cai, Q. *Acc. Chem. Res.* **2008**, *41*, 1450. (c) Surry, D. S.; Buchwald, S. L. *Chem. Sci.* **2010**, *1*, 13. (d) Evano, G.; Theunissen, C.; Pradal, A. *Nat. Prod. Rep.* **2013**, *30*, 1467. (e) Kuranaga, T.; Sesoko, Y.; Inoue, M. *Nat. Prod. Rep.* **2014**, *31*, 514.

(9) (a) Klapars, A.; Huang, X.; Buchwald, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 7421. (b) Jiang, L.; Job, G. E.; Klapars, A.; Buchwald, S. L. *Org. Lett.* **2003**, *5*, 3667.

(10) Yamashita, T.; Matoba, H.; Kuranaga, T.; Inoue, M. *Tetrahedron* **2014**, *70*, 7746.

(11) Friedrich-Bochnitschek, S.; Waldmann, H.; Kunz, H. *J. Org. Chem.* **1989**, *54*, 751.

(12) To prevent the palladium hydride-promoted isomerization of the double bond, 2-methyl-2-butene was utilized as its trapping agent.

(13) Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205.

(14) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.

(15) El-Faham, A.; Funosas, R. S.; Prohens, R.; Albericio, F. *Chem. - Eur. J.* **2009**, *15*, 9404.

(16) (a) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591. (b) Bhushan, R.; Brückner, H. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 3148.

(17) Takada, K.; Ninomiya, A.; Naruse, M.; Sun, Y.; Miyazaki, M.; Nogi, Y.; Okada, S.; Matsunaga, S. *J. Org. Chem.* **2013**, *78*, 6746.

(18) See Supporting Information for details.

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(19) One-pot deprotection procedure was adopted after the coupling with **6**, because the aqueous work-up appeared to cleave the Boc-protected amide bond between residues-3 and -4. For facile cleavage of the carbamate protected amide, see: Grehn, L.; Gunnarsson, K.; Ragnarsson, U. *J. Chem. Soc., Chem. Commun.* **1985**, 1317.
(20) Peptide natural products give variable NMR spectra depending on concentration, temperature, and purity. Thus, structural identity between natural and synthetic compounds is often difficult to be validated by the NMR spectral comparison. For recent examples, see: (a) Zou, B.; Long, K.; Ma, D. *Org. Lett.* **2005**, *7*, 4237. (b) Sugiyama, H.; Watanabe, A.; Teruya, T.; Suenaga, K. *Tetrahedron Lett.* **2009**, *50*, 7343. (c) Ma, B.; Litvinov, D. N.; He, L.; Banerjee, B.; Castle, S. L. *Angew. Chem., Int. Ed.* **2009**, *48*, 6104. (d) Ma, B.; Banerjee, B.; Litvinov, D. N.; He, L.; Castle, S. L. *J. Am. Chem. Soc.* **2010**, *132*, 1159.
(21) (a) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827. (b) Roehm, N. W.; Rodgers, G. H.; Hatfield, S. M.; Glasebrook, A. L. *J. Immunol. Methods* **1991**, *142*, 257.
(22) See Supporting Information for possible errors in the original structural determination.

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