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C_3 and 2D C_3 Marfey's Methods for Amino Acid Analysis in Natural Products

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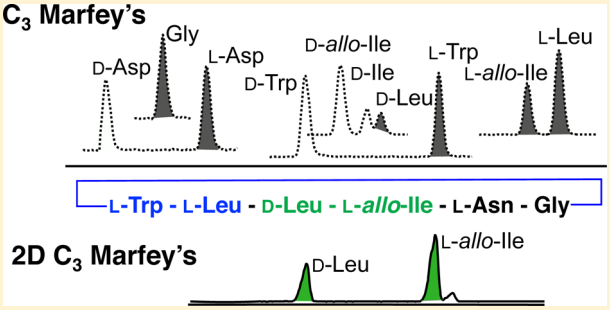
Supporting Information

ABSTRACT: We validate the improved resolution and sensitivity of the C_3 Marfey's method, including an ability to resolve all Ile isomers, against an array of amino acids commonly encountered in natural products and by comparison to an existing Marfey's method. We also describe an innovative 2D C_3 Marfey's method as an analytical approach for determining the regiochemistry of enantiomeric amino acid residues in natural products. The C_3 and 2D C_3 Marfey's methods represent valuable tools for probing and defining the stereocomplexity of hydrolytically accessible amino acid residues in natural products.



First described in 1984, Marfey's method[1] has emerged as a popular and effective procedure for determining the absolute configurations of hydrolytically accessible amino acid residues in natural products (typically but not exclusively peptides). This method employs four steps: (i) the natural product to be analyzed is subjected to acid hydrolysis (6 M HCl) to release amino acid residues, (ii) the hydrolysate is derivatized under alkaline conditions (1 M $NaHCO_3$) with *Nα*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA) or the enantiomer *Nα*-(2,4-dinitro-5-fluorophenyl)-D-alaninamide (D-FDAA), (iii) the neutralized derivatized analyte is subjected to HPLC-UV (340 nm) analysis, and (iv) HPLC retention times of derivatized amino acids in the analyte are compared with those of derivatized L and D amino acid standards. Key advantages of this method include improved detection sensitivity compared to underivatized amino acids, the use of readily available HPLC technology, and the commercial availability of both enantiomers of the chiral derivatizing agent (CDA) FDAA. The latter represents a significant but often underappreciated benefit. For example, where a D-amino acid standard is not available, the D-FDAA-L-amino acid derivative is a chromatographically (i.e., analytically) equivalent surrogate for the L-FDAA-D-amino acid derivative. Consequently, Marfey's analyses can be successfully performed with L-FDAA and D-FDAA derivatives of L-amino acid standards, without the need to source the frequently less accessible D-amino acid standards.

Over the last three decades several efforts have been made to improve Marfey's method.[2−6] One prominent example, the advanced Marfey's method, introduced the CDA *Nα*-(2,4-dinitro-5-fluorophenyl)-L-leucinylamide (L-FDLA), its racemate (L/D-FDLA), and a modified HPLC protocol supportive of ESIMS detection (Table 1).[7,8] Other adaptations incorporate *ad hoc* C_{18} HPLC protocols, herein collectively referred to as C_{18} Marfey's methods (Table 1).

Notwithstanding the widespread use of Marfey's, advanced Marfey's, and C_{18} Marfey's methods, an enduring shortcoming of all these methods has been the inability to differentiate L-Ile from L-*allo*-Ile, and D-Ile from D-*allo*-Ile.[3,6] With Ile commonly occurring in natural products, this limitation is problematic. For example, although the Marfey's method was correctly acknowledged as incapable of resolving Ile and *allo*-Ile diastereomers in the tetrapeptide asperterrestide A,[9] this did not preclude it being used to assign L-Ile residues in microcystilide A,[10] dichotomins J and K,[11] euryjanicin A,[12] C and D,[13] and E−G,[14] aeruginopeptins,[15] micropeptins 88-N and 88-Y,[16] kailuins A−F,[17−19] talaromins A and B,[20] callyaerins A, B, D, and J−L,[21,22] trichoderins A and A1,[23] falcitidin,[24] mullinamide A,[25] and stylissatin A[26] and D-Ile residues in anthranicine,[27] PF1171C,[28] PF1171F, and PF1171G.[29] Significantly these assignments focused on distinguishing L-Ile from D-Ile, without reference to *allo* isomers.

Alert to the *allo* option, other researchers employed adaptations of Marfey's method, including (i) chiral-phase HPLC to identify L-Ile residues in companeramides A and B,[30] L-*allo*-Ile in desomatides C and D,[31] myriastramides A−C,[32] and hirsutellide A,[33] D-*allo*-Ile in aspergillicins A, C, and D[34] and hytramycin I,[35] and L-*allo*-Ile and D-*allo*-Ile in aspergillicin E;[34] (ii) multiple (tandem) HPLC columns to identify D-*allo*-Ile in nostophicin[36] and yaku'amides A and B[37] and L-*allo*-Ile in lagunamides A−C;[38,39] and (iii) normal-phase (cellulose) HPLC to identify L-Ile and D-*allo*-Ile in teixobactin,[40] a very promising new class of antibiotic recently reported from an uncultured bacterium. Despite case-specific successes, none of these HPLC adaptations were systematically validated against all standard amino acids, and none have been adopted as a generic solution to

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Table 1. Summary of Prominent Marfey's Methods

	HPLC method[d]		
CDA	column (temp)	solvent elution	detection
Marfey's[a]			
L-FDAA or D-FDAA	reversed-phase (room temp)	linear gradient 10% to 50% MeCN in 50 mM triethyl ammonium phosphate at pH 3.0	UV (340 nm)
Advanced Marfey's[a]			
L-FDLA and D/L-FDLA)	C_{18} (40 °C)	1 mL/min, 45 min linear gradient elution from 25% to 65% MeCN/H_2O, with an isocratic TFA (0.01 M) modifier[b]	UV (340 nm) + ESIMS
C_{18} Marfey's[a,c]			
L-FDAA or D-FDAA	C_{18} (room temp)	1 mL/min, 45 min linear gradient elution from 15% to 45% MeCN in 0.1 M NH_4OAc/TFA buffer at pH 3.0	UV (340 nm)
C_3 Marfey's			
L-FDAA or D-FDAA	C_3 (50 °C)	1 mL/min, 55 min linear gradient elution from 15% to 60% MeOH/H_2O with a 5% isocratic 1% formic acid in MeCN modifier	UV (340 nm) + ESIMS

[a]Cannot resolve D/L-Ile from D/L-*allo*-Ile. [b]Flow rate and gradient profiles are optimized on a case-by-case basis. [c]This is an indicative HPLC method, as many variants have been reported.[4,5] [d]In all these methods, while absolute retention times of individual amino acids will vary between laboratories (e.g., HPLC configurations and columns), relative retention times are preserved. Retention times can be especially sensitive to pH and isocratic solvent modifier concentrations (note: as % TFA and formic acid can vary with storage, these should be prepared fresh).

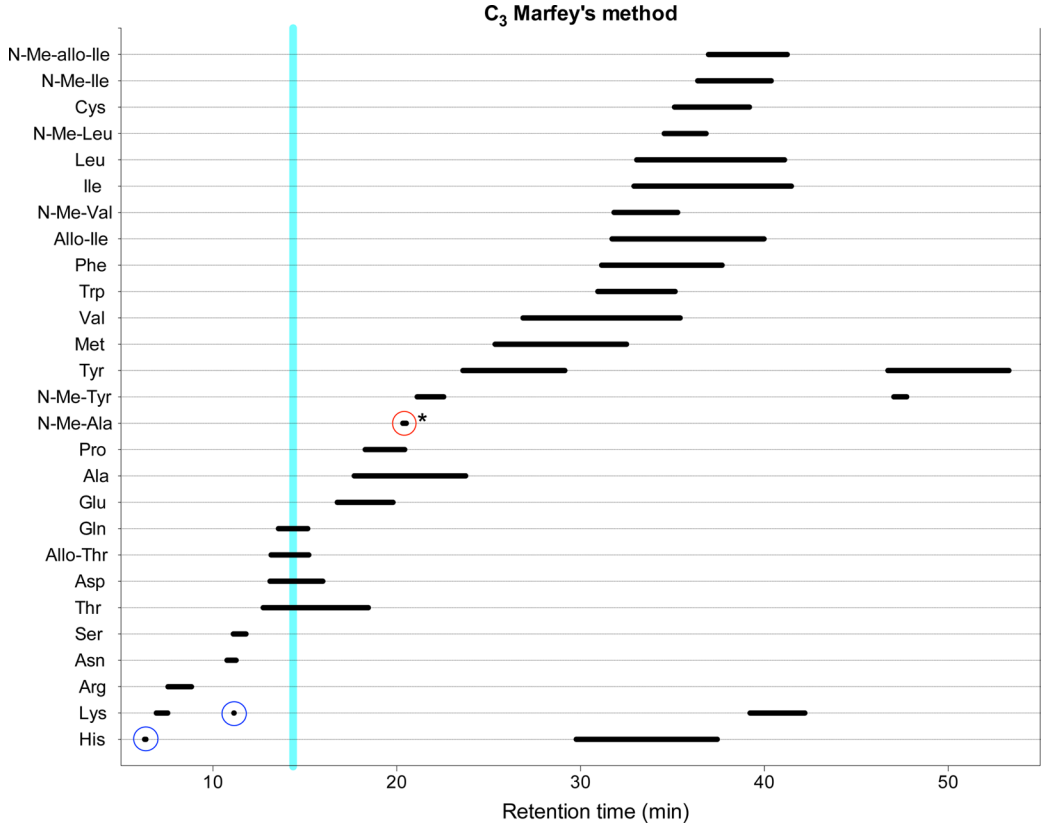

Figure 1. Derivatized amino acid standards analyzed by the C_3 Marfey's method. Black horizontal line: Difference in retention times between L-FDAA-L-AA/D-FDAA-D-AA (earlier t_R) and D-FDAA-L-AA/L-FDAA-D-AA (later t_R). Vertical cyan line: Residual Marfey's reagent. Red circle: Enantiomers are not resolved. Blue circle: Enantiomers are resolved by diderivatized analogues. *Not resolved.

the Ile challenge. Yet other researchers addressed the assignment of Ile configurations by modifying the CDA, for example, using 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate to identify L-Ile in sungsanpin[41] and L-Ile and D-Ile in surugamides,[42] or resorted to chemical degradation, identifying *N*-Me-L-Ile and L-Ile residues in epoxomicin,[43] or total synthesis, identifying L-*allo*-Ile in desotamide[44] and D-*allo*-Ile and L-Ile in aspergillicin F.[45] The aspergillicins are an especially noteworthy case, with the L-Ile-containing aspergillicin F being a suppressor of the innate immune system and its L-*allo*-Ile stereoisomer aspergillicin E exhibiting no suppressor activity, affirming a correlation between Ile configuration and biological activity. The examples cited above illustrate the need for a more effective analytical methodology, capable of rapid and cost-effective assignment of configurations to all amino acid residues encountered in natural products, inclusive of Ile.

Another enduring challenge in the structure elucidation of peptidic natural products has been assigning the regiochemistry of enantiomeric amino acid residues. For example, while the regiochemistry of L- and D-Ala residues in the cyclohexadepsipeptide paecilodepsipeptide A was inferred (unconvincingly) from NOESY correlations,[46] the regiochemistry of L- and D-Asn

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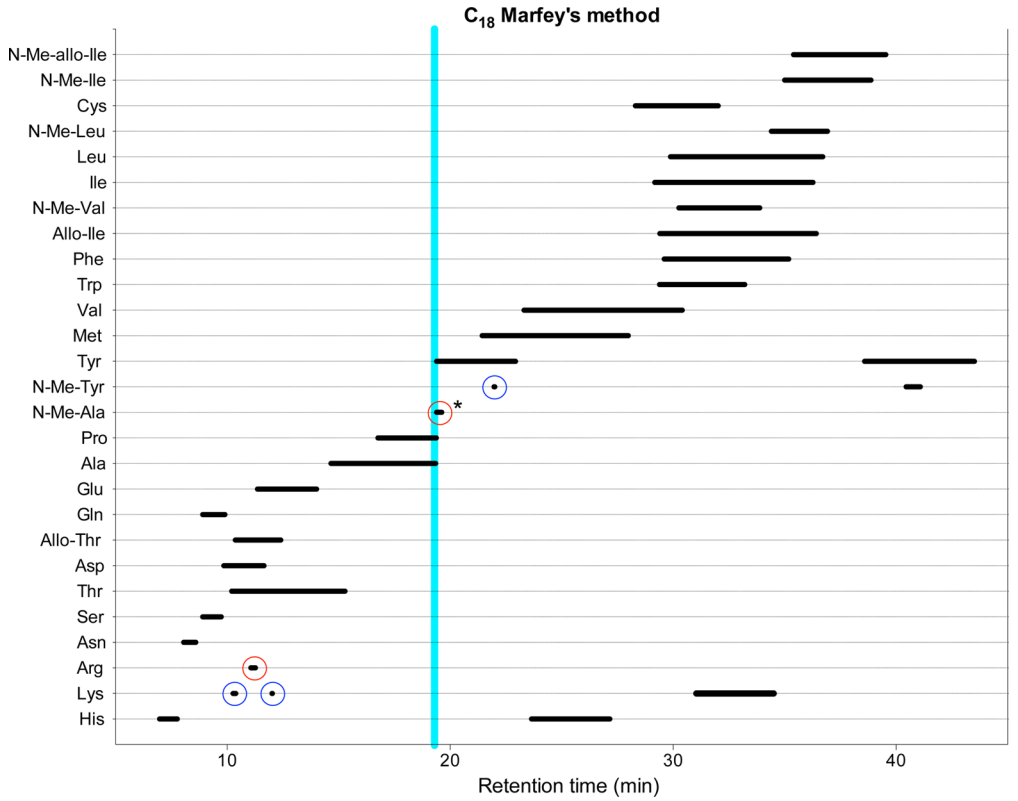

Figure 2. Derivatized amino acid standards analyzed by the C_{18} Marfey's method. Black horizontal line: Difference in retention times between L-FDAA-L-AA/D-FDAA-D-AA (earlier t_R) and D-FDAA-L-AA/L-FDAA-D-AA (later t_R). Vertical cyan line: Residual Marfey's reagent. Red circle: Enantiomers are not resolved. Blue circle: Enantiomers are resolved by diderivatized analogues. *Not resolved; however, enantiomer resolution can be achieved with a modified C_{18} Marfey's method [see Experimental Section].

in mojavensin A[47] and L- and D-Leu in the cyclohexapeptide desotamides B–D[31] and desotamide G[48] were asserted without comment, and the regiochemistry of L- and D-Leu residues in the lipopeptide hallobacillin was left unassigned.[49] As evidence of the effort required to secure unambiguous assignments, the regiochemistry of L- and D-Leu in the cyclohexapeptide desotamide A relied on the total synthesis of multiple isomers,[44] while the regiochemistry of L- and D-Ile in the cyclooctapeptide surugamides A–E relied on partial hydrolysis to tri- and dipeptide fragments, which were in turn identified by a combination of Marfey's analyses and HPLC comparison to six synthetic dipeptides.[42] The regiochemistry of L- and D-*N*-Me-Ala in companeramide B was determined by a combination of partial acid hydrolysis, followed by HPLC isolation of a diagnostic tetrapeptide fragment, and an advanced Marfey's analysis.[30] This regiochemical challenge is also well exemplified by kahalalide F, first isolated in 1993 as an exceptionally potent cytotoxic cyclic peptide from the sarcoglossan mollusk *Elysia rufescens* and its dietary green alga (*Bryopsis* sp.). The structure assigned to kahalalide F on the basis of NMR, acid hydrolysis, and chiral-phase GC-MS analysis was inclusive of five Val residues, including both L- and D-Val.[50] Although a 1999 chemical degradation proposed the regiochemistry of L- and D-Val (and all other amino acid) residues,[51] subsequent chemical synthesis[52] and degradation[53] studies led to a revision of regiochemistry. Significantly, these latter studies confirmed that the unnatural D/L-Val regioisomer was significantly less cytotoxic than the natural regioisomer, confirming the ecological/pharmacological importance of accurately determining regiochemistry. Significantly, to date no implementation of Marfey's method alone has proved sufficient to solve the regiochemical challenge posed by enantiomeric amino acid residues, and other approaches are labor intensive and costly in material and time.

Collectively, the examples cited above illustrate the need for improved analytical methodology capable of assigning both amino acid configurations and regiochemistry to enantiomeric amino acid residues in natural products. In responding to this need we set out to refine existing Marfey's methods, retaining the well-regarded L-FDAA and D-FDAA and focusing our efforts on developing a more effective, reproducible, and readily accessible achiral HPLC method with enhanced levels of resolution. Aware that natural products chemists deal with very small quantities of hard-won metabolites, we were also alert to the need for enhancing sensitivity. Illustrative of this latter point, the majority of Marfey's analyses cited above involved the acid hydrolysis of 0.5–5.0 mg of natural product, a level of consumption that is both excessive and in many instances impractical. Our aspiration was to develop a highly sensitive analytical Marfey's method capable of rapid and unambiguous assignment of configuration and enantiomer regiochemistry, across all commonly encountered amino acids.

RESULTS AND DISCUSSION

After extensive trials employing multiple achiral HPLC columns and numerous elution profiles (see Supporting Information), we settled on a C_3 HPLC column held at 50 °C, with a H_2O/MeOH gradient elution modified by isocratic 1% formic acid in MeCN, taking advantage of both UV (340 nm) and ESI(±)MS detection, the latter further enhanced by single ion extraction filters. To validate the resolution of this C_3 Marfey's method, we

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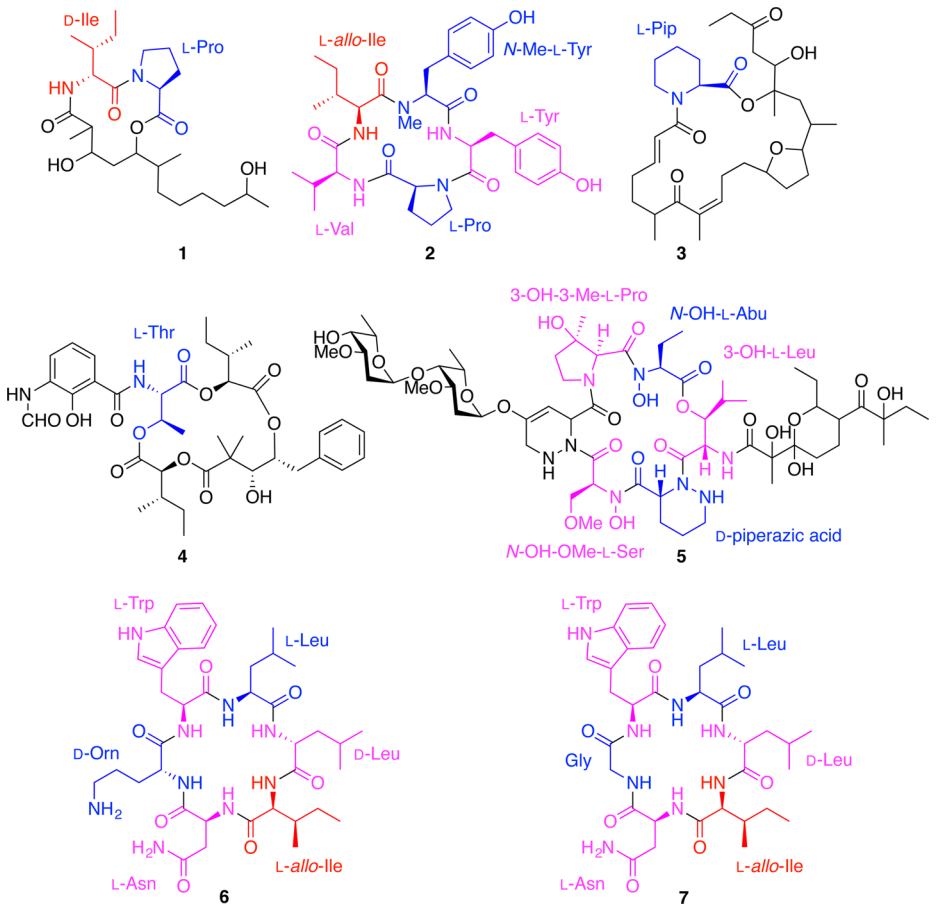

Figure 3. Published natural products incorporating amino acid residues identified by the C_3 Marfey's method.

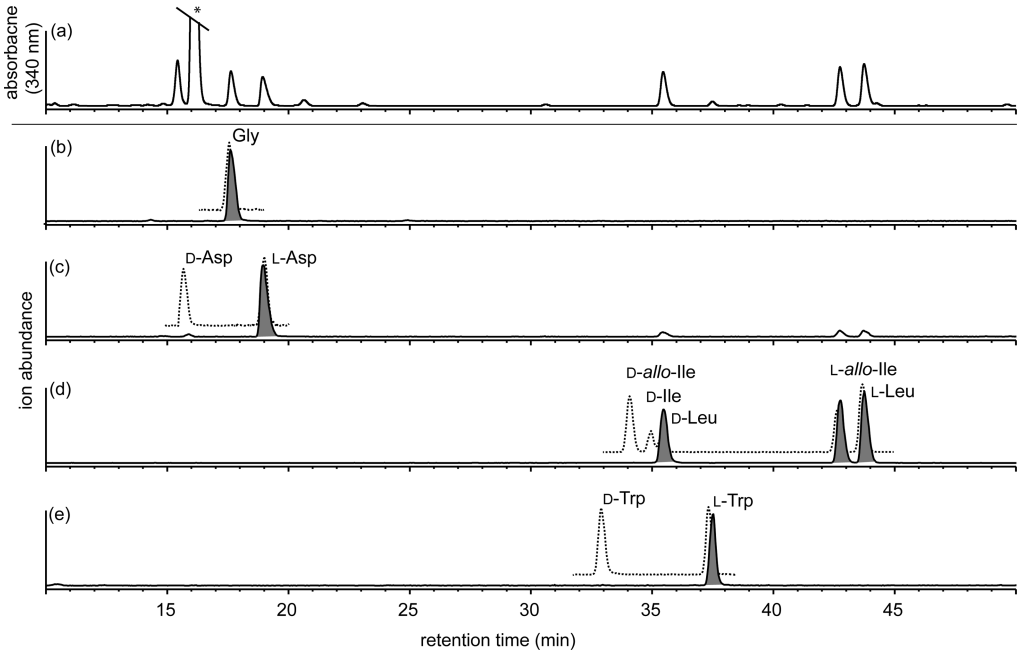

Figure 4. HPLC-DAD-MS chromatograms extracted from a C_3 Marfey's method analysis of 50 μg of desotamide A (7) derivatized with D-FDAA: (a) UV (340 nm), where * = residual D-FDAA; (b) SIE (m/z 328) for D-FDAA-Gly (shaded); (c) SIE (m/z 386) for D-FDAA-L-Asp (shaded); (d) SIE (m/z = 384) for D-FDAA-D-Leu, D-FDAA-L-*allo*-Ile, and D-FDAA-L-Leu (shaded); (e) SIE (m/z = 457) for D-FDAA-L-Trp (shaded). Note for (b)−(e) FDAA derivatives of authentic amino acid standards are also displayed (broken line).

applied it to all standard and several nonstandard (*N*-methylated) amino acids, with comparisons to a typical C_{18} Marfey's method (Figures 1 and 2, respectively). This analysis confirmed that the C_3 Marfey's method was capable of resolving

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all amino acids, including Ile stereoisomers (Figure S5). Moreover, in the C_3 Marfey's method the residual Marfey's reagent did not obscure any target amino acids, unlike the C_{18} Marfey's method, where residual reagent can mask L-FDAA-L-Tyr, L-FDAA-L-*N*-Me-Ala, D-FDAA-L-Pro, and D-FDAA-L-Ala.

During this development phase we applied the C_3 Marfey's method to assign amino acid configurations in the lipodepsipeptide acremolides [e.g., D-Ile and L-Pro in acremolide C (**1**)],[54] cyclopentapeptide cotteslosins [e.g., L-*allo*-Ile, L-Val, L-Tyr, *N*-Me-L-Tyr, and L-Pro in cotteslosin B (**2**)][55] and macrolide polyketide nocardiopsins [e.g., L-pipecolic acid in nocardiopsin A (**3**)],[56,57] as well as the anticancer *N*-formyl amino-salicylamide neoantimycins [e.g., L-Thr in neoantimycin G (**4**)],[58] the antimalarial glycol-hexadepsipeptide-polyketide mollemycin A (**5**),[59] and the antibacterial cyclohexapeptide desotamides and wollamides [e.g., L-Trp, L-Leu, D-Leu, L-*allo*-Ile, L-Asn, and D-Orn in wollamide A (**6**)][60] (Figure 3). Significantly, all these studies were performed at an analytical scale (50−100 μg) well below that routinely reported in the literature for Marfey's, advanced Marfey's, and C_{18} Marfey's methods. In addition to providing a comprehensive documentation and validation of our C_3 Marfey's method against a suite of amino acids commonly encountered in natural products (Figure 1), we take this opportunity to describe a new Marfey's derivatization, the 2D C_3 Marfey's method, which can assign regiochemistry to enantiomeric amino acid residues. The utility of the C_3 and 2D C_3 Marfey's methods is illustrated on desotamide A (**7**), as summarized below.

In a 1997 report describing the DeSota Falls soil *Streptomyces* cyclic hexapeptide desotamide A (**7**), the assignment of amino acid configuration and the regiochemistry of L- and D-Ile residues relied on the total synthesis of multiple isomers.[44] In 2014 the rediscovery of **7**, together with the new congeners desotamides E and F and wollamides A and B, from an Australian desert soil *Streptomyces* provided an opportunity to showcase the C_3 Marfey's method as a means to rapidly identify the configurations of all amino acid residues.[60] This process involved the acid hydrolysis of **7** (50 μg) followed by derivatization with D-FDAA and HPLC-DAD-MS analysis to generate the UV (340 nm) chromatogram illustrated in Figure 4a. While the UV detection approach employed by established Marfey's methods is an improvement over underivatized amino acids, it nevertheless suffers from several limitations. These include the relative instability of Trp to acid hydrolysis (i.e., leading to reduced sensitivity) and the masking effect of residual Marfey's reagent and near-identical retention times for several derivatized amino acids (i.e., leading to reduced resolution). In an effort to overcome these limitations, the C_3 Marfey's method employs ESIMS detection and single-ion extraction (SIE). For example, in the case of **7**, individual SIE chromatograms at *m*/*z* 328 (Figure 4b), 386 (Figure 4c), 384 (Figure 4d), and 457 (Figure 4e) readily distinguished the constituent amino acids by MW (i.e., providing enhanced sensitivity), with comparison to the retention times of L- and D-FDAA derivatives of L amino acid standards confirming these as Gly, L-Asp, D-Leu, L-*allo*-Ile, L-Leu, and L-Trp (i.e., providing enhanced resolution). Note, although Asn converts to Asp under acid hydrolysis, this does not preclude assignment of its absolute configuration.

With an effective C_3 Marfey's method in hand, we set out to develop a 2D Marfey's (partial hydrolysis followed by total hydrolysis) adaptation as a tool for assigning the regiochemistry of enantiomeric amino acid residues in peptidic natural products, which we refer to as the 2D C_3 Marfey's method. While partial hydrolysis Marfey's approaches have been used in the past, these have been relatively insensitive (i.e., operating on a 4−15 mg scale acid hydrolysis)[30,51,53] and often relied on the total synthesis and chromatographic and/or spectroscopic comparison to multiple putative hydrolysis products.[42] Significantly, none of these "partial hydrolysis" approaches have gained acceptance as a generally applicable solution. By contrast, the 2D C_3 Marfey's method operates at an analytical scale without recourse to chemical synthesis, as illustrated by its assignment of the L- and D-Leu regiochemistry in **7** (Figure 5). A short-duration

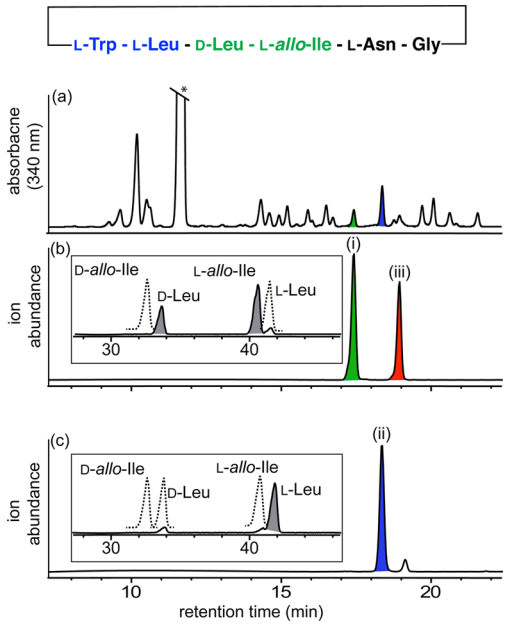

Figure 5. HPLC-DAD-MS chromatograms extracted from a 2D C_3 Marfey's method analysis of 150 μg of desotamide A (**7**) derivatized with D-FDAA: (a) UV (340 nm), where * = residual D-FDAA; (b) SIE (*m*/*z* 497) revealing (i) as D-FDAA-D-Leu-L-*allo*-Ile (green peak) and (iii) as D-FDAA-L-Leu-D-Leu (red peak). Inset corresponds to the SIE (*m*/*z* 384) for D-FDAA-derivatized D-*allo*-Ile, L-*allo*-Ile, D-Leu, and L-Leu standards (broken line) and the D-FDAA-derivatized acid hydrolysate of peak (i) (shaded). (c) SIE (*m*/*z* 570) revealing (ii) as D-FDAA-L-Trp-L-Leu (blue peak). Inset corresponds to the SIE (*m*/*z* 384) of the standards as per (b) and the D-FDAA-derivatized acid hydrolysate of peak (ii) (shaded).

partial hydrolysis of **7** (150 μg) generated peptide fragments that were derivatized with D-FDAA and analyzed by HPLC-DAD-MS. The UV (340 nm) chromatogram (Figure 5a) revealed an array of partial hydrolysis peptide fragments, with SIE chromatograms at *m*/*z* 497 (Figure 5b) and 570 (Figure 5c) selecting for the diagnostic dipeptides D-FDAA-L/D-Leu-L-*allo*-Ile and/or D-FDAA-L/D-Leu-D/L-Leu (i and iii) and D-FDAA-L-Trp-L/D-Leu (ii), respectively. The well-resolved peak (i) was purified by analytical HPLC and subjected (without further characterization) to acid hydrolysis and derivatization with D-FDAA. A subsequent C_3 Marfey's analysis (Figure 5b inset) confirmed the presence of both D-Leu and L-*allo*-Ile, unambiguously establishing the regiochemistry of the D- and L-Leu residues in **7**. As further evidence of this assignment, a comparable sequence of analyses carried out on peak (ii) confirmed that L-Leu (Figure 5c inset) was adjacent to L-Trp. While the broad utility of the 2D C_3 Marfey's method is conditional on the complexity of the partial hydrolysate of the target natural product, it is nevertheless a valuable analytical tool that should find general application in the field of natural products chemistry.

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In summary, this report provides a detailed account of the C_3 Marfey's method, documenting enhanced resolution and sensitivity in assigning configurations to hydrolytically accessible amino acid residues commonly encountered in natural products. This report also describes an innovative adaptation, the 2D C_3 Marfey's method, capable of determining the regiochemistry of enantiomeric amino acid residues in peptidic natural products, on an analytical scale and without recourse to chemical synthesis. These two methods represent valuable new analytical tools that can be used to probe the stereocomplexity of amino acid residues in natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. *Nα*-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA, synonym 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and *Nα*-(2,4-dinitro-5-fluorophenyl)-D-alaninamide (D-FDAA, synonym 1-fluoro-2,4-dinitrophenyl-5-D-alanine amide) were purchased from NovaBiochem. Amino acids and standards were purchased from NovaBiochem, BAChem Biosciences, Sigma, Fluka, or Merck. HPLC-grade MeCN and MeOH were degassed and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane prior to use. Water for HPLC was filtered through an ELGA PurelabUltra system prior to degassing and further filtration through a 0.45 μm hydrophilic polypropylene membrane. All other chemicals and solvents were of analytical grade.

HPLC-DAD-ESIMS. Analyses were performed on an Agilent 1100 series separations module equipped with a quaternary pump, vacuum degasser, well plate autosampler with thermostat control, thermostated column compartment, diode array detector detection (DAD) system with analytical flow cell, and a single quadrupole electrospray ionization mass detector operating in both positive and negative ionization modes, over m/z 100 to 1000.

C_3 Marfey's Method (Desotamide A). A sample of **7** (50 μg) in 6 M HCl (100 μL) was heated to 100 °C in a sealed vial for 8−12 h, after which the hydrolysate was concentrated to dryness at 40 °C under a stream of dry N_2. The hydrolysate was then treated with 1 M $NaHCO_3$ (20 μL) and D-FDAA (1% solution in acetone, 40 μL) at 40 °C for 1 h, after which the reaction was neutralized with 1 M HCl (20 μL) and filtered (0.45 μm PTFE) prior to HPLC-DAD-ESIMS analysis. An aliquot (10 μL) of the analyte was injected into an Agilent Zorbax SB-C_3 column, 5 μm, 150 × 4.6 mm, 50 °C, with a 1 mL/min, 55 min linear gradient elution from 15% to 60% MeOH/H_2O with a 5% isocratic modifier of 1% formic acid in MeCN. The analyte amino acid content was assessed by UV (340 nm) and ESI(±)MS monitoring, supported by SIE, with comparison to authentic standards.

2D C_3 Marfey's Method (Desotamide A). A sample of **7** (150 μg) in 2 M HCl (100 μL) was heated at 100 °C in a sealed vial for 2 to 3 h, after which the hydrolysate was concentrated to dryness at 40 °C under a stream of dry N_2. The hydrolysate was then treated with 1 M $NaHCO_3$ (20 μL) and D-FDAA (1% solution in acetone, 40 μL) at 40 °C for 1 h, after which the reaction was neutralized with 1 M HCl (20 μL), diluted with MeCN (100 μL), and filtered (0.45 μm PTFE) prior to HPLC-DAD-ESIMS analysis. An aliquot (2 μL) of the analyte was injected into an Agilent Zorbax SB-C_3 column, 5 μm, 150 × 4.6 mm, 30 °C, with a 1 mL/min, 25 min linear gradient elution from 10% to 65% MeCN/H_2O with a 5% isocratic modifier of 1% formic acid in MeCN. The amino acid content was assessed by UV (340 nm) and ESI(±)MS monitoring, supported by SIE to identify peaks of interest. The remaining analyte was subjected to HPLC-DAD purification using the same conditions as above. Peaks of interest were collected, concentrated to dryness at 40 °C under a stream of dry N_2, and subjected to C_3 Marfey's method as described above for desotamide A.

C_3 Marfey's Method (Amino Acid Standards). Amino acid standards (as listed in Figure 1) were subjected to derivatization with L- and D-FDAA (see Supporting Information) and HPLC-DAD-ESIMS analysis as detailed above for the C_3 Marfey's method.

C_{18} Marfey's Method (Amino Acid Standards). Amino acid standards (as listed in Figure 2) were subjected to derivatization with L- and D-FDAA as detailed in the Supporting Information. Aliquots (5 μL) of each analyte were injected into an Agilent Zorbax Eclipse XDB-C_{18} HPLC column, 5 μm, 150 × 4.6 mm, 25 °C (rt), with a 1 mL/min, 45 min linear gradient elution from 15% to 45% MeCN in an NH_4OAc/TFA buffer adjusted to pH 3.0. The amino acid content in analytes was assessed by UV (340 nm), with comparison to authentic standards.

A Modified C_{18} Marfey's Method (*N*-Me-Ala). Natural product acid hydrolysis and the synthesis of FDAA derivatives of the acid hydrolysate and of amino acid standards were performed as indicated above for the C_{18} Marfey's method. Aliquots (5 μL) of each analyte were injected into an Agilent Zorbax SB-C_{18} HPLC column, 5 μm, 150 × 4.6 mm, 50 °C, with a 1 mL/min, 35 min isocratic elution of 23% MeOH/H_2O with a 5% isocratic modifier of 1% formic acid in MeCN. These condition resolved L-FDAA-*N*-Me-L-Ala (t_R = 23.3 min) from D-FDAA-*N*-Me-L-Ala (t_R = 24.2 min).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01125.

HPLC chromatograms, development of the C_3 Marfey's method, and experimental details for 2D C_3 Marfey's method (PDF)

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Notes

The authors declare no competing financial interest.

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