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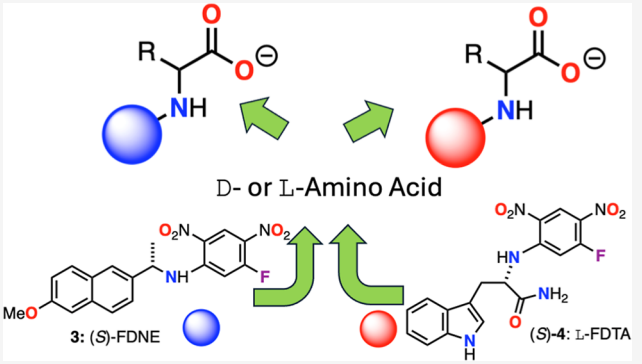
ABSTRACT: The configurational analysis of amino acids (AAs) in natural product peptides, often containing nonproteinogenic AAs, is mostly carried out by the venerable Marfey's method using a chiral derivatizing agent (CDA) 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA)—Marfey's reagent—which undergoes S_NAr reaction of the 1° amino group. The resulting AA-DAA derivatives are mostly well-separated by reversed-phase HPLC, but some DAA derivatives resist resolution. Here, we report the synthesis and characterization of two CDAs: L-FDTA (**4**) in which the L-alanine-derived auxiliary is replaced by L-tryptophanamide and (*S*)-FDNE (**3**) where the auxiliary is *S*-(6-methoxynaphth-2-yl)-1-ethylamine. Side-by-side comparisons of the two reagents were carried out by AA derivatization and reversed-phase HPLC analysis with variables such as organic solvent, additives, and the ionic strength of the mobile phase. L-DTA derivatives of L- and D-AAs were found to show superior HPLC performance and an improvement in resolutions. When incorporated into the mobile phase, the ammonium ion (NH_4^+, 0−100 mM) showed a dramatic influence on differential retention times [$\Delta t_R = \Delta t_{RD} - \Delta t_{RL}$] of several key AAs. We attributed the effect to π−cation interactions between the indole ring of DTA and the NH_4^+ counterion in the analyte, a hypothesis supported by ^{1}H NMR titrations and DFT calculations.



1. INTRODUCTION

Chiral derivatizing agents (CDAs) have found use for nonempirical analysis of enantiomeric compositions of amino acids (AAs), but perhaps none so widely utilized as the eponymous reagent of Marfey's method.[1] The challenge of assignment of AAs in peptides can be illustrated by the history of enantiomeric analysis of AAs in antitumor cyclic peptides from the Caribbean tunicate *Tridemnum solidum* (Figure 1). The didemnin family of peptides contain less-frequently encountered AAs including D-*N*-methylleucine, *N*,*O*-dimethyltyrosine, and the γ-AA isostatine. Early studies of the structure of didemnin B (**1a**), discovered in 1981 by the Rinehart group and the first marine natural product to enter anticancer clinical trials,[2] relied on classical methods based on peptide hydrolysis, conversion of the resulting AAs to suitable volatile derivatives, and GC identification of the analytes by comparison with standards. Contemporary HPLC analysis of AA composition and configuration of plitidepsin (dehydrodidemin B, **1b**, Aplidin)[3] from *Aplidium albicans*, and related tamandarins A and B from an unidentified didemnid ascidian,[4] were achieved efficiently using Marfey's analysis. Plitidepsin (**1b**), now approved for treatment of multiple myeloma,[5] was also

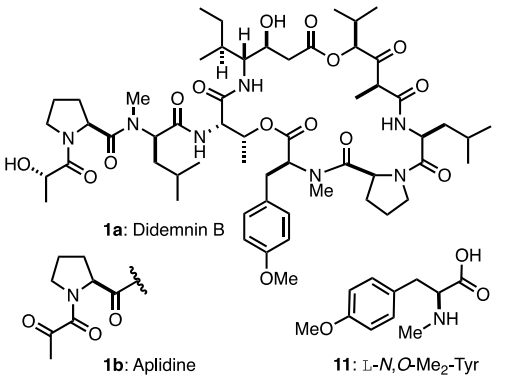


Figure 1. Structures of didemnin B (**1a**), aplidine (**1b**), and L-*N*,*O*-dimethyltyrosine (L-*N*,*O*-Me_2-Tyr, **11**).

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discovered to improve clinical outcomes of patients suffering from COVID-19.[6]

The assignment of absolute configuration of α-AAs by Marfey's method relies on reaction of the former with Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA, **2**), reversed-phase HPLC separation of the resulting diastereomers, and matching the retention times (t_R) against standards prepared in the same way from authentic L- and D-AAs. While Marfey's derivatives of most less-polar L- and D-AAs are well-resolved at long retention times, separations of Marfey's derivatives of polar AAs, such as Ser, Thr, Lys, and Arg, are more challenging. Even among nonpolar AAs, Marfey's method poorly resolves several N^{α}-Me-AAs, other nonproteinogenic AAs, the classic case of L-Ile and *allo*-D-Ile, while some others, not at all. Variants of Marfey's reagent have been introduced in which the L-alaninamide chiral auxiliary is replaced with L-valinamide,[7] L-leucinamide,[8] L-methioninamide,[9] L-prolinamide, L-phenylglycinamide,[9] or L-phenylalaninamide.[10] A chiral "diazaspiroketone", derived from (−)-menthone, glycinamide, and 2-fluoro-3,5-dinitrobenzoic acid, has also been assessed in AA analysis.[11] These and other Marfey's reagent variants have been extensively reviewed,[12−14] most recently in 2023 by the comprehensive retrospective by Kijjoa and coauthors.[15]

Typically, homologous CDA variants lead to longer and improved retention times, mostly with wider differential retentions on the HPLC column [$\Delta t_R = t_R$(D-AA) − t_R(L-AA)] due to increased hydrophobicity of the analyte. In most cases, the L-AA-Marfey's derivative elutes before the D-diastereomer. Improvements have also been achieved by altering analytical conditions; for example, the use of LCMS detection with custom mobile phase gradient profiles,[16] alkylammonium phosphate buffers,[10] or replacements of the HPLC C_{18} stationary phase with shorter-chain (C_3) bonded phase and expansion into the so-called "2D C_3" space.[17]

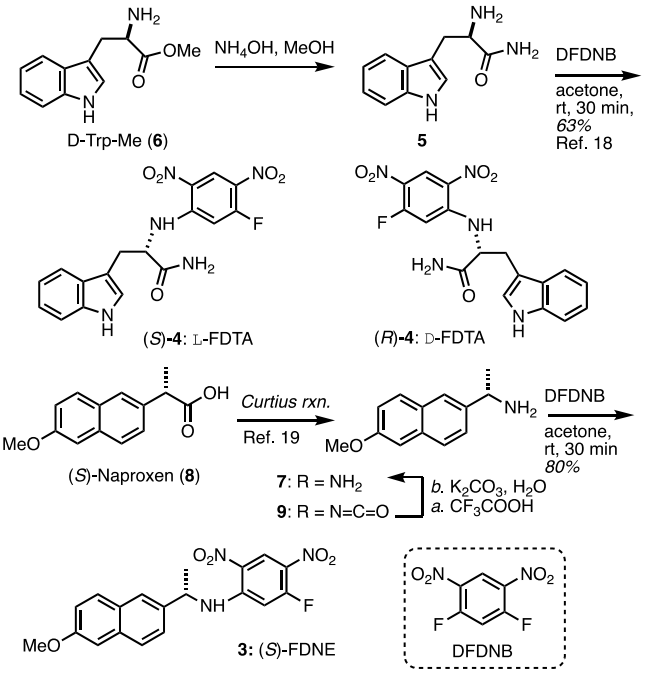
In our investigations of marine-derived peptides, reliable HPLC resolution was required for chiral derivatives of two critical classes of AAs: L- and D-*N*-Me-Ala and L- and D-Ile from their diastereomeric *allo*-isomers, all notoriously difficult problems with current Marfey-type reagents. We sought to transcend common replacements of the conventional AA-based auxiliaries, designed upon simple empirical leanings toward "more hydrophobic" derivatives, and focus on those containing π-rich aryl and heteroaromatic rings. Our guiding design principle was the introduction of a π-rich donor aromatic ring to the structure of the CDA that would enhance π interactions, alter the dynamics of analyte residency time between mobile and stationary phases during HPLC separation, and improve resolution. It was anticipated prospective replacement of a "π-neutral" phenyl substituent, found in such phenylalaninamide-derived Marfey-type reagents,[10] with a "π-rich ring" would enhance intramolecular π-acid−π-base interactions between the 2,4-DNP, augmented by the usual expected increase in hydrophobic interactions. Additionally, we projected that the inherent fluorescent properties of suitable aryl rings would confer the added advantage of sensitivity through fluorescence detection in critical analyses at the pmole level. Here, we describe the synthesis, characterizations, and deployment of two new CDAs, (*S*)-**3** and L-**4**, for analysis of enantiomeric compositions of AA derivatives and their scope and limitations for enantiomeric AA analyses.

The syntheses of (*S*)-**3** and L-**4** were achieved by replacement of L-alaninamide in **2** with one of two chiral amines—*S*-(6′-methoxynaphth-2′-yl)-1-ethylamine (**5**) and D-tryptophanamide (**6**), respectively. Both reagents were evaluated in side-by-side performance comparisons by derivatization-HPLC analysis of panels of representative AAs and shown to offer complementary advantages over FDAA. Derivatives **4a** of L-FDTA (**4**) with AAs provided superior performance in the most critical cases. A possible mechanism for enhanced HPLC separation is proposed, supported by NMR measurements and computational methods, that invokes π−cation interaction of the NH_4^+ ion with the aryl rings in HPLC analyte discrimination.

2. RESULTS AND DISCUSSION

Preparation of L-5-fluoro-2,4-dinitrophenyl-N^{α}-L-tryptophanamide (L-FDTA, *S*-**4**)[18] was achieved using a variant of Marfey's protocol.[1] D-Tryptophanamide (D-Trp-NH_2, **5**), obtained by ammonolysis of D-Trp methyl ester (**6**, Scheme 1), was

Scheme 1. Synthesis of (*S*)-FDNE (3) and D-FDTA (4)



converted to (*R*)-**4** by reaction with 1,5-difluoro-2,4-dinitrobenzene (DFDNB).[18] In a parallel manner, (*S*)-(6-methoxynaphth-2-yl)-1-ethylamine (**7**)—conveniently prepared by Curtius reaction of commercial (*S*)-(+)-naproxen (**8**) to give isocyanate **9**, followed by hydrolysis[19]—was reacted with DFDNB to give (*S*)-(1-fluoro-2,4-dinitrophenyl-naphth-2-yl)-1-ethylamine (FDNE, (*S*)-**3**).[20]

The UV−vis spectra of **3** and **4** exhibited long-wavelength absorptions attributed to the dinitrophenyl group identical to that of FDAA (λ_{max} 335 nm, ε 14,500); however, the additional chromophores present in **3** and **4** conferred modest improvements in sensitivity of detection due to larger extinction coefficients, ε, at shorter λ_{max} (Table S3). Unfortunately, the fluorescent properties of naproxen and Trp were abolished in their derivatives **3a** and **4a** (see Supporting Information) most likely through internal quenching by the 2,4-dinitrophenyl group.

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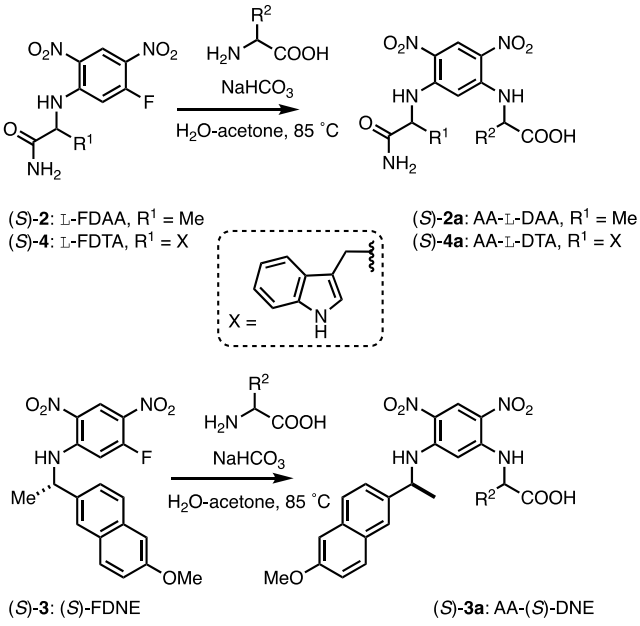
The use of complementary L- and D-Marfey's reagents is useful in cases where only one enantiomer of a particular standard AA is available, say L-. The L-AA-D-Marfey's diastereomer, of course, will have the same retention time as the unavailable and antipodal D-AA-L-Marfey's diastereomer. For the purposes of this study, comparative AA analyses were carried out with different AAs and single enantiomers of CDAs, L-FDAA (**2**), (*S*)-FDNE (**3**), or L-FDTA (**4**), to give their corresponding diastereomeric derivatives **2a**–**4a** (Scheme 2).

Scheme 2. Derivatization of AAs with L-FDAA (2), L-FDNE (*S*-3), or L-FDTA (4)



Derivatizations of AAs were carried out by S_NAr substitution with a CDA reagent under conditions slightly modified from Marfey's original protocol ($NaHCO_3$, H_2O/acetone, 85 °C, 30 min, Scheme 2),[1] which shortened reaction times without compromising efficiency (see Experimental Section).

The retention times t_R of L- and D-AA DTA and DNE derivatives, **4a** and **3a**, prepared from L-**4** and *S*-**3**, respectively (Table 1), increased as a function of increased hydrophobicity, in accordance with the mechanism proposed by Harada and co-workers for the separation of AA in the "advanced Marfey's method".[21,22] The differences in retention times of the L- and D-AA derivative, Δt_R, were also modulated by hydrophobicity of the aromatic rings.[8] Differential retention times, Δt_R, of diastereomeric derivatives, **3a**, prepared with (*S*)-**3** and a test panel of AAs ((±)-Ala, (±)-Phe, (±)-Glu, (±)-Leu, (±)-Ser, and (±)-Pro) showed poorer resolutions (Table 1) compared to those prepared from L-**4** (for conditions of analyses conducted by UHPLC and conventional analytical HPLC, see Experimental Section and table footnotes, below). In contrast, derivatization of AAs with L-**4** gave diastereomers with overall increased retention times and improved Δt_R. The order of elution of L-DTA derivatives **4a** mostly followed those of L-DAA derivatives **2a** (L-AA before D-AA), while—unexpectedly—reversal of retention times was observed for *S*-DNE derivatives of Leu, Ser, and Pro.

Direct comparative HPLC analysis of an expanded panel of AA derivatives, prepared from Marfey's reagent, L-FDAA (**2**) and L-FDTA (**4**), is shown in Tables 3 and 4.[23] The

Table 1. HPLC Analysis[a] of L-DNP- and L-DTA-Derivatives (3a and 4a) of Selected (±)-AAs Prepared from (*S*)-3 and L-4

	3a from *S*-FDNE (**3**)			**4a** from L-FDTA (**4**)		
	retention time, t_R/min		Δt_R[b]	retention time, t_R/min		Δt_R[b]
AA	L-	D-	/min	L-	D-	/min
(±)-Ala	7.43	7.43	0.00	8.08	8.86	0.78
(±)-Phe	8.50	8.93	0.43	9.95	10.81	0.86
(±)-Glu	5.53	5.69	0.16	7.49	7.73	0.24
(±)-Leu	8.40	8.14	−0.26	9.98	11.11	1.13
(±)-Ser	5.64	5.53	−0.11	11.08	11.46	0.38
(±)-Pro	7.15	6.95	−0.20	8.21	8.75	0.54

[a]Hypersil GOLD C_{18} column, 50 × 2.1 mm (1.9 μm), and a flow rate of 0.500 mL min^{-1}. Elution was conducted with a step gradient of 10% CH_3CN–H_2O-0.1% HCO_2H to 0.3 min to 50% CH_3CN–H_2O/0.1% HCO_2H to 11 min and 100% CH_3CN to 13 min, followed by re-equilibration with 10% CH_3CN–H_2O/0.1% HCO_2H for 2 min; void time, t_0 = 0.53 min. [b]$\Delta t_R = t_R$(D-AA) − t_R(L-AA). Negative values indicate reversal of the elution order.

diastereomers were judged to be "resolved" if they showed baseline separation, although partial separations were also considered significant. Similar elution patterns were observed for both the L-DAA and L-DTA derivatives of most of the neutral AAs; for example, the L-AA derivative eluted before the D-AA isomer. Under the same conditions (see footnote of Table 2), neither reagent resolved (±)-Asp, while only reaction with L-**4**, followed by HPLC, allowed resolution of both (±)-citrulline and (±)-Ser.

Major differences were observed for DTA derivatives of polar AAs (±)-Glu and (±)-*N*-Me-Asp: D-AA derivatives eluted before their L-diastereomers. For the basic AAs, (±)-Lys, (±)-Orn, and (±)-His, with two reactive amino groups (α-NH_2, ω-NH_2 groups, or imidazole), both mono- and diderivatives were obtained. Pairs of mono-α-*N* and di-*N*,*N*′-L-DAA derivatives of all three basic AAs were well-resolved, but in contrast, the corresponding L-DTA derivatives showed poor resolution (Δt_R) for L-and D-AAs, despite increased retention times. The L-DTA derivatives of (±)-Tyr, which has two nucleophilic groups (phenoxyl and α-NH_2), gave a similar result. The latter observations imply a change in the mechanism of separation for the AA derivatives. Notably, the L-DAA derivatives of the enantiomers of Ser, *iso*-Ser, *N*-Me-Ala, and citrulline (Table 2) failed to resolve, but were well-separated as their L-DTA derivatives (Table 3, see also S2). For example, L-DTA-Ser, L-DTA-*iso*-Ser, L-DTA-*N*-Me-Ala, and L-DTA-citrulline showed differential separations of Δt_R = 0.50, 0.37, 0.55, and −0.38 min, respectively. Some L-DTA derivatives (e.g., Asn and Lys) gave poor or no resolution which comes as no surprise as no one CDA can be expected to optimally resolve all AA derivatives across a range of polarities. Interestingly, mono-α-L-DTA-Orn showed no separation (Table 3), but di-α,γ-L-DTA-Orn diastereomers were resolved (Δt_R = 0.21 min).

A perennial challenge in AA analysis using modified Mosher's methods has been efficient separation of derivatives of Ile and D-*allo*-Ile. While Ile and L-*allo*-Ile are readily separated (e.g., using Capon's "2D C_3" variant of Marfey's method paired with DAA derivatives),[17] the resolution of L-Ile and D-*allo*-Ile has always been problematic. In natural product peptides, D-*allo*-Ile arises from L-Ile residues by α-epimerization

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Table 2. Analyses of Marfey's (L-DAA) Derivatives of (±)-AAs[a]

AA	elution order	retention time, min		Δt^b, min	resolved?	α^c
		L-AA	D-AA			
Ala	L → D	5.20	7.38	2.18	Y	1.42
norvaline	L → D	10.28	13.78	3.50	Y	1.34
Val	L → D	9.73	13.25	3.52	Y	1.36
Leu	L → D	13.25	16.55	3.30	Y	1.25
Ile	L → D	12.67	16.18	3.51	Y	1.28
Met	L → D	8.98	12.30	3.32	Y	1.37
Phe	L → D	13.28	16.16	2.88	Y	1.22
Tyr (di-*N*,*O*)	L → D	18.97	21.45	2.48	Y	1.13
Pro	L → D	5.23	6.10	0.87	Y	1.17
Ser	L → D	3.02	3.02	0.00	N	1.00
allo-Thr	L → D	3.57	4.40	0.83	Y	1.23
Asn	[d]	2.72	2.72	0.00	N	1.00
Glu	L → D	4.46	5.57	1.11	Y	1.25
Asp	L → D	3.57	4.55	0.98	Y	1.27
Lys (mono-α)	L → D	2.11	3.21	1.10	Y	1.52
Lys (di-α,ε)	L → D	19.19	21.36	2.17	Y	1.11
Orn (mono-α)	D → L	2.26	1.76	−0.50	N	1.28
Orn (di-α,δ)	D → L	16.93	15.25	−1.68	Y	1.11
His (mono-α)	D → L	1.72	1.50	−0.22	N	1.15
His (di-α,δ)	L → D	9.48	11.60	2.12	Y	1.22
Arg	L → D	1.76	2.07	0.31	N	1.18
Cit	[d]	4.15	4.15	0.00	N	1.00
N-Me-Ala	[d]	8.39	8.39	0.00	N	1.00
N-Me-Asp	D → L	3.49	4.29	0.80	Y	1.23
iso-Ser	[d]	3.82	3.82	0.00	N	1.00
N,*O*-Me$_2$-Tyr[e]	L → D	18.50	19.04	0.54	Y	1.03

[a]UHPLC conditions: Hypersil GOLD C_{18} column, 50 × 2.1 mm (1.9 μ particle), and a flow rate of 0.50 mL min^{-1}. Elution was completed with a linear gradient of 15%−45% CH_3CN−H_2O/0.1% HCOOH for 25 min, followed by 100% CH_3CN for 2 min and re-equilibration with 15% CH_3CN−H_2O/0.1% HCOOH for 3 min. [b]$\Delta t_R = t_R$(D-AA) − t_R(L-AA). [c]$\alpha = (t_RA - t_0)/(t_RB - t_0)$, where t_0 is void time and A and B are the slower and faster eluting components, respectively. For definitions of other parameters, see footnotes of Table 1. [d]Co-eluted diastereomers. [e]*N*-Methyl-*O*-methyl tyrosine.

(epimerase-mediated) in the cognate NRPS assembly line. We were pleased to observe excellent separation of the DTA derivatives of L-Ile and D-*allo*-Ile (Table 3) (Δt_R = 1.03 min), an improvement over the corresponding DAA diastereomers (Table 4, Δt_R = 0.16 min).[24]

Resolution of N^{α}-methyl AA derivatives can also be challenging. L- and D-*N*-Me-Ala DAA derivatives (**2a**, Table 3) failed to separate under conditions where the corresponding DTA derivatives, **4a**, were resolved (Δt_R = 1.03 min). L- and D-*N*-Me-Asp DTA and DAA derivatives (**4a** and **2a**) were both cleanly resolved (Tables 3 and 4). Notably, the L- and D-*N*-Me-Asp-DTA **4a** were well-separated (t_R = 11.67 and 10.51 min, Δt_R = −1.16 min, respectively; note reversal of elution order), albeit at retention times longer than their DAA counterparts, **2a** (t_R = 3.49 and 4.29 min, Δt_R = 0.80 min).

Further examination of the separation characteristics of those critical AAs with $\Delta t_R \leq 1.00$ min was carried out by testing the effect of ammonium acetate in the HPLC mobile phase (NH_4OAc, 20 mM) on the elution behavior of L-DTA-derivatives. Some literature variants of Marfey's method have employed salt in the mobile phase: either Et_3NH^+ phosphate or volatile NH_4OAc. In the present work, aqueous CH_3CN gradients (0.1% TFA or HCO_2H), without salt buffer, effected little or no separations of some AA-L-DAA derivatives (**2a**, e.g., AA = (±)-Ser, (±)-*iso*-Ser, and (±)-citrulline, Tables 3 and 4) in contrast to the literature results for (±)-Ser which resolved under similar elution conditions.[10] The latter observation suggests some dependency of separation upon the stationary phase, e.g., column particle homogeneity, differences in proprietary column composition, or simply age-related column degradation.

Improved resolution was observed for most of the AA L-DTA derivatives in the presence of NH_4OAc in the mobile phase (Table 4); these included (±)-Lys, (±)-Orn, (±)-His, (±)-Asp, and (±)-*N*-Me-Ala. The L-DAA-derivatives of AA with different charged side chains (±)-citrulline (neutral) and (±)-Arg (positive) failed to separate and coeluted as one peak. The largest improvement in separation—defined as $\Delta\Delta t_R$, the difference of absolute values of Δt_R in the presence and absence of NH_4OAc, respectively—occurred with (±)-Arg and (±)-Lys, which failed to resolve under the former conditions (Table 3) but were successfully resolved as DTA derivatives (Table 5). The diastereomers of (±)-Arg-L-DTA showed improved separation ($\Delta\Delta t_R$ = −0.62 min), while the $N^{\alpha,\varepsilon}$-double-derivative (±)-Lys-[L-DTA]$_2$ separated with the largest difference in retention times ($\Delta\Delta t$ = 10.11 min) under the latter conditions (Table 5).

The origin of the dramatic effect of NH_4OAc upon retention times, where the mobile phase is set to approximately pH ∼ 7,[25] appears to be related to two properties: the completely ionized RCO_2^- group of the AA and the presence of the indole heterocycle. Contrary to presumptions of the dominance of H-bonding interactions between stationary and mobile phases in HPLC separations, the key interactions of Marfey's derivatives,

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Table 3. HPLC Analyses[a] of L-DTA Derivatives of (±)-AAs

AA	DTA				
		t_R, min			
		L	D	Δt_R[b]	α[b]
Ala	L → D	13.08	15.52	2.44	1.19
Nva[c]	L → D	16.79	20.26	3.47	1.21
Val	L → D	16.22	19.85	3.63	1.22
Leu	L → D	18.85	22.36	3.51	1.19
Ile	L → D	18.40	22.06	3.66	1.20
Met	L → D	15.75	18.88	3.13	1.20
Phe	L → D	18.83	21.60	2.77	1.15
N,*O*-Me$_2$-Tyr (**11**)	L → D	25.87	26.10	0.23	1.01
Pro	L → D	13.57	15.28	1.71	1.13
Ser	L → D	10.43	10.93	0.50	1.05
allo-Thr	L → D	10.98	12.24	1.26	1.11
Asn	[d]	9.73	9.73	0.00	1.00
Glu	D → L	12.82	11.92	−0.90	1.08
Asp	L → D	10.79	11.76	0.97	1.09
Lys (mono-α)	[d]	9.91	9.91	0.00	1.00
Lys (di-a,e)	[d]	24.02	24.02	0.00	1.00
Orn (mono-α)	[d]	8.93	8.93	0.00	1.00
Orn (di-a,d)	L → D	22.30	22.51	0.21	1.01
His (mono-α)	D → L	7.53	6.34	−1.19	1.19
His (di)	L → D	19.65	20.37	0.72	1.04
Arg	L → D	8.12	8.74	0.62	1.08
Cit[e]	D → L	10.93	11.31	0.38	1.03
N-Me-Ala	L → D	19.40	19.95	0.55	1.03
N-Me-Asp	D → L	11.67	10.51	−1.16	1.11
iso-Ser[f]	L → D	16.67	17.04	0.37	1.02
Ile[g]	L → D	34.41			
allo-Ile[g]	L → D	33.38			

[a]Conditions: Reversed-phase C_{18} column, 50 × 2.1 mm (1.9 μm), flow rate 0.50 mL min^{-1}; elution completed with a linear gradient of 15−45% CH_3CN/H_2O-0.1% HCOOH for 25 min, followed by 100% CH_3CN for 2 min and re-equilibration with 15% CH_3CN/H_2O-0.1% HCOOH for 3 min before the next measurement; void time t_0 = 0.53 min. [b]For definition of parameters, see the footnotes of Tables 1 and 2. [c]Norvaline. [d]Co-elution. [e]Citrulline. [f]Isoserine (3-amino-2-hydroxypropanoic acid). [g]Conditions: Agilent Zorbax SB-Aq column, 4.6 × 250 mm (5 μm), stepped gradient, initial conditions 30−40% CH_3CN/H_2O/20 mM NH_4OAc-0.1% trifluoroacetic acid (TFA), 40 min; 40−50% CH_3CN/H_2O/20 mM NH_4OAc-0.1% TFA, flow rate 0.7 mL min^{-1}.

2a−**4a** with reversed-phase stationary phases (e.g., C_{18}), are hydrophobic in nature. It has been proposed that a combination of intramolecular H bonding and non-H bonding orders the structures of diastereomeric analytes, AA-DAA, creating a "more compact L−L diastereomer" compared to L−D.[26] An increase in hydrophobicity of the analyte molecule (supported by analysis of CPK models) is proposed and evidenced by comparative analysis of retention times of D- and L-AAs.[25] Conversely, Harada and coauthors argue a mechanism (supported by NOE measurements) independent of hydrogen bonding.[8] Either way, AA derivatives **4a** also show enhanced hydrophobicity (increased t_R) through a compaction of the analyte structure but with a secondary element of control absent from other Marfey-type reagents: π−π and cation−π interactions (see below) with the heterocyclic ring in Trp-NH_2 that fine-tune molecular discrimination of analytes **4a** (see Scheme 1).

Generally, the longer retention times observed during reversed-phase HPLC in the presence of NH_4^+ (Table 5) imply more hydrophobic mobile species than those in the absence of NH_4^+. We attribute the improved resolutions of **4a** to discrete intramolecular π-base properties of the electron-rich indole ring of Trp-NH_2. The latter may include intramolecular π−π interactions with aromatic analytes or attractive π−cation interactions[27] induced by Na^+, R_3NH^+, or NH_4^+ counterions in the HPLC mobile phase, carboxylate ion pair, or a combination of both.

^{1}H NMR data supports the conformation presented in structure **10** (Figure 2). The chemical shift of H-5 in the dinitrophenyl ring is unusually high (δ 5.6 ppm, cf. H-2, δ 9.8), which is explained by diamagnetic shielding induced by the indole ring current in a conformation that is folded (compacted). We briefly investigated the conformation of the L-DTA derivative of L-Ala (**10**, Figure 2) by geometry-minimized structures (MMFF) further refined by DFT quantum mechanical calculations (Spartan '14, EDF2 6-31G* 6-31G(D)). The lowest-energy conformers of neutral **10** (Figure 2b depicts the most stable conformer) adopt a conformation that stacks the electron-poor DNP ring above the electron-rich benzenoid ring of indole. Although both aromatic rings are *syn* to each other, their planes are displaced by an angle of approximately 33°. Importantly, the constraints imposed by attractive electrostatic dipole−dipole interactions and torsional angles actively cooperate to compact the structure in a way that disposes the side chain of the Ala side chain to the exterior. Introduction of the NH_4^+ counterion is expected to build stabilization through a π−cation interaction, Figure 2a; however, accurate calculations of this entity would, of necessity, include solvation of both charged groups, with appropriate parameterization, requisites that are beyond the scope of this report. Nevertheless, a detailed understanding of this phenomenon may inform design of new optimized Marfey's-type reagents based on non-natural AAs and is currently a subject of exploration in our laboratory.[28]

As reported earlier by Fujii and co-workers,[8] several lines of evidence suggest that the diastereomeric discrimination between Marfey's derivatives has less to do with hydrogen bonding in solvent−analyte interaction and more with hydrophobicity of analyte−stationary phase association.[8] We hypothesize that a pronounced intramolecular π−cation interaction between the NH_4^+ counterion and the electron-rich indole ring presents a more compact, hydrophobic mobile species to the stationary phase that enhances molecular discrimination between D- and L-AAs derivatives. In both the neutral carboxylic acid (for simplicity we refer to this structure as **10**•H, Figure 2, minimized geometry, MMFF, and DFT-calculated energy) and the corresponding ammonium salt as **10**•NH_4^+. Strong dipole or charge interactions, respectively, between the carboxyl terminus and the electron-rich indole ring shield the polar groups within two hydrophobic "walls" comprising the two aryl ring systems—indole and 2,4-dinitrophenyl ring (DNP). The deviation in conformation leads to compaction of the analyte molecular structure in high-dielectric aqueous HPLC mobile species. The DFT-calculated molecular geometry of the ammonium salt **10**•NH_4^+ (ωB97X-D, 6-31G*, polar solvent) shows an even tighter association of the 2,4-dinitrophenyl ring (DNP) and indole rings; the two planes deviate only ∼16° from parallel; (see Supporting Information). In the NH_4^+ salts of traditional Marfey's derivatives obtained with FDAA, such a π−cation interaction

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Table 4. HPLC Analyses of Critical L-DAA-AAs and L-DTA-AAs, 2a and 4a in the Presence of NH_4OAc[a]

	L-DAA				L-DTA			
	t_R, min				t_R, min			
AA	L	D	Δt_R[b]	α[c]	L	D	Δt_R[b]	α[c]
N,O-Me_2-Tyr (**11**)	45.54	46.38	0.83	1.02	37.76	39.28	1.53	1.05
Ser	29.67	29.99	0.32	1.01	21.39	22.36	0.97	1.06
Asn	28.35	28.35	0.00	1.00	20.74	21.85	1.11	1.07
Glu	31.21	31.51	0.30	1.01	19.67	20.08	0.41	1.03
Lys (mono-α)	25.86	24.38	−1.48	1.08	22.04	22.04	0.00	1.00
Lys (di-α,ε)	26.33	26.33	0.00	1.00	24.88	34.99	10.11	1.52
Orn (mono-α)	25.48	25.48	0.00	1.00	24.06	24.06	0.00	1.00
Orn (di-α,δ)	41.98	41.98	0.00	1.00	33.81	32.98	−0.83	1.03
His (mono-α)	24.93	23.44	−1.49	1.08	22.34	21.80	−0.54	1.03
His (di-α,δ)	34.34	34.69	0.35	1.01	32.42	32.99	0.57	1.02
Arg	26.61	25.17	−1.44	1.07	23.14	23.14	0.00	1.00
Cit[d]	29.23	28.12	−1.11	1.05	22.02	22.02	0.00	1.00
N-Me-Ala	34.32	34.88	0.57	1.02	24.26	25.53	1.28	1.07
N-Me-Asp	30.43	29.68	−0.74	1.03	18.99	18.54	−0.45	1.03
iso-Ser[e]	29.70	29.98	0.28	1.01	23.28	23.28	0.00	1.00
allo-Ile	39.33	42.56	3.23	1.10	26.62	30.01	3.39	1.16
Ile	39.43	42.58	3.16	1.09	26.52	29.85	3.33	1.16

[a]Conditions: Reversed-phase C_{18} column, 250 × 4.6 mm (5 μm), flow rate 0.70 mL min^{-1}; elution completed with a linear gradient of 15−65% CH_3CN/0.1 M NH_4OAc−H_2O-0.1% TFA for 40 min, followed by 100% CH_3CN for 5 min; void time t_0 = 5.31 min. [b]$\Delta t = t_R$(D-AA) − t_R(L-AA). [c]$\alpha = [t_R(B) - t_0]/[t_R(A) - t_0]$, where A and B are the faster and slower eluting components, respectively. [d]Citrulline. [e](±)-Amino-2-hydroxypropanoic acid.

Table 5. HPLC Analyses of Differential Retention Times (t_R, Δt_R, and $\Delta\Delta t_R$) of Critical L-DTA Derivatives, 4a, of AAs in the Presence of NH_4OAc

AA[a]	t_R, min				
	L-AA	D-AA	Δt_R[b], min	$\Delta\Delta t_R$[c], min	resolved?
N,O-Me_2-Tyr (**11**)	37.76	39.28	1.53	1.3	Y
Ser	21.39	22.36	0.97	0.47	Y
Asn	20.74	21.85	1.11	1.11	Y
Glu	20.08	19.67	−0.41	−0.49	Y
Lys (mono-α)	22.04	22.04	0.00	0	N
Lys (di-α,ε)	24.88	34.99	10.11	10.11	Y
Orn (mono-α)	24.06	24.06	0.00	0	N
Orn (di-α,δ)	33.81	32.98	−0.83	0.62	Y
His (mono-α)	22.34	21.80	−0.54	−0.65	Y
His (di-α,δ)	32.42	32.99	0.57	−0.15	Y
Arg	23.14	23.14	0.00	−0.62	N
Cit	22.02	22.02	0.00	−0.38	N
N-Me-Ala	24.26	25.53	1.28	0.73	Y
N-Me-Asp	18.99	18.54	−0.45	−0.71	Y
iso-Ser	23.28	23.28	0.00	−0.37	N
allo-Ile	26.62	30.01	3.39	−0.3	Y
Ile	26.52	29.85	3.33	−0.19	Y

[a]Conditions: Luna C_{18} column (250 × 4.6 mm, 5 μm) and a flow rate of 0.70 mL min^{-1}. Elution was carried out with a linear gradient of 15%−65% CH_3CN-0.10 M NH_4OAc-0.1% TFA for 40 min, followed by 100% CH_3CN for 5 min. [b]$\Delta t = t_R$ (D-AA) − t_R (L-AA). Negative values indicate reversal of the elution order. [c]$\Delta\Delta t_R = |\Delta t_R$ (Table 4)| − |Δt_R (Table 3)|. Negative values indicate poorer resolution with DTA derivatives.

would, of course, be unlikely as the only delocalized π-system available is the electron-deficient 2,4-DNP ring; here, an "ammonium ion effect" would be prohibited. This intriguing

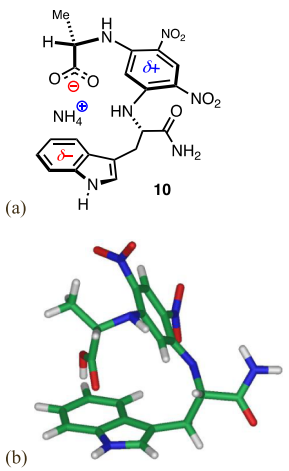

Figure 2. π−cation interactions in the L-DTA-derivative of L-alanine (**10**. See Scheme 2). (a) Representational model of the NH_4^+ salt of **10**. (b) Calculated model (DFT) of neutral **10**•H (Spartan '14, EDF2 6-31G* 6-31G) (D). See Supporting Information for the DFT z file of calculated structure **10**•NH_4^+ (ωB97X-D, 6-31G*, polar solvent).

property is the subject of ongoing investigation in our laboratory.

Evidence for the π−cation interaction was obtained from ^1H NMR measurements of **10** with titration by ammonium acetate (600 MHz, DMSO-d_6, NH_4OAc, Figure 3). In the absence of NH_4OAc, the downfield region of the ^1H NMR spectrum (δ 6−9 ppm) was populated only by aromatic signals of the indole and dinitrophenyl rings and the NH signal (δ 7.41, br s). Upon addition of NH_4OAc (0.1−1.0 equiv), a new 1:1:1 triplet signal grew in (δ 7.08, t, $^1J_{1H-14N}$ = 47.6 Hz), corresponding to the NH_4^+ ion signal, which increased in intensity up to 1.0 equiv of NH_4OAc. Observation of discrete one-bond ^1H−^{14}N heteronuclear coupling constants to the ^{14}N nucleus (spin I = 1) implied the presence of NH_4^+ in the ion

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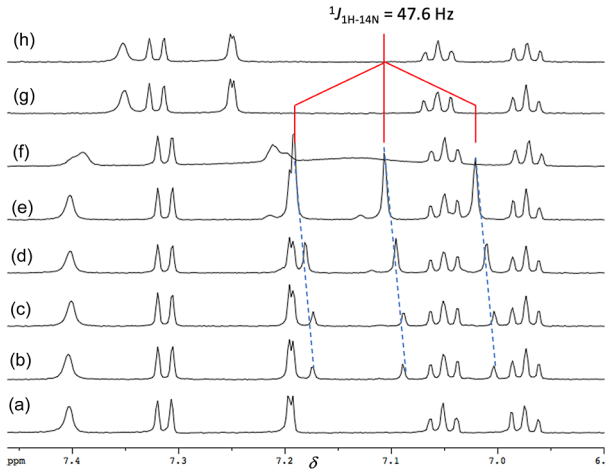
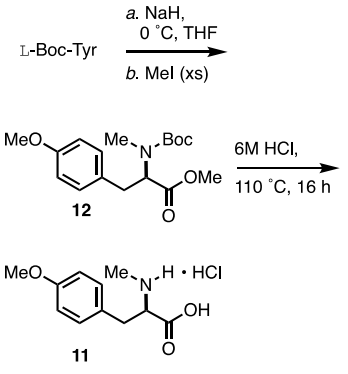


Figure 3. ^{1}H NMR titration of L-Ala-L-DTA (**10**, 600 MHz, DMSO-d_6) (a), no NH_4OAc (b), 0.1% TFA and 0.1 equiv. NH_4OAc (c), 0.2 equiv. (d), 0.5 equiv. (e), 1.0 equiv. (f), 2.0 equiv. (g), 5.0 equiv., and (h) 10.0 equiv. Dashed lines and ^{1}H NMR "splitting tree" indicate a discrete NH_4^+ ^{1}H−^{14}N couplet (1:1:1, $^1J_{NC}$ = 47.6 Hz).).

pair **10**•NH_4^+ (here, **10** is in its conjugate base form) within a relatively symmetrical electronic environment and in a slow exchange regime. Addition of excess NH_4OAc (2.0−10.0 equiv) resulted in progressive broadening of the NH_4^+ signal, until it merged completely into the baseline. Other dramatic changes in the indole ^{1}H chemical shifts were observed. This is consistent with disruption of the discrete stoichiometric species (1:1 NH_4^+:**10**) undergoing rapid intermolecular exchange with NH_4^+ outside of the first sphere of coordination with concomitant signal broadening.

Finally, we revisited the historical antitumor cyclodepsipeptide, didemnin B (**1a**), and applied **4** to the analysis of the constituent AA residues. Standard L-*N*,*O*-Me_2-Tyr hydrochloride (**11**) was synthesized by N-methylation of *N*-Boc-Tyr (NaH, excess MeI, THF, Scheme 3) to give ester **12**

Scheme 3. Synthesis of L-*N*,*O*-Me_2-Tyr



followed by acid hydrolysis (6 M HCl, 110 °C, see Experimental Section) to deliver **11**. Hydrolysis of **1b** (6 M HCl, 110 °C, 16 h), followed by derivatization of the resultant AA mixture with L-**4** and D-**4** and LCMS analysis of the products **4a**, revealed diastereomers arising from L-Pro, *N*-Me-D-Leu, and, *N*,*O*-Me_2-L-Tyr, consistent with prior analyses,[4] but with improved Δt_R of L-**11**-DTA versus L-**11**-DAA (Δt_R 1.53 and 0.83 min, respectively. See Table 4), albeit slightly less in the presence of NH_4OAc ($\Delta\Delta t_R$ = 1.3, Table 5).

3. CONCLUSIONS

Two new CDAs, both Marfey-type reagents, **3** and **4** for derivatization and chiral analysis of L- and D-AAs, were designed and synthesized from optically pure amines, (*S*)-**5** (derived from *S*-naproxen) and L-Trp, respectively. Evaluation of HPLC analysis of AA derivatives **3a** and **4a** of the two CDAs, reagent **3** was outperformed by the tryptophanamide-derived reagent **4** in side-by-side comparisons. The new reagent FDTA (**4**) was also shown to be superior to Marfey's reagent (**2**) for HPLC analysis of the corresponding derivatives of several polar proteinogenic AAs and key nonproteinogenic AAs, including *iso*-Ser and N^{α}-methylated AAs including *N*-Me-Ala and *N*,*O*-Tyr. A working hypothesis was proposed for the basis of the diastereomeric discrimination of DTA derivatives on HPLC. A notable 'NH_4^+ cation effect' of DTA derivatives was observed and supported by ^{1}H NMR titration of **10** with NH_4OAc (Figure 3, **4a**, R^2 = Me), which invokes intramolecular π−cation interaction of the NH_4^+ counterion between the electron-poor and electron-rich aromatic 2,4-dinitrophenyl and Trp indole ring systems, respectively.

4. EXPERIMENTAL SECTION

4.1. General Experimental Procedures. 1,5-Difluoro-2,4-dinitrobenzene and L- and D-AAs were purchased from Sigma-Aldrich (St. Louis, MO). FDAA, L-tryptophan, and HCOOH were purchased from Thermo Fisher Scientific (Waltham, MA). Ammonium hydroxide and TFA, AR grade, were purchased from EMD Millipore (Chicago, IL). Ammonium acetate (AR, >99%) was purchased from Macron Fine Chemicals (Avantor, Center Valley, PA). All other solvents were of HPLC or LCMS grade and purchased from Thermo Fisher Scientific (Waltham, MA). Optical rotations were measured on a JASCO P-2000 at the D-double emission line of Nå. Analytical HPLC was carried out on an integrated JASCO system consisting of dual-pumps (PU-2086 Plus), a dynamic mixer (MX-2080-32), and a UV−vis detector (UV-2075). UV−vis spectra were measured on a JASCO V-630 spectrometer using a quartz cell (1 mm path length). FTIR spectra were collected on thin film samples using a JASCO FTIR-4100 fitted with an ATR accessory (ZnSe plate). 1D-NMR and inverse-detected 2D NMR spectra were measured on a Bruker Avance III (600 MHz) NMR spectrometer with a 1.7 mm ^{1}H{^{13}C/^{15}N} microcryoprobe. Other NMR spectra were measured on a JEOL ECA 500 spectrometer equipped with a 5 mm ^{1}H{^{13}C} room temperature probe at 500 MHz. ^{13}C NMR spectra were measured using a Varian VX 500 NMR spectrometer equipped with a 5 mm Xsens ^{13}C{^{1}H} cryoprobe at 125 MHz. NMR spectra are referenced to residual solvent signals ($CDCl_3$, δ_H 7.26, δ_C 77.00 ppm; DMSO-d_6, δ_H 2.50, δ_C 39.52 ppm; acetone-d_6, δ_H 2.05, δ_C 205.87 ppm). High-resolution ESITOF analyses were carried out on an Agilent 6350 TOF MS coupled to an Agilent 1200 HPLC at the Small Molecule MS Facility (UCSD). Low-resolution MS measurements were made using a Thermoelectron Surveyor UHPLC coupled to a UV−vis detector (PDA) and an MSD single-quadrupole detector.

4.2. Animal Material. *T. solidum* Van Name, 1902, was collected from Little San Salvador, Bahamas, in 1999 by scuba at a depth of −20 m and stored at −20 °C until required. A voucher sample is archived at UC San Diego.

4.3. Extraction and Isolation of Didemnin B (1a) from *T. solidum*. Didemnin B (**1a**) was isolated from extracts of a frozen sample of *T. solidum* that had been stored at −20 °C as previously described.[29]

4.4. *S*-(+)-Naproxen. Commercial "over-the-counter" Naproxen Sodium tablets (CVS brand, 250 mg, ∼50 count, 13 g) were ground to a fine powder and dispersed/dissolved in water (∼500 mL). The mixture was made basic (NaOH, pH ∼ 10) and stirred briefly, and the suspension filtered (Whatman no. 1 paper) to remove excipients. The filtrate was cooled to 0 °C and carefully acidified (6 M HCl), and the precipitate of *S*-(+)-Naproxen filtered and dried under reduced

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pressure in a desiccator over KOH. The crude solid was ground in a mortar and pestle to a fine colorless powder [$[\alpha]_D$ +65 ($CHCl_3$), lit.[30] +66 ($CHCl_3$)]. ^{1}H NMR matched literature values.[31] *S*-(+)-Naproxen was used in the next step without further purification.

4.5. (6-Methoxynaphth-2-yl)-1-ethylamine (6). *S*-(+)-Naproxen (0.531 g, 2.30 mmol) was subject to Curtius rearrangement according to the method of Mutschler and co-workers,[19] to give (*S*)-2-(1-isocyanatoethyl)-6-methoxynaphthalene (**5a**) as a colorless solid (0.319 g, 61%). Conversion of **5a** to **5** was completed by hydrolysis (CF_3COOH, reflux, followed by K_2CO_3 H_2O, rt), and extractive workup (EtOAc) gave the optically pure amine (*S*)-1-(6-methoxynaphth-2-yl)-1-ethylamine, (*S*)-**5**[32] which was carried forward to the next reaction.

4.6. (*S*)-5-Fluoro-*N*-(1-(6-methoxynaphth-2-yl)ethyl)-2,4-dinitroaniline (3).[1] A solution of (*S*)-**5** (44.5 mg, 0.221 mmol) in 1 N NaOH (0.2 mL) and acetone (3 mL) was treated with anhydrous $MgSO_4$ (0.5 mg) with continuous stirring over for 15 min. The mixture was filtered and the residue washed with a little cold acetone, and the filtrate and washings combined with a solution of 1,3-difluoro-2,4-dinitrobenzene (45.0 mg, 0.221 mmol): *Caution! Acutely toxic, skin irritant!* in acetone (2.0 mL) and stirred at 23 °C for 45 min during which the color changed to a bright orange. H_2O was added to precipitate the crude product, and the entire suspension passed through a short column of Celite. The column was washed with a little H_2O, and the product was dissolved and eluted with acetone. After removal of the volatiles and drying under high vacuum, (*S*)-**3** was obtained as a bright-yellow-orange solid (68.1 mg, 80%). (*S*)-**3**; $[\alpha]_D^{24.2}$ +200 (*c* 0.22, CH_3CN); UV (MeOH) λ_{max} 232 nm (ε log 4.75), 262 (4.05), 334 (4.10); FTIR (ATR, ZnSe plate) ν 3357, 2936, 1630, 1606, 1582, 1542, 1521, 1484, 1422, 1366, 1330, 1287, 1267, 1177, 1103, 1055, 1029, 918, 855, 833, 740, and 711. ^{1}H NMR (600 MHz, acetone-d_6): δ 9.01 (2H, d, 7.8), 7.97 (1H, s), 7.85 (1H, d, 8.4), 7.78 (1H, d, 8.4), 7.61 (1H, dd, 8.4, 1.8), 7.30 (1H, d, 2.4), 7.15 (1H, dd, 9.0, 2.4), 6.89 (1H, d, 14.4), 5.21 (1H, m), 3.91 (3H, s), 1.82 (3H, d, 6.6). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6): δ (ppm): 159.9, 157.8, 157.4, 148.3 {148.2}, 137.3, 133.8, 129.4, 128.3, 127.6, 127.4, 124.9, 124.5, 119.0, 105.9, 102.7, 102.5, 55.3, 52.9, and 23.2. HRMS(ESI-TOF) *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{16}N_3O_5FNa$ 408.0972; found 408.0968.

4.7. L-5-Fluoro-2,4-dinitrophenyl-N^{α}-L-tryptophanamide (L-FDTA, 4). Reagent **4** was prepared from L-tryptophan as previously described.[18]

4.8. Analytical Chromatography. LC APCI-MS and ESI-MS spectra were obtained on a Thermo Fisher UHPLC, coupled to a Thermo Fisher MSD quadrupole detector. Separations were carried out using a Hypersil GOLD C_{18} column, 50 × 2.1 mm (1.9 μm particle), and a flow rate of 0.500 mL min^{-1}. Elution was conducted with a linear gradient of 15% to 45% CH_3CN/H_2O-0.1% HCOOH for 25 min, followed by 100% CH_3CN for 2 min and re-equilibration with 15% CH_3CN/H_2O-0.1% HCOOH for 3 min before the next measurement. Analytical HPLC measurements were carried out using a Luna C_{18} column (250 mm × 4.60 mm, particle size 5 μm) and 0.700 mL flow rate with UV detection (λ = 335 nm). Elution was completed with a linear gradient of 15% to 65% CH_3CN/H_2O-0.1% TFA or CH_3CN/0.1 M NH_4OAc-0.1% TFA for 40 min followed by a 5 min wash with 100% CH_3CN and re-equilibration with 15% CH_3CN/H_2O-0.1% TFA or CH_3CN/0.1 M NH_4OAc, 0.1% TFA, respectively. See also the conditions listed in the table footnotes.

4.9. Derivatization of L-, D-, and DL-AAs by 2−4. The following is a representation of AA derivation by **2**−**4**. To a 3.8 mL vial equipped with a magnetic stir bar was added an aqueous solution of DL-AA (10 mM, 250 μL, 2.5 μmol), followed by a solution of L-FDTA (**4**) or *S*-FDNE (**3**) or L-FDAA (**2**) in acetone (1% w/v, 140 μL, 3.6 μmol), aqueous $NaHCO_3$ (1 M, 20 μL, 20 μmol), and acetone (200 μL). The mixtures were stirred at 85 °C for 30 min, cooled to room temperature, and neutralized with 1 M HCl (20 μmol). For LC−MS analysis, aliquots of the derivatized AAs (20 μL) were diluted with MeOH (80 μL) and centrifuged, and an aliquot of each solution (10 μL) analyzed by HPLC under the conditions described in the footnotes of Tables 3−5. Retention times and peak areas were determined by using native software. Standard AAs, racemic (±)- or L- and D-AAs, were prepared and analyzed in the same manner. See Tables 3−5.

4.10. L-*N,O*-Dimethyltyrosine (11). A stirred solution of L-*N*-Boc-Tyr (2.00 g, 7.1 mmol) in THF (40 mL) was cooled to 0 °C. NaH (60% w/w dispersion in mineral oil, 4.0 equiv) was added in one portion, and the solution was stirred for an additional 15 min at 0 °C. Iodomethane (0.574 g, 14.36 mmol, 5.0 equiv. *Caution! Acute toxicity, inhalation hazard!*) was added slowly, and the mixture allowed to warm to 23 °C over 20 h. The solution was carefully quenched by dropwise addition of H_2O and diluted with additional H_2O (50 mL) before extraction with EtOAc (×3). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated to a yellow oil which was purified by flash chromatography (SiO_2, 3:1 hexanes-Et_2O to 100% Et_2O) and gave *N*-Boc-**11** as a colorless oil (1.57 g, 71%). Compound **11** (124.2 mg) was subjected to hydrolysis in 4 M HCl in dioxane (6.0 mL) at 23 °C for 5 h. Removal of the volatiles gave **11**·HCl as fine colorless needles (quant.). ^{1}H NMR matched the literature values.[33]

4.11. Hydrolysis of Didemnin B and Derivatization of *N,O*-Dimethyltyrosine with L- and D-FDTA. A sample of didemnin B (**1a**, 1.0 mg) was subjected to acid hydrolysis (6 M HCl, 110 °C, ∼16 h). The sample was cooled to 23 °C, and the volatiles were removed under a stream of N_2. The mixture was derivatized with L- or D-FDTA (**4**) as described above. After centrifugation, an aliquot of the supernatant (20 μL) was analyzed by LCMS. t_R (min) and *m*/*z* [M + H]$^+$ of DTA derivatives **4a**: t_R (min, *m*/*z* [M + H]$^+$), AA = L-Thr (10.14, *m*/*z* 487.10), L-Pro (13.55, *m*/*z* 483.19), D-*N*-Me-Leu (21.57, *m*/*z* 512.92), L-Leu (18.21, *m*/*z* 499.18), L-*N,O*-Me_2-Tyr (18.40, *m*/*z* 577.18). For the t_R values of standard AA-L-DAA (**2a**) and D-DTA derivatives (**4a**), see Table 4.

4.12. DFT Calculations of L-Alaninyl-DTA 10 and Its Ammonium Salt 10•NH_4^+. Optimized geometry and energy of L-alaninyl-DTA (**4a** R = Me, **10**) and the corresponding ammonium salt, **10**•NH_4^+, were calculated using DFT (functional and basis set ωB97X-D, 6-31G*, polar solvent, Spartan '20 Wave function Inc., Irvine, CA, USA). See Supporting Information for results and citation.

■ ASSOCIATED CONTENT

Data Availability Statement

. The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site at DOI: xxxx The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.4c02882.

^{1}H and ^{13}C NMR of **3** and DFT-calculated structure of **10**•NH_4^+ salt (PDF)

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Notes

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(24) Separations of L-DAA and *S*-DTA derivatives of the diastereomeric L-Ile and D-*allo*-Ile (Table 4)—a common encounter in natural product peptides—were Δt_R = 3.13 and 3.49 min, respectively, on a conventional column (Table 4, C_{18}, 3 μm). The critical separation L-Ile-DTA and allo-L-Ile-DTA dramatically

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improved with a UHPLC column (Table 3, particle size = 1.9 μm, Δt_R = 1.03 min) compared to a conventional column. Both L-DAA and L-DTA derivatives of enantiomeric L- and D-Ile were well separated (Δt_R = 3.16 and 3.33 min, respectively, Table 4).

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