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Integramides A and B, Two Novel Non-Ribosomal Linear Peptides Containing Nine C^{α}-Methyl Amino Acids Produced by Fungal Fermentations That Are Inhibitors of HIV-1 Integrase

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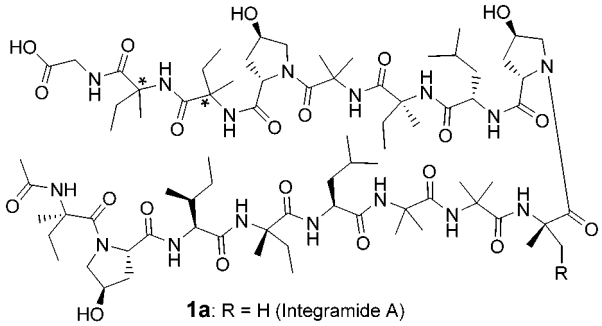
ABSTRACT



1a: R = H (Integramide A)
1b: R = CH_3 (Integramide B)

Integramides A and B are two novel 16-mer linear peptides rich in C^{α}-methyl amino acids that were isolated from fungal extracts of *Dendrodochium* sp. by employing a bioassay-guided isolation procedure using recombinant HIV-1 integrase. The structure and stereochemistry were elucidated by a combination of 2D NMR and ESI- and FAB-MS including MS/MS studies and by Marfey's method. Integramides A and B inhibited the coupled reaction of HIV-1 integrase with IC_{50} values of 17 and 10 μM, respectively.

AIDS is caused by HIV infections. In the past decade, clinical treatments of HIV-infected patients with inhibitors of reverse transcriptase and protease have led to significant improvements in reducing the viral load and the progression of AIDS. However, these treatments can become ineffective due to rapid mutations to the virus. HIV-1 integrase is another important enzyme that is critical for the integration of the HIV genome into the host genome via a three-step process that includes assembly of proviral DNA onto integrase, endonucleolytic processing of the proviral DNA, and strand transfer of the proviral DNA into host cell DNA.[1] This process is unique to the virus and is absent in the host and, therefore, presents a safe target for the development of single and/or combination anti-HIV therapy. Several HIV-1 integrase inhibitors have been reported, particularly diketo acid (DKA) based inhibitors that display antiviral potency essentially equal to HIV-1 protease inhibitors. This discovery

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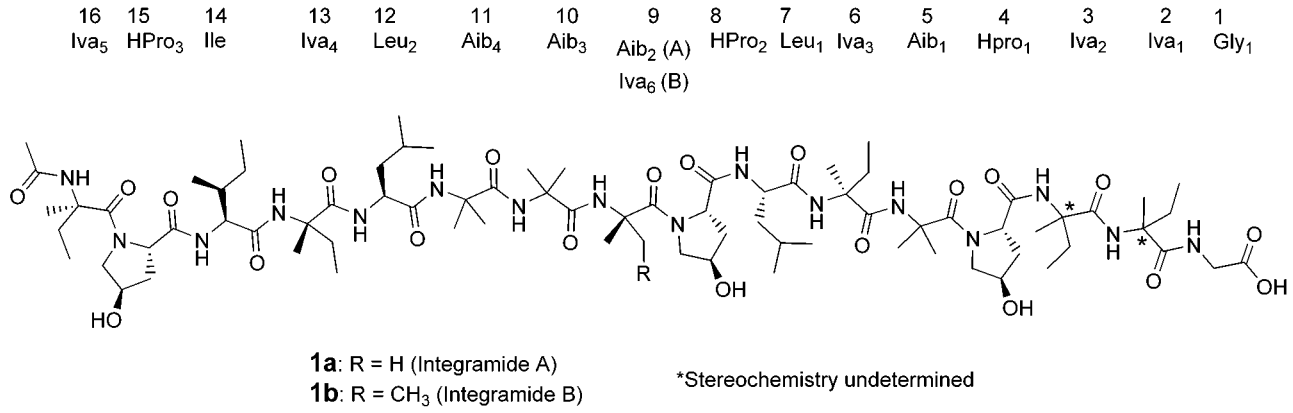

Figure 1. Structures of integramides A and B with amino acid designations.

helped validate that inhibition of integrase activity could potently block replication of HIV-1 in whole cells[2] and could potentially lead to clinical candidates. However, clinical efficacy and tolerability of these compounds could not be predicted with certainty and therefore new classes of inhibitor leads are needed.

Natural product extracts continue to be important sources of leads for various biological targets, particularly for cancer and viral targets. Screening of microbial extracts against recombinant HIV-1 integrase led to the discovery of several novel natural product inhibitors including equisetin,[3] integric acid,[4] and complestatin.[5] Continued screening of fungal extracts led to the discovery of two novel linear peptides containing 16 amino acids including nine α-methyl amino acids terminating with an *N*-acetyl group. These compounds, herein named integramides A (**1a**) and B (**1b**, Figure 1), inhibited the HIV-1 integrase coupled reaction with IC_{50} values of 17 and 10 μM, respectively. The isolation, structure, stereochemistry, and biological activities of integramides A and B are described.

Isolation. The fungus *Dendrodochium* sp. (MF6888), isolated from leaf litter collected in the Osa Peninsula of Costa Rica, was grown on a vermiculite-based media and extracted with 1.2 volumes of methyl ethyl ketone. Gel permeation on Sephadex LH20 in methanol produced an active fraction which was purified by reversed phase HPLC to give integramides A (50 mg/L, $[\alpha]^{23}_D$ +7.7° (*c*, 1.3, MeOH)) and B (23 mg/L, $[\alpha]^{23}_D$ +5.7° (*c*, 0.7, MeOH)) as white powders.

Integramide A (1a). HRESI-FTMS of integramide A produced a $[M + NH_4]^+$ ion at *m/z* 1649.0208 that afforded a molecular formula of $C_{78}H_{134}N_{16}O_{21}{\cdot}NH_4$ (calcd for 1649.0253) which was fully supported by NMR spectral data (Table 1). Examination of ^1H (600 MHz) and ^{13}C NMR spectra of integramide A indicated that it was a peptide containing all aliphatic amino acids. Analysis of the ^{13}C NMR spectrum of **1a** indicated the presence of 17 carbonyls that were quickly assigned to 16 amino acids and an *N*-acetyl group. The ^1H and ^{13}C NMR signals were reasonably dispersed in C_5D_5N and produced minimal overlaps. This was particularly true for all methyl groups, α-protons, and NH groups. The COSY, TOCSY, and HMQC experiments of **1a** produced spin systems that were easily assigned for two leucine (Leu), one isoleucine (Ile), three 4-hydroxyproline (Hpro), and one glycine (Gly) residues. In addition, these experiments indicated the presence of five isolated ethyl groups that were assembled into five 2-amino-2-methylbutyric acids (isovaline, Iva) by HMBC correlations of C^{α}- and C^{β}-methyl groups to respective α and carbonyl carbons. The remaining eight methyl groups were assigned to four 2-aminoisobutyric acids (Aib) by similar HMBC correlations (Table 2, provided as Supporting Information). The HMBC correlations of the α-H and NH protons to the two flanking carbonyls helped in establishment of the amino acid sequence of **1a**. For example, both α-methyl (δ 1.93, CH_3-5) and β-methyl (δ 1.25, CH_3-4) groups of Iva_2 gave HMBC correlations to C-2 (δ 61.31) and the α-methyl showed a correlation to the C-1 carbonyl (δ 175.20) which was also correlated to the NH groups of both Iva_1 and Iva_2. These correlations established the amino acid identity of Iva_2 and its firm linkage to Iva_1. The identity and sequence of other amino acids were accordingly established and were further verified by an F_1-selective HMBC experiment[6] using a 10 ppm F_1 spectral width spanning just the carbonyl region δ 170−180 that helped in unambiguous assignment of HMBC

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Table 1. ^{1}H (600 MHz) and ^{13}C NMR Assignments of Integramide A (**1a**) in C_5D_5N

Position	δ_c	δ_H, J in Hz	Position	δ_c	δ_H, J in Hz
		Gly	3	26.44	1.85, s
1	173.28	---	4	24.48	2.03, s
2	42.73	4.29, dd, 18, 5	NH	---	8.41, s
		4.68, dd, 18, 6			
NH	---	8.51, t, 5.6			**Aib_3**
		Iva_1	1	177.65	---
1	177.03	---	2	57.76	---
2	60.95	---	3	28.83	1.91, s
3	27.84	2.40, m	4	23.75	1.98, s
		3.02, m			
4	8.33	1.08, t, 7.6	NH	---	7.80, s
5	24.53	2.10, s			**Aib_4**
NH	---	8.10, s	1	175.29*	---
		Iva_2	2	57.54	---
1	175.20	---	3	27.20	1.88, s
2	61.31	---	4	24.03	1.80, s
3	33.33	2.42,m	NH	---	8.35, s
4	9.38	1.25, t, 7.2			**Leu_2**
5	20.80	1.93, s	1	175.32*	---
NH	---	8.445, s	2	56.24	4.46, m
		$Hpro_1$	3	40.53	1.91, m
					2.24, m
1	174.76	---	4	25.83	2.05, m
2	63.89	5.18, dd, 10.4, 7.6	5	23.42	1.00, d, 6.8
3	38.35	2.14, m	6	21.55	0.89, d, 6.0
		2.60, m			
4	70.85	4.86, brs	NH	---	7.76, d, 5.6
5	58.39	4.36, brd, 13			**Iva_4**
		4.55, brd, 13			
		Aib_1	1	176.24	---
1	174.63	---	2	60.84	---
2	57.52	---	3	33.51	2.08, m
					2.30, m
3	26.44	1.78, s	4	8.89	1.11, t, 7.6
4	24.73	1.81, s	5	19.99	1.79, s
NH	--	8.04, s	NH	---	7.59, s
		Iva_3			**Ile**
1	176.46	----	1	173.86	---
2	61.15	----	2	62.65	4.10, dd, 8.4, 6.0
3	33.97	2.10, m	3	36.06	2.31, m
		2.25, m			
4	8.68	1.14, t, 7.6	4	27.48	1.38, m
					1.88, m
5	20.20	1.98, s	5	11.69	1.03, t, 7.6
NH	---	7.67, s	6	16.09	1.18, d, 6.8
		Leu_1	NH	---	8.45, d, 5.2
1	174.46	---			**$Hpro_3$**
2	55.84	4.55, m	1	175.65	---
3	40.30	2.02, m	2	63.28	5.12, dd, 10.4, 7.6
		2.24, m			
4	25.68	2.05, m	3	38.41	2.20, m
					2.76, m
5	23.69	1.04, d, 6.0	4	70.64	4.81, brs
6	21.65	0.84, d, 6.0	5	58.06	3.85, brd, 11
					4.42, brd, 11
NH	---	8.36, d, 7.2			**Iva_5**
		$Hpro_2$	1	175.57	---
1	175.12	----	2	59.64	---
2	63.12	5.09, dd, 10.4, 7.6	3	29.73	2.14, m
					2.56, m
3	38.38	2.13, m	4	8.29	1.02, t, 7.6
		2.73, m			
4	70.85	4.86, brs	5	20.31	1.55, s
5	58.61	4.45, brd, 11	NH	---	9.90, s
		4.64, brd, 12			
		Aib_2			**Acetate**
1	175.12	---	1	171.56	---
2	57.56	---	2	22.72	2.26, s

correlations to the respective α-H, NH, and methyl groups (Table 2). The sequence was further supported by NOESY correlations of NH protons of the two adjoining amino acids (Table 2).

The peptide sequence was corroborated by FAB-MS fragmentation, which was confirmed by ESI-MS/MS analysis (Figure 2). The ESI-MS/MS of $[M + H]^+$ at *m/z* 1631 of **1a** produced major fragment ions at *m/z* 1556, 1457, 1245, 1160, 835, 750, 665, 580, 467, 368, and 255 corresponding to the acylium ions due to the sequential cleavage of amide bonds as shown in Figure 2. The FABMS showed three exclusive fragment ions at *m/z* 142, 948, and 1358 (Figure 2).

Acid hydrolysis of integramide A with 6 N HCl for 12 h at 110 °C followed by reaction of the hydrolysate with Marfey's reagent[7] for 5 days and analysis by RPHPLC furnished Gly, 4*S*-hydroxy-2*S*-Pro (*trans*-HPro), *S*-Leu, 2*S*,3*S*-Ile, *S*-Iva, *R*-Iva, and Aib in a ratio of 1:3:2:1:3:2:4, respectively. Partial hydrolysis of **1a** with 1 N HCl for 1 h at 110 °C afforded four products with molecular weights of 1489, 1121, 711, and 456, produced mostly due to the cleavage of secondary amide bonds consisting of residues 1−15, 5−15, 9−15, and 12−15 in a ratio of 1:3:16:9, respectively. The former two hydrolytic products were essentially exclusively produced in a 1:1 ratio upon heating with 0.75 N HCl at 110 °C for 30 min. HPLC purification of hydrolytic products followed by exhaustive hydrolysis of the three largest pieces (1−15, 5−15, and 9−15) and analysis of Marfey's derivatives established Iva_3, Iva_4 as *S* and Iva_5 as *R* configuration. Unfortunately, the stereochemistry of the remaining two Iva's (i.e., Iva_1 and Iva_2) could not be determined (Figure 1).

Integramide B (1b). HRESI-FTMS analysis of **1b** afforded a $[M + NH_4]^+$ at *m/z* 1663.0464 good for a molecular formula of $C_{79}H_{136}N_{16}O_{21}\cdot NH_4$ (calcd for 1663.0410) and indicated that it has an additional methylene group. The substitution of Aib_2 (ninth residue) with an Iva attributed to this difference, which was verified by the MS fragmentation of **1b** (Figure 2) that showed a +Δ14 Da difference in the fragment ions that contained the ninth residue. This amino acid substitution was confirmed by analogous complete acid hydrolysis of **1b**, which gave an additional *S*-Iva at the expense of an Aib residue compared to **1a**. Thus *S*-Iva was assigned to the ninth residue of integramide B (Figure 1).

^{1}H and ^{13}C NMR spectra of both compounds were essentially superimposable except for the corresponding differences reflecting the substitution of ninth amino acid. The ^{1}H NMR spectrum of **1a** including the amide protons was unaffected by differing solute concentrations (5−15 mM) indicating strong intramolecular H-bonding. Integramide A exhibited sequential $H^N_i - H^N_{i+1}$ NOESY cross-peaks indicating an $\alpha/3_{10}$ helical structure that is known to be consistently adapted by most C^α-methyl rich peptides.[8] It appears that the methyl substitutions do not allow it to adapt a β-sheet structure (lack of interstrand NOEs and lower $^{3}J_{NH}$ (<7 Hz) values of Leu_2 and Ile)[9] despite the presence of three β-turn (Hpro) residues. The β-turn residues simply break the linearity of the helix.

HIV-1 Integrase Activity. Integramides were evaluated against coupled, strand transfer, and viral spread assays.[2a] Integramide A inhibited coupled and strand transfer reactions

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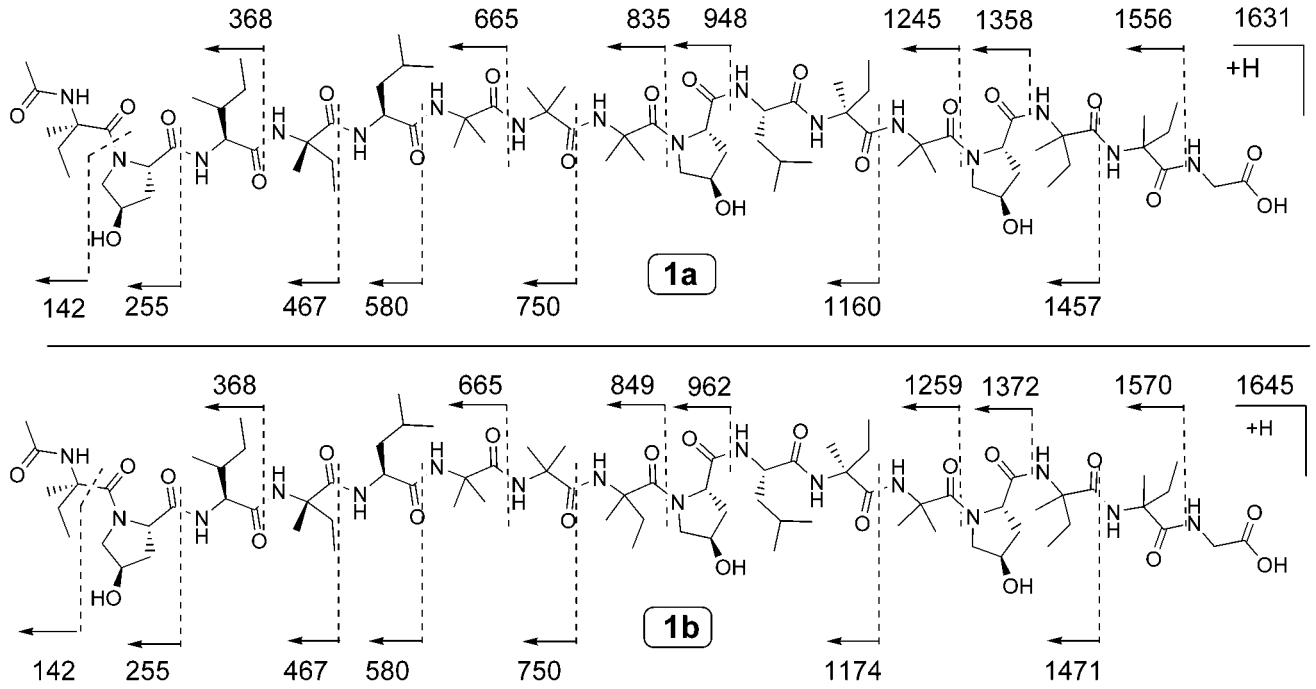

Figure 2. ESI-MS/MS and FAB-MS fragmentation of integramides A (**1a**) and B (**1b**).

with IC_{50} values of 17 and 60 μM, respectively. Integramide B was slightly more active against the coupled reaction and showed corresponding IC_{50} values of 10 and 60 μM. These compounds exhibited toxicity in viral spread assay at the IC_{50} levels. Fungally derived peptaivirins,[10] peptaibiol type C^{α}-methyl peptides with a CH_2OH C-terminus, have been reported to inhibit TMV infection of tobacco plants. No HIV integrase data were reported.

In summary, we have reported two novel 16-mer linear peptides containing nine C^{α}-methylated amino acids that are produced by fungal fermentations and are novel inhibitors of HIV-1 integrase, new targets of anti HIV therapy.

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Supporting Information Available: Table 2 with detailed NMR assignments and HMBC and NOESY correlations of integramide A. This material is available free of charge via the Internet at http://pubs.acs.org.

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