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Note

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Isolation and Structural Elucidation of Cyclic Tetrapeptides from *Onychocola sclerotica*

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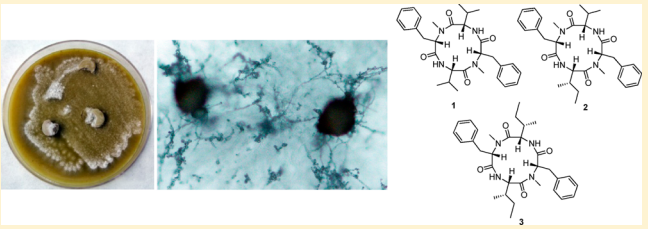
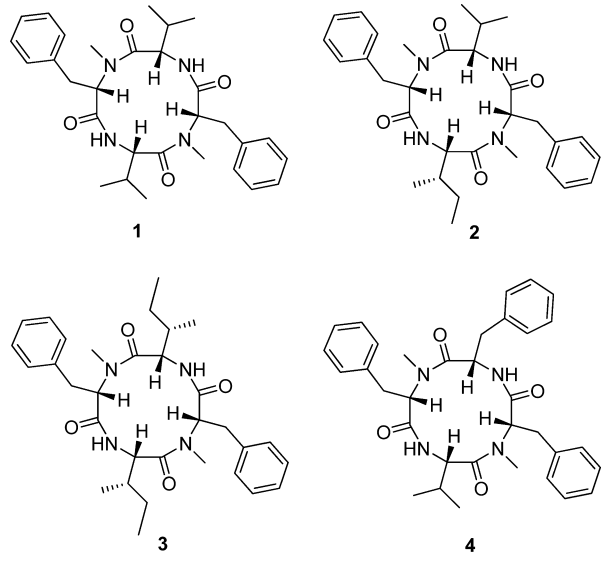
Supporting Information

ABSTRACT: Three new cyclic tetrapeptides (**1**–**3**) have been isolated from the crude fermentation extract of *Onychocola sclerotica*. The planar structures of **1**–**3** were elucidated by detailed spectroscopic analyses using one- and two-dimensional NMR experiments and high-resolution mass spectrometry. The absolute configuration of the amino acid residues in each cyclotetrapeptide was established by Marfey's method. Compounds **1**–**3** displayed activity as cardiac calcium channel blockers (Cav1.2) but did not inhibit the hERG potassium channel and were not cytotoxic. These peptides are the first secondary metabolites ever reported from fungi of the order Arachnomycetales.



Medium ring-sized cyclic peptides containing the amino acids leucine, isoleucine, valine, and *N*-methylphenylalanine have been reported from diverse microorganisms. The tetrapeptide *cyclo*-(L-Ile-L-Pro-L-Leu-L-Pro) was isolated from an actinomycete (*Nocardiopsis* sp.) collected from a Pacific deep-sea sediment.[1] Crews et al. isolated fenestins A (*cyclo*-(L-Pro-L-Pro-L-Leu-L-Ile)) and B (*cyclo*-(L-Pro-L-Val-L-Pro-L-Leu-L-Ile)) from the marine sponge *Leucophloeus fenestrata* and suggested that these metabolites might be of bacterial origin.[2] The peptide *cyclo*-(L-Ile-L-Ile-L-Ile-L-Phe) and four related cyclotetrapeptides were isolated from a fermentation broth of an actinomycete strain (*Nonomuraea* sp.) obtained from a soil sample collected in Henan Province, China.[3] A marine sediment-derived *Halobacillus litoralis* strain (from Huanghai Sea) yielded halolitoralins B (*cyclo*-(L-Ile-L-Leu-L-Ile-L-Leu)) and C (*cyclo*-(L-Ile-L-Val-L-Ile-L-Leu)) by fermentation.[4] Cyclic tetrapeptides have also been isolated from marine bacteria associated with the seaweed *Diginea* sp. (*cyclo*-(L-Phe-L-Pro-L-Leu-L-Pro) and *cyclo*-(L-Ile-L-Pro-L-Leu-L-Ala)) and the sponge *Halisarca ectofibrosa* (*cyclo*-(L-Phe-L-Leu)$_2$ and *cyclo*-(L-Leu-L-Ile)$_2$).[5] *Lactobacillus helveticus* produces the tyrosinase inhibitor *cyclo*-(L-Pro-L-Tyr-L-Pro-L-Val).[6] The only microbial cyclic tetrapeptides reported to date containing *N*-methylphenylalanine residues are hirsutide (*cyclo*-(L-*N*MePhe-L-Phe-L-*N*MePhe-L-Val), isolated from the spider-derived entomopathogenic fungus *Hirsutella* sp.,[7] and dihydrotentoxin (*cyclo*-(L-Leu-L-*N*MePhe-Gly-L-*N*Me-L-Ala), a phytotoxin produced by the fungi *Alternaria alternata*,[8] *A. citri*,[9] and *A. porri*.[10] In this note, we report the isolation and structure elucidation of three new cyclic tetrapeptides (**1**–**3**) each containing two *N*-methylphenylalanine residues. Biological activities in cardiac ion channel influx assays and cytotoxicity are also reported herein. These new peptides have been isolated from a fermentation broth of the fungus *Onychocola sclerotica* and are structurally related to hirsutide (**4**).[7]



The investigation of new fungal orders constitutes an appropriate approach for discovering new natural products. Secondary metabolites from Arachnomycetales, Eurotiomycetes, and Ascomycota,[11] to our knowledge, remain unstudied.

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For this reason and based on its taxonomy, the type strain of *O. sclerotica* (CBS 201.92) was selected for chemical investigation. The fungus was isolated from a poultry farm soil from Sulawesi, Indonesia, sent to the Institute of Tropical Medicine in Antwerp, Belgium.[12] The fungus was first described as *Malbranchea sclerotica* and later was reclassified in the genus *Onychocola* in the new order Arachnomycetales on the basis of new phylogenetic and mating data.[11,13] The strain was first grown as microfermentations in an eight-medium nutritional array to more thoroughly survey its metabolic capacity.[14−17] LC-UV-MS analysis of the corresponding fermentation extracts revealed, in one of the eight media, the presence of three major compounds not included in our in-house microbial natural products library.[15,18−20] The compounds were detected in a mannitol-based medium supplemented with yeast extract and mineral salts. The culture was then scaled up to 1 L in a static fermentation of the same medium. Three new cyclic tetrapeptides (**1**−**3**) were isolated from the acetone extract of this culture by a combination of reversed-phase flash chromatography and LH-20 chromatography followed by semipreparative HPLC.

Compound **1** was assigned a molecular formula of $C_{30}H_{40}N_4O_4$ using HRESIMS. Its ^{1}H NMR and ^{13}C NMR spectra (Table 1) contained signals that suggested a peptidic nature for the molecule, with valine, phenylalanine, and an *N*-methyl group being the only constituents as observed by analysis of the COSY, TOCSY, HSQC, and HMBC spectra. The presence of only 13 signals in its ^{13}C NMR spectrum pointed to the existence of molecular symmetry in the structure of **1**. The constituent amino acids (two valine residues, two phenylalanine residues), the presence of two *N*-methyl groups, and the number of double-bond equivalents indicated by the molecular formula were compatible only with a cyclic structure, indicating that **1** was a cyclotetrapeptide. The amino acid sequence and the position of the *N*-methyl groups were determined from HMBC correlations (Figure 1). Long-range correlations of the *N*-methyl group at δ_H 2.84 ppm with the valine carbonyl group at δ_C 173.4 and the C_α of phenylalanine at δ_C 64.6 established the presence of two *N*-methylphenylalanine (*N*-MePhe) residues in the cyclotetrapeptide. On the other hand, the α-proton of valine at δ_H 4.22 ppm correlated to its carbonyl group at δ_C 173.4 and to that of *N*-methylphenylalanine at δ_C 172.2 and confirmed the sequence, *cyclo*-(*N*-MePhe-Val)$_2$, corroborating the C_2 symmetrical conformation for **1**. ESIMS/MS experiments further confirmed the complete amino acid sequence of this cyclotetrapeptide, with the presence of an ion at *m*/*z* 261 corresponding to the *N*-MePhe-Val fragment.

Table 1. NMR Data (500 MHz, CD_3OD, at 24 °C) for 1[a]

amino acid	position	δ_C, mult.	δ_H, mult. (*J* in Hz)	COSY	HMBC
L-*N*-MePhe	CO	172.2, C			
	α	64.6, CH	4.47, dd (11.2, 2.8)	β	
	β	35.1, CH_2	a 3.66, dd (15.0, 2.5)	α, geminal	
			b 3.00, dd (14.2, 12.2)	α, geminal	C_δ (with b)
	γ	138.8, C			
	δ	129.5, CH	7.28, m	ε, ζ	C_ζ, C_δ, C_β
	ε	129.9, CH	7.33, m	δ, ζ	Cγ, C_ε
	ζ	128.1, CH	7.24, m	δ, ε	C_δ
	N-CH_3	31.2, CH_3	2.84, s		Val-CO, C_α
L-Val	CO	173.4, C			
	α	57.3, CH	4.22, d (7.8)	β	CO, Phe-CO, C_β, C_γ
	β	30.2, CH	2.10, m	α, γ, γ'	
	γ	20.8, CH_3	0.71, d (6.4)	β	C_α, C_β, $C_{\gamma'}$
	γ'	18.5, CH_3	0.86, d (6.8)	β	C_α, C_β, C_γ

[a]Due to molecular symmetry, both L-Val and both L-*N*-MePhe are chemically equivalent.

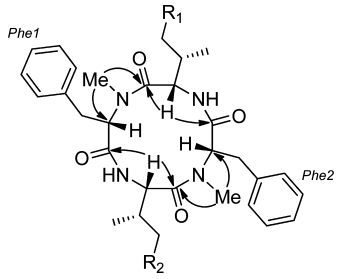

Figure 1. Key HMBC correlations indicative of the amino acid sequence of **1** (R_1 = R_2 = H), **2** (R_1 = H, R_2 = Me), and **3** (R_1 = R_2 = Me).

HRESIMS indicated a molecular formula of $C_{31}H_{42}N_4O_4$ for compound **2**. This molecular formula suggested the presence of an additional methylene unit in **2** with respect to **1**, in agreement with the higher hydrophobicity observed by reversed-phase HPLC and suggesting a closely related structure for the compound. The ^{1}H NMR spectrum contained signals attributable to four α-proton amino acid units, indicating the absence of symmetry in this case. Signals similar to those of **1** due to the presence of valine, phenylalanine (×2), and *N*-methyl groups (×2) were observed in the ^{1}H and ^{13}C NMR spectra of **2** (Table 2). The fourth residue was identified as isoleucine by analysis of the COSY, TOCSY, and HMBC spectra (Table 2). The amino acid sequence was established from the HMBC spectrum via the same key long-range correlations already observed in **1** (Figure 1). The determined sequence, *cyclo*-(*N*-MePhe-Val-*N*-MePhe-Ile), revealed a quasi-symmetrical structure for **2**, which explained the large number of isochronous resonances observed in the ^{1}H and ^{13}C NMR spectra. ESIMS/MS experiments further confirmed the complete amino acid sequence of this cyclotetrapeptide, with the presence of an ion at *m*/*z* 261 corresponding to the *N*-MePhe-Val fragment and a second ion at *m*/*z* 275 consistent with the *N*-MePhe-Ile fragment.

The molecular formula determined for **3** using HRESIMS was $C_{32}H_{44}N_4O_4$, indicating an additional methylene unit in **3** with respect to **2**. Once again this characteristic was consistent with the higher hydrophobicity observed by reversed-phase HPLC and suggested a compound closely related to both **1** and **2**. As expected, the ^{1}H NMR spectrum of **3** confirmed this, showing a similar pattern to the one observed for **1** and **2** with characteristic peptidic signals between 4 and 5 ppm. The ^{1}H and ^{13}C spectra (Table 3) showed signals characteristic of phenylalanine, isoleucine, and *N*-methyl residues. A 2-fold symmetry was clearly observed in the ^{13}C NMR spectrum, where only 14 signals appeared. The amino acid sequence and the positions of the *N*-methyl groups were established by analysis of the HMBC spectrum, via similar key long-range

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Table 2. NMR Data (500 MHz, CD_3OD, at 24 °C) for 2[a]

amino acid	position	δ_C, mult.	δ_H, mult. (J in Hz)	COSY	HMBC
L-*N*-MePhe1	CO	172.2, C			
	α	64.6, CH	4.48, m	β	
	β	35.1, CH_2	a 3.66, d (14.4)	α, geminal	
			b 3.00, t (13.1)	α, geminal	C_δ (with b)
	γ	138.8, C			
	δ	129.5, CH	7.28, m	ε, ζ	C_ζ, C_δ, C_β
	ε	129.9, CH	7.33, m	δ,ζ	Cγ, C_ε
	ζ	128.1, CH	7.24, m	δ, ε	C_δ
	N-CH_3	31.3, CH_3	2.83, s[b]		Val-CO, C_α
L-Val	CO	173.5, C			
	α	57.3, CH	4.21, d (7.8)	β	CO, Phe-CO C_β, C_γ
	β	30.3, CH	2.10, m	α, γ, γ'	
	γ	20.9, CH_3	0.72, d (6.1)	β	C_α, C_β, $C_{\gamma'}$
	γ'	18.5, CH_3	0.86, d (6.7)	β	C_α, C_β, C_γ
L-*N*-MePhe2	CO	172.2, C			
	α	64.6, CH	4.48, m	β	
	β	35.1, CH_2	a 3.66, d (14.4)	α, geminal	
			b 3.00, t (13.1)	α, geminal	C_δ (with b)
	γ	138.8, C			
	δ	129.5, CH	7.28, m	ε, ζ	C_ζ, C_δ, C_β
	ε	129.9, CH	7.33, m	δ, ζ	Cγ, C_ε
	ζ	128.1, CH	7.24, m	δ, ε	C_δ
	N-CH_3	31.3, CH_3	2.85, s[b]		Ile-CO, C_α
L-Ile	CO	173.5, C			
	α	56.6, CH	4.28, d (7.8)	β	CO, Phe-CO, C_β
	β	36.8, CH	1.84, m	α, β-CH_3, γ_b	
	γ	25.7, CH_2	a 1.48, m	geminal, δ	
			b 1.00, m	geminal, β, δ	
	δ	11.9, CH_3	0.87, t (6.7)	γ	C_β, C_γ
	β-CH_3	17.0, CH_3	0.69, d (6.1)	β	C_α, C_β, C_γ

[a]Due to the quasi-symmetrical structure of **2**, both L-*N*-MePhe resonances are perfectly isochronous except for the proton N-Me singlets. [b]Assignments may be interchanged.

Table 3. NMR Data (500 MHz, CD_3OD, at 24 °C) for 3[a]

amino acid	position	δ_C, mult.	δ_H, mult. (J in Hz)	COSY	HMBC
L-*N*-MePhe	CO	172.2, C			
	α	64.6, CH	4.48, d (10.3)	β	
	β	35.1, CH_2	a 3.65, d (13.9)	α, geminal	
			b 3.00, t (13.0)	α, geminal	C_δ (with b)
	γ	138.8, C			
	δ	129.5, CH	7.28, m	ε, ζ	C_ζ, C_δ, C_β
	ε	129.9, CH	7.33, m	δ, ζ	Cγ, C_ε
	ζ	128.1, CH	7.24, m	δ, ε	C_δ
	N-CH_3	31.3, CH_3	2.84, s		Ile-CO, C_α
L-Ile	CO	173.4, C			
	α	56.6, CH	4.28, d (7.8)	β	CO, Phe-CO, C_β
	β	36.9, CH	1.84, m	α, β-CH_3, γ_b	
	γ	25.7, CH_2	a 1.48, m	geminal, δ	
			b 1.00, m	geminal, β, δ	
	δ	11.9, CH_3	0.87, t (7.2)	γ	C_β, C_γ
	β-CH_3	17.0, CH_3	0.69, d (5.9)	β	C_α, C_β, C_γ

[a]Due to molecular symmetry, both L-Ile and both L-*N*-MePhe are chemically equivalent.

correlations to those observed in **1** and **2** (Figure 1). The sequence *cyclo*-(*N*-MePhe-Ile)$_2$ also corroborated the C_2 symmetrical conformations for **3**. ESIMS/MS experiments further confirmed this sequence with the presence of an ion at m/z 275, corresponding to the *N*-MePhe-Ile fragment.

Marfey's analysis was used to determine the absolute configuration of the amino acids present in the three cyclotetrapeptides. Acid hydrolysis of each peptide followed by HPLC analysis of the hydrolysate after derivatization with Marfey's reagent and comparison with the retention times obtained for standards revealed the *S*- (or L-) configuration for all the residues.[21] In addition, an *S*-configuration was also determined for the second chiral center of isoleucine, confirming the presence of L-isoleucine in compounds **2** and **3**. The absolute stereochemistry determined for **1**−**3** was consistent with the symmetrical features already observed by NMR.

Cyclotetrapeptides **1**−**3** were tested for activity as cardiac ion channel blockers and showed activity as calcium channel blockers (IC_{50} ca. 6 μM) in a Ca^{2+} influx assay (Table 4). This potency is close to that of diltiazem (IC_{50} ca. 2 μM), a benzothiazepine drug that selectively blocks vascular calcium channels, reducing arterial pressure.[22] None of the compounds displayed activity as hERG potassium channel blockers in a K^+ influx assay, indicating the potential absence of cardiotoxicity.[23] Finally, the three peptides revealed no cytotoxic activity when tested against the Fa2N4 cell line (IC_{50} > 20 μM). These results suggest the potential of the new cyclotetrapeptides as vasodilator drugs.

Table 4. Biological Activity of Compounds 1−3 As Cardiac Ion Channel Blockers (reference drugs included for comparison)[a]

compound	Cav1.2 IC_{50} (μM)	hERG IC_{50} (μM)
1	6.2 ± 0.6	>24.0
2	7.1 ± 0.2	>23.4
3	5.0 ± 0.9	>22.7
diltiazem	2.2 ± 0.5	
verapamil	0.9 ± 0.2	
nitrendipine	0.003 ± 0.001	
astemizole		0.008 ± 0.002
haloperidol		0.23 ± 0.01

[a]Cav1.2 (calcium ion channel); hERG (potassium ion channel).

In conclusion, three new compounds (**1**−**3**) have been isolated from static fermentations of *O. sclerotica*. They represent a family of new methylene homologue cyclo-

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tetrapeptides. The structural similarity between the three new cyclotetrapeptides and hirsutide (**4**) is remarkable, all of them having two *N*-MePhe residues in the same relative "opposite" positions within the sequence. Interestingly, the three new peptides showed activity as cardiac calcium channel blockers but did not inhibit the hERG potassium channel and were not cytotoxic, thus suggesting their potential as vasodilators. Although hirsutide (**4**) has not been tested in these ion channel blocker assays, it has been reported to display no cytotoxicity (against P388 cells) or antimicrobial activity.[7] Cyclotetrapeptides **1**−**3** represent the first secondary metabolites described from a fungus of the order Arachnomycetales. Their discovery from one among eight growth conditions demonstrates the value of microfermentation arrays as a strategy for identifying nutritional conditions to produce novel compounds.[14]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. IR spectra were measured with a JASCO FT/IR-4100 spectrometer. NMR spectra were recorded on a Varian DirectDrive spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent as internal references (δ_H 3.31 and δ_C 49.0 ppm for CD_3OD). LC-UV-MS analysis was performed on an Agilent 1100 single quadrupole LC-MS system, using a Zorbax SB-C_8 column (2.1 × 30 mm, 5 μm), maintained at 40 °C and with a flow rate of 300 μL min^{-1}. Solvent A consisted of 10% acetronitrile and 90% water with 1.3 mM trifluoroacetic acid and ammonium formate, and solvent B was 90% acetronitrile and 10% water with 1.3 mM trifluoroacetic acid and ammonium formate. The gradient started at 10% B and went to 100% B in 6 min, was kept at 100% B for 2 min, and returned to 10% B for 2 min to initialize the system. Full diode array UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 s/scan. The eluting solvent was ionized using the standard Agilent 1100 electrospray ionization source adjusted to a drying gas flow of 11 L min^{-1} at 325 °C and a nebulizer pressure of 40 psig. The capillary voltage was set to 3500 V. Mass spectra were collected as full scans from 150 *m*/*z* to 1500 *m*/*z*, with one scan every 0.77 s, in both positive and negative modes. Database searching was performed using an in-house developed application where the DAD (UV−vis) spectra, retention time, and positive and negative mass spectra of the samples were compared to the corresponding UV-LC-MS data of known microbial metabolites stored in the proprietary database.[15,18−20] (Merck reference library containing metabolite standards data obtained under identical conditions to those for the samples under analysis. The library is comprised of 365 fungal metabolites and 423 metabolites from bacteria and actinomycetes.) HRESIMS and MS/MS spectra were acquired using a Bruker maXis QTOF mass spectrometer coupled to the same HPLC system as described above. The mass spectrometer was operated in positive ESI mode. The instrumental parameters were 4 kV capillary voltage, drying gas flow of 11 L min^{-1} at 200 °C, and nebulizer pressure at 2.8 bar. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Prerun calibration was by infusion with the same TFA-Na calibrant. Acetone used for extraction was analytical grade. Solvents employed for isolation were HPLC grade. Marfey's reagent analogue (FDVA, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-valinamide) and amino acid standards were from Sigma-Aldrich.

Strain and Fermentation. *Onychocola sclerotica* (CBS 201.92) was purchased from the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl). The strategy and protocols for fermentation of fungi on nutritional arrays have been described previously.[14,16,24] To scale up the fermentation to 1 L, 10 mycelial discs were used to inoculate 50 mL of SMYA (Difco neopeptone 10 g, maltose 40 g, Difco yeast extract 10 g, agar 4 g, distilled H_2O 1 L). After 7 days incubation at 22 °C and 220 rpm, 3 mL aliquots of this culture were used to inoculate MMK2 medium (mannitol 40 g, yeast extract 5 g, Murashuge & Skoog salts (Sigma-Aldrich) 4.3 g, distilled H_2O 1 L) distributed among 10 × 100 mL in 500 mL Erlenmeyer flasks. The flasks were incubated statically at 22 °C, 70% relative humidity for 21 days.

Extraction and Isolation. The initial 1 mL microfermentations were extracted with an equal volume of acetone, and the acetone was removed by vacuum evaporation as described previously.[16,24] The scaled-up fermentation broth (1 L) was extracted with acetone (1 L) under continuous shaking at 220 rpm for 1 h. The mycelium was then separated by centrifugation, and the supernatant (ca. 2 L) was concentrated to 1 L under a stream of nitrogen. This solution was loaded (with continuous 1:1 water dilution, discarding the flow-through) on a column packed with SP-207SS reversed-phase resin (brominated styrenic polymer, 65 g) previously equilibrated with water. The loaded column was further washed with water (1 L) and afterward eluted at 8 mL min^{-1} on an automatic flash-chromatography system (CombiFlash Rf, Teledyne Isco) using a linear gradient from 10% to 100% acetone in water (in 12.5 min) with a final 100% acetone step (for 15 min), collecting 11 fractions of 20 mL.[25] Fractions were concentrated to dryness on a centrifugal evaporator, and fractions 7 and 8, containing the target compounds (as revealed by LC-UV-MS analysis), were each fractionated by Sephadex LH-20 chromatography (gel bed 35 × 120 mm) using MeOH as eluent to generate seven subfractions each. The LH-20 subfractions containing the target compounds (subfractions 3, 4, and 5) were pooled and further purified by reversed-phase semipreparative HPLC (Agilent Zorbax SB-C_8, 9.4 × 250 mm, 7 μm; 3.6 mL min^{-1}, UV detection at 210 nm) with a linear gradient of water−CH_3CN from 5% to 100% CH_3CN over 37 min to yield **1** (4.0 mg), **2** (11.4 mg), and **3** (8.5 mg) eluting at 26.6, 27.7, and 28.8 min, respectively.

*Compound **1** (cyclo-(L-N-MePhe-L-Val)$_2$):* white solid; $[\alpha]^{20}_D$ −202 (*c* 0.2, CH_2Cl_2); IR (ATR) ν_{max} 3326, 3062, 3028, 2963, 2928, 2872, 1661, 1627, 1496, 1454, 1402, 1385, 1318, 1176, 1088, 976, 733, 700 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 521.3141 [M + H]$^+$ (calcd for $C_{30}H_{41}N_4O_4$, 521.3128).

*Compound **2** (cyclo-(L-N-MePhe-L-Val-L-N-MePhe-L-Ile):* white solid; $[\alpha]^{20}_D$ −208 (*c* 0.3, CH_2Cl_2); IR (ATR) ν_{max} 3326, 3062, 3028, 2964, 2931, 2874, 1661, 1629, 1496, 1454, 1402, 1384, 1318, 1174, 1088, 974, 733, 700 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 2); HRESIMS *m*/*z* 535.3301 [M + H]$^+$ (calcd for $C_{31}H_{43}N_4O_4$, 535.3284).

*Compound **3** (cyclo-(L-N-MePhe-L-Ile)$_2$):* white solid; $[\alpha]^{20}_D$ −211 (*c* 0.3, CH_2Cl_2); IR (ATR) ν_{max} 3326, 3063, 3028, 2963, 2931, 2874, 1660, 1630, 1496, 1454, 1402, 1318, 1295, 1173, 1088, 972, 734, 699 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 3); HRESIMS *m*/*z* 549.3461 [M + H]$^+$ (calcd for $C_{32}H_{45}N_4O_4$, 549.3441).

Preparation and Analysis of Marfey Derivatives. Compounds **1**−**3** (150 μg each) were hydrolyzed by heating in HCl (6 N, 300 μL) at 110 °C for 24 h. After cooling, the solutions were evaporated to dryness and redissolved in H_2O (50 μL). To each peptide acid hydrolysate solution (or to 50 μL of a 50 mM solution of the respective amino acid standard) was added $NaHCO_3$ (1 M; 20 μL) and then a 1% (w/v) solution (100 μL) of FDVA (N^{α}-(2,4-dinitro-5-fluorophenyl)-L-valinamide, a variant of Marfey's reagent) in acetone. The mixture was incubated for 1 h at 40 °C. The reaction was stopped by addition of HCL (1 N, 20 μL). The derivatized peptide hydrolysate solutions were diluted by addition of 300 μL of MeCN, and the derivatized amino acid standards solutions were diluted by addition of 770 μL of MeCN. An aliquot of these solutions (4 μL for **1**, **2**, or **3** and 2 μL for the standards) was analyzed by HPLC-UV-MS (Zorbax SB-C_8 column, 2.1 × 30 mm, 5 μm, 40 °C, 300 μL min^{-1}; linear gradient: 0 min 25% B, 30 min 35% B, mobile phase and detection as described above for the general LC-UV-MS analysis). Retention times (min) of the FDVA amino acid derivatives used as standards were as follows: L-Val (2.55), D-Val (6.12), *N* Me-L-Phe (6.60), *N* Me-D-Phe (7.55), L-Ile (4.18), D-Ile (10.35), L-*allo*-Ile (4.03), D-*allo*-Ile (10.19). Retention times (min) of the observed peaks in the HPLC trace of the FDVA-derivatized hydrolysis products of **1** were as follows: L-Val (2.49), *N* Me-L-Phe (6.52); for **2** as follows: L-Val (2.57), *N* Me-L-Phe (6.71), L-Ile (4.17); and for **3** as follows: *N*-Me-L-Phe (6.64), L-Ile (4.15). Due to the close retention times observed for L-Ile and L-*allo*-

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Ile, the presence of L-Ile in **2** and **3** was confirmed using a second HPLC method with isocratic elution (15% B during 50 min). Under these conditions, retention times of 20.1 and 21.0 min were obtained for L-*allo*-Ile and L-Ile, respectively. Analyses of the compound **2** and **3** hydrolysates derivatized with Marfey's reagent revealed the presence of only one peak, at 21.0 min in both cases. Co-injection of these hydrolysates with the FDVA derivative of L-*allo*-Ile showed the presence of a second peak at 20.1 min, confirming that L-*allo*-Ile was not present in the structures of **2** and **3**.

Biological Assays. Cytotoxicity tests against the Fa2N4 cell line were performed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay.[26] Cardiac ion channel blocker activity was determined by two assays. The first one measured calcium influx into the cells (wild-type HEK or C1-6-37-3 cells) in a FLIPR^{TETRA} (Molecular Devices) as previously described.[27] The second one measured potassium influx using the FluxOR potassium channel assay as outlined in the Invitrogen information sheet and performed on the FLIPR^{TETRA}.[28]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR, ESIMS/MS, and DAD (UV−vis) spectra for compounds **1**−**3**, HPLC traces corresponding to Marfey's analyses, photographs of the microorganism in culture, and details of the biological assays are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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