Pentaminomycins F and G, first non-ribosomal peptides containing 2-pyridylalanine

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ABSTRACT

Pentaminomycins F-H (**1**-**3**), a group of three new hydroxyarginine-containing cyclic pentapeptides, were isolated from cultures of a *Streptomyces cacaoi* subsp. *cacaoi* strain along with the known pentaminomycins A-E. The structures of the new peptides were determined by a combination of mass spectrometry and NMR and Marfey´s analyses. Among them, pentaminomycins F (**1**) and G (**2**) were shown to contain in their structures the rare amino acid 3-(2-pyridyl)-alanine. This finding represents the first reported examples of non-ribosomal peptides containing this residue. The LDLLD chiral sequence found for the three compounds was in agreement with that reported for previously isolated pentaminomycins and consistent with the epimerization domains present in the putative non-ribosomal peptide synthetase (NRPS) biosynthetic gene cluster.

Microbial secondary metabolites are a major source of novel chemical entities with privileged structures, many of which have become lead compounds for clinical use as antibiotics, immunosuppressive or anticancer agents. Among them, cyclic peptides are of great importance due to their structural variety and multiple functions, ranging from therapeutic to ecological roles.[1] Recently, a family of novel cyclic pentapeptides characterized by the presence of N^5-hydroxy-arginine, pentaminomycins A-E (**4**-**8**), has been uncovered. [2–4] The putative biosynthetic gene cluster (BGC) for both, pentaminomycins and the previously known BE-18257 cyclic peptides,[5] was identified in two closely related *Streptomyces cacaoi* strains. This BGC was shown to harbor two different NRPSs, a P450 enzyme accounting for the hydroxylation of the arginine residue and a putative hydrolase presumably responsible for the release and cyclization of the peptides, along with regulatory and transport-related genes. The number and location of the epimerisation (E) domains within the two different NRPS proteins were shown to be fully consistent with the LDLLD or the enantiomeric DLDDL chiral sequences in pentaminomycins and BE-18257 peptides, respectively. [3,4]

Interestingly, a recent work on a *Streptomyces cacaoi* subsp. *cacaoi* strain CA-170360 developed by our group led to the discovery of cacaoidin, a novel glycosylated lanthipeptide with unprecedented structural features[6] which represents the first member of class V lanthipeptides (lanthidins).[7] The easy access to this strain and its whole genome sequence, prompted us to search the BGC of pentaminomycins within the genome of the strain CA-170360 in order to confirm its presence, compare it with that previously reported, and explore the possibility of producing new cyclic pentapeptides of this interesting family. Fortunately, the gene cluster of pentaminomycins was found in the genome of CA-170360[8] and the subsequent culture of the strain under different growth conditions allowed the detection of all the pentaminomycins previously described together

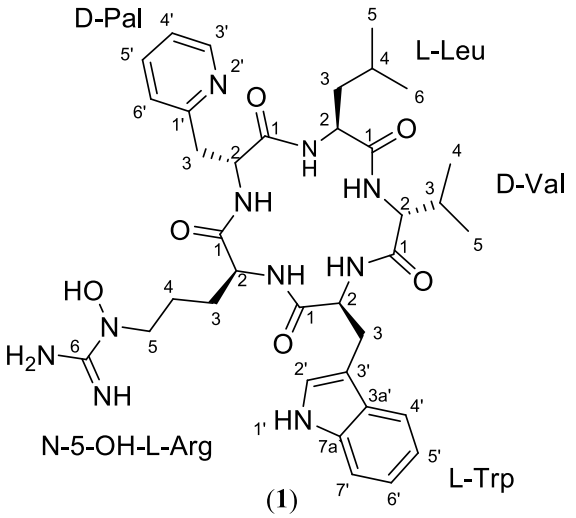
with additional unreported members of the family. Targeted isolation of these new analogues led to the identification of pentaminomycins F-H (**1-3**). Among them, **1** and **2** were revealed as unprecedented non-ribosomal peptides containing the unusual amino acid 3-(2-pyridylalanine) (2-Pal). The isolation and structural elucidation of these three new cyclic pentapeptides are described herein.

RESULTS AND DISCUSSION

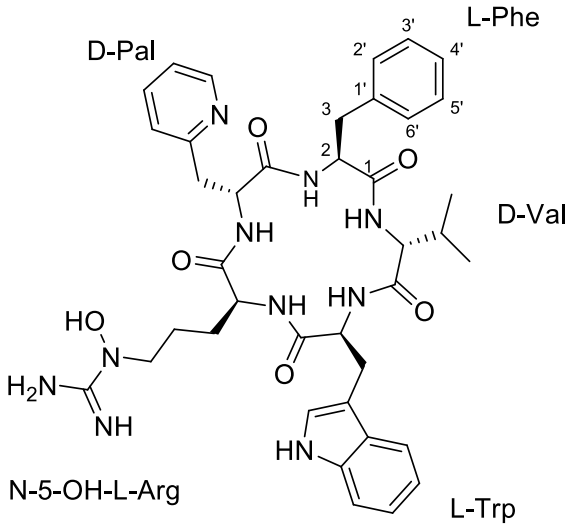
The strain CA-170360 was identified as *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748^T on the basis of its 16S rDNA sequence (GenBank accession number MW131875), as described previously.[6] The draft genome sequence of CA-170360 was analyzed with antiSMASH 5.1.2[9] and the putative BGC of pentaminomycins and BE-18257 peptides was searched within it. As expected, a nearly identical BGC containing two NRPS genes (*cppB, cppM*) with high homology to those previously reported, was identified in the genome sequence of CA-170360.[8] These NRPS enzymes were individually linked to the biosynthesis of BE-18257 cyclic peptides (CppB) and pentaminomycins (CppM) on the basis of their E domains, as previously reported. [3,4]

The strain CA-170360 was subsequently cultured in three different growth media (MPG, YEME and R2YE) for 21 days at 28ºC and the culture broths and mycelia were extracted with acetone and processed as described previously.[10] These three extracts were analyzed by LC-DAD-MS and the chromatograms interrogated for the presence of pentaminomycins and/or BE-18257 peptides. Different components with molecular formulae matching those of some known members of both families were detected in the three extracts. Thus, cyclic peptides BE-18257A, BE-18257B/C and pentaminomycin D (**7**) were detected in the acetone extract of YEME (SI, Figure S1), while that

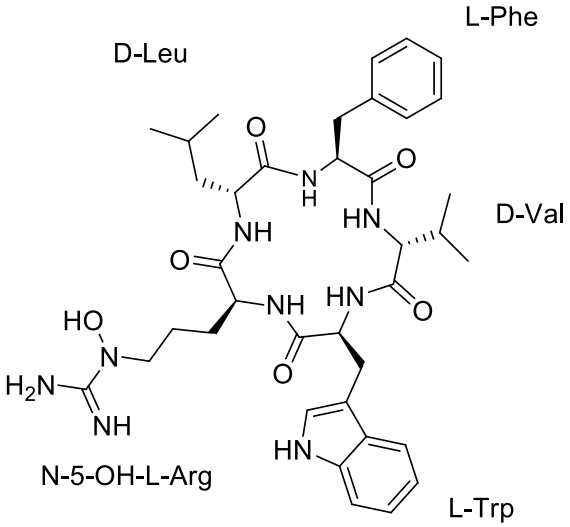
of the MPG culture contained mainly the BE-18257 peptides (SI, Fig. S2). On the other hand, the culture in R2YE medium showed the richest profile in pentaminomycins content (SI, Fig. S3). Interestingly, the analysis of the R2YE extract also revealed the presence of two components with $[M+H]^+$ ions at 719.3980 and 753.3830 *m/z*, which were indicative of compounds having molecular formulae of $C_{36}H_{50}N_{10}O_6$ (Δ -1.11 ppm) and $C_{39}H_{48}N_{10}O_6$ (Δ -0.20 ppm), respectively (SI, Fig. S4). These previously unreported molecular formulae indicated the presence of compounds with a close structural relationship with pentaminomycins but containing an additional nitrogen atom that suggested the presence of a basic amino acid residue not present in previously isolated members of the family. This finding prompted us to isolate these new pentaminomycins and determine their structures. For this purpose, the producing strain was cultivated in a 3L-scale (R2YE, 21 days, 28 ºC) and the broth and mycelia were extracted with acetone as described in the experimental section. After evaporation of the organic solvent, the mycelial debris was discarded by filtration. Considering the basicity of previously known pentaminomicyns (pKa was calculated as 9.6 by using the Marvin suite, ChemAxon)[11], the pH of the aqueous broth was therefore adjusted to 10 and extracted with MEK. The organic extract was purified by MPLC (reversed phase C18) and the resulting fractions were analysed by LC-MS. The more polar fractions containing the two new compounds mentioned above were pooled and repurified by semipreparative RP-HPLC (SI, Fig. S5) to yield pentaminomycins F (**1**) and G (**2**). Pentaminomycins A-E (**4**-**8**) were also found in different less polar adjacent fractions of this chromatography.



(**1**)



(**2**)



(**3**)

Compound **1** was isolated as an off-white amorphous solid (1.5 mg) and the molecular formula $C_{36}H_{50}N_{10}O_6$ previously identified was confirmed based on ESI-TOF MS data (SI, Fig. S6). The peptidic nature of **1** was evidenced by the presence of different exchangeable amide NH and α-amino acid hydrogen signals in the ^{1}H-NMR spectrum (Table 1; SI, Fig. S7), as well as of characteristic amide-type carbonyl signals in the ^{13}C-NMR spectrum (Table 1; SI, Fig. S8).

Analysis of 2D-NMR spectroscopic data (COSY, HSQC, HMBC and TOCSY) (SI, Figs. S9-S12) of **1** revealed the presence of valine (Val), tryptophan (Trp), N^5-hydroxyarginine (N5-OH-Arg) and leucine (Leu), as well as of an additional non-proteinogenic aromatic amino acid residue. Along with the similar chemical shift values reported for N5-OH-Arg in the previously reported pentaminomycins A-E,[2-4] the hydroxylation at N-5 of the arginine residue in **1** was supported by two different experimental evidences: the downfield shift of C-5 (δ_C: 50.5 ppm; see Table 1), which strongly suggested the hydroxy group to be located at the adjacent atom (N-5), and the detection of key fragment ions in the HRMS/MS spectrum of **1** (SI, Fig. S13).

As mentioned above, compound **1** showed signals accounting for a second aromatic amino acid in the 2D-NMR spectra. (SI, Figs. S9–S12). A spin system in the TOCSY spectrum (SI, Fig. S12) comprising four aromatic proton signals at δ_H 7.25 (H-6'; δ_C: 123.9 according to the HSQC spectrum), 7.65 (H-5'; δ_C: 136.1), 7.19 (H-4'; δ_C: 121.7) and the low-field signal at δ_H 8.43 (H-3'; δ_C: 148.4), along with a quaternary carbon signal at δ_C 157.8 ppm (C-1') in the ^{13}C-NMR spectrum (Table 1), jointly suggested the presence of a 2-substituted pyridine ring, which was further supported by a set of key HMBC correlations (Fig.1; SI, Fig. S11). Another spin system was identified in the TOCSY spectrum (SI, Fig. S12) consisting of a NH amide hydrogen at δ_H 8.91, along with an α-amino acid hydrogen at δ_H 4.81 (H-2) and one diastereotopic methylene group at δ_H 3.14 and 2.94 (H-3) coupled to carbon signals at δ_C 51.8 (C-2) and 36.1 (C-3), respectively, in the HSQC spectrum (SI, Fig. S10). HMBC correlations from H-2 and H-3 to C-1' at δ_C 157.8, and from H-3 to C-6' at δ_C 123.9 , revealed the connection between this spin system and the pyridine ring, thus establishing the presence of the rare 3-(2-pyridylalanine) amino acid (2-Pal) residue in **1**.

The planar structure of **1** was elucidated based on the key HMBC correlations from NH- to -CO (C-1) within each amide bond (Fig. 1; SI, Fig. S11). HMBC correlations from Trp NH (δ_H 8.60) to Val C-1(δ_C 171.4), from Val NH (δ_H 8.51) to Leu C-1(δ_C 172.4), from Leu NH (δ_H 7.51) to 2-Pal C-1 (δ_C 170.5), from 2-Pal NH (δ_H 8.91) and H-2 (δ_H 4.81) to N5-OH-Arg C-1 (δ_C 170.3) and from N5-OH-Arg NH (δ_H 7.31) to Trp C-1 (δ_C 171.6) allowed to determine the amino acid sequence of **1** as cyclo-(Leu-Val-Trp-N5-OH-Arg-2-Pal). This sequence was in agreement with the molecular formula of **1** and was furtherly supported by HRMS/MS fragmentation (Fig. 2).

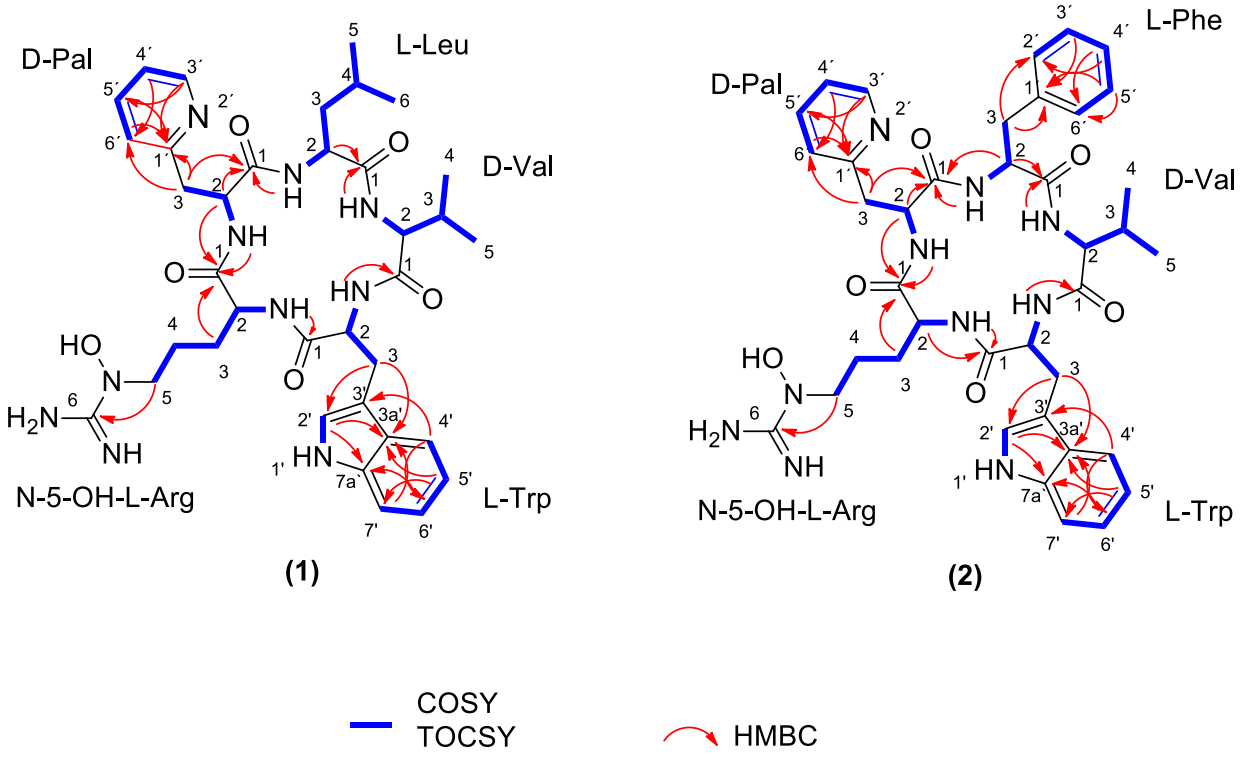


Figure 1. Key COSY/TOCSY and HMBC correlations observed in the spectra of **1** and **2**.

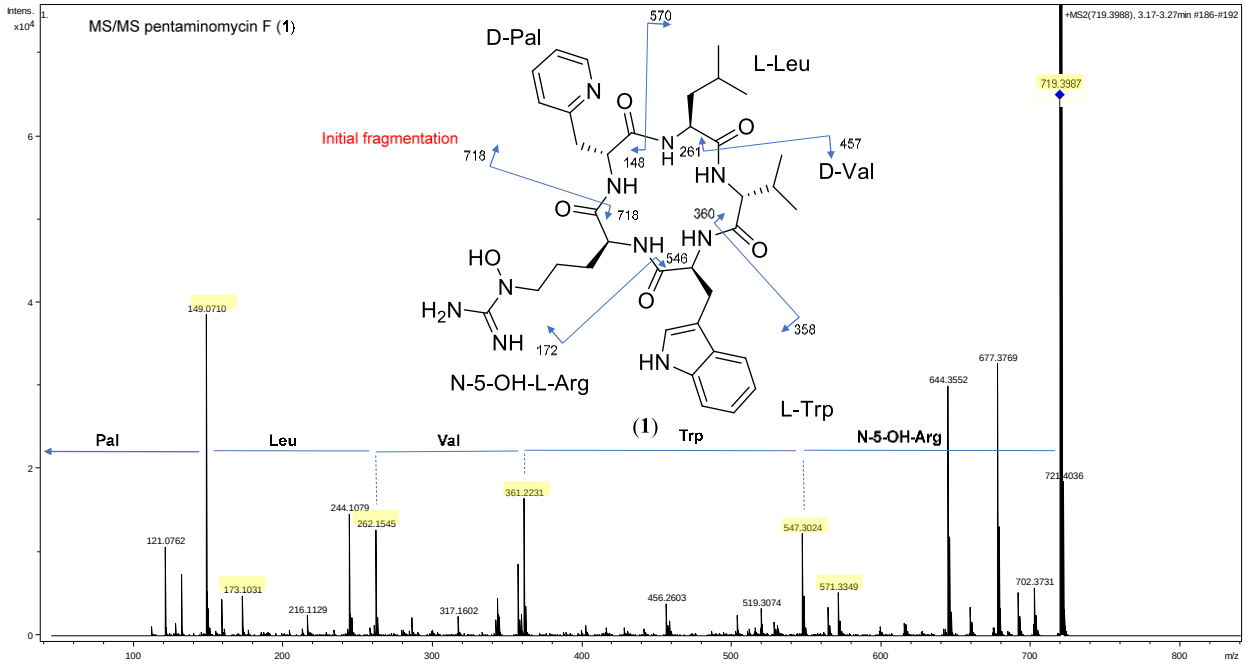


Figure 2. HRMS/MS spectrum and amino acid sequencing of **1**.

The absolute configuration of the amino acid residues in **1** were determined by advanced Marfey´s analysis[12] under two different conditions: A sample of **1** was hydrolyzed (HCl 6 N, 110 ºC) in presence of 5% thioglycolic acid to prevent Trp degradation.[13] In parallel, the hydroxy group of N5-OH-Arg was removed by using hydriodic acid (HI) for the hydrolysis, as previously reported.[14] Each hydrolyzate was separately derivatized with L-FDVA, analyzed by HPLC and the retention times compared to those of standard amino acids derivatives. The results jointly showed that the absolute configurations of the amino acids in **1** were L-Leu, D-Val, L-Trp, N5-OH-L-Arg (detected as L-Arg in the second hydrolyzate with HI) and D-2-Pal (SI, Figs. S32-S38). Compound **1** was therefore elucidated as cyclo-(L-Leu-D-Val-L-Trp-N5-OH-L-Arg-D-2-Pal) and named pentaminomycin F. The LDLLD chiral sequence found in **1** is, not surprisingly, the same as that reported for pentaminomycins A-E, some of which were also detected in the culture broth.

Compound **2** was isolated as an amorphous solid (1.3 mg) and its ESI-TOF MS data confirmed the molecular formula $C_{39}H_{48}N_{10}O_6$ previously assigned (SI, Fig. S14). Combined analysis of 1D and 2D NMR set data (Table 1, SI. Figs. S15-S20) identified Val, Trp, N5-OH-Arg, Phe and 2-Pal as the constituent amino acid residues in **2**, thus advancing the closely relatedness to **1.**
Detailed analysis of HMBC correlations (Fig 1; SI, Fig. S19) confirmed the replacement of Leu by Phe (with respect to **1**) and established its planar structure as cyclo-(Phe-Val-Trp-N5-OH-Arg-2-Pal), which was in concordance with the HRMS/MS data (SI, Fig. S21) The absolute configuration of the amino acid residues in **2** was determined by advanced Marfey's analysis (SI, Figs. S39-S43) and the same LDLLD chiral sequence was found, as expected. Consequently, the structure of **2** was established as cyclo-(L-Phe-D-Val-L-Trp-N5-OH-L-Arg-D-2-Pal) and the compound was named pentaminomycin G.

Remarkably, although the non-proteinogenic amino acid 3-(2-pyridyl)-L-alanine (also named 2′aza-L-phenylalanine) has been previously isolated from cultures of *Streptomyces sp.* SF2538,[15] the presence of 2-Pal (either in its L or D form) as an amino acid residue within a peptide is reported herein for the first time, thus highlighting the structural novelty of pentaminomycins F and G.

Table 1. ^{1}H NMR (500 MHz in DMSO-d$_6$) and ^{13}C NMR (125 MHz in DMSO-*d$_6$*) data for compounds **1**-**3**.

Pentaminomycin F (**1**)				Pentaminomycin G (**2**)				Pentaminomycin H (**3**)			
position		δ^{13}C	δ^{1}H (mult, J, Hz)	position		δ^{13}C	δ^{1}H (mult, J, Hz)	position		δ^{13}C	δ^{1}H (mult, J, Hz)
Leu	**1**	172.4, C		**Phe**	**1**	171.2		**Phe**	**1**	171.1, C	
	2	50.4, CH	4.41, dd (16.0, 8.2)		**2**	53.0, CH	4.66, dd (7.6, 7.3)		**2**	53.2, CH	4.61, m
	3	41.4, CH$_2$	1.34, m		**3**	38.4, CH$_2$	2.83, m		**3**	37.4, CH2	2.82, m
	4	24.3, CH	1.44, m				2.77, m				
	5	22.7, CH$_3$	0.85, d (6.5)		**1′**	137.1, C			**1′**	137.5, C	
	6	22.0, CH$_3$	0.81, d (6.5)		**2′**	129.1, CH	7.16, m		**2′**	129.2, CH	7.21, m
	NH		7.51, d (7.6)		**3′**	128.0, CH	7.22, m		**3′**	128.0, CH	7.22, m
					4′	126.2, CH	7.16, m		**4′**	126.2, CH	7.15, m
					5′	128.0, CH	7.22, m		**5′**	128.0, CH	7.22, m
					6′	129.1, CH	7.16, m		**6′**	129.2, CH	7.21, m
					NH		7.66, m		**NH**		7.93, d (9.1)
Val	**1**	171.4, C		**Val**	**1**	171.3		**Val**	**1**	171.2, C	
	2	60.1, CH	3.68, dd (9.9, 7.4)		**2**	60.1, CH	3.61, dd (9.8, 7.7)		**2**	59.6, CH	3.72, dd (9.3,7.6)
	3	28.2, CH	1.66, m		**3**	28.2, CH	1.56, m		**3**	29.0, CH	1.57, m
	4	19.1, CH$_3$	0.75, d (6.6)		**4**	19.3, CH$_3$	0.54, d (6.5)		**4**	18.9, CH$_3$	0.56, d (6.5)
	5	18.5, CH$_3$	0.33, d (6.6)		**5**	18.8, CH$_3$	0.29, d (6.5)		**5**	18.5, CH$_3$	0.32, d (6.5)
	NH		8.51, d (7.4)		**NH**		8.38, d (7.7)		**NH**		8.08, d (7.6)
Trp	**1**	171.6		**Trp**	**1**	171.6		**Trp**	**1**	171.7, C	
	2	55.3, CH	4.30, m		**2**	55.3, CH	4.28, m		**2**	55.5, CH	4.25, m
	3	27.0, CH$_2$	3.20, dd (14.8, 3.3)		**3**	26.9, CH$_2$	3.18, m		**3**	26.9, CH$_2$	3.17, br d (4.3)
			2.92, m				2.90, m				2.91, dd (14.2, 11.8)
	2-NH		8.60, d (7.9)		**2-NH**		8.59, d (8.3)		**2-NH**		8.56, d (7.7)
	1′(NH)		10.76, s		**1′(NH)**		10.76, br s		**1′(NH)**		10.77, s
	2′	123.9, CH	7.17, m		**2′**	123.9, CH	7.16, m		**2′**	123.9, CH	7.16, br d (2.2)
	3′	110.2, C			**3′**	110.2, C			**3′**	110.2, C	
	3a′	126.9, C			**3a′**	126.8, C			**3a′**	126.9, C	
	4′	117.9, CH	7.51, br d		**4′**	117.8, CH	7.51, br d		**4′**	117.9, CH	7.51, br d (7.9)
	5′	118.3, CH	6.98, dd (7.2, 7.2)		**5′**	118.2, CH	6.97, dd (7.4, 7.3)		**5′**	118.3, CH	6.98, dd (7.9, 7.3)
	6′	120.8, CH	7.05, br dd (7.6, 7.2)		**6′**	120.8, CH	7.04, m		**6′**	120.9, CH	7.04, m
	7′	111.3, CH	7.30, m		**7′**	111.3, CH	7.29, m		**7′**	111.4, CH	7.30, br d (8.1)
	7a′	126.9, C			**7a′**	136.1, C			**7a′**	136.2, C	
N5-OH-Arg	**1**	170.3		**N5-OH-Arg**	**1**	170.4		**N5-OH-Arg**	**1**	170.6, C	
	2	52.7, CH	4.16, dd (14.2, 7.2)		**2**	52.7, CH	4.16, m		**2**	52.6, CH	4.20, dd (14.4, 7.3)
	3	28.3, CH$_2$	1.54. m		**3**	28.2, CH$_2$	1.54, m		**3**	28.5, CH$_2$	1.61, m
	4	22.0, CH$_2$	1.33, m		**4**	22.0, CH$_2$	1.32, m		**4**	22.3, CH$_2$	1.48, m
			1.24, m				1.19, m				

	5	50.5, CH_2	3.44, m		**5**	50.5, CH_2	3.44, m		**5**	50.8, CH_2	3.52, m
	6	157.5, C			**6**	157.4, C			**6**	157.6, C	
	2-NH		7.31, m		**2-NH**		7.28, m		**2-NH**		7.25, m
	N-OH		10.47, s		**N-OH**		10.51, s		**N-OH**		10.52, s
	NH_2				**NH_2**				**NH_2**		
2-Pal	**1**	170.5		**2-Pal**	**1**	170.3		**Leu**	**1**	171.1, C	
	2	51.8, CH	4.81, dd (15.1, 8.0)		**2**	51.7, CH	4.8, dd (8.0,7.8)		**2**	50.8, CH	4.24, m
	3	36.1, CH_2	3.14. m		**3**	36.2, CH_2	3.09, dd (14.2, 6.6)		**3**	37.4, CH_2	1.37, m
			2.94, m				2.91, m		**4**	24.1, CH	1.33, m
	NH		8.91, d (8.2)		**NH**		8.91, d (7.6)		**5**	22.0, CH_3	0.73, d (6.2)
	1′	157.8, C			**1′**	157.4, C			**6**	22.5, CH_3	0.82, d (6.0)
	2′ (N)				**2′ (N)**				**NH**		8.58, d (7.9)
	3′	148.4, CH	8.43, br d (4.2)		**3′**	148.7, CH	8.42, br d (4.2)				
	4′	121.8, CH	7.19, m		**4′**	121.6, CH	7.19, m				
	5′	136.1, CH	7.65, ddd(7.6,7.6,1.6)		**5′**	136.3, CH	7.64, m				
	6′	123.9, CH	7.25, br d (7.9)		**6′**	123.7, CH	7.22, m				

Further LC-HRESIMS and tandem mass spectrometry analysis of the MPLC fractions containing the known pentaminomycins A-E (**4**-**8**) revealed the presence of a new compound (**3)** isobaric to pentaminomycin C (**6**). Detection of different key fragment ions in the HRMS/MS spectrum of compound **3** in comparison with those of pentaminomycin C (**6**) strongly suggested a different order in the amino acids sequence between them (SI, Fig. S22). The new peptide (**3**) was then isolated by semipreparative HPLC along with all the other known pentaminomycins A-E (**4**-**8**) (SI, Figs. S23, S59). Interpretation of 2D NMR data of compound **3** (Table 1; Figs. S25-S31) unambiguously determined **3** as a sequence isomer of pentaminomycin C (**6**), in which the Leu and Phe residues and their chiralities were interchanged in the peptide backbone. The planar structure of **3** was then established as cyclo-(Phe-Val-Trp-N5-OH-Arg-Leu). The absolute configurations of the amino acid residues in **3** were determined by advanced Marfey′s analysis

(SI, Figs. S44-S48) and again, the same LDLLD chiral sequence was found. Consequently, **3** was elucidated as cyclo-(L-Phe-D-Val-L-Trp-N5-OH-L-Arg-D-Leu) and named as pentaminomycin H.

The co-isolation during this work of three new pentaminomycins (**1**-**3**) along with the known pentaminomycins A-E (**4**-**8**) strongly supports a common biosynthetic origin for all these compounds. According to previous results,[3,4] bioinformatic analysis [9,16] of the adenylation (A) domains of the CppM protein accurately predicted the incorporation of Val and Arg for the second and fourth A domains, while suggested a polar aromatic amino acid as the substrate of the third A domain, the latter being consistent with a Trp residue at this position in the backbone for all pentaminomycins A-H. For the first and fifth A domains, the substrates predicted were in both cases hydrophobic amino acids such as Val, Leu or Ile among others, thus advancing the promiscuity of these domains. The variety of residues found at these positions in the backbone of pentaminomycins A-H is an experimental evidence of this promiscuity, and the acceptance of 2-Pal as a substrate of the fifth A domain (**1** and **2**) further highlights it.

Regarding the production of pentaminonycins F (**1**) and G (**2**), it is logical to assume that the amino acid 2-Pal must be being biosynthesized by the strain under the culture conditions, and then recruited by the biosynthetic pathway of pentaminomycins. Thus, 2 -Pal would be accepted as a substrate by CppM (more precisely, by the A domain of its fifth module) and eventually incorporated as a D amino acid residue in **1** and **2,** because of the action of the E domain in this module.

The question that immediately came up was the origin of 2-Pal. As mentioned above, this amino acid (in its L form) has been previously isolated from *Streptomyces* sp. SF2538.[15] However,

nothing is known about its biosynthesis. The authors of a previous work on the BGC of kedarcidin chromophore and related enediyne compounds, postulated a type II PKS locus as possibly responsible for the biosynthesis of 2′-aza-L-phenylalanine (i.e,; 2-L-Pal) and the related amino acid 2′-aza-L-tyrosine (which would ultimately result in the formation of the aza-β-tyrosine substructure present in the kedarcidin chromophore), but this hypothesis could not be sufficiently proved.[17] Apart from that set of genes, which were not found anywhere within the genome of CA-170360, no other obvious candidate genes could be considered for search. Also, none of the putative proteins encoded in the BGC of pentaminomycins seems to be related to the synthesis of 2-Pal, so the question about its biosynthetic origin remains unclear.

Finally, compounds **1**–**8** were evaluated for their antibacterial properties against a panel of Gram-negative pathogens such as *Escherichia coli* ATCC 25922, *Acinetobacter. baumannii* MB5973, *Klebsiella pneumoniae* ATCC700603 or *Pseudomonas aeruginosa* MB5919. Two of them, compounds **5** and **6**, showed a modest bioactivity against a clinical isolate of *A. baumannii*, presenting MIC values of 16-32 μg/mL.

In summary, the use of different culture conditions for the strain *Streptomyces cacaoi* subsp. *cacaoi* CA-170360 led to the isolation of the three new pentaminomycins F-H (**1-3**). Of major significance, pentaminomycins F (**1**) and G (**2**) reported herein are the first non-ribosomal peptides incorporating in their structures the rare 3-(2-pyridylalanine) amino acid residue. This work highlights the importance of exploring different conditions to extend the microorganism′s biosynthetic potential and thus have access to novel and privileged structures.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P-2000 polarimeter. IR spectra were recorded with a JASCO FT/IR-4100 spectrometer equipped with a PIKE MIRacle single reflection ATR accessory. NMR spectra were recorded on a Bruker Avance III spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe. Chemical shifts were reported in ppm using the signals of the residual solvents as internal reference (δ_H 2.51 and δ_C 39.5 for DMSO-d_6). LC-UV-LRMS analysis were performed on an Agilent 1100 single quadrupole LC-MS system as previously described.[18] ESI-TOF and MS/MS spectra were acquired using a Bruker maXis QTOF mass spectrometer coupled to an Agilent Rapid Resolution 1200 LC. 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 of 2.8 bar. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Pre-run calibration was done by infusion with the same TFA-Na calibrant. Medium pressure liquid chromatography (MPLC) was performed on semiautomatic flash chromatography (CombiFlash Teledyne ISCO Rf400×) with a precast reversed-phase column. Semi-preparative HPLC separation was performed on Gilson GX-281 322H2 with a semi-preparative reversed-phase column (Zorbax SB-C18, 250 × 9.4 mm, 5 µm). Acetone used for extraction was analytical grade. Solvents employed for isolation were all HPLC grade. Chemical reagents and standards were purchased from Sigma-Aldrich.

Strain identification. The taxonomic identification of the strain was reported previously.[6] According to the EzTaxon server analysis[19] (http://www.ezbiocloud.net/eztaxon), the strain was identified as *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748T (similarity of 99.93%) based on its nearly complete 16S rDNA sequence (1410 bp) (GenBank accession number MW131875).

Culture conditions. The strain CA-170360 was cultivated in EPA vials (10 mL of medium/ 40 mL vial) in three different media: YEME, consisting of yeast extract (3 g/L), bacto-peptone (5 g/L), oxoid malt extract (3 g/L), glucose (10 g/L), sucrose (340 g/L), $MgCl_2$-$6H_2O$ 2mL/L; R2YE, consisting of yeast extract (5 g/L), sucrose (103 g/L), K_2SO_4 (0.25 g/L), $MgCl_2$-$6H_2O$ (10.12 g/L), glucose (10 g/L), casamino acids (0.1 g/L), KH_2PO_4 (0.5% 1 mL), $CaCl_2$-$2H_2O$ (3.68% 8 mL), L-proline (20% 1.5 mL), TES buffer 5.73% adjusted to pH 7.2 (10 mL), trace element solution 0.2 mL, NaOH 1N (0.5mL), growth factors for auxotrophs (0.75 mL), trace element solution: $ZnCl_2$ (40 mg/L), $FeCl_3$-$6H_2O$ (200 mg/L), $CuCl_2$-$2H_2O$ (10 mg/L), $MnCl_2$-$4H_2O$ (10 mg/L), $Na_2B_4O_7$-$10H_2O$ (10 mg/L), $(NH_4)6Mo_7O_{24}$-$4H_2O$ (10 mg/L); MPG, consisting of glucose (10 g/L), millet meal (20 g/L), cottonseed flour (20 g/L), MOPS (20 g/L), pH 7.0. The EPA vials were incubated at 28 °C for 21 days in a rotary shaker at 220 rpm and 70% humidity before harvesting. The whole broth of each one was extracted with an equal volume of acetone under shaking for 1h. After evaporation of the organic solvent, the mycelial debris was discarded by centrifugation and filtration. The aqueous broth was then extracted with MEK (2 × 5 mL). The organic extract was evaporated to dryness and a portion of the residue was reconstituted in 20% DMSO: H_2O (2 × WBE, Whole Broth Equivalent units).

Large-scale fermentation, extraction and isolation. The producing strain CA-170360 was cultured in a 3L-scale as follows: A first seed culture of the producing organism was prepared by inoculating 10 mL of seed medium ATCC-2 [soluble starch (20 g/L), dextrose (10 g/L), NZ amine

EKC (Sigma) (5 g/L), Difco beef extract (3 g/L), Bacto peptone (5 g/L), yeast extract (5 g/L), and CaCO3 (1 g/L), adjusted to pH 7.0 with NaOH before addition of $CaCO_3$], in a 40 mL tube with 0.5 mL of a frozen inoculum stock of the producing strain and incubating the tube at 28 °C with shaking at 220 rpm for about 48 h. A second seed culture was prepared by inoculating 50 mL of seed medium in two 250 mL flasks with 2.5 mL of the first seed. A 5% aliquot of the second seed culture was transferred to each of the 20 × 500 mL Erlenmeyer flasks containing 150 mL of the production medium R2YE. The 3 L whole broth was then extracted with an equal volume of acetone under continuous shaking for 1h. After evaporating most of the organic solvent (concentration to initial volume) under a N_2 stream, the mycelial debris was discarded by centrifugation and vacuum filtration. The resulting aqueous residue was adjusted to pH 10.0 with NaOH 4 N, extracted with MEK (3 × 800 mL), and the extract was rotary evaporated to dryness to afford a crude extract of 0.910 g.

A portion of this MEK extract (250 mg) was loaded onto a Reversed-Phase C18 (Phenomenex Luna) column (32 × 100 mm) that was eluted with a H_2O-CH_3CN gradient , with both solvents containing 0.1% trifluoroacetic acid (TFA) (25% to 55% ACN in 36 min + 100% ACN in 10 min, 10 mL/min, 18 mL/fraction) to afford 14 fractions. Fractions containing the target compounds were pooled into two different groups according to their LC-UV-MS profiles and evaporated to dryness in a centrifugal evaporator to yield fractions A (18.5 mg) and B (35.2 mg).

Fraction A (18.5 mg) was further chromatographed by semipreparative reverse-phase HPLC (Zorbax SB-C18, 9.4 × 250 mm, 5 µm; 3.6 mL/min, UV detection at 210 and 280 nm) applying a linear H_2O-CH_3CN gradient (3.6 mL/min; 20-32% CH_3CN in 30 min; UV detection at 210 nm), both solvents containing 0.1% trifluoroacetic acid (TFA) yielding **1** (1.5 mg, rt 23 min) and **2** (1.3 mg, rt 27.5 min).

Fraction B (35.2 mg) was chromatographed by semipreparative reversed-phase HPLC (Zorbax SB-C18, 9.4 × 250 mm, 5 μm; 3.6 mL/min, UV detection at 210 and 280 nm) applying a linear H_2O-CH_3CN gradient (3.6 mL/min; 30-45% CH_3CN in 30 min; UV detection at 210 nm), both solvents containing 0.1% trifluoroacetic acid (TFA) yielding **3** (1.6 mg, rt 26.5 min), **4** (0.6 mg, rt 19 min), **5** (0.8 mg, rt 25.5 min), **6** (2.1 mg, rt 27.5 min), **7** (3.8 mg, rt 22 min), and **8** (0.8 mg, rt 28 min).

Pentaminomycin F (**1**): $[\alpha]^{25}_D$ -8.7 (c 0.30, MeOH); UV (MeOH) λ_{max} 219, 267; IR (ATR) ν_{max} 3286, 2964, 2875, 1667, 1546, 1439, 1203, 1136 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; (+)-ESI-TOFMS m/z 719.3992 $[M + H]^+$ (calcd. for $C_{36}H_{50}N_{10}O_6^+$, 719.3993), 360.2031 $[M + 2H]^{2+}$ (calcd. for $C_{36}H_{51}N_{10}O_6^{2+}$, 360.2008).

Pentaminomycin G (**2**): $[\alpha]^{25}_D$ -5.7 (c 0.24, MeOH); UV (MeOH) λ_{max} 219, 267; IR (ATR) ν_{max} 3276, 2971, 2936 1668, 1639, 1545, 1438, 1202, 1134 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; (+)-ESI-TOFMS m/z 753.3829 $[M + H]^+$ (calcd. for $C_{39}H_{48}N_{10}O_6^+$, 753.3837), 377.1947 $[M + 2H]^{2+}$ (calcd. for $C_{39}H_{49}N_{10}O_6^{2+}$, 377.1930).

Pentaminomycin H (**3**): $[\alpha]^{25}_D$ -0.6 (c 0.24, MeOH); UV (MeOH) λ_{max} 219, 279; IR (ATR) ν_{max} 3274, 2959, 2872, 1659, 1635, 1543, 1439, 1202, 1135 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; (+)-ESI-TOFMS m/z 718.4042 $[M + H]^+$ (calcd. for $C_{37}H_{52}N_9O_6^+$, 718.4035), 359.7048 $[M + 2H]^{2+}$ (calcd. for $C_{37}H_{53}N_9O_6^{2+}$, 359.7054)

Pentaminomycin A (**4**): UV (MeOH) λ_{max} 219, 279; ^1H and ^{13}C NMR data (SI, Figs. S49, S50); (+)-ESI-TOFMS m/z 670.4036 $[M + H]^+$ (calcd. for $C_{33}H_{52}N_9O_6^+$, 670.4035), 335.7051 $[M + 2H]^{2+}$ (calcd. for $C_{33}H_{53}N_9O_6^{2+}$, 335.7054)

Pentaminomycin B (**5**): UV (MeOH) λ_{max} 219, 279; ^{1}H and ^{13}C NMR data (SI, Figs. S51, S52); (+)-ESI-TOFMS m/z 684.4199 $[M + H]^+$ (calcd. for $C_{34}H_{54}N_9O_6^+$, 684.4192), 342.7129 $[M + 2H]^{2+}$ (calcd. for $C_{34}H_{55}N_9O_6^{2+}$, 342.7132)

Pentaminomycin C (**6**): UV (MeOH) λ_{max} 219, 279; ^{1}H and ^{13}C NMR data (SI, Figs. S53, S54); (+)-ESI-TOFMS m/z 1436.8028 $[2M + H]^+$ (calcd. for $C_{74}H_{103}N_{18}O_{12}^+$, 1436.8031), 718.4035 $[M + H]^+$ (calcd. for $C_{37}H_{52}N_9O_6^+$, 718.4036),

Pentaminomycin D (**7**): UV (MeOH) λ_{max} 219, 279; ^{1}H and ^{13}C NMR data (SI, Figs. S55, S56); (+)-ESI-TOFMS m/z 736.3615 $[M + CH_3OH + H]^+$ (calcd. for $C_{37}H_{54}N_9O_7^+$, 736.4141), 704.3880 $[M + H]^+$ (calcd. for $C_{36}H_{50}N_9O_6^+$, 704.3879), 352.6968 $[M + 2H]^{2+}$ (calcd. for $C_{36}H_{50}N_9O_6^{2+}$, 352.6954).

Pentaminomycin E (**8**): UV (MeOH) λ_{max} 219, 279; ^{1}H and ^{13}C NMR data (SI, Figs. S57, S58); (+)-ESI-TOFMS m/z 752.3883 $[M + H]^+$ (calcd. for $C_{40}H_{50}N_9O_6^+$, 752.3884), 376.6970 $[M + 2H]^{2+}$ (calcd. for $C_{40}H_{50}N_9O_6^{2+}$, 376.6954).

Marfey's Analysis of Compounds 1-3. Samples (300 μg) of compounds **1-3** were separately dissolved in 0.6 mL of 6 N HCl containing 5% (v/v) of thioglycolic acid and heated at 110ºC for 16 h in a sealed vial. Additionally, a second 300 μg batch of compound **1** was dissolved in 0.6 mL of 4 N HI and heated at 150ºC for 20 h in a sealed vial. The crude hydrolysates were evaporated to dryness under a nitrogen stream and each residue was dissolved in 100 μL of water. A 1% (w/v) solution (100 μL) of L-FDVA (Marfey's reagent,N-(2,4-dinitro-5-fluorophenyl)-L-valinamide) in acetone was added to an aliquot (50μL) of a 50 mM solution of each amino acid standard (leucine, phenylalanine, valine, tryptophan, arginine and 3-(2-Pyridyl)-L-alanine, D, L, or DL mixture) and to the aqueous solution of each compound hydrolysate. After addition of 20 μL of 1 M $NaHCO_3$

solution, each mixture was incubated for 60 min at 40 ºC. The reactions were quenched by addition of 10 µL of 1 N HCl and the crude mixtures were diluted with 700 µL of acetonitrile and analyzed by ESI LC/MS on an Agilent 1100 single Quadrupole LC/MS. Separations were carried out on an Agilent Zorbax SB-C8 column (2.1 × 30 mm, 5µm), maintained at 40 ºC. A mixture of two solvents, A (10% CH_3CN, 90% H_2O) and B (90% CH_3CN, 10% H_2O), both containing 1.3 mM trifluoroacetic acid and 1.3 mM ammonium formate, was used as the mobile phase under a linear gradient elution mode (10-26% B in 6 min, isocratic 26% B for 2 min, 26-28% B in 0.1 min, isocratic 28% B for 2 min, 28-40% B in 1 min, isocratic 40% B for 2 min, 40-100% B in 0.1 min and then isocratic 100% B for 2 min) at a flow rate of 0.3 mL/min. For D and L arginine the separation was carried out on Waters XBridge C_{18} column (4.6 × 150 mm, 5µm), maintained at 40 ºC. A mixture of two solvents, A (100% H_2O) and B (100% CH_3CN), both containing 0.1% of trifluoroacetic acid, was used as the mobile phase under a linear gradient elution mode (isocratic 32% B for 8 min, 32-40% B in 27 min, 40-100% B in 9 min and then isocratic 100% B for 2 min) at a flow rate of 1 mL/min. Retention times (min) for the derivatized (L-FDVA) amino acid standards and for the observed peaks in the HPLC trace of each L-FDVA-derivatized hydrolysis product under the reported conditions were as follows: retention times (min) for the derivatized (L-FDVA) standards amino acids (both L- and D- forms) present in **1** were: L-Trp: 9.18, D-Trp: 11.36, L-Val: 7.04, D-Val: 10.37, L-Leu: 8.70, D-Leu: 12.83, L-2-Pal: 3.03, D-2-Pal: 4.59, L-Arg: 5.18, D-Arg: 4.21. Retention times for the observed peaks in the HPLC trace of the L-FDVA derivatized hydrolysis product of **1** were: L-Trp: 9.29, D-Val: 10.50, L-Leu: 8.83, D-2-Pal: 4.64, L-Arg: 5.37. Retention times (min) for the derivatized (L-FDVA) standards amino acids (both L- and D- forms) present in **2** were: L-Trp: 9.19, D-Trp: 11.34, L-Val: 7.06, D-Val: 10.33, L-Phe: 8.84, D-Phe: 12.12, L-Pal: 2.93, D-2-Pal: 4.65. Retention times for the observed peaks in the HPLC trace

of the L-FDVA derivatized hydrolysis product of **2** were: L-Trp: 9.31, D-Val: 10.52, L-Phe: 8.95, D-2-Pal: 4.57. Retention times (min) for the derivatized (L-FDVA) standards amino acids (both L- and D- forms) present in **3** were: L-Trp: 9.54, D-Trp: 11.86, L-Val: 7.33, D-Val: 10.82, L-Phe: 9.52, D-Phe: 12.58, L-Leu: 9.17, D-Leu: 13.04. Retention times for the observed peaks in the HPLC trace of the L-FDVA derivatized hydrolysis product of **3** were: L-Trp: 9.64, D-Val: 10.96, L-Phe: 9.42, D-Leu: 13.23.

Antibacterial Assays. Compounds **1**–**8** were tested in antimicrobial assays for the growth inhibition of Gram-negative pathogens (*E. coli* ATCC 25922, *A. baumannii* MB5973, *K. pneumoniae* ATCC700603 or *P. aeruginosa* MB5919) following previously described methodologies.[20,21] Briefly, each compound was serially diluted in DMSO with a dilution factor of 2 to provide 10 concentrations starting at 96 μg/mL for all the antimicrobial assays. The MIC was defined as the lowest concentration of compound that inhibited ≥ 95% of the growth of a microorganism after overnight incubation. The Genedata Screener software (Genedata, Inc., Basel, Switzerland) was used to process and analyze the data and also to calculate the RZ' factor, which predicts the robustness of an assay.[22] In all experiments performed in this work the RZ' factor obtained was between 0.87 and 0.98.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI

1D and 2D NMR, COSY, TOCSY, NOESY and HMBC correlations, HR-ESIMS, HR-ESIMS/MS spectrum, LC-UV semipreparative chromatogram, LC-MS results from the Marfey's analysis of compounds **1**-**3** and structures of all pentaminomycins isolated from CA-170360 (**1**-**8**) (PDF)

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Notes

The authors declare no conflict of interest.

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