Isolation, structure and synthesis of mahafacyclin B, a cyclic heptapeptide from the latex of *Jatropha mahafalensis*

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Received (in Cambridge, UK) 23rd October 2000, Accepted 22nd June 2001
First published as an Advance Article on the web 16th August 2001

Mahafacyclin B is a cyclic heptapeptide with antimalarial activity isolated from the latex of *Jatropha mahafalensis*. The structure is elucidated by chemical degradation, tandem mass spectrometry, homo- and heteronuclear NMR experiments, and confirmed by synthesis.

Introduction

The latex of *Jatropha* species (Euphorbiaceae) has been shown to be a rich source of bioactive cyclic peptides which contain seven to ten residues with a high proportion of hydrophobic amino acids.[1–8] In our search for antimalarial peptides from plants, we have studied the latex composition of *J. mahafalensis*, the endemic bottle tree of Madagascar, and which is used to treat infected wounds in folk medicine.[9]

Natural cyclopeptides such as apicidins[10] and cyclosporin[11] have potent antiparasitic effects against *Plasmodium* and in our previous studies we have shown that two cyclopeptides, chevalierin A from *J. chevalieri* and curcacyclin B from *J. curcas*, possessed activity (IC_{50} < 10 mM) against *Plasmodium falciparum*.

This paper deals with the isolation, structure elucidation and synthesis of a cyclic heptapeptide containing four phenylalanines, mahafacyclin B (Fig. 1), a minor component of the *J. mahafalensis* latex,[12] which showed antimalarial activity (IC_{50} = 2.2 μM).

Results and discussion

Isolation and characterization of mahafacyclin B

The dry latex of *J. mahafalensis* (250 g) was extracted with CH_2Cl_2–MeOH (9 : 1) to give 2.72 g of extract. This crude material was chromatographed on Sephadex LH20 (MeOH) to yield a crude peptide fraction (673 mg) which was then analyzed by C_{18} reversed-phase HPLC, and exhibited one major component, mahafacyclin A[12] and a minor one, mahafacyclin B. The minor peptide was purified by repetitive semi-preparative HPLC (6.7 mg) and proved to be homogeneous by further mass spectrometry and NMR analyses. Mahafacyclin B gave a positive reaction with the hydrogen chloride–*o*-toluidine reagent, suggesting the presence of amide groups and a negative reaction with ninhydrin, indicating the absence of a free amino group and thus a cyclic peptide structure.

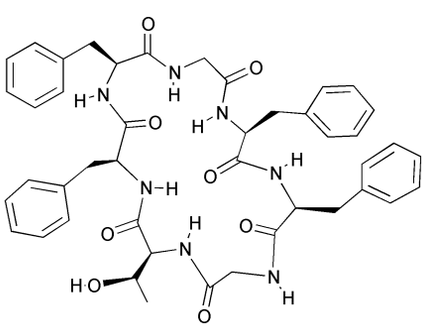

Fig. 1 Structure of mahafacyclin B.

The amino acid composition was determined from acid hydrolysis of the cyclic peptide (HCl 6 M; 110 °C; 24 h) followed by HPLC analysis: Gly (2), Phe (4), Thr (1). The absolute configuration of the chiral amino acids was shown to be L by derivatization of the acid hydrolysate to *N*-trifluoroacetyl isopropyl esters, followed by GC analysis on a chiral capillary column. The molecular weight M = 803 was deduced from the positive LSIMS spectrum where the protonated molecule MH^+ and the adduct ion $[M + Na]^+$ were observed at *m*/*z* 804 and 826, respectively. In the high-resolution mass spectrum, the protonated molecule MH^+ at *m*/*z* 804.3743 corresponding to the molecular formula $C_{44}H_{50}N_7O_8$ (calc. *m*/*z*, 804.3721), was in agreement with the above amino acid composition in a cyclic heptapeptide.

Sequence determination of mahafacyclin B

The NMR sequence determination of mahafacyclin B was obtained by assignments of different spin systems to residue types by analysis of COSY, TOCSY and ROESY experiments, and sequential assignments were further afforded by exploitation of dαN(i,i + 1) and dNN(i,i + 1) ROESY connectivities and confirmed by long-range ^1H–^{13}C HMBC experiments. Complete assignments of ^1H and ^{13}C NMR signals were obtained in DMSO-d_6. In such a polar solvent, mahafacyclin B gave sharp well-resolved signals at 298 K and the presence of minor conformers was not observed. The lowest-field region showed 7 amide protons together with the signals for phenylalanine aromatic protons. Two doublets, at δ 5.11 (1H) and δ 0.93 (3H), were assigned to the hydroxy and methyl groups of the threonine residue, respectively.

Complete assignment of ^1H chemical shifts to specific protons of individual residues was obtained by 2D homonuclear COSY and TOCSY experiments, showing complete spin systems of 2 Gly, 4 Phe and 1 Thr. The aromatic protons of the four phenylalanine residues, which resonated in the region

 DOI: 10.1039/b008538n

Table 1 ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR data for mahafacyclin B (DMSO-d_6; 298 K)

		δ(^{1}H) (mult; J in Hz)	δ(^{13}C)			δ(^{1}H) (mult; J in Hz)	δ(^{13}C)
Gly[1]	NH	8.29 (t; 5.2)[d]		**Gly**[5]	NH	8.02 (t; 5.8)	
	α	3.84 (dd; 5.2; 16.5)	42.5		α	3.84 m	42.5
		3.50 (dd; 5.2; 16.5)				3.30 m	
	CO		168.9		CO		168.8
Thr[2]	NH	7.58 (d; 8.7)		**Phe**[6]	NH	7.40 (d; 8.0)	
	α	4.28 m	58.1		α	4.47 m	54.0
	β	4.07 m	67.8		β	2.84 m	37.0
	γ	0.93 (d; 6.2)	19.2			2.64 (dd; 9.9, 13.7)	
	OH	5.11 (d; 8.3)			γ		137.2[a]
	CO		170.3		δ	[7.10–7.30] m	129.0[b]
					ε	[7.10–7.30] m	128.2
Phe[3]	NH	8.30 (d; 7.4)			ξ	[7.10–7.30] m	126.3
	α	4.40 (ddd; 3.8; 7.4; 10.8)	54.8		CO		172.2[c]
	β	3.02 (dd; 3.8; 14.5)	36.2				
		2.71 (dd; 10.8; 14.5)		**Phe**[7]	NH	8.68 (d; 5.9)	
	γ		137.8[a]		α	4.25 m	55.3
	δ	[7.10–7.30] m	128.8[b]		β	3.12 m	35.9
	ε	[7.10–7.30] m	128.2			2.94 (dd; 9.2; 14.0)	
	ξ	[7.10–7.30] m	126.3		γ		137.6[a]
	CO		170.9[c]		δ	[[7.10–7.30] m	129.0[b]
					ε	[7.10–7.30] m	128.2
Phe[4]	NH	7.84 (d; 7.8)			ξ	[7.10–7.30] m	126.3
	α	4.45 m	54.5		CO		170.8[c]
	β	3.12 m 2.82 m	37.4				
	γ		137.7[a]				
	δ	[7.10–7.30] m	129.1[b]				
	ε	[7.10–7.30] m	128.2				
	ξ	[7.10–7.30] m	126.3				
	CO		171.0[c]				

[a,b,c] Assignments may be reversed within the same column. [d] Determined at 313 K.

δ 7.10–7.30, were not differentiated. The assignments of the ^{13}C nuclei were obtained on the basis of J-modulated ^{13}C, HMQC and HMBC experiments (Table 1). The connectivities between adjacent residues were determined from the ROESY spectrum. The lowest-field NH proton triplet of glycine at δ 8.29 was assigned to Gly[1]. Inter-residue dαN(i,i + 1) connectivities were found between each adjacent residue, and together with the CO_i–NH_{i+1} correlations observed in the HMBC spectrum, the sequence of mahafacyclin B was determined as cyclo(-Gly[1]-Thr[2]-Phe[3]-Phe[4]-Gly[5]-Phe[6]-Phe[7]-). Inter-residual dNN(i,i + 1) correlations observed between Gly[1] and Thr[2], then from Phe[3] to Phe[7], were in agreement with the proposed structure.

Synthesis of mahafacyclin B in homogeneous phase

In order to confirm the proposal sequence and to make available a sufficient amount of cyclic peptide for bioassays, mahafacyclin B was synthesized by a solution-phase method in the presence of the (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling reagent in DMF at room temperature, according to the synthetic route shown in Fig. 2. Due to the repetition of the Phe-Phe-Gly motif in the sequence, this tripeptide was built in a stepwise manner using a Boc/OMe strategy with the glycine residue at the C-terminal position to prevent racemization during the dimerization and cyclization steps. No protection was used for the side chain of the Thr residue.

The cyclization step was accomplished under high-dilution conditions (10^{-3} M), with 1.5 equivalents of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and 10 equivalents of triethylamine in DMF. The reaction product was dissolved in methanol and chromatographed by steric exclusion. The peptidic fraction was then purified by semi-preparative reversed-phase HPLC. The analytical HPLC retention time and the ^{1}H NMR spectrum of synthetic mahafacyclin B were identical with those of the natural cyclic heptapeptide.

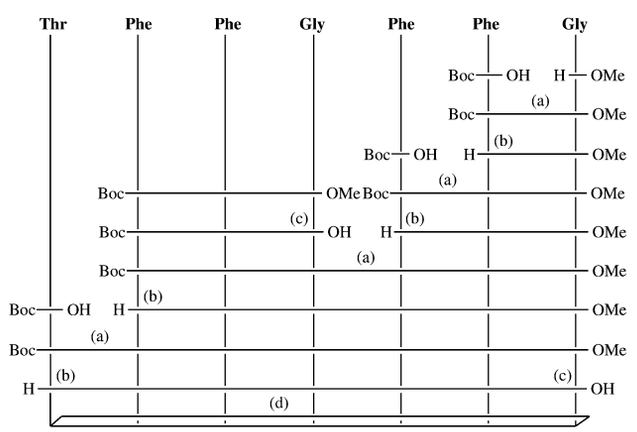

Fig. 2 Scheme for the total synthesis of HB1. *Reagents and conditions*: (a) BOP, NEt_3, DMF (2 h, rt); (b) TFA (30 min, rt); (c) KOH (2 M), MeOH (273 K); (d) HBTU, NEt_3, DMF (30 min, rt).

Conformational analysis of mahafacyclin B

The cyclic heptapeptides are a particular case of compounds possessing in solution a stable and rigid backbone conformation[13] forming a typical motif termed β-bulge.[14] This motif is defined by two β-turns with a mixed hydrogen bond. Such a structure is generally observed for cycloheptapeptides containing at least one proline residue. This is the case with evolidine[15] and pseudostellarin D,[16] the Xxx-Pro peptidic-bond geometry being *cis* in the first one and *trans* in the second one. We earlier reported the β-bulge[17] characteristics of pohlianins A–C, cyclic heptapeptides of *J. pohliana*, in DMSO-d_6 solution. To our knowledge, mahafacyclin A[12] is the first natural cyclic heptapeptide, without a proline residue, exhibiting the β-bulge characteristics. In order to know if mahafacyclin B, with its four phenylalanine residues, takes this particular conformation, we analyzed the temperature coefficients of amide protons, the $^{3}J_{\mathrm{NH\text{-}H}\alpha}$ coupling constants and molecular modeling using the

Table 2 Distance Geometry Calculations data for mahafacyclin B

Structural parameters	
Number of constraints	
distance	68
torsion	7
Number of converted conformers	45
Mean RMSROE (Å)	0.2
RMSD for backbone heavy atoms of mean structure (Å)	0.7

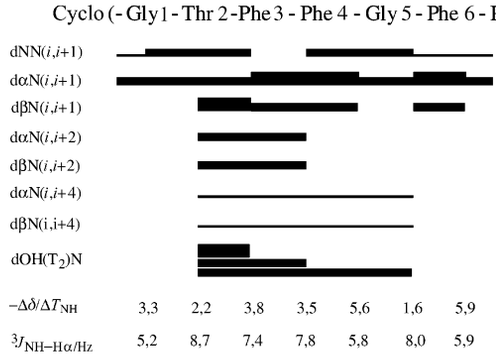

Fig. 3 Amino acid sequence of mahafacyclin B and a survey of the NOE connectivities involving NH and CαH, of the $^3J_{NH-\ C\alpha H}$ coupling constants, and of the temperature coefficients (10^{-3} ppm K^{-1}) of the amide protons. The observed NOEs are classified as strong, medium and weak and shown by thick, medium and thin lines, respectively.

Distance Geometry Calculation (DGC) method with distance constraints deduced from nuclear Overhauser effects (NOEs).

The existence of possible intramolecular hydrogen bonds was determined by examination of the variation of the NH chemical shifts with temperature. The absence of association between the molecules was previously checked by recording 1D ^{1}H NMR spectra at several concentrations (0.6–12 mM) for which no shift variation was observed. The measurements of the chemical shifts for the amide protons were carried out by steps of 5 K between 298 and 323 K. The linear variation of these chemical shifts indicated that the conformation was stable in this temperature range. The strong temperature coefficients measured for Gly^5 and Phe^7 amide protons were indicative of solvent-accessible NH groups, while the weaker values obtained for those of Thr^2 and Phe^6 suggested their implication in intramolecular hydrogen bonds. The intermediate values observed for the other NH groups did not allow any conclusions to be drawn. The values of the temperature coefficients, as well as the $^3J_{NH-H\alpha}$ coupling constants and the NOEs observed, are reported in Fig. 3.

The solution conformation of mahafacyclin B was studied by DGC, using as constraints the distances derived of the NOEs observed on the ROESY experiment. These constraints were classified in three ranges, 1.86–2.50, 1.86–3.50 and 2.50–4.50 Å corresponding to strong, medium and weak NOEs, respectively. The initial structures consistent with the experimental data were generated by Distance Geometry (DG) calculations with the DISGEOM program.[18] The generated conformers were then subjected to energy-minimization constraints with the AMBER all-atom force field[19] (Table 2). Among the 300 structures arising from these DG calculations, 45 superposable conformers possessing a root-mean-square deviation (RMSD) coefficient for the backbone heavy atoms of less than 0.2 Å were obtained. The RMSD between each individual structure and the mean structure was 0.7 Å for the backbone heavy atoms.

The implication of NH-Phe^6 and NH-Thr^2 intramolecular H-bonds suggested the presence of two β-turns around Phe^4-Gly^5 and Phe^7-Gly^1. The φ and ψ torsion angles measured for these residues (Table 3) show a type-I β-turn around Phe^4-Gly^5, stabilized by a 4→1 hydrogen bond between CO-Phe^3 and NH-Phe^6, and a type II β-turn around Phe^7-Gly^1 stabilized by a 4→1 hydrogen bond between CO-Phe^6 and NH-Thr^2. In addition to this information, the results of the computer modelling allowed the identification of a third intramolecular hydrogen bond (5→1) between CO-Phe^6 and NH-Phe^3, which was not possible by analysis of the temperature coefficients, and thus to characterize the β-bulge motif (Table 4). The mean structure of mahafacyclin B is represented in Fig. 4.

Table 3 Backbone dihedral angles in the mean structure of mahafacyclin B calculated according to the Distance Geometry Calculations

Residues	φ/°	ψ/°	ω/°
Gly^1	55.6	13.3	−175.6
Thr^2	−141.3	−8.7	−179.2
Phe^3	67.5	−57.3	−175.3
Phe^4	−62.0	−44.3	177.5
Gly^5	−97.8	21.4	−172.3
Phe^6	62.9	−80.8	−177.3
Phe^7	−51.3	108.8	172.2

Table 4 Intramolecular hydrogen bonds in the mean structure of mahafacyclin B

From	To	Distance/Å	Angle/°
NH–Phe^6	CO–Phe^3	2.26	159.9
NH–Thr^2	CO–Phe^6	2.44	161.2
NH–Phe^3	CO–Phe^6	2.39	147.7

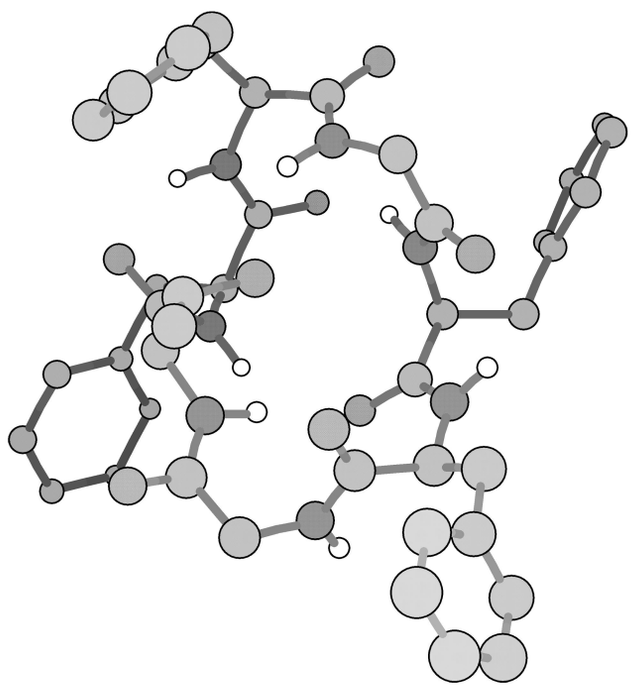

Fig. 4 Proposed conformation of mahafacyclin B in solution. The three broken lines represent intramolecular hydrogen bonds.

The activity of mahafacyclin B against *Plasmodium falciparum* was evaluated according to the Desjardins method.[20] Mahafacyclin B showed a moderate antimalarial activity with an IC_{50} value of 2.2 μM, which is, however, slightly superior to the antiparasitic effect observed for the cycloheptapeptide chevalierin A (8.9 μM).[8]

Experimental

Amino acid composition

Mahafacyclin B (1 mg) was hydrolyzed with 6 M HCl (0.5 ml) in a sealed tube under an argon atmosphere and heated at

110 °C for 24 h. After hydrolysis, the reagents were removed under reduced pressure. For the determination of the amino acid composition, the crude residues were dissolved in a 0.2 M sodium citrate buffer (pH 2.2) and separated by cation-exchange chromatography on a Liquimat 2 Amino Acid Analyser (Kontron) using OPA as detection reagent.

Absolute configuration of amino acids

Hydrolysates of the peptides obtained with 6 M HCl as described above were dried over potassium hydroxide pellets. The crude residues were dissolved in an anhydrous solution of 3 M HCl in propan-2-ol and heated at 110 °C for 20 min. The reagents were evaporated off under reduced pressure, the residues were dissolved in CH_2Cl_2 (0.5 ml), and 0.5 ml of trifluoroacetic anhydride was added. The mixtures were kept in a screw-capped tube at 100 °C for 5 min. The reagents were evaporated off, and GC analyses were realized on a Hewlett Packard series II 5890 gas chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.) with He (1.1 bar[1 bar = 10^5 Pa]) as carrier gas and a temperature program of 50–130 °C at 3 °C min^{-1}, then 130–190 °C at 10 °C min^{-1}. The retention times (min) of L-Leu (19.1), L-Phe (25.3), were compared with those of commercial references.

NMR spectroscopy

^1H and ^{13}C NMR spectra were carried out in DMSO-d_6, on a Bruker AC 300 spectrometer [$n_O(^1H)$ = 300.13 MHz and $n_O(^{13}C)$ = 75.47 MHz] equipped with an ASPEC 3000 computer using the DISNMR software. A reverse-probe head for 1D ^1H and 2D ^1H–^1H homonuclear and heteronuclear ^1H–^{13}C experiments, and a Dual probe head for 1D ^{13}C, were used. The measurements were realized at 298 K, with 6.7 mg of mahafacyclin B dissolved in 0.5 ml of degassed DMSO-d_6, and in a 5 mm sealed tube. *J*-values are given in Hz.

The one-dimensional ^1H and ^{13}C spectra were registered with 160 and 13 949 scans, respectively. All the homonuclear bidimensional spectra were carried out with 256 experiments, with a memory size of 2K in F2 (SI2) and in artificially improving the resolution of the first dimension by zero filling (memory size SI1 = 1K). The COSY spectrum was run with 48 scans per experiment, with a sweep width of 2857 Hz. Sine bell weighting functions were applied in F1 and F2. The TOCSY spectrum was recorded with 144 scans per experiment, with a sweep width of 3067 Hz. A mixing time of 100 ms allowed us to observe the couplings all along the side chains of the residues. The ROESY spectrum was obtained with 112 scans per experiment, with a sweep width of 3067 Hz and a mixing time of 150 ms. For the TOCSY and ROESY spectra, the calculations were realized applying sine bell weighting functions shifted by $\pi/2$ in F_1 and F_2.

The heteronuclear ^1H–^{13}C HMQC and HMBC spectra were carried out in the reverse detection mode (^1H in F_2 and ^{13}C in F_1), with a memory size of 2K. For both, the calculations were made applying a Q-sine function shifted by $\pi/3$ in F_1 and F_2. The direct-correlation ^1H–^{13}C spectrum (HMQC) was registered with sweep widths of 2702 Hz in F_2 and 5319 Hz in F. The coupling constant used to establish the neccesary delay for the selection of the protons coupled to the carbon was estimated to be equal to 135 Hz. This value corresponds to a delay of 3.7 ms. The HMBC spectrum was recorded with 384 scans per experiment, and with sweep widths of 2763 Hz in F_2 and 6944 Hz in F_1. The delay preceding the ^{13}C pulse for the creation of the multiple quanta coherences through the bonds was set to 70 ms, this delay corresponds to a long-range coupling constant of 7 Hz. The direct ^1H–^{13}C correlations were suppressed by the utilization of a low-pass J-filter.

Mass spectrometry

The positive LSIMS spectrum of mahafacyclin B was recorded on a ZAB2-SEQ (VG Analytical, Manchester, UK) mass spectrometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. The sample was prepared by dissolving the peptide in a methanolic solution, then by mixing with α-monothioglycerol as matrix. The positive HR-LSIMS was recorded on a ZAB-HF spectrometer, with a resolution of 2000.

LSIMS data: *m*/*z* (relative intensity): 804 (MH^+, 100), 826 ($[M + Na]^+$, 3).

HR-LSIMS data: *m*/*z* 804.3743 (calc. *m*/*z* 804.3721 for $C_{44}H_{50}N_7O_8$).

Computational methods

The molecular modelling as well as all the calculations were performed with the software package TINKER. The initial structure satisfying the experimental conditions were generated by Distance Geometry Calculations (DGC) using the DISGEOM program. DG, SA and molecular mechanics calculations were carried out with the AMBER all-atom force field. The conformers were submitted to energy minimization constraints by simulated annealing. The structures were first equilibrated at 200 °C for 1000 steps of molecular dynamics at a time-step of 0.04 ps, then were submitted to cooling in 10 000 steps (time step 0.02 ps) to 0 °C. The energy minimization was continued until a value of less than 0.01 kcal mol^{-1} $Å^{-2}$† was reached for the structures. In addition to the intermolecular distances, the peptidic bond-torsion constraints were also included in the calculations. The solvent molecules were not taken into consideration.

Peptide synthesis

The linear precursor of mahafacyclin B was prepared by homogeneous-phase peptide synthesis, by a succession of deprotection and coupling reactions, using Boc as protecting group and BOP as coupling reagent. After each coupling (or deprotection reaction), the structural integrity and the purity of the products were checked by electrospray ionization mass spectrometry (Micromass Platform II spectrometer) and analytical RP-HPLC, respectively. The main intermediates were also characterized by ^1H NMR (1D ^1H and 2D COSY ^1H–^1H).

C-Terminal deprotection (procedure A). Removal of the Boc groups were carried out by acidolysis in the presence of pure TFA. The solution of TFA was added to the peptide until complete dissolution of the latter, and the mixture was stirred at room temperature for 30 min, then the solution was concentrated *in vacuo* and the by-products were eliminated by evaporation under reduced pressure after addition of hexane. The deprotected peptide was then precipitated in cold diethyl ether (−30 °C) and filtered off.

Coupling (procedure B). The coupling steps were performed, for 1 equivalent of the peptide in elongation, with BOP (1 eq.) NEt_3 (2 eq.) and the residue (or peptidic fragment) to condense (1 eq.). To the peptide dissolved in DMF (5 ml per mmol of peptide) were successively added the amino acid, BOP and NEt_3. The solution (pH 7–8) was shaken at room temperature for 2.5 h. The oil obtained after concentration of the mixture was then dissolved in ethyl acetate and washed successively with the following solutions: aq. citric acid (0.1 M), saturated aq. NaCl, saturated aq. $KHCO_3$, saturated aq. NaCl and distillated water. The organic phase was dried on Na_2SO_4, filtered and concentrated by evaporation under reduced pressure. The crude

† 1 cal = 4.184 J.

peptide was obtained by filtration after precipitation in cold diethyl ether.

N-Terminal deprotection (procedure C). The removal of the C-terminal extremity was performed by saponification using 0.33 mmol of peptide, 4 ml of MeOH and 5 eq. of aq. KOH (2 M). The KOH solution was added dropwise to the cooled (0 °C) methanolic solution containing the peptide. When the mixture had reached room temperature, the end of the reaction was verified by TLC with ninhydrin as detection reagent, and the solvent was removed by evaporation under reduced pressure, and the reaction product was dissolved in distilled water and washed with ethyl acetate. The pH was set to 4–5 with hydrochloric acid (0.1 M) and the aqueous phase was extracted several times with ethyl acetate, then dried over Na_2SO_4, filtered, and concentrated. The crude deprotected peptide was obtained by precipitation with cold diethyl ether.

Cyclization (procedure D). For the cyclization step, realized on the crude peptide, the precursor was dissolved in 4–5 ml of DMF and added to a solution containing NEt_3 (1.5 eq.) and HBTU (10 eq.) in DMF (100 ml for 0.1 mmol of precursor). The peptide was introduced quickly, using a syringe, to the solution of the reagent under argon. The mixture was stirred for 2 h at room temperature. After removal of the solvent, 60 ml of ethyl acetate were added to the obtained oil and the solution was successively washed with the following solutions: aq. citric acid (0.1 M), saturated aq. NaCl, saturated aq. $KHCO_3$, saturated aq. NaCl and distillated water. The organic phase was dried on Na_2SO_4, filtered, and the crude cyclic peptide was obtained after removal of the solvent under reduced pressure. Purification of mahafacyclin B by reversed-phase HPLC (Kromasil C_{18}; 5 μm, 7.8 mm i.d.; 250 mm length, detection at 220 nm; MeOH–water 78 : 22) led to 8.2 mg of the cyclopeptide (yield 10%).

Boc-Phe-Gly-OMe 1. H-Gly-OMe·HCl (2.00 g, 16 mmol) and Boc-Phe-OH (4.24 g,16 mmol) were treated with BOP (7.06 g, 16 mmol) and 4.5 ml of NEt_3 (32 mmol) in 80 ml of DMF according to procedure A. This afforded crude peptide (4.60 g, 13.7 mmol), HPLC (MeOH–water 78 : 22) t_R 22.0 min; ESMS (%) *m/z* 337 (100) MH^+, *m/z* 359 (48) $[M + Na]^+$, *m/z* 375 (7) $[M + K]^+$; δ_H 8.34 (1H, t, *J* 5.8, NH-Gly), 3.86 (2H, dd, *J* 5.8, 9.1, Hαα′-Gly), 6.91 (1H, d, *J* 8.7, NH-Phe), 4.19 (1H, m, Hα-Phe), 2.99 (1H, dd, *J* 3.9, 13.8, Hβ-Phe), 2.71 (1H, dd, *J* 10.6, 13.7, Hβ′-Phe), 1.27 (9H, s, CH_3-Boc), 3.62 (3H, s, OCH_3).

Boc-Phe-Phe-Gly-OMe 2. The N-terminal extremity of Boc-Phe-Gly-OMe (4.00 g, 11.9 mmol) was removed according to procedure A. The tripeptide was obtained by coupling of the crude TFA salt (4.00 g, 11.9 mmol) with Boc-Phe-OH (3.16 g, 11.9 mmol) according to procedure B in the presence of BOP (5.26 g, 11.9 mmol) and NEt_3 (3.5 ml, 25 mmol) in 60 ml of DMF. After precipitation in diethyl ether, 5.07 g (10.5 mmol) of **2** were obtained, HPLC (MeOH–water 78 : 22) t_R 25.2 min; ESMS (%) *m/z* 484 (11) MH^+, *m/z* 506 (100) $[M + Na]^+$, *m/z* 522 (4) $[M + K]^+$; δ_H 8.50 (1H, t, *J* 5.6, NH-Gly), 3.85 (2H, d, *J* 5.4 Hαα′-Gly), 8.10 (1H, d, *J* 8.3, NH-Phe), 4.58 (1H, m, Hα-Phe), 2.84 (1H, dd, *J* 3.7, 13.8, Hβ-Phe), 3.03 (1H, dd, *J* 4.7, 13.9, Hβ′-Phe), 6.86 (1H, d, *J* 8.7, NH-Phe), 4.10 (1H, m, Hα-Phe), 2.83 (1H, dd, *J* 9.3, 13.8, Hβ-Phe), 2.62 (1H, dd, *J* 10.3, 13.6, Hβ′-Phe), 1.26 (9H, s, CH_3-Boc), 3.62 (3H, s, OCH_3).

Boc-Phe-Phe-Gly-Phe-Phe-Gly-OMe 3. A part of the tripeptide **2** (2.45 g, 5.07 mmol) was treated by acidic hydrolysis (procedure A) to obtain the corresponding TFA salt **2a** (2.55 g, 5.13 mmol). The other part (2.45 mg, 5.07 mmol) was subjected to saponification (procedure C), by dilution in 60 ml of methanol and addition of 13 ml of KOH (2 M), which led to 2.55 g (5.4 mmol) of Boc-Phe-Phe-Gly-OH **2b** after several washing steps. The fragments **2a** (2.5 g, 5.0 mmol) and **2b** (2.36 g, 5.0 mol) were treated by BOP (2.23 g, 5.0 mmol) and NEt_3 (1.5 ml, 10.6 mmol) in 25 ml of DMF according to procedure B to lead to 3.52 g (4.2 mmol) of the hexapeptide **3**, HPLC (MeOH–water 78 : 22) t_R 27.0 min; ESMS (%) *m/z* 835 (100) MH^+, *m/z* 857 (40) $[M + Na]^+$, *m/z* 873 (13) $[M + K]^+$; δ_H 8.36 (1H, t, *J* 5.9, NH-Gly), 3.85 (2H, d, *J* 6.6, Hαα′-Gly), 8.14 (1H, ββ, NH-Gly), 3.70 (1H, dd, *J* 5.5, 17.0, Hα-Gly), 3.57 (1H, dd, *J* 5.5, 17.0, Hα′-Gly), 6.86 (1H, d, *J* 8.9, NH-Phe), 4.10 (1H, m, Hα-Phe), 2.60 (1H, Hβ-Phe), 2.84 (1H, Hβ′-Phe), 7.98 (1H, d, *J* 8.3, NH-Phe), 4.50 (1H, m, Hα-Phe), 2.95 (1H, Hβ-Phe), 2.70 (1H, Hβ′-Phe), 7.95 (1H, d, *J* 6.0, NH-Phe), 4.55 (1H, m, Hα-Phe), 3.00 (1H, m, Hβ-Phe), 2.82 (1H, m, Hβ′-Phe), 8.23 (1H, d, *J* 8.7, NH-Phe), 4.55 (1H, m, Hα-Phe), 3.00 (1H, m, Hβ-Phe), 2.82 (1H, m, Hβ′-Phe), 7.15–7.25 (20 H, ArH-Phe), 1.25 (9H, s, CH_3-Boc), 3.62 (3H, s, OCH_3).

Boc-Thr-Phe-Phe-Gly-Phe-Phe-Gly-OMe 4. After acidic hydrolysis (procedure A) of the above hexapeptide (3.3 g, 3.95 mmol), the TFA salt (1.0 g, 1.17 mmol) and Boc-Thr-OH (258 mg, 1.17 mmol) were coupled by treatment with BOP (520 mg, 1.17 mmol) and NEt_3 (370 μl, 2.5 mmol) in 15 ml of DMF (procedure B), leading to the linear protected precursor Boc-Thr-Phe-Phe-Gly-Phe-Phe-Gly-OMe (945 mg, 1.0 mmol), HPLC (MeOH–water 78 : 22) t_R 25.5 min; ESMS (%) *m/z* 936 (35) MH^+, *m/z* 958 (100) $[M + Na]^+$, *m/z* 974 (6) $[M + K]^+$.

CF_3CO_2·H-Thr-Phe-Phe-Gly-Phe-Phe-Gly-OH 5. The C-terminal extremity was deprotected by a saponification reaction (procedure C) performed by the addition of 2.5 ml of KOH to 920 mg (0.98 mol) of the TFA salt dissolved in 12 ml of methanol, leading to 806 mg (0.86 mmol) of Boc-Thr-(Phe-Phe-Gly)$_2$-OH. The Boc group was removed by acidic hydrolysis of the heptapeptide (806 mg, 0.87 mol). After acidic hydrolysis, 793 mg (0.85 mol) of the totally free heptapeptide were obtained, HPLC (MeOH–water 78 : 22) t_R 19.1 min; ESMS (%) *m/z* 822 (100) MH^+, *m/z* 844 (9) $[M + Na]^+$, *m/z* 860 (3) $[M + K]^+$.

cyclo(-Thr-Phe-Phe-Gly-Phe-Phe-Gly-), mahafacyclin B. The cyclization step was performed on 32 mg (0.03 mmol) of linear precursor, with 45 μl of NEt_3 (3.2 mmol) and 20 mg (0.05 mmol) of HBTU in 35 ml of DMF. The progress of the reaction was checked by HPLC every half-hour, and after 2 h the product was concentrated, then dissolved in methanol (5 ml) and finally purified by semi-preparative reversed-phase HPLC (water–MeOH 22 : 78), leading to 8.2 mg (0.02) of pure mahafacyclin B (cyclization yield 30%; global yield 10%).

Acknowledgements

We thank Professor Ph. Grellier (Laboratoire de Biologie Parasitaire, MNHN) for antimalarial tests and Dr M. Becchi (Centre de Spectroscopie de Masse du CNRS, Lyon) for LSIMS measurements, and Dr J. Verducci (Laboratoire des Amino acides, Peptides et Proteines, Montpellier) for his help in peptide synthesis. We are indebted to Dr R. Gerold (Protanex) for the gift of *J. mahafalensis* latex.

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