# Sequences and Antimycoplasmic Properties of Longibrachins LGB II and LGB III, Two Novel 20-Residue Peptaibols from *Trichoderma longibrachiatum*

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Longibrachins are members of the class of natural Aib-containing peptides designated as peptaibols. Six longibrachins, LGA I−IV and LGB II and III, were purified from a *Trichoderma longibrachiatum* strain by a procedure employing several chromatography steps including reversed-phase HPLC. The amino acid sequence determination was based on a combination of liquid secondary ion mass spectrometry (LSIMS) and two-dimensional $^{1}H$ and $^{13}C$ NMR spectroscopy. Longibrachins are 20-residue peptaibols with a C-terminal phenylalaninol and either neutral (LGA; $Gln^{18}$) or acidic (LGB; $Glu^{18}$) character. Longibrachins LGB II and III have novel sequences. Both longibrachins LGA and LGB show significant bactericidal activity against mycoplasmas (*Acholeplasma*, *Mycoplasma*, and *Spiroplasma*), with minimal inhibitory concentrations in the range 1.56−12.5 $\mu$M (3−25 $\mu$g/mL), and also perturb the permeability of membrane bilayers. Longibrachin LGA IV is the most potent of the presently known 18−20-residue peptaibols. The antimicrobial and membrane-perturbing properties of longibrachins, which are described here for the first time, were shown to be correlated.

Antibiotic peptides of the peptaibol class are widely synthesized by *Trichoderma* soil fungi. These linear hydrophobic peptides, with an acetylated N-terminus and a C-terminal amino alcohol, have a high content in α,α-dialkylated amino acids, such as α-aminoisobutyric acid (Aib) and isovaline (Iva). They result from a nonribosomal biosynthesis pathway involving peptide synthetases as multienzymic templates[1] and are thus produced as microheterogeneous mixtures of strongly related peptide analogues. Three subclasses of peptaibols have been defined according to the number of amino acids: the long-sequence peptaibols contain 18−20 residues, including a single central proline,[2−6] the short-sequence peptaibols have 11−16 residues and several Aib-Pro motifs,[7−10] and the lipopeptaibols are characterized by 7 or 11 residues, a high amount of glycine, and an N-terminal amino acid acylated by an 8−10-carbon linear fatty acid[11,12] instead of the usual acetyl group. Peptaibol antibiotics are known as membrane-modifying and pore-forming peptides. They are organized in amphipathic helices, which interact with phospholipid bilayers and increase their permeability, either in the presence[13,14] or in the absence of voltage.[15] Their antibiotic activity is mainly directed against Gram-positive bacteria, and the long-sequence peptaibols exemplified by the prototypic alamethicin[1] are the most potent. Recently, we have reported that several species of mollicutes, the smallest and simplest self-replicating prokaryotes,[16,17] are sensitive to peptaibols containing 18 residues, such as trichorzins PA.[18,19] As many mollicute genera (*Acholeplasma*, *Mycoplasma*, *Spiroplasma*) are pathogens of humans, animals, arthropods, or plants and are resistant to a number of conventional antibiotics, the search for new antimycoplasmic compounds is an important challenge.

From the culture broth of a strain of *Trichoderma longibrachiatum*, we characterized a group of 20-residue peptaibols, termed longibrachins, which were shown to form voltage-dependent channels through transbilayer helix bundles of amphiphilic helical peptide monomers.[20] In this paper, we report the production, isolation, and sequence determination of these peptides. Longibrachins LGB II and III were shown to consist of novel sequences (Figure 1), due to the presence of a glutamic acid at position 18, which also confers an acidic character. The neutral longibrachins LGA I−IV, with Gln at position 18, have the same sequences as peptaibols previously isolated from other *Trichoderma* strains (Figure 1).[21] The potent activity of longibrachins LGA I−IV and LGB II, III against *Acholeplasma*, *Mycoplasma*, and *Spiroplasma* cells and their strong membrane-permeabilizing action are described herein for the first time.

## Results and Discussion

**Isolation, Purification, and Structure Elucidation of Longibrachins.** *T. longibrachiatum* (LCP-853431) was cultivated for 15 days on liquid synthetic medium. Exclusion chromatography through Sephadex LH 20 of the culture broth butanolic extract afforded a crude peptaibol mixture, from which two peptide groups, the neutral longibrachins LGA and the acidic longibrachins LGB, were further separated by silica gel chromatography. When analyzed by reversed-phase HPLC, the LGA and LGB groups each appeared as mixtures of four components (Figure 2), which were isolated by repetitive semipreparative reversed-phase HPLC. Six of the components, longibrachins LGA I−IV and LGB II and III, were isolated as single homogeneous peptides and were submitted to sequence analysis, while the others remained as mixtures and were not analyzed.

The absence of reactivity of the peptides with ninhydrin and the presence of sharp singlets at ∼2.0 ppm in the 1D $^{1}H$ NMR spectra were in agreement with the presence of

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| | 1 | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LGB II** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Aib** | **Glu** | Gln | Pheol |
| **LGB III** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Iva** | **Glu** | Gln | Pheol |
| **LGA I** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Aib** | **Gln** | Gln | Pheol |
| **LGA II** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Iva** | **Gln** | Gln | Pheol |
| **LGA III** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Aib** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Aib** | **Gln** | Gln | Pheol |
| **LGA IV** | Ac | Aib | **Ala** | Aib | Ala | Aib | **Aib** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Iva** | **Gln** | Gln | Pheol |
| **Alm F50I** | Ac | Aib | **Pro** | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **Aib** | **Gln** | Gln | Pheol |
| **PA VI** | Ac | Aib | | **Ser** | Ala | Aib | **Iva** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | **Leu** | Aib | **Aib** | | Gln | Pheol |

**Figure 1.** Sequences of longibrachins LGB II and LGB III. The sequences of longibrachins LGA and of reference peptaibols used in this paper, alamethicin F50 I (Alm F50 I,[2] identical to atrovirin A[29]) and trichorzin PA VI taken as example of trichorzin PA, are given for comparison; the positions of amino acids that differ in the sequences are identified by bold characters. The sequences of the neutral LGA have been found here to be identical to the previously isolated gliodeliquescin Gl-A,[21] trichobrachins TIIb A−D,[22] and trichokonins TK VI, VII, VIII,[23] with LGA I = Gl-A = TIIb A = TK VI; LGA II = TIIb B = TK VII; LGA III = TIIb C = TK VIII; LGA IV = TIIb D.

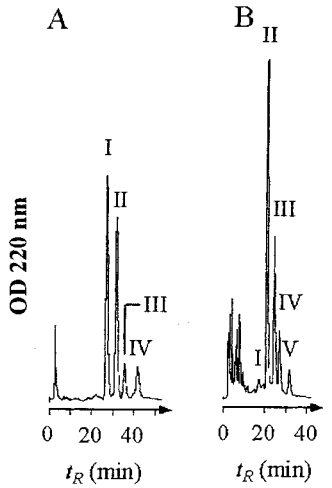


**Figure 2.** HPLC elution profiles of the longibrachins LGA (A) and LGB (B) natural microheterogeneous mixtures isolated from *T. longibrachiatum* LCP-853431: (A) Kromasil $C_{18}$ (5$\mu$m), 4.6 × 250 mm, MeOH−$H_2O$ (85:15), flow rate 1 mL/min; (B) Spherisorb-ODS2 (5 $\mu$m), 4.6 × 250 mm, MeOH−$H_2O$−TFA (86:14:0.05), flow rate 1 mL/min. Absorption monitored at 220 nm.

acetylated N-terminal residues.[4−6,8−10] Complete amino acid composition and absolute configurations were determined from GLC analyses of the total acidic hydrolysates, after derivatization as *N*-trifluoroacetyl isopropyl esters and comparison with standards. The six peptides all contained Glx (3), Gly (1), Leu (1), Pheol (1), Pro (1), and Val (2) and are distinguished from each other by different contents of Aib, Ala, and Iva, with Aib (8), Ala (3) for LGA I and LGB II, Aib (7), Ala (3), Iva (1) for LGA II and LGB III, Aib (9), Ala (2) for LGA III, and Aib (8), Ala (2), Iva (1) for LGA IV. The three Glx residues were assigned to two Gln and one Glu in the LGB peptides, based on their reactivity with diazomethane and from observation of the *syn* and *anti* $\epsilon$-protons of the two carboxamide groups in the $^1$H 1D NMR spectra. In the LGA peptides, three Gln were present. Finally, the chirality of the monoalkylated amino acids and of the phenylalaninol was shown to be L, and that of the dialkylated isovaline to be D. Analysis of the positive ion liquid secondary ion (LSI) mass spectra of peptaibols, either in the absence or in the presence of added lithium ions, has proven to be a powerful method for sequence determination of peptaibols.[4,5,8,9,24,25] Molecular masses of the longibrachins were obtained from the molecular ion species, either $MH^+$, $[M + Na]^+$, and $[M + K]^+$ in the absence of added lithium (Table 1) or $[M + Li]^+$ and $[M + 2Li]^{2+}$ in the presence of lithium (Table 2). Amide bond cleavages afforded a series of $b_n$ acylium ions,[4,5,8,9,24,25] in the absence of $Li^+$, while $[a_n + Li-H]^+$ ions prevailed when LiCl was added to the matrix.[26] Sequence determination thus resulted from the mass differences between two consecutive ions of the same series. The preferential cleavage at the Aib−Pro bond(s), which generally prevails in the absence of $Li^+$ and is typical of peptaibols,[24,25] giving rise to the complementary N-terminal acylium $N^+$ ion and C-terminal diprotonated ammonium $[HC,H]^+$ ion (Table 1), was no longer observed in the presence of $Li^+$ (Table 2). The spectra obtained with the lithiated adducts each showed a single series of $[a_n + Li - H]^+$ ions, starting from the lithiated adduct ions[26−28] and accompanied in some cases by $[d_n + Li]^+$ ions, which arose from the loss of a radical from the side chains as a result of a $\beta$-cleavage. Such ions are useful to differentiate between isomeric residues such as Leu/Ile, from the mass differences characterizing the couples of $[a_n + Li - H]^+/[d_n + Li]^+$ ions. Leu and Ile are thus defined from such differences of 42 and 28 amu, respectively. These ions here indicated the presence of leucine at positions 12 in the longibrachins, in agreement with the amino acid composition. However, Val cannot be differentiated from Iva in this way.

Determination of the complete sequences of the longibrachins required the specification of the location of a single glutamic acid at position 7, 18, or 19 in LGB II and LGB III and the assignment of the location of the isomeric Val/Iva residues in LGB III and in LGA II and LGA IV. The mass spectra of LGB II and LGB III appeared very similar to those of LGA I and LGA II, respectively, but a mass difference of 1 amu was observed for the ions arising from fragmentation of the $[HC, H]^+$ ions. This favored the presence of glutamic acid at position 18 or 19 in LGB II and LGB III, but the very low intensities of these ions prevented determination of the precise location of this residue. The methyl ester derivatives of LGB II and LGB III (LGB II−OMe and LGB III−OMe) were thus prepared and their fragmentation pattern was studied in the absence and in the presence of $Li^+$. Glutamic acid was unambiguously assigned at position 18 by the mass values of ions $[a_{18} + Li - H]^+$ and the mass differences between $[a_{18} + Li - H]^+$ and $[d_{18} + Li]^+$, which were 58 for LGB II and III and 72 for LGB II−OMe and LGB III−OMe (Table 2).

An NMR study was then undertaken to assign the location of Val/Iva residues at positions 15 and 17 in the sequences of LGB III, LGA II, and LGA IV. Sequential assignments of the peptides were obtained from the combined use of COSY/TOCSY experiments to assign the spin systems to amino acid types, and of ROESY data to determine sequential $d_{NN(i, i+1)}$ and $d_{\alpha N(i, i+1)}$ connectivities and, thus, sequential positions. By this way, Iva was located unambiguously at position 17 in the sequences of LGB III, LGA II, and LGA IV. Location of the two glutamines at positions 7 and 19 in LGB II and LGB III

**Table 1.** Pseudomolecular Ion Species and Sequence-Specific Fragment Ions Arising in the (+) Ion LSI Mass Spectra of Longibrachins[a]

| | peptide | LGB II 1936 $C_{90}H_{148}N_{22}O_{25}$ | | LGB III 1950 $C_{91}H_{150}N_{22}O_{25}$ | | LGB II-OMe 1950 $C_{91}H_{150}N_{22}O_{25}$ | | LGA IV 1963 $C_{92}H_{153}N_{23}O_{24}$ | |
|---|---|---|---|---|---|---|---|---|---|
| ion type | | *m/z* | % | *m/z* | % | *m/z* | % | *m/z* | % |
| $[M + K]^+$ | | 1975 | 9 | 1989 | 3 | 1989 | 1 | 2002 | 4 |
| $[M + Na]^+$ | | 1959 | 47 | 1973 | 12 | 1973 | 100 | 1986 | 18 |
| $MH^+$ | | 1937 | 1 | 1951 | 1 | 1951 | 1 | 1964 | 1 |
| $N^+$ | $b_{13}$ | 1163 | 15 | 1163 | 11 | 1177 | 18 | 1163 | 11 |
| $[HC, H]^+$ | $y_7$ | 775 | 24 | 789 | 14 | 788 | 33 | 789 | 7 |
| *N* | $b_{12}$ | 1078 | 3 | 1078 | 1 | 1092 | 2 | 1078 | 1 |
| *N* | $b_{11}$ | 965 | 2 | 965 | 1 | 979 | | 965 | 1 |
| *N* | $b_{10}$ | 908 | 16 | 908 | 9 | 922 | 8 | 908 | 6 |
| *N* | $b_9$ | 823 | 6 | 823 | 4 | 837 | 4 | 823 | 3 |
| *N* | $b_8$ | 724 | 26 | 724 | 14 | 738 | 15 | 724 | 9 |
| *N* | $b_7$ | 639 | 1 | 639 | 1 | 653 | 1 | 639 | 1 |
| *C* | | 624 | 2 | 638 | 1 | 637 | 2 | 638 | 1 |
| *N* | $b_6$ | 511 | 13 | 511 | 11 | 525 | 30 | 511 | 6 |
| *C* | | 496 | 2 | 510 | 1 | 509 | 2 | 510 | 3 |
| *N* | $b_5$ | 440 | 50 | 440 | 43 | 440 | 50 | 440 | 22 |
| *C* | | 367 | 4 | 381 | 3 | 381 | 6 | 367 | 2 |
| *N* | $b_4$ | 355 | 74 | 355 | 76 | 355 | 78 | 355 | 48 |
| *N* | $b_3$ | 284 | 10 | 284 | 100 | 284 | 100 | 284 | 62 |
| *C* | | 282 | 12 | 282 | 13 | 282 | 17 | 282 | 5 |
| *N* | $b_2$ | 199 | 47 | 199 | 51 | 199 | 44 | 199 | 26 |
| *C* | | 197 | 10 | 197 | 9 | 197 | 11 | 197 | 3 |
| *N* | $b_1$ | 128 | 48 | 128 | 53 | 128 | 54 | 128 | 35 |

[a] α-Thioglycerol as matrix; the origin of the observed ions, from $N^+$ or $[HC, H]^+$ is indicated by *N* or *C*, respectively (H = 1.000).

**Table 2.** Pseudomolecular Ion Species and Sequence-Specific Fragment Ions Arising in the (+) Ion LSI Mass Spectra of Longibrachins[a]

| | LGB II | | LGB III | | LGB II-OMe | | LGA IV | |
|---|---|---|---|---|---|---|---|---|
| ion | *m/z* | % | *m/z* | % | *m/z* | % | *m/z* | % |
| $[M + 2Li - H]^+$ | 1949 | 100 | 1963 | 100 | | | | |
| $[M + Li]^+$ | 1943 | 53 | 1957 | 58 | 1957 | 100 | 1970 | 100 |
| $[M + 3Li - H]^{2+}$ | 978 | 12 | 985 | 9 | | | | |
| $[M + 2Li]^{2+}$ | 975 | 10 | 982 | 8 | 982 | 11 | 988.5 | 9 |
| $[y_7 + Li - 3H]^+$ | 779 | 8 | 793 | 6 | 793 | 7 | 792 | 5 |
| $[a_{18} + Li - H]^+$ | 1636 | 1 | 1650 | 1 | 1650 | 2 | 1663 | 2 |
| $[d_{18} + Li]^+$ | 1578 | 2 | 1592 | 2 | 1578 | 1 | 1606 | 1 |
| $[a_{17} + Li - H]^+$ | 1507 | 4 | 1521 | 4 | 1507 | 3 | 1535 | 3 |
| $[a_{16} + Li - H]^+$ | 1422 | 5 | 1422 | 4 | 1422 | 3 | 1436 | 3 |
| $[a_{15} + Li - H]^+$ | 1337 | 6 | 1337 | 5 | 1337 | 3 | 1351 | 4 |
| $[a_{14} + Li - H]^+$ | 1238 | 4 | 1238 | 4 | 1238 | 2 | 1252 | 3 |
| $[a_{13} + Li - H]^+$ | 1141 | 8 | 1141 | 7 | 1141 | 4 | 1155 | 5 |
| $[a_{12} + Li - H]^+$ | 1056 | 10 | 1056 | 9 | 1056 | 5 | 1070 | 7 |
| $[d_{12} + Li]^+$ | 1014 | 10 | 1014 | 9 | 1014 | 4 | 1028 | 7 |
| $[a_{11} + Li - H]^+$ | 943 | | 943 | | 943 | | 957 | 1 |
| $[a_{10} + Li - H]^+$ | 886 | 10 | 886 | 8 | 886 | 5 | 900 | 3 |
| $[a_9 + Li - H]^+$ | 801 | 8 | 801 | 7 | 801 | 4 | 815 | 3 |
| $[a_8 + Li - H]^+$ | 702 | 8 | 702 | 6 | 702 | 4 | 716 | 3 |
| $[a_7 + Li - H]^+$ | 617 | 8 | 617 | 5 | 617 | 3 | 631 | 2 |
| $[d_7 + Li]^+$ | 560 | 10 | 560 | 8 | 560 | 4 | 574 | 3 |
| $[a_6 + Li - H]^+$ | 489 | 5 | 489 | 4 | 489 | 3 | 503 | 2 |
| $[a_5 + Li - H]^+$ | 418 | 8 | 418 | 6 | 418 | 4 | 418 | 2 |
| $[a_4 + Li - H]^+$ | 333 | 9 | 333 | 6 | 333 | 3 | 333 | 2 |
| $[a_3 + Li - H]^+$ | 262 | 13 | 262 | 9 | 262 | 6 | 262 | 5 |

[a] (H = 1.000); 3-nitrobenzyl alcohol saturated with LiCl as matrix.

was confirmed from observation of ROEs between the *syn* and *anti* ϵ protons of the carboxamide groups and the γ protons of the lateral chains of these glutamines. Stereospecific assignments, especially those of the Aib β-methyl groups were obtained from further analyses of the ROE data and of the $^1H-^{13}C$ HSQC/HMBC experiments. Taking into account the *S* configuration of the α carbon, the proline stereochemical assignments were obtained from the intraresidue dipolar correlations between the $H_\alpha$, *pro-S* $H_\beta$, *pro-R* $H_\gamma$, and *pro-R* $H_\delta$. Complete $^{13}C$ NMR assignments of the amino acid lateral chains were possible from the $^1J_{CH}$ connectivities observed in the $^1H-^{13}C$ HSQC spectra. Quaternary carbons, such as carbonyl groups and Aib α carbons, were assigned from $^1H-^{13}C$ HMBC correlations optimized for $J_{CH}$ long-range couplings of 5 Hz. For LGB II, the nitrogen chemical shifts, except the proline one, were obtained from the $^1J_{NH}$ couplings in the $^1H-^{15}N$ HSQC experiment. The $^{15}NH$ of the α,α-dialkylated Aib residues appeared more deshielded than those of the Gly ones and of the monoalkylated amino acids, as expected. The complete sequences (Figure 1) were thus obtained for the six longibrachins. The NMR assignments of LGB II and LGB III are given in Tables 3 and 4. Longibrachins LGB II and LGB III have unique sequences that distinguish them from other recently described related 20-residue peptaibols, atrovirins B and C,[29] and from alamethicins,[2] gliodeliquescin,[21] trichobrachins,[22] and trichokonins.[23]

**Antimycoplasmic Activity of Longibrachins LGA and LGB.** The antimycoplasmic activities of the uncharged longibrachins (LGA natural mixture and purified LGA IV) and of the negatively charged longibrachins (LGB natural mixture and purified LGB II and LGB III) were measured on several strains of mollicutes and compared with those of related peptaibols. As previously noticed for the 18-residue trichorzins PA,[19] the natural microheterogeneous mixtures of longibrachins LGA and LGB exhibited an activity in the same range as the peptides purified to homogeneity, indicating neither a positive nor negative significant influence of the minor sequence modifications on the antimycoplasmic properties.

Both LGA IV and LGB II and LGB III inhibited the growth of the six species of mollicutes used in this experiment, with MICs ranging from 1.56 to 12.5 μM (3 to 25 μg/mL; Table 5). These values are of the same order of magnitude as those reported for the other peptaibols previously examined, specifically the neutral alamethicin F50 (1.56 to 12.5 μM) and trichorzins PA (3.12 to 25 μM).[19] LGA and LGB peptides were also bactericidal toward the mollicutes with MLCs ranging from 3.12 to 100 μM (6 to 195 μg/mL).

When comparing the MICs obtained with longibrachins LGA (Ala2, Gln18), LGB (Ala2, Glu18), and Alm F50 (Pro2,

**Table 3.** $^{1}H$, $^{13}C$, and $^{15}N$ Chemical Shifts for Longibrachin LGB II ($CD_3OH$, 298 K)[a]

| | $\delta^{1}H$; mult.; *J* (Hz) | | | $\delta^{13}C$ | | | $^{15}N$ |
|---|---|---|---|---|---|---|---|
| residue | N*H* | Hα | other groups (*J*, Hz) | CO | Cα | other groups | *N*H |
| Ac | | | Me 2.050 | 173.1 | | Me 23.1 | |
| $U_1$ | 8.436; s | | *β* Me *pro-S* 1.46/*β* Me *pro-R* 1.47 | 178.4 | 57.1 | *β* Me *pro-S* 26.5/*β* Me *pro-R* 24.1 | 132.5 |
| $A_2$ | 8.301; d, 4.4 | 4.01 | *β* Me 1.417 | 176.6 | 53.9 | Me *β* 16.6 | 111.2 |
| $U_3$ | 7.664; s | | *β* Me *pro-S* 1.53/*β* Me *pro-R* 1.50 | 178.2 | 57.2 | *β* Me *pro-S* 26.9/*β* Me *pro-R* 22.9 | 121.7 |
| $A_4$ | 7.727; d, 5.1 | 4.05 | *β* Me 1.49 | 177.2 | 54.3 | Me *β* 16.8 | 112.4 |
| $U_5$ | 8.017; s | | *β* Me *pro-S* 1.57/*β* Me *pro-R* 1.51 | 178.1 | 57.2 | *β* Me *pro-S* 26.9/*β* Me *pro-R* 23.2 | 123.0 |
| $A_6$ | 7.954; d, 4.5 | 4.01 | *β* Me 1.54 | 178.2 | 53.9 | Me *β* 16.9 | 110.9 |
| $Q_7$ | 8.069; d, 4.9 | 3.94 | *β* 2.15/*β′* 2.29 | 175.9 | 58.1 | *β* 27.2/*γ* 32.6 | 112.0 |
| | | | *γ* 2.36/*γ′*2.55 | 177.4[b] | | | 101.5[b] |
| | | | $\epsilon_{anti}$ 7.446/$\epsilon_{syn}$ 6.764 *a* | | | | |
| $U_8$ | 8.113; s | | *β* Me *pro-S* 1.52/*β* Me *pro-R* 1.60 | 178.2 | 58.5 | Me *pro-R* 27.5/Me *pro-S* 27.4 | 122.8 |
| $V_9$ | 7.519; d, 5.3 | 3.59 | *β* 2.24/*γ*1 Me 1.135; d, 6.7/*γ*2 Me 1.001; d, 6.5 | 175.3 | 65.9 | *β* 30.2/*γ*1 Me 20.9/*γ*2 Me 19.6 | 109.0 |
| $U_{10}$ | 8.224; s | | *β* Me *pro-S* 1.47/*β* Me *pro-R* 1.55 | 179.0 | 57.6 | *β* Me *pro-S* 26.6/*β* Me *pro-R* 23.5 | 124.8 |
| $G_{11}$ | 8.339; t, 5.7 | *pro-S* 3.95/ *pro-R* 3.68 | | 173.0 | 45.0 | | 95.0 |
| $L_{12}$ | 8.113; d, 7.8[a] | 4.46 | *β* 1.60/*β′* 1.95/*γ* 1.95 | 175.8 | 54.0 | *β* 41.5/*γ* 25.6 | 113.0 |
| | | | *δ*1Me 0.934; d, 6.4/*δ*2 Me 0.912; d, 6.5 | | | *δ*1 Me 23.4/*δ*2 Me 21.3 | |
| $U_{13}$ | 8.382; s | | *β* Me *pro-S* 1.62/*β* Me *pro-R* 1.52 | 174.9 | 58.0 | Me *pro-R* 23.8/Me *pro-S* 23.1 | 128.0 |
| $P_{14}$ | | 4.37 | *β pro-S* 2.32/*β pro-R* 1.81 | 176.3 | 64.6 | *β* 30.0/*γ* 28.0/*δ* 50.5 | |
| | | | *γ pro-S* 2.09/*γ pro-R* 1.99 | | | | |
| | | | *δ pro-S* 3.74/*δ pro-R* 3.88 | | | | |
| $V_{15}$ | 7.589; d, 7.5 | 3.71 | *β* 2.35/*γ*1 Me 1.071; d, 6.7/*γ*2 Me 0.976; d, 6.5 | 175.4 | 64.3 | *β* 30.0/*γ*1 Me 20.3/*γ*2 Me 19.4 | 110.0 |
| $U_{16}$ | 7.568; s | | *β* Me *pro-S* 1.59/*β* Me *pro-R* n.d. | 177.9 | 57.3 | Me *pro-R* 27.4/Me *pro-S* n.d. | 124.1 |
| $U_{17}$ | 7.826; s | | *β* Me *pro-S* 1.59/*β* Me *pro-R* n.d. | 178.9 | 57.6 | Me *pro-R* 27.4/Me *pro-S* n.d. | 119.9 |
| $E_{18}$ | 7.904; d, 5.4 | 4.11 | *β β′* $CH_2$ 2.25 | 176.6 | 57.0 | *β* 27.9/*γ* 32.9 | 109.2 |
| | | | *γ* 2.50/*γ′* 2.56 | 177.3[c] | | | |
| $Q_{19}$ | 7.966; d, 7.7[a] | 4.19 | *β β′* $CH_2$ 2.03 | 174.2 | 55.8 | *β* 27.9/*γ* 32.6 | 111.3 |
| | | | *γ* 2.33/*γ′* 2.20 | 177.4[b] | | | 102.0[b] |
| | | | $\epsilon_{anti}$ 7.381/$\epsilon_{syn}$ 6.686[a] | | | | |
| $Fol_{20}$ | 7.407; d, 9.2 | 4.17 | *β β′* $CH_2OH$ 3.65 | | 54.5 | *β* $CH_2OH$ 64.9 | 114.5 |
| | | | *β* 2.74/*β′* 2.96 | | | *β* 38.0 | |
| | | | Arom. 2′, 6′ 7.227 / 3′, 5′ 7.287 / 4′ 7.147 | | | Arom. 1′ 139.6 / 2′, 6′ 128.9 3′, 5′ 130.2 / 4′ 126.9 | |

[a] Determined at 311 K. [b] $\epsilon$ $^{15}NH$ and $\delta$ $^{13}CO$ chemical shifts of glutamine lateral chains. [c] $\delta$ $^{13}CO$ chemical shift of glutamic acid lateral chain; n.d. not determined; chemical shifts are given to the nearest three decimals or two decimals when obtained from 1D or 2D spectra, respectively.

Gln18) on the three *Spiroplasma* strains, the Ala2→Pro substitution did not appear to influence the antimycoplasmic activity, while the Gln18→Glu replacement led to a 2-fold decrease in the MIC values. Suppression of the two residues at positions 2 and 18 (Ala/Pro and Gln/Glu) in the trichorzins PA sequences resulted in the same effect. The genera *Acholeplasma* and *Mycoplasma* did not appear to exhibit the same sensitivity to the presence of the negatively charged glutamic acid, since all the peptides exhibited identical MIC values for these organisms.

**Liposome Permeabilization.** Long-sequence peptaibols, such as trichorzins PA[18] (18 residues), trichorzianins[4,5] (19 residues), or saturnisporins[30] (20 residues), have been shown to bind to membrane bilayers and increase their permeability to small molecules and ions.[31] A correlation between these properties and the antimycoplasmic activity has been demonstrated for trichorzins PA.[19] We thus examined the permeabilization of liposomes composed of egg phosphatidylcholine (ePC)/cholesterol (Chol), 7:3, by the neutral (LGA I−IV) and acidic (LGB II, III) 20-residue longibrachins. The efficiency of longibrachin-induced membrane bilayer permeabilization was followed by fluorescence measurements of carboxyfluorescein (CF) released from the liposomes (Table 6), according to the protocol previously described.[19,32] The permeabilization efficiency, described as $R_{i50}$ values, of LGA peptides ranged from 1600 to 4500, in agreement with previous data on related neutral long-sequence peptaibols[31] and indicating a strong permeabilizing activity. The permeabilization process appeared to be strongly related to the global hydrophobicity of the peptide. The LGA IV peptide, which contains the more hydrophobic residues (Aib6, Iva17, Gln18), is the more active peptide among those examined here and among all the peptaibols tested thus far.[31] A most important point is the unfavorable effect of glutamic acid at position 18, which decreases the activity by a factor of 3−5. The requirement of neutral uncharged residues for permeabilization activity, previously demonstrated for trichorzianins,[15] was indicated here by observation that LGB II−OMe and LGB III−OMe, formed by methylation of the carboxylic acid function of Glu18 in LGB II and LGB III, are both active (Table 6). These findings are in agreement with the antimycoplasmic activity, which indicates that 2-fold higher concentrations of longibrachins LGB are required for the same inhibition as the LGA peptides. These results suggest that the bactericidal activity of longibrachins toward mollicutes is related to the permeabilization of the plasma membrane, in agreement with our previous results obtained with trichorzins PA.[19] More generally, the same assumption may be extended to other peptaibols.

## Experimental Section

**Isolation of Longibrachins LGA and LGB.** *T. longibrachiatum* (strain LCP-853431, Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, Paris, France), was cultivated in Roux flasks for 14 days at 27 °C on liquid synthetic medium, the composition of which was given previously.[6] A typical 20 L culture was filtered to separate mycelium from culture broth. The filtered broth was extracted three times with *n*-butanol, leading to 2 g of crude extract, which

**Table 4.** $^1$H and $^{13}$C Chemical Shifts for Longibrachin LGB III ($CD_3OH$, 298 K)[a]

| | $\delta^1$H; mult.; *J* (Hz) | | | $\delta^{13}$C | | |
|---|---|---|---|---|---|---|
| residue | N*H* | Hα | other groups (*J*, Hz) | CO | Cα | other groups |
| Ac | | | Me 2.020 | 173.5 | | Me 23.0 |
| $U_1$ | 8.434; s | | *β* Me *pro-S* 1.47/*β* Me pro-R 1.47 | 178.4 | 57.2 | *β* Me *pro-S* 26.6/*β* Me *pro-R* 24.3 |
| $A_2$ | 8.289; d, 4.5 | 4.02 | *β* Me 1.42 | 176.6 | 53.8 | Me *β* 16.7 |
| $U_3$ | 7.659; s | | *β* Me *pro-S* 1.52/*β* Me *pro-R* 1.50 | 178.3 | 57.2 | *β* Me *pro-S* 26.9/*β* Me *pro-R* 23.2 |
| $A_4$ | 7.714; d, 5.3 | 4.06 | *β* Me 1.48 | 177.2 | 54.0 | Me *β* 16.8 |
| $U_5$ | 8.006; s | | *β* Me *pro-S* 1.57/*β* Me *pro-R* 1.50 | 178.3 | 57.6 | *β* Me *pro-S* 26.9/*β* Me *pro-R* 23.2 |
| $A_6$ | 7.940; d, 4.5 | 4.03 | *β* Me 1.54 | 178.1 | 53.9 | Me *β* 16.9 |
| $Q_7$ | 8.062; d, 4.9 | 3.98 | *β* 2.15/*β′* 2.29<br>*γ* 2.37/*γ′* 2.55<br>$\epsilon_{anti}$ 7.445/$\epsilon_{syn}$ 6.757[a] | 175.9<br>177.5[b] | 57.9 | *β* 27.1/*γ* 33.5 |
| $U_8$ | 8.100; s | | *β* Me *pro-S* 1.51/*β* Me *pro-R* 1.61 | 178.3 | 57.6 | Me *pro-R* 27.6/Me *pro-S* 27.4 |
| $V_9$ | 7.501; d, 5.5 | 3.60 | *β* 2.25/*γ*1 Me 1.131; d, 6.7/*γ*2 Me 1.000; d, 6.5 | 175.3 | 65.4 | *β* 30.5/*γ*1 Me 20.8/*γ*2 Me 19.6 |
| $U_{10}$ | 8.201; s | | *β* Me *pro-S* 1.48/*β* Me *pro-R* 1.56 | 179.1 | 57.6 | *β* Me *pro-S* 26.6/*β* Me *pro-R* 23.5 |
| $G_{11}$ | 8.335; t, 5.6[a] | *pro-S* 3.95/<br>*pro-R* 3.68 | | 173.0 | 44.9 | |
| $L_{12}$ | 8.097; d, 8.0[a] | 4.46 | *β* 1.60/*β′* 1.94/*γ* 1.94<br>*δ*1Me 0.932; d, 6.4/*δ*2 Me 0.908; d, 6.5 | 175.7 | 53.8 | *β* 41.3/*γ* 25.5<br>*δ*1 Me 23.3/*δ*2 Me 21.2 |
| $U_{13}$ | 8.331; s | | *β* Me *pro-S* 1.63/*β* Me *pro-R* 1.51 | 175.0 | 58.0 | Me *pro-R* 23.8/Me *pro-S* 23.2 |
| $P_{14}$ | | 4.39 | *β pro-S* 2.34/*β pro-R* 1.83<br>*γ pro-S* 2.09/*γ pro-R* 2.01<br>*δ pro-S* 3.75/*δ pro-R* 3.89 | 176.2 | 64.4 | *β* 30.0/*γ* 27.0/*δ* 50.4 |
| $V_{15}$ | 7.559; d, 7.3 | 3.80 | *β* 2.35/*γ*1 Me 1.065; d, 6.6/*γ*2 Me 0.977; d, 6.5 | 175.2 | 63.8 | *β* 30.1/*γ*1 Me 20.1/*γ*2 Me 19.5 |
| $U_{16}$ | 7.624; s | | *β* Me *pro-S* 1.59/*β* Me *pro-R* n.d. | 177.8 | 57.2 | Me *pro-R* 27.4/Me *pro-S* n.d. |
| $J_{17}$ | 7.726; s | | *β* Me *pro-S* 1.59/*β′* $CH_2$ 2.40, 1.80/<br>*γ* Me 0.844, t, 7.6 | 179.5 | 60.4 | Me *pro-S* 23.5/*β′* $CH_2$ 26.8/*γ*Me 7.7 |
| $E_{18}$ | 7.920 | 4.17 | *β β′* $CH_2$ 2.25<br>*γ* 2.50/*γ′* 2.56 | 176.6<br>177.3[b] | 57.0 | *β* 27.9/*γ* 32.9 |
| $Q_{19}$ | 7.920 | 4.17 | *β β′* $CH_2$ 2.03<br>*γ* 2.37/*γ′* 2.25<br>$\epsilon_{anti}$ 7.445/$\epsilon_{syn}$ 6.628[a] | 174.3<br>177.5[b] | 55.8 | *β* 27.9/*γ* 32.6 |
| $Fol_{20}$ | 7.343; d, 9.3 | 4.16 | *β β′* $CH_2OH$ 3.63<br>*β* 2.74/*β′* 2.94<br>Arom. 2′, 6′ 7.231 / 3′, 5′ 7.273 / 4′ 7.148 | | 54.5 | *β* $CH_2OH$ 64.9<br>*β* 38.0<br>3′, 5′ 130.2 / 4′ 126.9 |

[a] Determined at 311 K. [b] *δ* $^{13}$CO chemical shifts of glutamine or glutamic acid lateral chains; n.d. not determined; chemical shifts are given to the nearest three decimals or two decimals when obtained from 1D or 2D spectra, respectively.

**Table 5.** Minimal Inhibitory Concentrations (*μ*M) of Longibrachins LGB II, LGB III, and LGA IV and of Reference Peptaibols[a] against Six Strains of Mollicutes

| | peptides | | | | |
|---|---|---|---|---|---|
| bacterial strain | LGB II | LGB III | LGA IV | PA VI | Alm F50 |
| *Acholeplasma laidlawii* A-PG8 | 1.56 | 1.56 | 0.78 | 3.12 | 1.56 |
| *Mycoplasma gallisepticum* S6 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| *Mycoplasma mycoides ssp mycoides* SC KH3J | 12.5 | 6.25 | 6.25 | 12.5 | 12.5 |
| *Spiroplasma apis* B31 | 12.5 | 12.5 | 6.25 | 12.5 | 6.25 |
| *Spiroplasma citri* R8A2 | 6.25 | 6.25 | 3.12 | 12.5 | 3.12 |
| *Spiroplasma floricola* BNR1 | 12.5 | 12.5 | 6.25 | 25.0 | 6.25 |

[a] PA VI, trichorzin PA VI;[19] Alm F50, alamethicin F50 natural mixture.[19,34]

was chromatographed over Sephadex LH 20, eluted with methanol. From the crude peptide mixture (140 mg), separated over silica gel ($SiO_2$, Merck, $CH_2Cl_2$−MeOH (9:1 to 0:10), longibrachins LGA (60 mg) were eluted with $CH_2Cl_2$−MeOH (7:3) and LGB (38 mg) with $CH_2Cl_2$−MeOH (1:1). Thin-layer chromatograms of the fractions ($SiO_2$, Merck 60 F 254, $CH_2Cl_2$−MeOH (7:3)) were visualized by spraying anisaldehyde reagent (anisaldehyde−$H_2SO_4$−AcOH 1:1:50).

**HPLC Separations.** The HPLC separations were carried out with a Waters liquid chromatograph: 600 controller, 717 autosampler, and 486 tunable absorbance detector. A semi-preparative reversed-phase Kromasil $C_{18}$ column (5 *μ*m, 300 × 7.5 mm, AIT France) with MeOH−$H_2O$ (85:15) as eluent and a flow rate of 2.0 mL/min was used for the LGA group: LGA I: $t_R$ 24 min, 3 mg; LGA II: $t_R$ 28 min, 5 mg; LGA III: $t_R$ 31 min, 7 mg; LGA IV: $t_R$ 37 min, 10 mg. A semipreparative reversed-phase Spherisorb ODS2 $C_{18}$ column (5 *μ*m, 300 × 7.5 mm, AIT France) with MeOH−$H_2O$−TFA (86:14:0.05) as eluent and a flow rate of 2.0 mL/min was used for the LGB group: LGB II: $t_R$ 23.5 min, 11 mg; LGB III: $t_R$ 27 min, 8 mg. Purity of the isolated peptides was checked on analytical columns (250 × 4.6 mm) in the same phase and solvent systems, at a flow rate of 1.0 mL/min.

**Amino Acid Composition.** One milligram of each peptide was hydrolyzed according to the general procedure (6 M HCl, 110 °C, Ar, 24 h) in a sealed tube. The residue was evaporated to dryness under vacuum in the presence of NaOH pellets. Crude amino acid and amino alcohol mixtures were derivatized to *N*-trifluoroacetylisopropylesters, as previously described.[5] GC analyses of the derivatives were performed on a Hewlett-Packard series II 5890 gas chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxan) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.), with He (0.7 bar) as carrier gas and a temperature program of 50−130 °C, 3 °C/min; 130−190 °C, 10 °C/min, as previously described.[5,18]

**LSI Mass Spectrometry.** Positive ion LSI mass spectra were recorded on a VG analytical ZAB-2 SEQ mass spectrometer (VG Analytical, Manchester, UK), equipped with a standard FAB source and a cesium ion gun operating at 35

**Table 6.** Liposome Permeabilization of Longibrachins as Compared with Selected Reference Peptaibols

| peptide | no. of residues/ no. of negative charges | $R_{i50}$[a] | peptide concentration ($\mu$M) |
|---|---|---|---|
| LGB II | 20/1 | 500 | 1.20 |
| LGB III | 20/1 | 540 | 1.10 |
| LGB II−OMe | 20/0 | 1335 | 0.45 |
| LGB III−OMe | 20/0 | 2500 | 0.36 |
| LGA I | 20/0 | 1667 | 0.36 |
| LGA II | 20/0 | 2860 | 0.21 |
| LGA III | 20/0 | 1820 | 0.33 |
| LGA IV | 20/0 | 4545 | 0.13 |
| saturnisporin SA IV[30] | 20/0 | 1000 | 0.40 |
| trichorzianin TA IIIc[4,15] | 19/0 | 2220 | 0.18 |
| trichorzianin TB IIIc[5,15] | 19/1 | 295 | 1.36 |
| trichorzin PA VI[19] | 18/0 | 1220 | 0.49 |

[a] [Lipid]/[peptide] ratios allowing 50% leakage in 20 min of the entrapped CF from ePC−Chol (7:3) liposomes ([lipid] = 0.6 mM) as compared with reference long-sequence saturnisporin SA IV (Ac-Aib[1]-Ala-Aib-Ala-Aib Gln-Aib-Leu-Aib-Aib[10]-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol[20]), trichorzianin TA IIIc (Ac-Aib[1]-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser[10]-Leu-Aib-Pro-Val-Aib-Ile-Gln-Gln-Trpol; Trpol = tryptophanol), trichorzianin TB IIIc (Ac-Aib[1]-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser[10]-Leu-Aib-Pro-Val-Aib-Ile-Gln-Glu-Trpol), trichorzin PA VI (Ac-Aib[1]-Ser-Ala-Aib-Iva-Gln-Aib-Val-Aib-Gly[10]-Leu-Aib-Pro-Leu-Aib-Aib-Gln-Pheol).

kV. Peptide samples dissolved in methanol were mixed with the matrix, either α-thioglycerol or 3-nitrobenzyl alcohol saturated with LiCl. The resolution was about 1000.

**NMR Spectroscopy.** Half milliliter amounts of 5−20 mM $CD_3OH$ solutions of longibrachins in 5 mm tubes (Wilmad) were used for the NMR experiments, which were conducted at 298 K unless otherwise specified. Spectra were recorded either on a Bruker AVANCE 400 equipped with an $^1H-^{13}C$ dual probehead and a Silicon Graphics O2 computer using XWIN NMR 2.5 software or on a Bruker DMX 500 spectrometer, equipped with a quadruple resonance $^1H-^{31}P-^{13}C-^{15}N$ gradient probehead and an ASPECT Station 1 computer. $^1H$ spectra were referenced to the central signal of the quintuplet due to the $CD_2H$ resonance of methanol at 3.313 ppm, downfield from TMS. $^1H$ 1D COSY experiments were recorded at 400 MHz, with solvent presaturation. TOCSY and ROESY experiments were recorded at 500 MHz, with solvent signal suppression by the WATERGATE scheme included in the pulse sequences, with a mixing time of 120 ms and a spin lock delay of 250 ms, respectively. $^{13}C$ spectra were referenced to the central signal of methanol at 49.0 ppm, downfield from TMS. $^1H-^{13}C$ HSQC and HMBC experiments were optimized for $^1H-^{13}C$ coupling constants of 135 and 5 Hz, respectively. The $^1H-^{15}N$ HSQC experiment was referenced to formamide in 10% acetone-$d_6$, at 112.4 ppm, downfield to $NH_3$, used as secondary external reference.[33] The $^{13}C$ and $^{15}N$ 2D spectra were processed using sine-bell squared functions in F1 and F2 dimensions shifted by 6−2 ($^1H-^{13}C$ HSQC), 2−2 $^1H-^{13}C$ HMBC), and 6−2 ($^1H-^{15}N$ HSQC).

**Antimicrobial Assays.** The antimicrobial activities of the peptides were determined as described previously[19,34] using six strains (Table 4) of bacteria belonging to the class Mollicutes (Collection of UMR 6026 CNRS, University of Rennes 1). Melittin from bee venom and alamethicin from *T. viride* (Sigma, St. Louis, MO) were used as positive controls. Briefly, minimal inhibitory concentrations (MICs) were determined in appropriate liquid culture media under microaerobic conditions using 96-well microtitration plates by following the color change of phenol red resulting from acidification of the culture medium during the cell growth. The MIC was defined as the lowest concentration completely inhibiting the growth of a cell suspension containing $10^6$ colony-forming units (CFUs)/mL. The assays were performed in triplicate. Bactericidal activities were assessed by spreading on agar plates cells treated for 2 h with different peptide concentrations. The minimal lethal concentration (MLC) was defined as the lowest peptide concentration capable of killing more than 99% of the cells in a suspension containing $10^6$ CFUs/mL.

**Liposome Permeabilization.** Permeabilization of liposomes (small unilamellar vesicles, SUV) was measured at 20 °C on an Aminco SPF 500 spectrofluorometer, using a fluorescent probe (carboxyfluorescein, CF) entrapped at a self-quenched concentration, according to the method described by Weinstein.[35] Excitation and emission wavelengths were set at 488 and 520 nm (1 nm band-pass), respectively. Each assay was performed five times. The peptides were added as aliquots of methanolic solutions to a mixture of 0.2 mL of vesicular suspension and 1.2 mL of Hepes buffer, the methanol concentration in the final solutions being kept below 0.5 vol %. The peptide concentrations were calculated to give peptide/lipid molar ratios ranging between 0 and $3 \times 10^{-3}$. The kinetics were monitored after rapid stirring and stopped at 20 min; 50 $\mu$L of 10% (vol/vol) Triton X-100 was then added to determine the total fluorescence ($F_T$) by disrupting the SUV. Percentages of released CF at 20 min were determined as $(F_{20} - F_0)/(F_T - F_0) \times 100$, with $F_0$ and $F_{20}$ the fluorescence intensities in the absence and in the presence of peptide, respectively. The permeabilization efficiency of the peptides was determined, as previously described,[19,30,32] and expressed as $R_{i50}$ values, defined as the [lipid]/[peptide] ratios allowing 50% leakage in 20 min of the SUV-entrapped CF. The activities of Triton X100 and of the reference peptaibols, trichorzianins TA IIIc and TB IIIc,[15] were used as positive controls.

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