Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 811 (2004) 243–251
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Preparative high-performance liquid chromatographic separation and analysis of the Maltacine complex – a family of cyclic peptide antibiotics from *Bacillus subtilis*

Gunnar Hagelin[a,*], Inger Oulie[a], Åse Raknes[a], Kjell Undheim[b], Ole G. Clausen[c,✠]

[a] *MYCOCOMB, Arnulf Øverlands vei 185, 0764 Oslo, Norway*
[b] *Department of Organic Chemistry, University of Oslo, Post Box 1033, Blindern 3, Oslo, Norway*
[c] *Department of Microbiology, Institute of Pharmacy, University of Oslo, Post Box 1032, Blindern 3, Oslo, Norway*

Received 15 June 2004; accepted 13 September 2004
Available online 14 October 2004

Abstract

Purification of secondary metabolites from fermentation broths can be a challenging task both due to the complexity of the medium, inherently unstable molecular structures or by the action of enzymes present in the fermentation broth leading to poor isolation yield and loss of antibiotic activity. A combination of different purification techniques has usually been used to arrive at acceptable purities for characterisation of the target molecules. Due to rapid decay of antimicrobial activity a rapid preparative high-performance liquid chromatography (HPLC) method was developed that provided separation and resolution of a family of 18 closely related cyclic peptides within 110 min with minimal loss of activity. Characterisation of the peptides with LC–MS, UV/IR spectroscopy and amino acid analysis disclosed 20 different peptides with cyclic structures (lactones) with molecular weights between 1447.7 and 1519.8 Da. No peptide antibiotics with identical molecular weights have previously been reported in the literature, which lead us to conclude that this peptide complex has not been discovered before. We have named them Maltacines.

Keywords: Preparative HPLC; LC–MS; Cyclic peptide antibiotics; *Bacillus subtilis*; Maltacines

1. Introduction

In the search for antimicrobial compounds from micro organisms, a strain of *Bacillus subtilis* isolated from a soil sample was found to produce antimicrobial substances upon fermentation. After fermentation for two days good activity against *Candida albicans*, *Thricophyton mentagrophytes* and *Aspergillus fumigatus* was observed. Several strains of *B. subtilis* has been known for a long time as a producers of many peptide antibiotics [1], which comprise both linear and cyclic molecules [2] ranging in molecular weight from 270 Da (Bacilycin) to 4500 Da (Licheiniformin). Most of the cyclic structures are lactams, but some lactones are also reported [3–6]. These compounds, named secondary or special metabolites [1] often contain rare and unusual amino acids with both D and L configurations that is crucial for biological activity. Most often a family of closely related substances are produced by an organism [2] and most of them are composed entirely of amino acids, but aminoglycoside and phosphorous containing components have also been found [2].

Purification of secondary metabolites from fermentation broth can be a challenging task due to the complexity of the medium, occasionally inherently unstable molecular structures or by the action of enzymes present in the fermentation broth, leading to poor isolation yield and loss of biological activity. This was also the case for the Maltacines. Several methods for isolation of the substances were investigated,

* Corresponding author. Tel.: +47 23 18 53 50; fax: +47 23 18 60 14.
E-mail address: gunnar.hagelin@amersham.com (G. Hagelin).
✠ Deceased.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.09.008

but none were found suitable as severe loss of activity was observed during the purification steps.

In need for a rapid and gentle separation method, a preparative high-performance liquid chromatography (HPLC) method was developed where lyophilised fermentation broth was applied to a reversed phase column eluted with a gradient of acetonitrile and water containing 0.1% trifluoroacetic acid (TFA). Base line separation of the components of the growth medium from the antibiotics was achieved with minimal loss of antibiotic activity. The hitherto unknown composition of the Maltacines was disclosed and resolved into seven separate chromatographic peaks. The method was superior to any of the other previously tried isolation techniques and required only filtration and lyophilisation of the fermentation broth prior to chromatographic separation.

Analysis of the isolated peaks identified the Maltacines to be a family of 20 novel cyclic peptides not previously described in the literature. This paper describes the production of Maltacines by fermentation, their isolation and partial characterisation. Determination of the amino acid sequences and the cyclisation patterns of 17 of them have been worked out in the authors laboratory and will be the subject of another paper.

2. Experimental

2.1. Production of the Maltacine complex by fermentation

The fermentation was carried out in 2-l Erlenmeyer flasks containing 0.5 l medium consisting of peptone (from Casein, Merck, Germany) 10 g, soluble starch (from potato, Merck, Germany) 5 g and calcium carbonate (Fluka) 5 g diluted to a total of 1 l with distilled water. After inoculation with the strain of *B. subtilis*, the flasks were incubated at 27–29 °C on a rotary shaker. The maximum antibiotic activity was reached after two days.

2.2. Determination of antibiotic activity

To determine the antimicrobial activity, a diffusion method on surface inoculated dishes was employed [7] whereby *C. albicans* (ATCC no. 10231) and *Staphylococcus aureus* (ATCC no. 6538) were used as routine test organisms. Ten-millilitre wells were punched out in the agar and filled with test solution. Antibiotic sensitivity medium containing 5% horse blood (AB Biodisc Sweden) was used for assay against *S. aureus* and Saborauds medium was used for assay against *C. albicans*.

2.3. Purification of the members of the Maltacine complex by preparative high-performance liquid chromatography

The fermentation broth was filtered through MediaKap-5 (0.2 μm, Microgon™) and lyophilised. The freeze-dried powder was re-dissolved in water, 0.1% trifluoroacetic acid (>98% Fluka) and purified on a preparative HPLC system (LC-8A, Shimadzu, Japan); column: Wydac C18, 25 mm × 250 mm; mobile phases: A = water, 0.1% trifluoroacetic acid, B = acetonitrile, 0.1% TFA; flow: 10.0 ml/min; gradient: 0–50% B for 60 min and 50–100% B for 120 min. Sample: 0.4 mg sample dissolved in 1.0 ml water, 0.1% TFA. Ten millilitres of fractions were collected by an autosampler every minute.

2.4. Characterisation by LC–MS

The peaks isolated by preparative liquid chromatography were analysed by a LC–PDA/MS instrument from Thermo Finnigan consisting of a Surveyor MS pump, autosampler and PDA detector. The mass spectrometer (LCQ Classic, Ion Trap Mass Spectrometer) was equipped with an electrospray ion source operated in positive and negative mode.

2.5. Chemoselective ring opening with NaOH

A selective cleavage of an ester in the presence of an amide can be achieved by reaction with base [8]. Samples (50–100 ng) were dissolved in 50 μl 90% acetonitrile, water and added 50 μl 1 M NaOH and left at room temperature (20 °C) for 16 h. The mixture was neutralised by addition of 50 μl 1 M HCl and evaporated to dryness (Speed Vac SVC-200, Savant).

2.6. Amino acid analysis

The isolated Maltacines were hydrolysed in gas phase (Pico Tag Workstation, Waters, USA). The samples (approximately 50 nmol) were placed in separate tubes in a reaction vial. Two hundred microlitres of 6 M HCl (suprapur, Pierce Company) was placed in the bottom of the reaction vial that was flushed with nitrogen and evacuated before heating to 110 °C for 16–18 h. The amino acid phenyl thiocarbamyl derivatives (PTC) were prepared by adding the sample to a mixture of 700 μl ethanol, 100 μl water, 100 μl triethyl amine and 100 μl of phenylisothiocyanate at room temperature. The mixture was allowed to react for 20 min. The amino acids were determined by the following HPLC method: column: Supelcosil LC-18, 0.46 cm × 25 cm, 5 μm (Supelco, USA); column temperature: 48 °C; mobile phase: A = 50 mM sodium acetate (Fluka) pH 6.2, mobile phase B = 100 mM sodium acetate pH 6.2:acetonitrile:methanol (4:5:1); flow rate: 1.0 ml/min; gradient: 6% B for 8 min, 6–40% B for 12 min, 40% B for 3 min, 40–50% B for 5 min, 50–100% B for 1 min and 100% B for 5 min; detection: 254 nm.

2.7. Determination of chirality of the amino acids

The chirality of the amino acids was determined by LC–MS using a cyclodextrine based chromatographic column and isocratic elution. The following method was em-

ployed: column: Crownpac CR+, 150 mm × 4 mm, 5 μm particles (Daicel Chemical Industries, Ltd.); mobile phase: 0.1% formic acid; flow: 0.4 ml/min; temperature: 7–8 °C; detection: ESI–MS full scan 50–200 Da (LCQ Classic, Ion Trap Mass Spectrometer, Thermo Finnigan, USA); injection volume: 5 μl, 5–7 nmol amino acid standards and 2–10 nmol samples.

2.8. UV and IR-spectra

Recording of the UV-spectra was done by a photo diode array detector (Surveyor PDA detector, Thermo Finnigan) coupled to the liquid chromatograph. Recording of the IR-spectra (solid state) was done on dry samples obtained by preparative HPLC by diffuse reflectance on a Perkin-Elmer Spectrum 2000 FTIR-instrument.

3. Results

3.1. Antimicrobial activity of fermentation broth

Typical growth inhibition zones (mm) were found to be: *C. albicans* (42), *T. mentagrophytes* (30), *A. fumigatus* (40) and *S. aureus* (35). The relative effect on the test organisms could vary between the fermentation batches.

3.2. Purification of the members of the Maltacine complex by preparative high-performance liquid chromatography

The isolation of the Maltacine complex from the fermentation broth components is given in Fig. 1. Despite the complexity of the sample both isolation from the medium components and a chromatographic resolution leading to the disclosure of the composition of the Maltacine complex was achieved (Fig. 2). The isolated peaks were the subjects to further analysis.

3.3. Analysis of the native Maltacines by LC–MS

Analysis of the peaks **B1** through **E1** showed that they contained more components (Fig. 3). The chromatograms of **B1**, **B2**, **C1**, **C2** and **E1** all showed two closely eluting substances. The compositions of Maltacine **D1** and **D2** were more complex and showed four chromatographic peaks. Appearance of MH^+ and $(MH_2)^{2+}$ in the ESI–MS spectra provided correct assignment of the molecular weights of the peptides (Figs. 4–8). The following components of the Maltacine complex were identified: **B1a** = 1477.7 (MH^+), t_R = 18.4 min; **B1b** = 1491.8 (MH^+), t_R = 18.1 min; **B2a** = 1477.8 (MH^+), t_R = 18.6 min; **B2b** = 1491.9 (MH^+), t_R = 18.3 min; **C1a** = 1505.8 (MH^+), t_R = 19.6 min; **C1b** = 1519.9 (MH^+), t_R = 19.3 min; **C2a** = 1505.8 (MH^+), t_R = 19.7 min; **C2b** = 1519.7 (MH^+), t_R = 19.4 min; **D1a** = 1447.8 (MH^+), t_R = 20.3 min; **D1b** = 1461.8 (MH^+), t_R = 20.0 min; **D1c** = 1475.8 (MH^+),

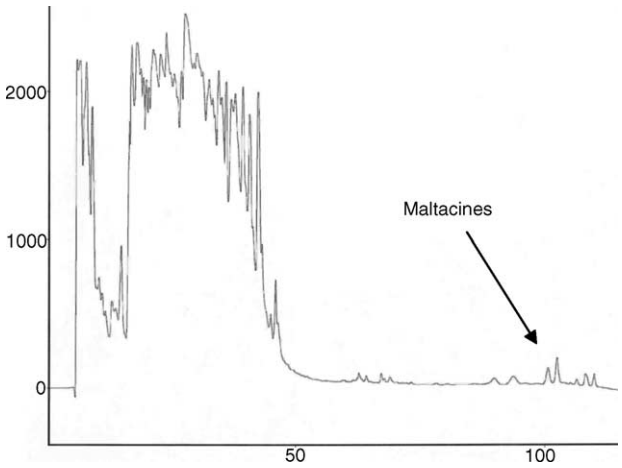

Fig. 1. Purification of the members of the Maltacine complex by preparative high-performance liquid chromatography. The fermentation broth was filtered through MediaKap-5 (0.2 μm, Microgon™) and lyophilised. The freeze-dried powder was re-dissolved in water, 0.1% trifluoroacetic acid (TFA, >98% Fluka) and purified on a preparative HPLC system (LC-8A, Shimadzu, Japan); column: Wydac C18, 25 mm × 250 mm; mobile phases: A = water, 0.1% trifluoroacetic acid, B = acetonitrile, 0.1% TFA; flow: 10.0 ml/min; gradient: 0–50% B for 60 min, 50–100% B for 120 min and 100% B to 200 min. Sample: 0.4 mg sample dissolved in 1.0 ml water, 0.1% TFA. Ten millilitres of fractions were collected by an autosampler every minute.

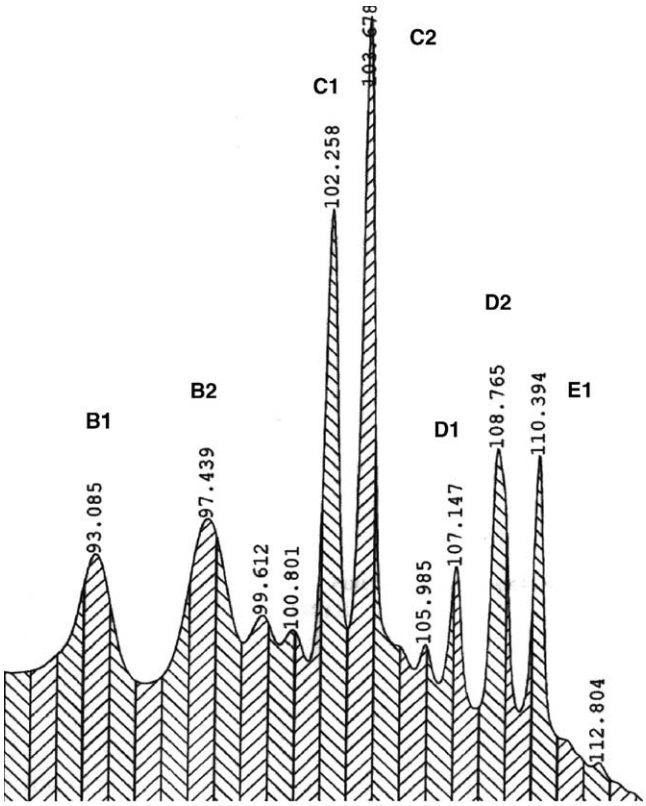

Fig. 2. Expanded region of the preparative HPLC chromatogram from 80 to 115 min (in Fig. 1), showing resolution of the Maltacine complex into seven peaks.

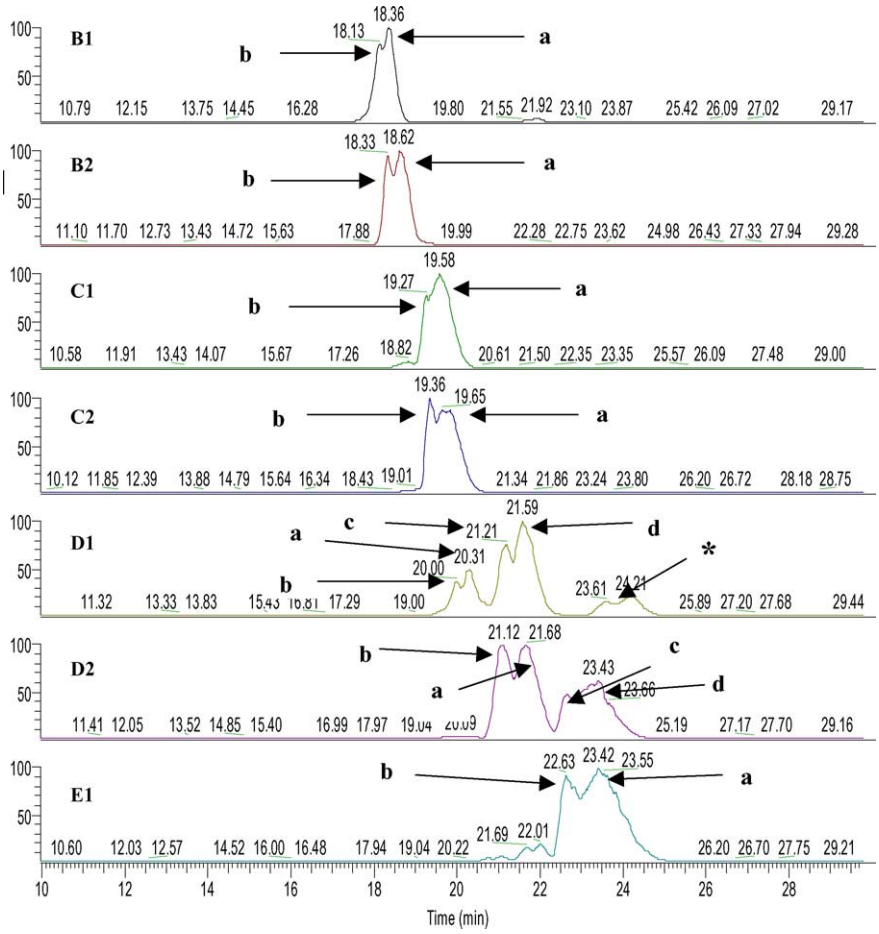

Fig. 3. LC–MS analysis (base peak) of the native cyclic Maltacines. Column: 4 μl, Hydro-RP-18, 80 Å, 150 mm × 2 mm (Phenomenex, Synergi); mobile phases: A = water, 0.1% formic acid (>98% Merck, Germany); B = acetonitrile, 0.1% formic acid; gradient: 0–50% B over 15 min and 50% B to 30 min. Detection, PDA: 200–800 nm full scan; MS: full scan 300–2000 Da; heated capillary: 220 °C; capillary voltage: 4.5 kV; spray current: 15–50 μA; tube lens voltage: 20 V; skimmer voltage: 10 V; sheet gas: 80 bar (nitrogen); aux. gas: 20 bar (nitrogen). *Impurities of **E1a** and **E1b**.

t_R = 21.2 min; **D1d** = 1489.8 (MH^+), 21.6 min; **D2a** = 1461.6 (MH^+), t_R = 21.7 min; **D2b** = 1475.7 (MH^+), t_R = 21.1 min; **D2c** = 1489.8 (MH^+), t_R = 22.7 min; **D2d** = 1503.8 (MH^+), t_R = 23.4 min; **E1a** = 1489.8 (MH^+), t_R = 23.6 min; **E1b** = 1503.8 (MH^+), t_R = 22.6 min.

3.4. Analysis of the ring opened Maltacines by LC–MS

Treatment with NaOH resulted in more hydrophilic molecules eluting earlier than their native species (Fig. 9), which increased by 18 Da in molecular weights (Fig. 10). The result indicates an intramolecular addition of water consistent with a cyclic structure formed by lactones. The ring opening resulted also in the appearance of more peaks in the chromatogram reflecting the presence of peptides with chiral variations on the amino acids.

3.5. Determination of amino acid composition and their chirality

Because the samples were mixtures of more peptides no integral ratios between the amino acids were found. The amino acid compositions of the peaks obtained by preparative chromatography is given in Table 1 and shows the presence of both D and L enantiomers. All efforts to isolate the closely eluting the peptides of **B1**, **B2**, **C1**, **C2**, **D1**, **D2** and **E1** were unsuccessful and the analyses were therefore performed on the mixture of them.

3.6. UV and IR-spectra

The UV-spectra of all the members of the complex were identical and is shown in Fig. 11, represented by the spectrum of **B1**. The 216 and 234 nm: NHCO absorption; 276 nm: aryl-OH of tyrosine.

The IR-spectra of all the members of the complex were also identical and is shown in Fig. 12, represented by the spectrum of **B1**. A dramatic change in the IR spectrum was observed after the chemoselective ring opening with NaOH. In the spectrum of native Maltacine (Fig. 12a), the absorption band at 1681 cm^{-1} is a composite of C=O stretch in amide and cyclic ester/lactone and 1216 and 1139 cm^{-1} is due to C–O stretch of cyclic ester/lactone and is evidence for the

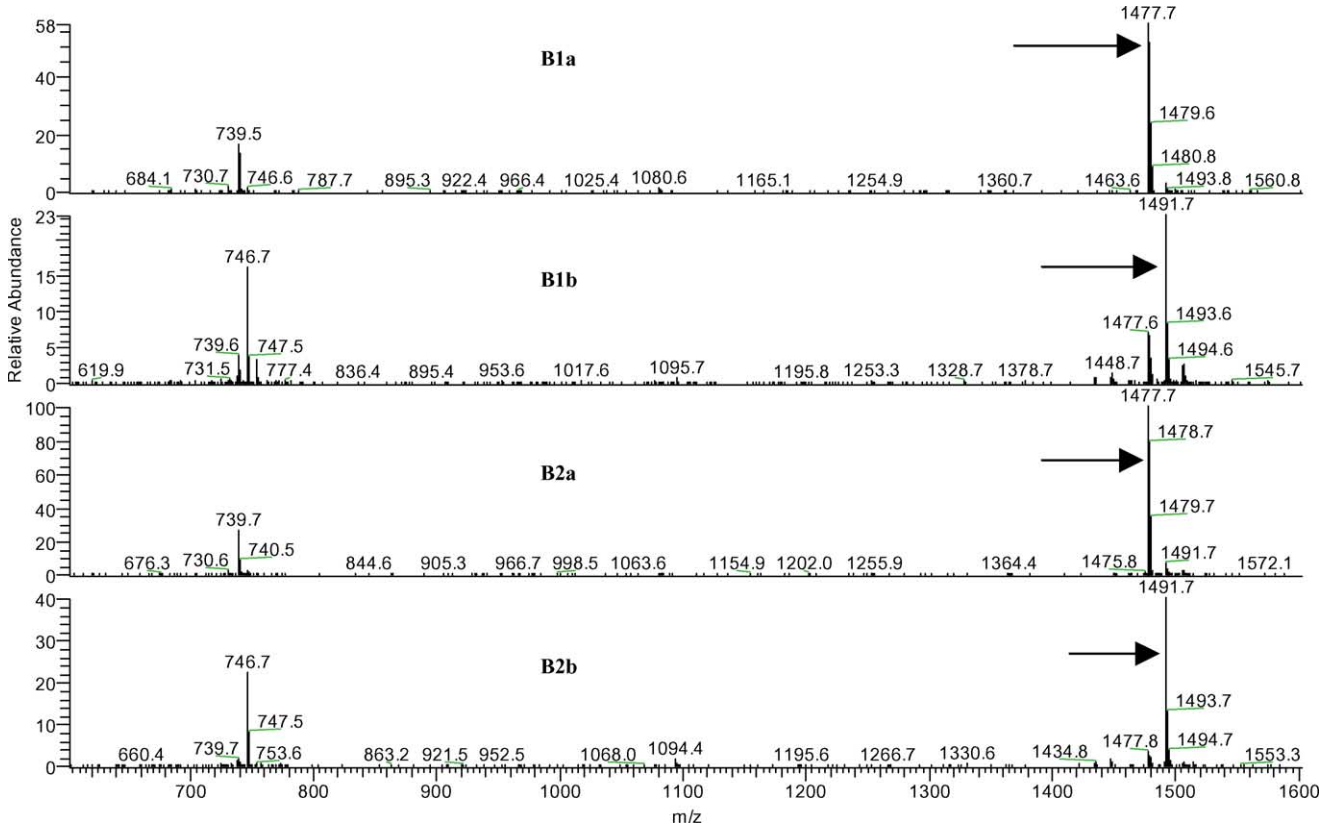

Fig. 4. The ESI mass spectrum of Maltacine **B1** and **B2** showing the MH^+ and $(MH_2)^{2+}$. Due to incomplete chromatographic resolution the signals for **B1a** appear in the spectrum for **B1b** and the signal for **B2b** appear in the spectrum for **B2a**.

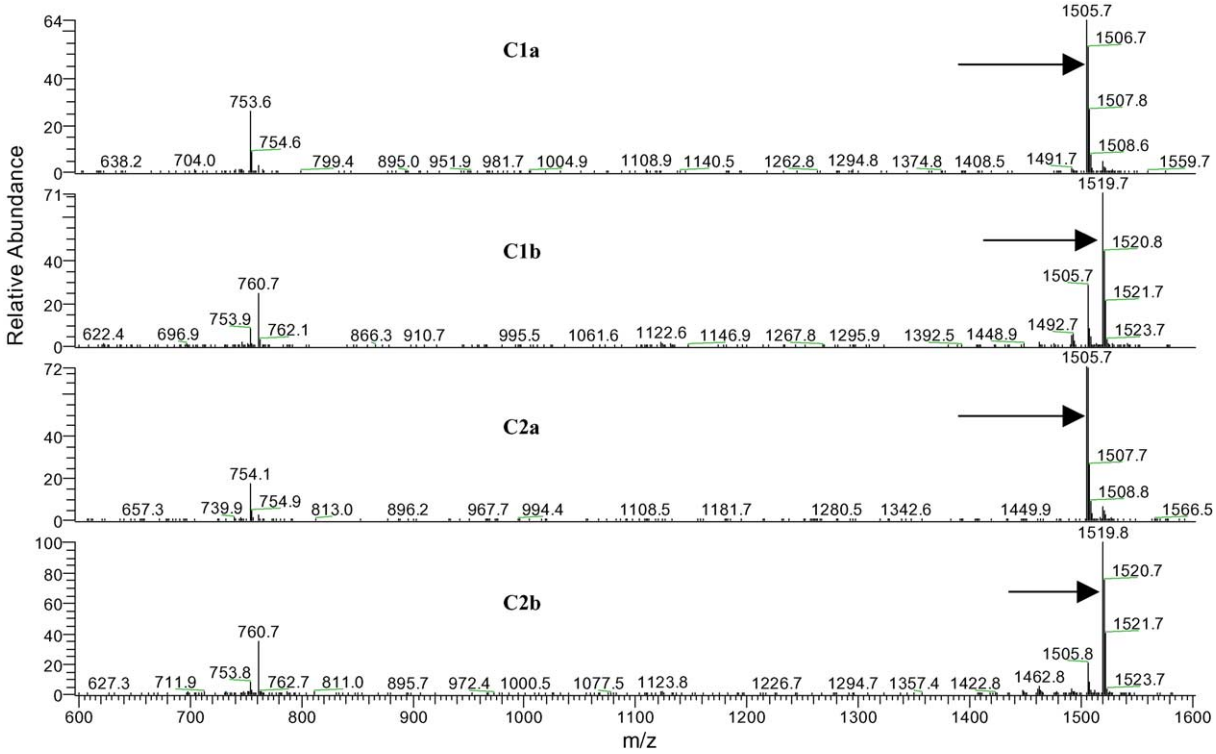

Fig. 5. The ESI mass spectrum of Maltacine **C1** and **C2** showing the MH^+ and $(MH_2)^{2+}$. Due to incomplete chromatographic resolution the signals for **C1a** appear in the spectrum for **C1b** and the signal for **C2b** appear in the spectrum for **C2a**.

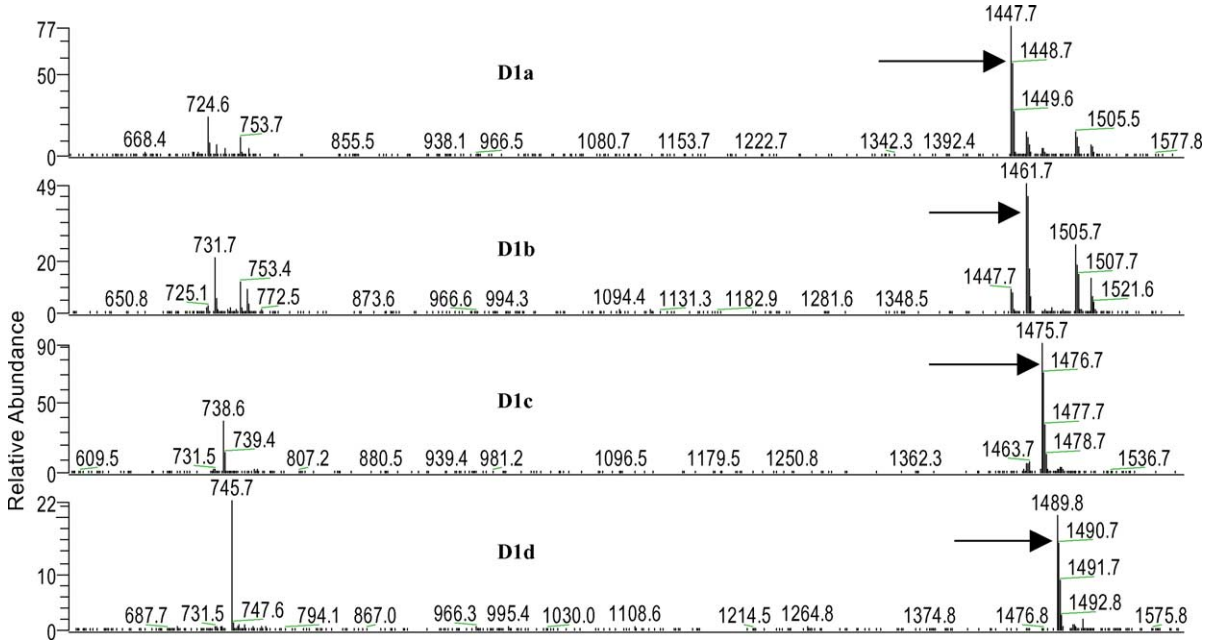

Fig. 6. The ESI mass spectrum of Maltacine **D1** showing the MH^+ and $(MH_2)^{2+}$. Due to incomplete chromatographic resolution the signals for **D1a** appear in the spectrum for **D1b**. The signals at *m/z* 1505.7 and 1519.6 in the spectra of **D1a** and **D1b** are due to contamination of **C2a** and **C2b**.

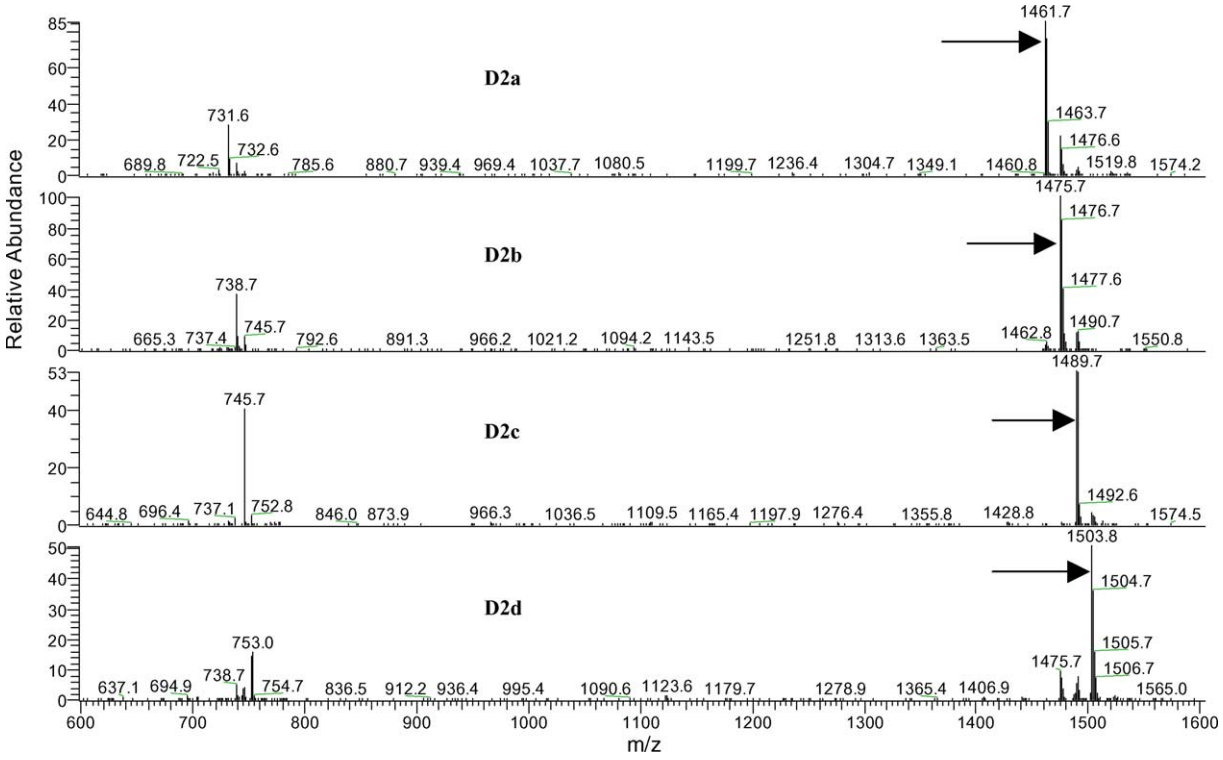

Fig. 7. The ESI mass spectrum of Maltacine **D2** showing the MH^+ and $(MH_2)^{2+}$. Due to incomplete chromatographic resolution the signals for **D2a** appear in the spectrum for **D2b** and vice versa which is also observed for **D2c** and **D2d**.

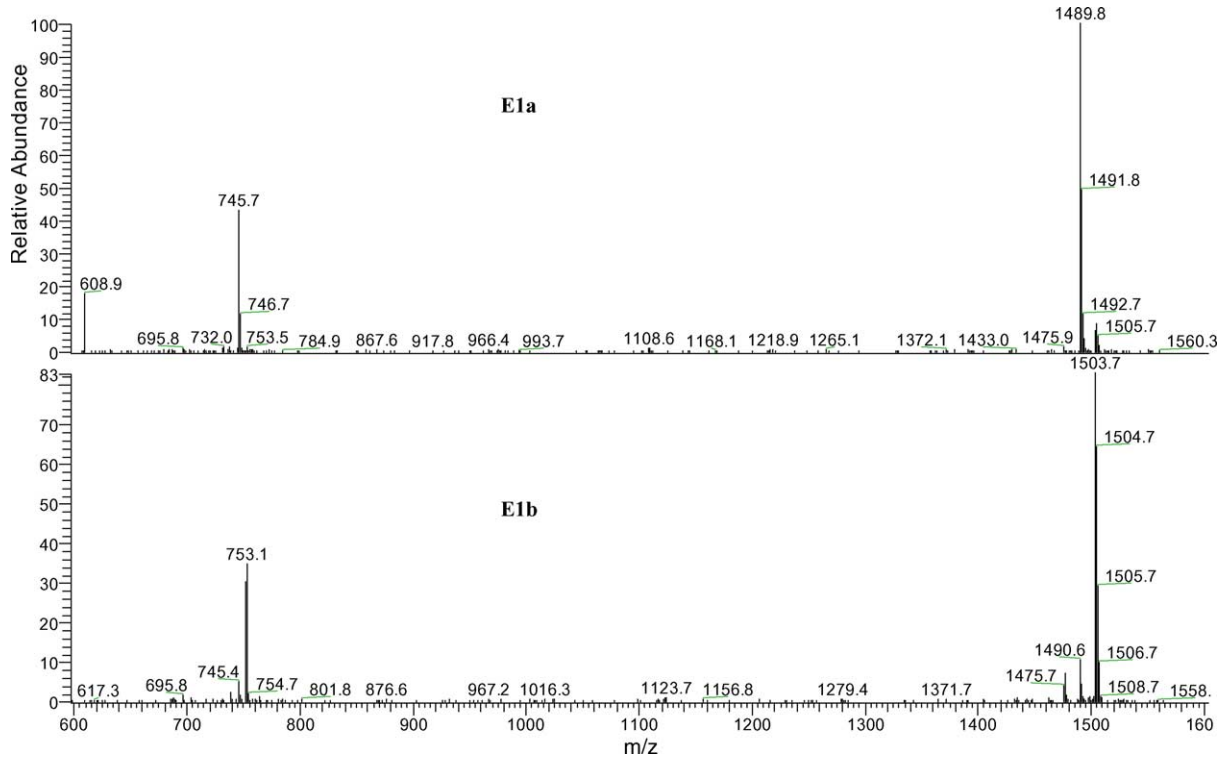

Fig. 8. The ESI mass spectrum of Maltacine **E1** showing the MH^+ and $(MH_2)^{2+}$. Due to incomplete chromatographic resolution the signals for **E1a** appear in the spectrum for **E1b** and vice versa.

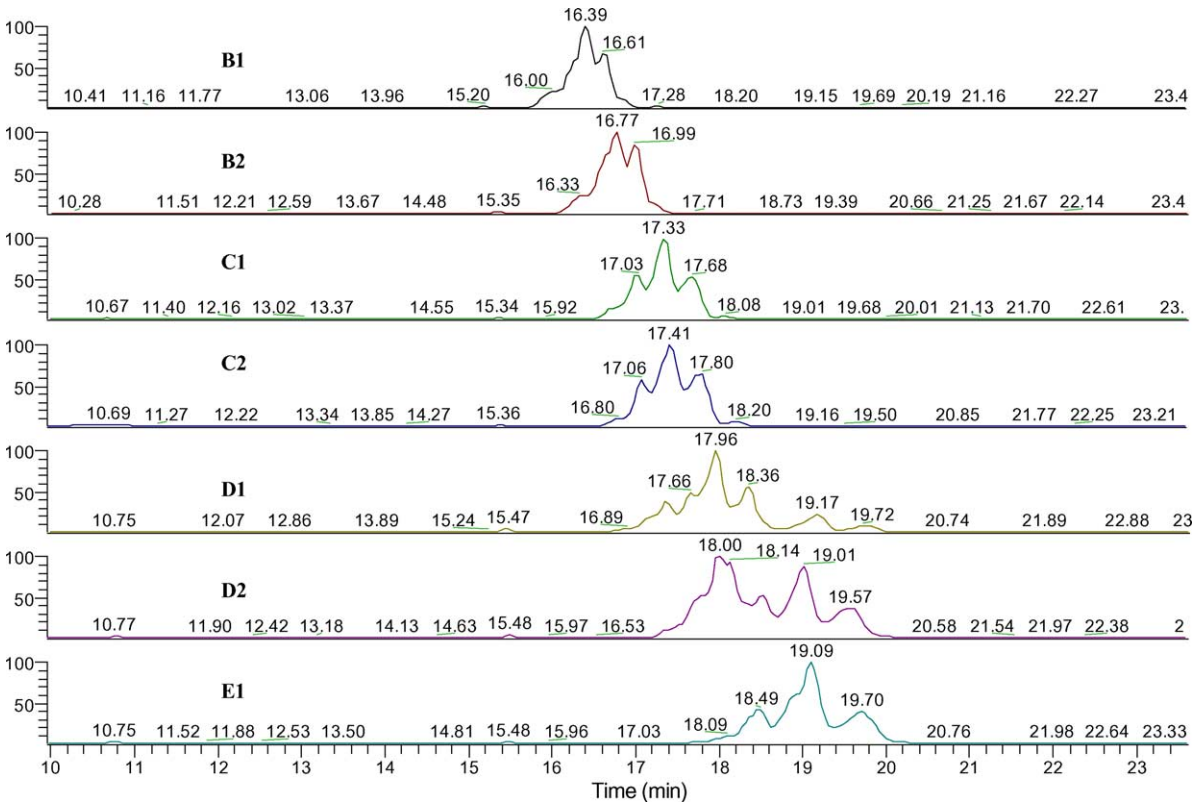

Fig. 9. LC–MS (base peak) of the ring opened Maltacines showing more hydrophilic molecules eluting earlier than their cyclic analogues (conf. Fig. 3).

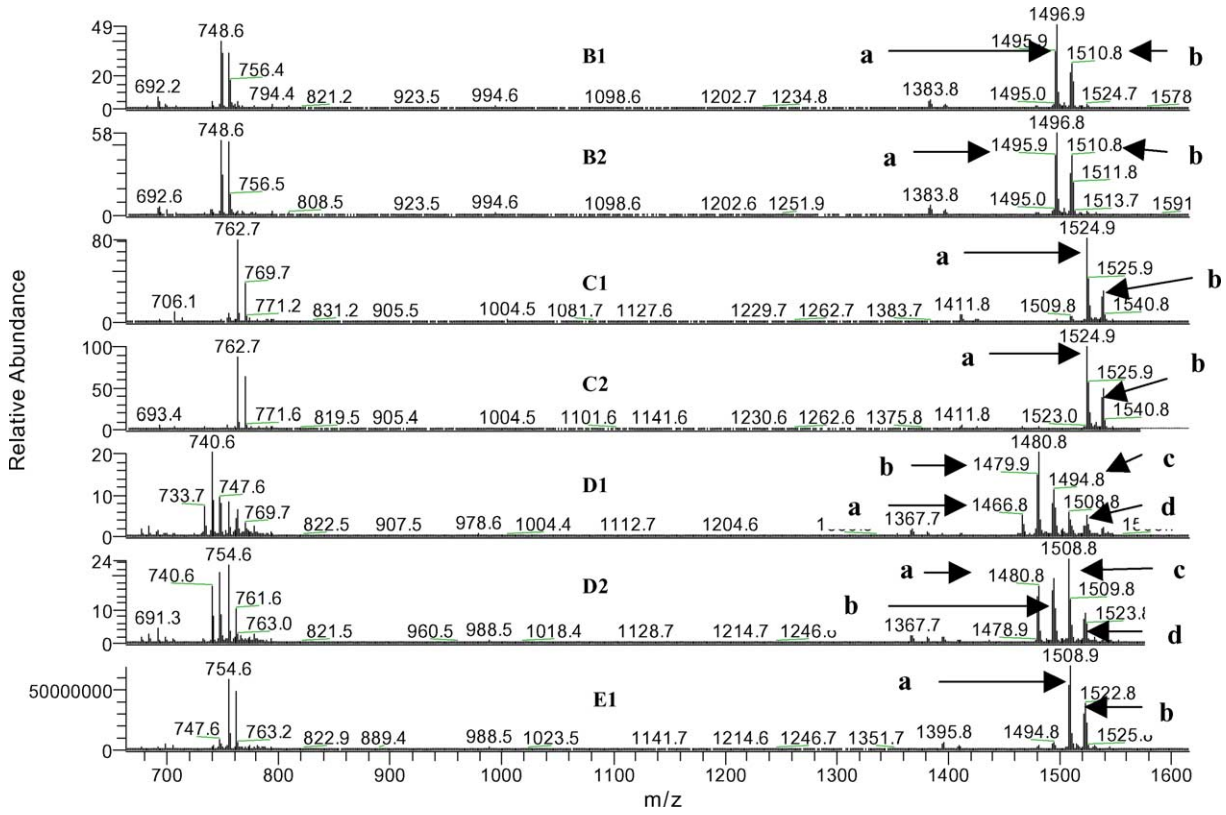

Fig. 10. ESI mass spectra of the ring opened Maltacines showing a mass increase of 18 Da relative to the cyclic analogues.

Table 1
The chiralities of the constituent amino acids of the various Maltacines

Amino acids	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**	**E1**
A	3.0 D	2.9 D	1.0 D	0.7 D	3.2 D	2.5 D	2.2 D
C	0	0	0	0	0	0	0
E/Q	2.5 L	3.6 L	2.1 L	0.5 L	2.4 L	3.2 L	3.1 L
H	0.9 nd	1.1 nd	0.9 nd	0.6 nd	0.9 nd	1.2 nd	1.0 nd
I	1.0 L	1.1 L	0.9 L	1.00 L	0.8 L	1.5 L	0.9 L
K/Orn	0.4/0.7 L + L	0.5/1.0 L + L	0.4/1.2 L + L	0.2/0.6 L + L	0.3/0.8 L + D	0.6/1.5 L + D	0.5/1.2 L + D
M	0	0	0	0	0	0	0
P	1.4 *	1.5 *	1.2 *	1.5 *	1.2 *	1.6 *	1.3 *
S	0 *	0 *	0 *	0 *	1.3 *	1.3 *	1.3 *
T	1.0 D	1.0 D	1.0 D	1.0 D	1.0 L	1.0 D + L	1.0 D
V	0	0	1.1 D	1.3 D	0.4 nd	0.9 nd	1.1 D
Y	2.1 D + L	2.3 D + L	1.9 D + L	1.5 D + L	1.9 D + L	2.5 D + L	2.0 D + L

* Chirality could not be determined by this column.

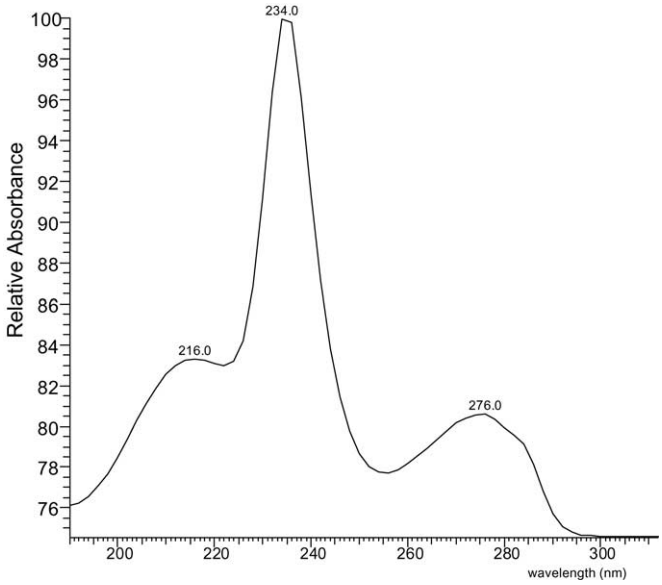

Fig. 11. Ultraviolet absorption spectrum of the Maltacines represented by Maltacine **B1**. Wavelength step: 5 nm; filter bandwidth: 1 nm; sampling rate: 5.0 Hz.

presence of a lactone in the molecule. Loss of the stretching frequencies at 1681, 1216 and 1139 cm^{-1} and appearance of the C=O absorption band at 1735 cm^{-1} shows the formation of a carboxylic acid and a linear molecule (Fig. 12b).

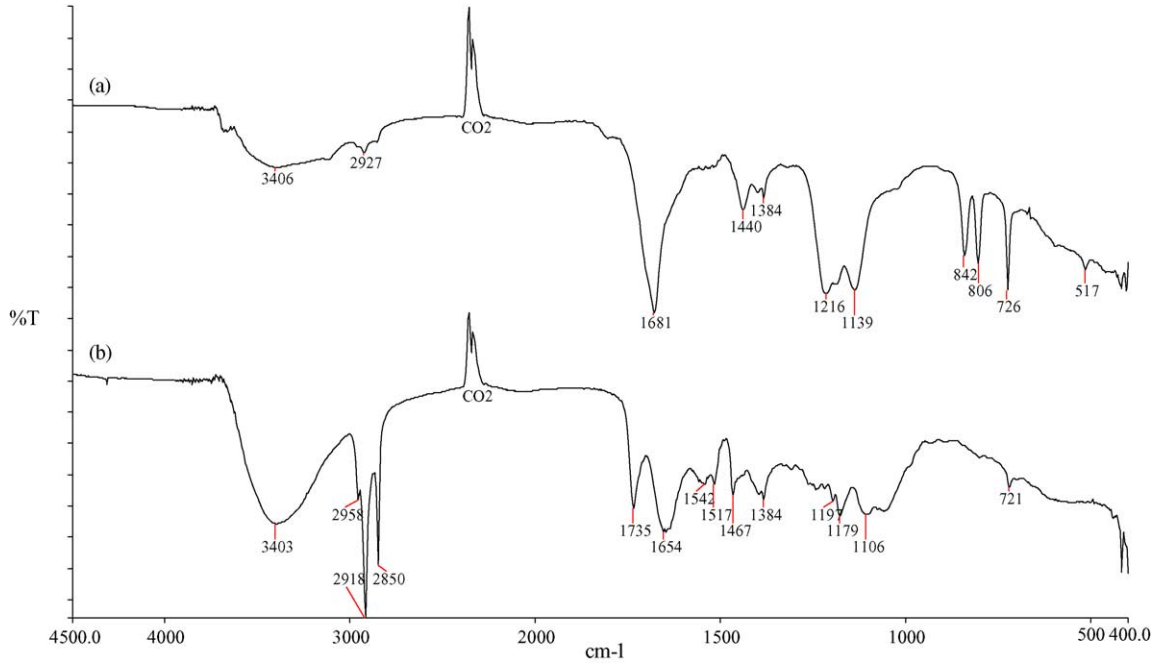

Fig. 12. IR-spectrum of Maltacines represented by the spectrum of **B1**: (a) (native Maltacine): 2927, 2857 cm^{-1} C—H stretch of alkyl groups; 3406 cm^{-1} N—H stretch of secondary amide and O—H of alcohol and acid; 1681 cm^{-1} composite of C=O stretch in amide and ester/lactone; 1216 and 1139 cm^{-1} C—O stretch of ester/lactone which is an evidence for the presence of an ester in the molecule; (b): (ring opened **B1**): 3403 cm^{-1} N—H stretch of secondary amide and O—H of alcohol and acid: 2958, 2918 and 2850 cm^{-1} C—H stretch of alkyl groups; 1735 cm^{-1} C=O stretch of carboxylic acid. The 1654 cm^{-1}: N—H stretch, amide I band.

4. Conclusion

The isolation of a new family of 18 closely related cyclic peptides from a growth medium rich in peptides has been obtained by preparative HPLC. The method provided samples pure enough for characterisation of the Maltacine complex. The members of the family were found to have cyclic structures formed by lactones. Their molecular weights, amino acid composition and chirality's have been determined. The position of either the D or L enantiomer in the peptides could not be determined. The finding of a family of closely related substances is in agreement with what is reported for other antibiotics produced by species of *Bacillus*. No peptide antibiotics with molecular weights identical to those found for the Maltacines have been reported so far which lead to the conclusion that this peptide complex has not been discovered before. We have named them Maltacines. Elucidation of their amino acid sequences and cyclisation patterns will be the subject of other publications.

References

[1] A.L. Sonensheim, J.A. Hoch, R. Losick (Eds.), *Bacillus subtilis* and Other Gram Positive Bacteria, American Society for Microbiology, Washington, DC, 1993.
[2] E. Katz, A. Demain, Bacteriol. Rev. 41 (2) (1977) 449.
[3] U. Koch, Fengycin: Strukturaufklarung eines mikroheterogenen Lipopeptidantibiotikums, Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften der Fakultet fur Chemie und Pharmazie der Eberhard-Karls Universitet zu Tubingen, 1988.
[4] M. Aydin, et al., Liebigs Annalen der Chemie 2285–2300 (1985).
[5] M. Ubukata, et al., Tetrahedron Lett. 25 (4) (1984) 423.
[6] M. Nishii, et al., Tetrahedron Lett. 21 (1980) 4627.
[7] O.G. Clausen, Drug Res. 12 (1962) 948.
[8] J. Schneider, et al., Zeitscrift fur Naturforschung, Sec. c 54 (11) (1999) 859.