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Use of synthetic analogues in confirmation of structure of the peptide antibiotics Maltacines

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Abstract

Maltacines comprise a family of cyclic peptide lactone antibiotics produced by a strain of *Bacillus subtilis*. The previously proposed amino acid sequences of the linear ring-opened molecules show similarity to the lipopeptide antibiotic Fengycin IX that is also produced by a strain of *B. subtilis*. There were some discrepancies in the Maltacin data that could not be explained. To address this and gain more information into the structure of the linear ring-opened Maltacines, the two members D1c, E1b and Fengycin IX acid were synthesised and their MS^2, MS^3 and MS^4 spectra compared.

The similarity of the product ion spectra of Maltacin and Fengycin IX acid revealed that proline occupies an internal position in Maltacin. This finding led to revision of the interpretation of the amino acid sequences of the Maltacines. The proposed new structures of the Maltacines shows that the cyclic part of the molecules is the same as in Fengycin IX acid and Fengycin XII acid, but they have unique N-terminal sequences not found in Fengycins, and thus represent novel lipopeptide antibiotics.

Keywords: Maltacin; Amino acid sequence; Peptide antibiotic; Synthetic analogue

1. Introduction

The Maltacines comprise a family of peptide lactone antibiotics produced by a strain of *Bacillus subtilis* [1] of which 13 of the molecules have been studied [1–5]. The Maltacines are previously suggested by the author to be cyclo-4,12 peptide lactones where the members have major parts of their primary sequences in common. Position 4 in all members was thought to be occupied by a hydroxyamino-acid (residue mass 131 Da) that is part of the lactone ring. The identity of this moiety has not been verified, but a tentative isomer of this moiety is 2-amino-4,5-dihydroxypentanoic acid (Adi). The amino acids found in Maltacines are of both L- and D-configuration. Maltacine D1c (MW = 1492.9 Th) was found to contain: D-Ala, D-Lys and D-Tyr and Maltacine E1b (MW = 1520.9 Th) were found to contain: D-Val, D-Lys, D-Thr and D-Tyr in addition to the other component L-amino acids [1].

The previous interpretation of the MS^n spectra of the Maltacines was based on the finding of two unknown components in the amino acid hydrolysates. The finding of rare amino acids or non-amino acid components in secondary metabolites from microorganisms is not uncommon and has been known for a long time [6]. Since the product ion mass spectra of the Maltacines gave almost uninterrupted series of homologous Y''_n and B_n ions, a high confidence was given to the proposed sequences that included Adi in position 4. Nevertheless, there are some discrepancies in the data, which indicate that there might be another possible solution to the linear structures of the Maltacines.

The chance of rediscovery of already known chemical structures is present when studying antibiotics from microorganisms. The question of whether a new candidate actually is novel or already described can be a real challenge. Comprehensive literature searches were performed in order to investigate whether this family of peptide lactones were already described. No obvious match could be found. Nevertheless, the families of Plipastatins [7,8] and Fengycins [9–11] caught attention. These lipopeptides are acylated decapeptides with the amino acid

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sequence β-hydroxyfatty acid-E-O-Y-T-E-A/V-P-Q-Y-I. In Plipastatin A (MW = 1480.6) and Plipastatin B (MW = 1494.6), the acyl moieties 3-hydroxy-hexadecanoyl and 3-hydroxy-14-methyl-hexadecanoyl have been identified [7]. In Fengycins the main acyl groups were found to be anteiso-pentadecanoic acid (ai-C_{15}), isohexadecanoic acid (I-C_{16}) or *n*-hexadecanoic acid (*n*-C_{16}) and traces of unsaturated fatty acids [11], but Koch reports to have found only 3-hydroxy fatty acids in Fengycins (Fengycin IX) [9]. There is also disagreement between Schneider et al. [10] and Vanittanacom and Loeffler [11] regarding the nature of the fatty acids occurring in Fengycin! Plipastatins and Fengycins are cyclic molecules where a lactone is formed between the C-terminal isoleucine and the side chain of tyrosine in position 3. Plipastatins differ from Fengycins only in the configuration on the two tyrosines, which are interchanged [10].

The aim of this work was to gain evidence for the correctness of the interpretation of the product ion mass spectrometric data of the two linear ring-opened Maltacine D1c and E1b. Linear Maltacine D1c and E1b having N-terminal Pro were therefore synthesised and their product ion spectra compared to the native peptides.

There are no product ion mass spectra available in the literature for Plipastatin [7,8] or Fengycin [9–11]. Schneider et al. [10] described that MS/MS of FAB produced molecular ions of Fengycin IX was performed, but no product ion spectra were shown. Whether the parent ion was MH^+ or $(MH_2)^{2+}$ was also not specified. It was thus not possible to compare the product ion spectra of Fengycin IX acid and Maltacines, so it was also necessary to synthesise Fengycin IX acid (MW = 1480.6) as a model peptide. Fengycine IX acid has an internal proline compared to the synthetic D1c and E1b, where proline occupies the N-terminus.

2. Experimental

2.1. Materials

Ultra-pure Milli-Q water (Millipore, Bedford, MA, USA) was used in the preparation of mobile phases for liquid chromatography. HPLC-grade acetonitrile and p.a. formic acid were obtained from Merck, Darmstadt, Germany. The N^{α}-Fmoc(tBu) and N^{α}-Fmoc(Trt) protected amino acids were purchased from Novabiochem. Diisopropyl carbodiimide (DIC) was obtained from Fluka and triisopropyl silane (TIS) was obtained from Aldrich. *N*-methylpyrrolidone (NMP) and the coupling agent HOAt were obtained from Applied Biosystems.

2.2. Synthesis of Fmoc-Adi(Trt)$_2$

This amino acid is not commercially available and was synthesised as described [12]. The applied Fmoc-Adi(Trt)$_2$ consists of a 60:40 mixture of the two chiral isomers at the δ-carbon.

2.3. Peptides synthesis

For unknown reasons attempts to prepare the three model peptides with D-amino substitutions at the anticipated correct positions was unsuccessful, and only the all-L peptides were therefore synthesised.

The target peptides: Maltacine D1c (P-Q-Y-Adi-A-E-T-Y-K-S-Y-I, MW = 1492.9 Th), Maltacine E1b (P-Q-Y-Adi-V-E-T-Y-K-S-Y-I, MW = 1520.9 Th) and Fengycin IX acid (3-hydroxy-hexadecanonoyl-E-O-Y-T-E-A-P-Q-Y-I, MW = 1480.6 Th) were synthesised by solid phase peptide synthesis using microwaves (Emrys Creator microwave instrument from Personal Chemistry, Uppsala, Sweden). The peptides were synthesised on a Wang resin by using N^{α}-Fmoc protected amino acids. The side chains of tyrosine, serine and glutamic acid were protected as tBu-groups and the side chains of glutamine and Adi were protected as Trt-groups. Couplings of all amino acids were achieved by using 3 equiv. of both amino acid and HATU and 6 equiv. of DIEA assisted by microwaves at 60 °C for 15 min. The availability of Fmoc-Adi(Trt)$_2$ was very limited and coupling of this amino acid was performed using 1.3 equiv. amino acid and diisopropyl carbodiimid (DIC)/HOAt at 60 °C for 2 h with microwave assistance. Coupling of 3-hydroxy-hexadecanoic acid to the Fengycin peptide was performed with PyAOP and DIEA.

Simultaneous cleavage of the side chain protecting groups and release of the peptides from the resin was achieved by TFA/TIS/water (95/2.5/2.5 vol.%) at room temperature for 60 min. After removal of the resin, the solvent was reduced to a small volume and the peptides precipitated by addition of diethyl ether. The crude products were washed four times with diethyl ether.

2.4. Analytical LC–MS

The synthetic peptides were analysed by a Surveyor HPLC coupled on line to a LCQ DECA XP Plus (Thermo Electron Corporation, San Jose, USA) Ion Trap mass spectrometer equipped with an electrospray ionisation source (ESI) operated at 4.5 kV. Column: C_{18} Luna, Phenomenex, 3 μm, and 150 mm × 2 mm; mobile phases: A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid; gradient: 0–50% B over 15 min and then 50% B to 20 min. Flow: 0.3 ml/min; detection: UV 214 and 256 nm and MS full scan 150–2000 Th.

2.5. Collision induced dissociation (CID) mass spectrometry

The ring-opened native Maltacines represents mixtures of two peptides with 1 Da in mass difference. The extra peptide of one higher unit mass was formed during the ring-opening reaction with NaOH and caused a de-amidation of glutamine to glutamic acid [2–5]. The combined isotopic clusters for these two peptides cause the A + 1 isotope in the B_n ion series to become the most abundant.

MS^n product ion spectra were recorded on the LCQ DECA XP Plus (Thermo Electron Corporation) spectrometer equipped with a nano-ESI ion source from Protana (Odense, Denmark). Instrument settings were: full MS-target value = 2×10^7, scan rate = 5500 Da/s, isolation with = 5 Da, *Q*-value = 0.25 and activation time = 30 ms. Approximately 50 ng of the native

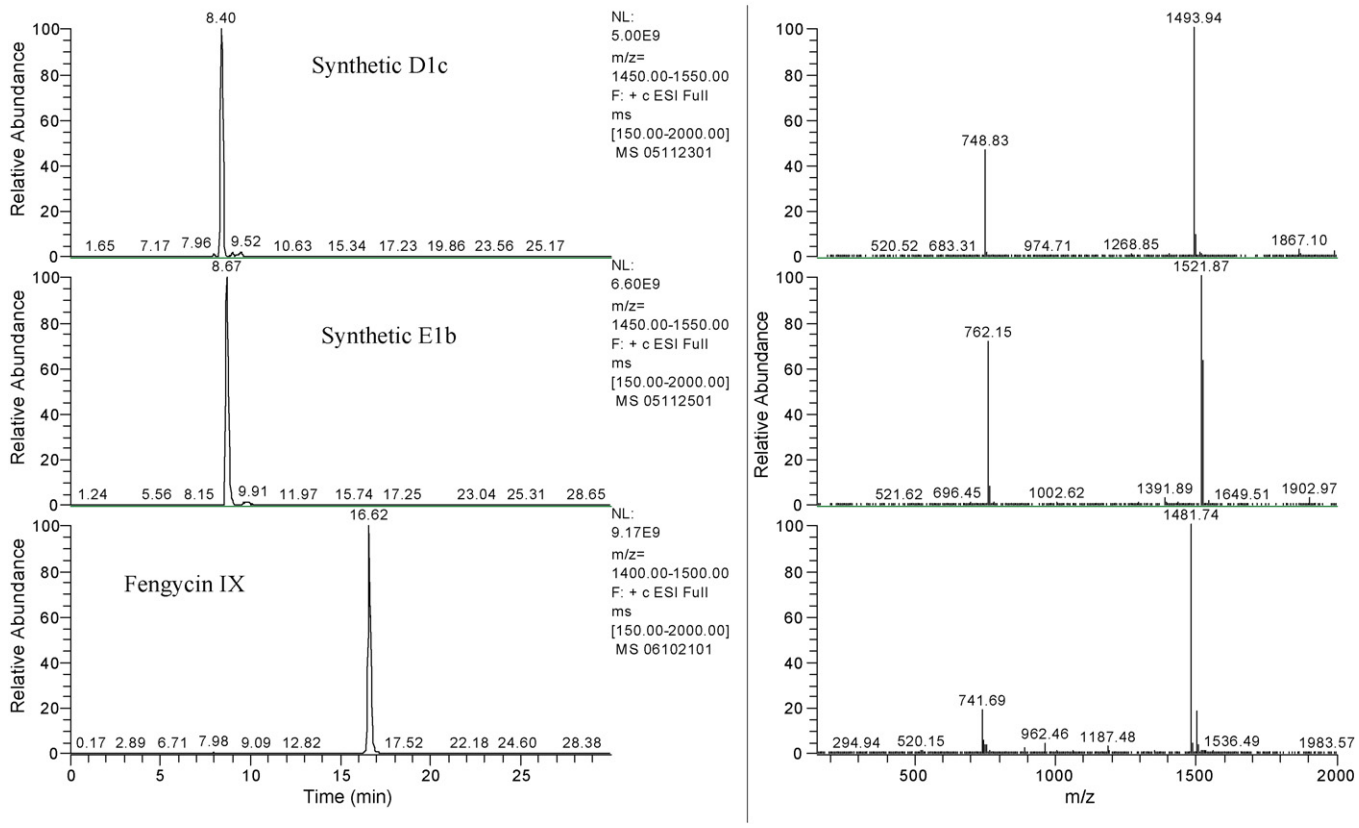


Fig. 1. HPLC–MS of synthetic linear Maltacin D1c, MH^+ = 1493.9 Da (top traces); E1b, MH^+ = 1521.8 Da (middle traces); and Fengycin IX acid, MH^+ = 1481.7 Da (bottom traces).

Maltacines were dissolved in 50% acetonitrile in water containing 0.1% formic acid and sprayed from a silver coated glass capillary supplied by Protana (Odense, Denmark). All spectra were recorded in the positive ion mode with unit mass resolution. The potential applied to the nano-flow tip was 0.8–1 kV. The collision energies for the CID experiments were the same as those used earlier in the studies of the native Maltacines [2–5] (Table 1).

High purity helium (Scientific helium 6.0, AGA, Norway) was used as collision gas in the trap.

3. Results

3.1. Analytical results

The synthetic peptides D1c, E1b and Fengycin IX acid were analysed by HPLC–UV/MS (Fig. 1). All three products have the required high purity. The retention times of D1c and E1b differ substantially from that of Fengycin IX acid. The longer t_R is indicative for the presence of the hydrophobic fatty acid moiety in the Fengycin molecule.

All three synthetic products show the expected values for the singly and doubly charged molecular ions. For D1c, *m/z* 1493.9 (MH^+) and 748.2 $(MH_2)^{2+}$ is observed. For E1b, *m/z* 1521.9 (MH^+) and 762.2 $(MH_2)^{2+}$ is observed. For Fengycin IX acid, *m/z* 1481.6 (MH^+) and 741.3 $(MH_2)^{2+}$ is observed.

3.2. Comparison of fragmentation patterns of the molecules

The product ion spectra (MS^2 of MH^+ and $(MH_2)^{2+}$) of synthetic linear D1c and E1b and their native counterparts are shown in Figs. 2–5. The expected B_n/Y''_n ion series and the observed internal fragments are shown in Scheme 1. Due to their identical

Table 1
Collision energies used for CID

Peptide	*m/z*	Charge state	Collision energy (% on the arbitrary scale of the instrument)
D1c	1493.8	MH^+	32
	747.4	$(MH_2)^{2+}$	30
	520.1	+	28
	226.1	+	27
E1b	1521.8	MH^+	31
	761.4	$(MH_2)^{2+}$	30
	520.1	+	29
	226.1	+	28
Fengycin	1481.6	MH^+	32
	741.3	$(MH_2)^{2+}$	30
	520.1	+	28
	226.1	+	28

On the thermo-ion trap instrument, the collision energies cannot be obtained in eV—only as % on the instruments arbitrary scale.

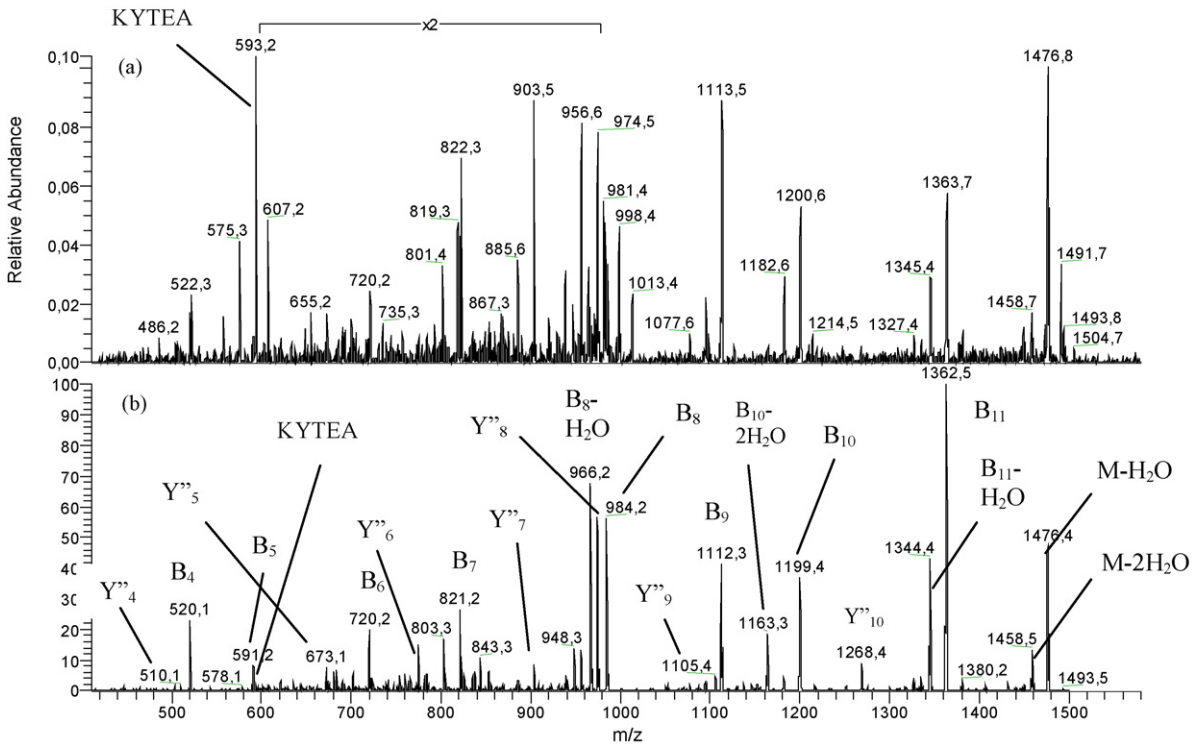


Fig. 2. MS^2-spectra of native ring-opened Maltacin D1c (a) and synthetic D1c (b). (CID of MH^+, m/z 1493.8) $Y''_6 = m/z$ 774.2. Due to the low mass cut-off of the ion-trap instrument, the product ion mass spectra were recorded down to m/z 400. See also Scheme 1. See Scheme 2 for assignment of the ions in the spectrum of native Maltacin that are in accordance with the revised structures.

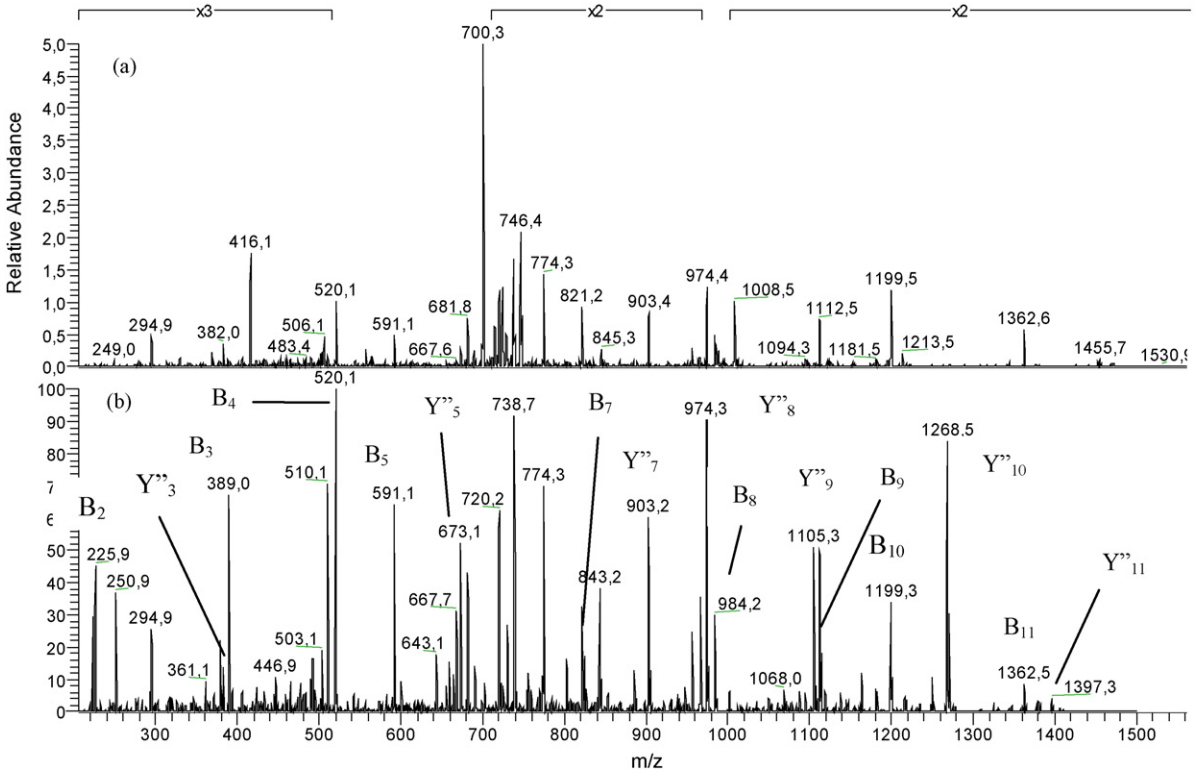


Fig. 3. MS^2-spectra of native ring-opened Maltacin D1c (a) and synthetic D1c (b). (CID of MH_2^{2+}, m/z 747.4). $(MH_2–H_2O)^{2+}$ (m/z 738.7), B_6 (m/z 720), B_7 (m/z 821), B_9 (m/z 1112), Y''_2 (m/z 295), Y''_4 (m/z 510) and Y''_6 (m/z 774). See also Scheme 1. See Scheme 2 for assignment of the ions in the spectrum of native Maltacin that are in accordance with the revised structures.

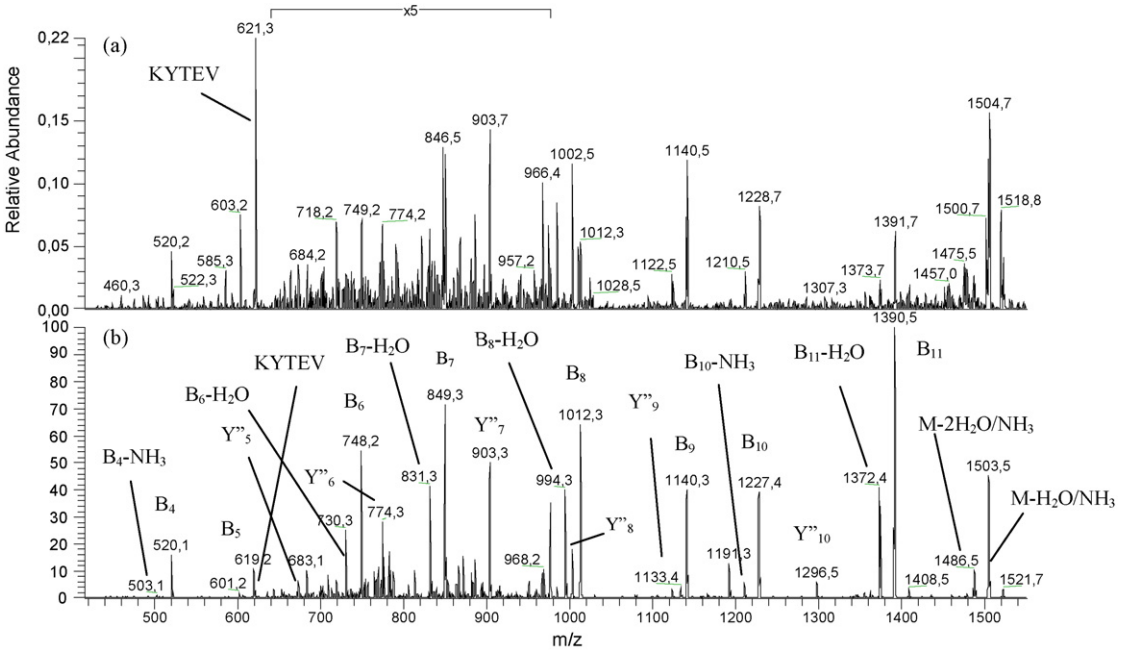


Fig. 4. MS^2-spectra of native ring-opened Maltacin E1b (a) and synthetic E1b (b). (CID of MH^+, *m/z* 1521.8). $B_{10}–2H_2O$ = *m/z* 1191.3. Y''_4 (*m/z* 510) is absent, Y''_5 (*m/z* 673), Y''_8 (*m/z* 1002), and Y''_{11} (*m/z* 1424) is absent. Due to the low mass cut-off of the ion-trap instrument, the product ion mass spectra were recorded down to *m/z* 400. See also Scheme 1. See Scheme 2 for assignment of the ions in the spectrum of native Maltacin that are in accordance with the revised structures.

M_r values, the spectra of synthetic and native D1c/E1b can be compared directly. The M_r of Fengycin IX acid is different from any of the Maltacines, so its product ion spectra cannot be compared directly with any of the Maltacines. Its structural relation to Maltacines must therefore be based on the comparison of the general fragmentation pattern. The product ion spectra of the ion at *m/z* 520 and the subsequent CID of the fragment at *m/z* 226 of all three peptides are shown in Figs. 6 and 7.

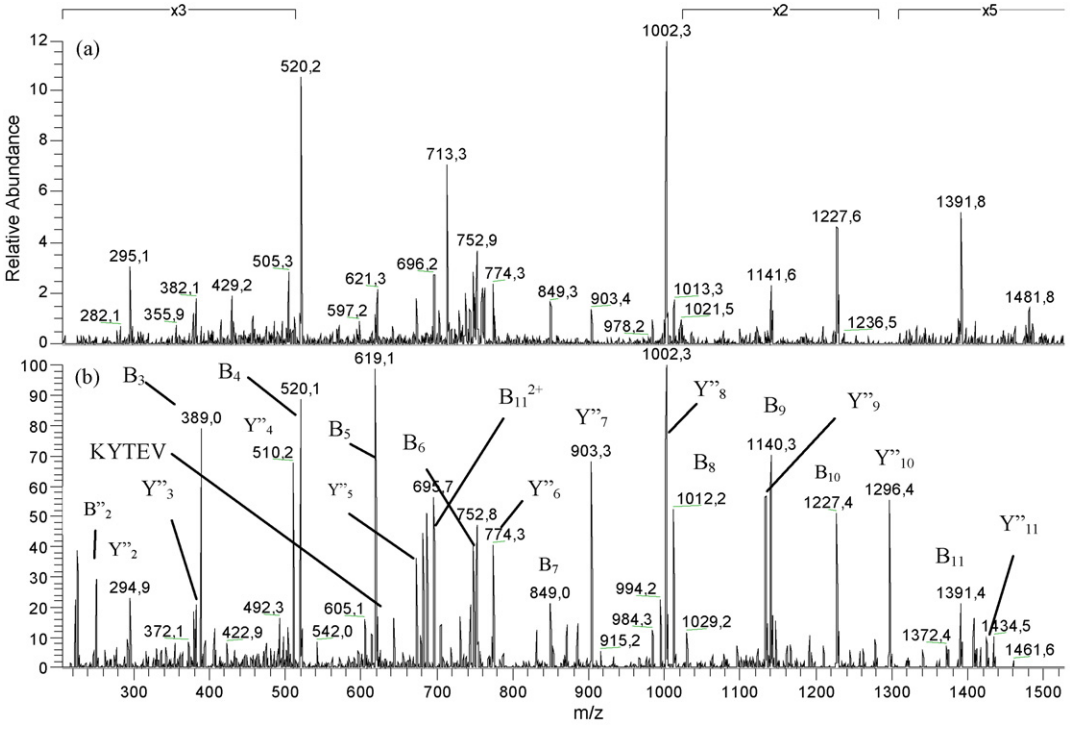
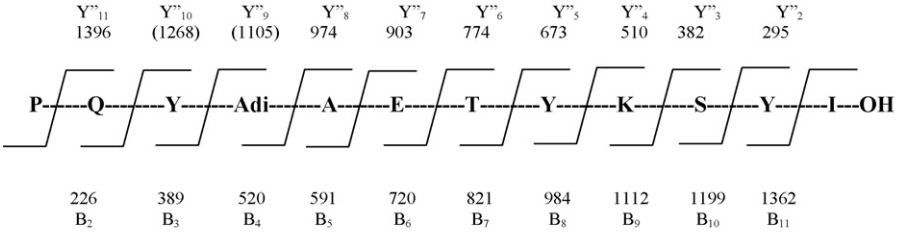


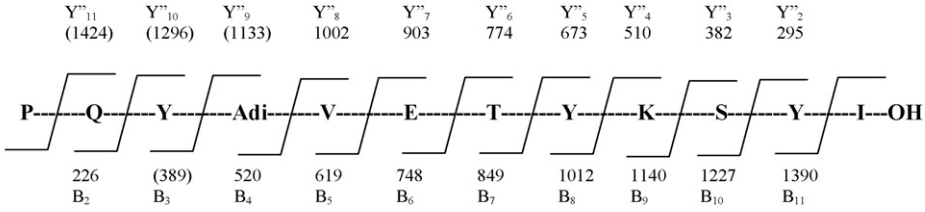
Fig. 5. MS^2 spectra of native ring-opened Maltacin E1b (a) and synthetic E1b (b). (CID of MH_2^{2+} at *m/z* 761.4) $B_{11}{}^{2+}$ (*m/z* 695.7), B_6 (*m/z* 748), Y''_5 (*m/z* 673), and Y''_9 (*m/z* 1133). See also Scheme 1. See Scheme 2 for assignment of the ions in the spectrum of native Maltacin that are in accordance with the revised structures.

(a) **Maltacin D1c, M_r=1492.8 Da.**



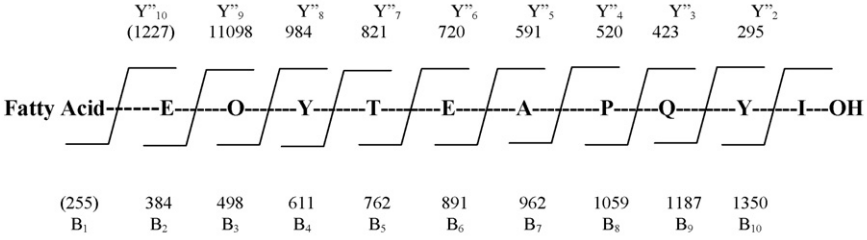
Internal fragments: m/z 295 $(Y\text{-}Adi)^+$, m/z 593 $(KYTEA)^+$ and m/z 575 $(KYTEA\text{-}H_2O)^+$.

(b) **Maltacin E1b, M_r=1520.8 Da.**



Internal fragments: m/z 295 $(Y\text{-}Adi)^+$, and m/z 621.3 $(KYTEV)^+$.

(c) **Linear sequence of Fengycin IX acid, M_r =1480.6 Da**



Internal fragments: m/z 579: $(O\text{-}Y\text{-}T\text{-}E\text{-}A^+)$; m/z 676: $(O\text{-}Y\text{-}T\text{-}E\text{-}A\text{-}P^+)$; m/z 853: $(Y\text{-}T\text{-}E\text{-}A\text{-}P\text{-}Q\text{-}Y^+)$ and m/z 295 (YI^+).

Scheme 1. Fragmentation scheme for synthetic Maltacin D1c (a), E1b (b) and Fengycin IX acid (c). Numbers in parentheses refer to ions not found in the MS^2 spectra of the native peptides.

3.2.1. Comparison of fragmentation patterns for native and synthetic linear D1c

MS^2 of MH^+ (CID of *m/z* 1493.8, Fig. 2). B ions: B_4–B_{11} are found in both spectra.

Y″ ions: both have Y''_4 (low abundant in both) to Y''_8 plus Y_{10}; Y''_9 is absent in the product ion spectrum of native D1c and is of very low abundance in the spectrum of synthetic D1c. *Loss of neutral molecules*: $(M–H_2O)^+$, $(M–2H_2O)^+$, $(B_{11}–H_2O)^+$, $(B_{10}–H_2O)^+$, $(B_9–H_2O)^+$ and $(B_8–H_2O)^+$ is observed for both peptides. *Internal fragments*: KYTEA is present in both spectra (low abundant in the spectrum of synthetic peptide). There is a relatively good match between the MS^2 spectra of the two peptides; most B_n and Y''_n ions are present, but ion abundances vary.

MS^2 of $(MH_2)^{2+}$ (Fig. 3). B ions: B_4–B_{11} is present in both spectra; B_2 and B_3 are present only in the spectra of the synthetic peptide. Y″ ions: Y''_2–Y''_8 are found in both spectra; Y''_9, Y''_{10} and Y''_{11} are lacking in the spectrum of native D1c. The spectrum of the native Maltacine does not show the B_2 and B_3 ions for the suggested N-terminal sequence PQY, as the spectrum of the synthetic analogue does. Likewise, the Y-Adi-A sequence is not covered by the homologues Y''_8, Y''_9 and Y''_{10}. This observation can indicate that the PQYAdi sequence is not at the N-terminus of native D1c as previously suggested [4].

Native D1c bears a stronger resemblance to Fengycin IX acid, but the presence of ornitine (residue mass 114) in position 2 is not found. Instead, the residue mass of 128 between Y''_8 and Y''_9 can indicate the presence of lysine in position 2.

3.2.2. Comparison of fragmentation patterns for native and synthetic E1b

MS^2 of MH^+ (CID of *m/z* 1521.8, Fig. 4). B ions: B_4–B_{11} are present in both spectra. Y″ ions: Y''_4 (*m/z* 510) is missing in the spectrum of synthetic E1b; Y''_5–Y''_8 are present in both spectra; Y''_9 and Y''_{10} are absent in the spectrum of native E1b and of low abundance in the spectrum of synthetic peptide. *Loss of neutral*

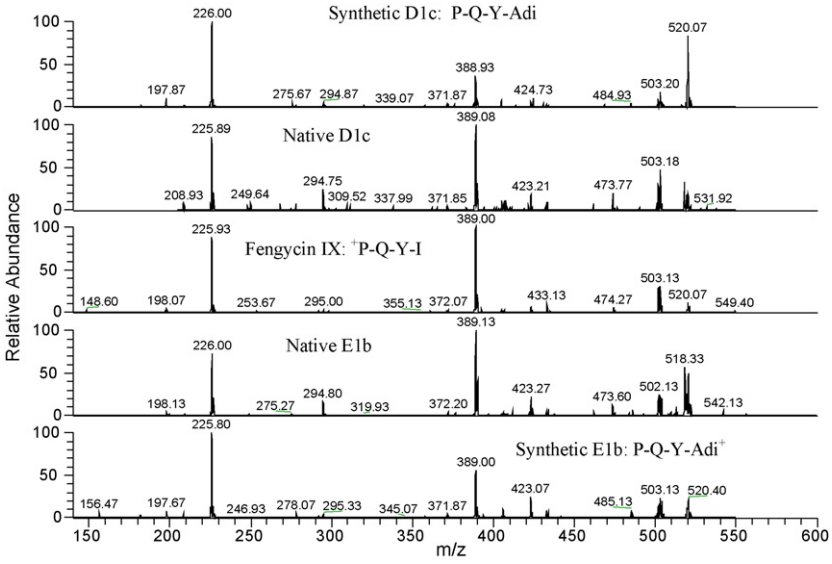


Fig. 6. MS^3 of synthetic and native D1c, E1b and synthetic Fengycin IX acid (CID of *m/z* 520).

molecules: $(M–H_2O/NH_3)^+$, $(M–2H_2O/NH_3)^+$, $(B_{11}–H_2O)^+$, $(B_{10}–NH_3)^+$, $(B_9–H_2O)^+$, $(B_7–H_2O)^+$ and $(Y''_8–H_2O)^+$ are found in both spectra. *Internal fragments*: KYTEV are present in both spectra (low abundant in the spectrum of synthetic peptide).

MS^2 of $(MH_2)^{2+}$ (Fig. 5). B ions: B_4–B_{11} are present in both spectra. B_2 and B_3 is found only in the spectrum of the synthetic E1b. Y″ ions: Y''_2–Y''_8 are present in both spectra; Y''_9 are found in both spectra (low abundant for the native peptide) and support the presence of a residue of 131 (Adi?) in position 4. Y''_{10} is present only in the spectrum of synthetic peptide. *Loss of neutral molecules*: only $(MH_2–NH_3)^{2+}$ is seen in the spectra of both peptides. *Internal fragments*: KYTEV is present in both spectra (low abundant in the spectrum of synthetic peptide).

Doubly charged ions: B_{11}^{2+} is seen in both spectra. Apart from the fact that B_3 and Y''_{10} are missing in the spectrum of native E1b, the match is relatively good for both B_n, Y''_n and the other ions.

3.2.3. Comparison of MS^3 (CID of m/z 520) and MS^4 (CID of m/z 226) for all peptides

MS^3 (CID of *m/z* 520, Fig. 6). All five spectra are almost identical, showing loss of NH_3 and H_2O (*m/z* 502/503) from the precursor ion. Likewise the ions at *m/z* 389 (B_3) and 226 (B_2) indicating loss of Adi and Y respectively from synthetic D1c/E1b, and loss of Y and I from Fengycin IX acid are present. *m/z* 295 is slightly more abundant in the spectra of

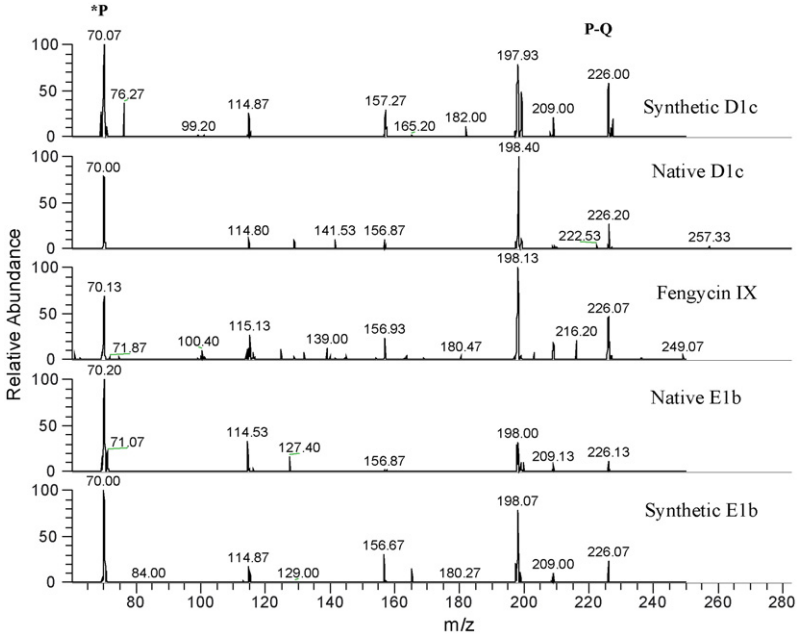


Fig. 7. MS^4 of synthetic and native D1c, E1b and Fengycin IX acid, respectively. (CID of *m/z* 226 of the dipeptides (P–Q, *m/z* 226) *P = immonium ion of proline.

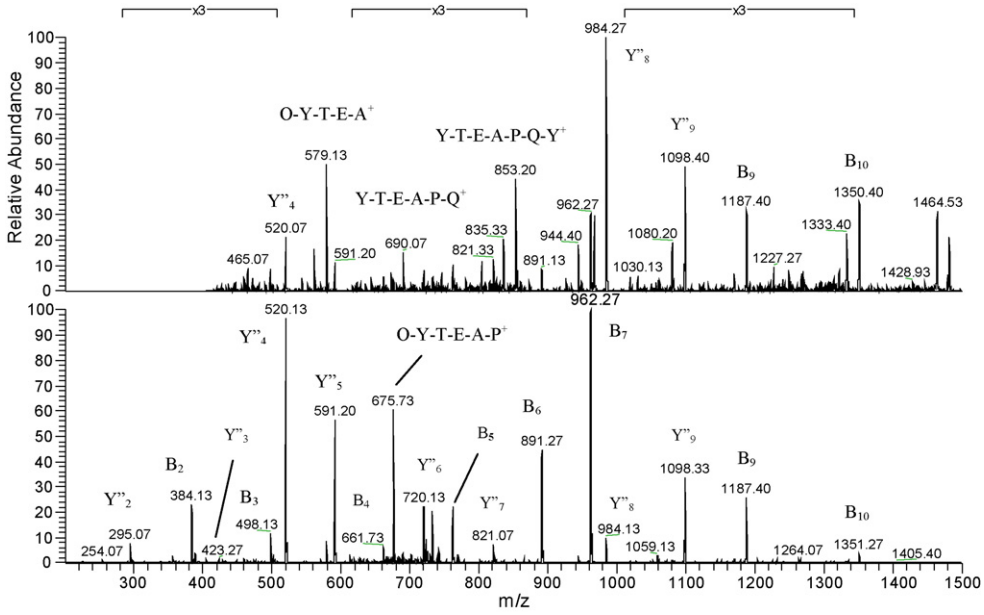


Fig. 8. MS^2 of L-Fengycin IX acid. CID of MH^+ *m/z* 1481.6 (top) and of $(MH_2)^{2+}$ *m/z* 741.3 (bottom). Due to the low mass cut-off of the ion-trap instrument, the product ion mass spectra of MH^+ were recorded down to *m/z* 400. See also Scheme 1c.

native D1c and E1b than in Fengycin IX, where it is of very low abundance.

MS^4 (CID of *m/z* 226, Fig. 7). The appearance of the spectra are almost identical for all five peptides, showing the prominent immonium ion of proline (*m/z* 70) and the immonium ion at *m/z* 198 due to loss of CO from the precursor ion. CID of *m/z* 520 and 226 shows that it is not possible to discern the two tetra-peptide fragments PQYAdi and PQYI, as they appear identical.

3.2.4. *Fragmentation pattern of* L-*Fengycin IX acid*

MS^2 of MH^+ (CID of *m/z* 1481.6) and *$(MH_2)^{2+}$* (CID of *m/z* 741.3) Fig. 8). B_n ions: B_2–B_{10} are present; Y'' ions: Y''_2–Y''_9 are present. *Internal fragments* $OYTEA^+$, $OYTEAP^+$, $YTEAPQ^+$ and $YTEAPQY^+$ are present. In the product ion spectrum of the doubly charged species, cleavage N-terminal to proline is the dominant fragment as expected.

4. Discussion

For comparison of the MS^n spectra it is important to bear in mind that the spectra of native Maltacines represents mixtures of two peptides with 1 Da in mass difference. The extra peptide with one mass unit higher was formed during the ring-opening reaction with NaOH and caused a de-amidation of glutamine to glutamic acid [2–5]. The combined isotopic clusters for these two peptides cause the A + 1 isotope in the B_n ion series to become the most abundant. The native Maltacines also contain D-amino acids in the sequence and are here compared with synthetic peptides composed of only L-amino acids that have consequences for the ion abundances. As natural Fengycin is not commercially available, it is not possible to obtain the absolutely correct product ion spectra that reflect the effect of D-amino acid substitution on the fragmentation pattern.

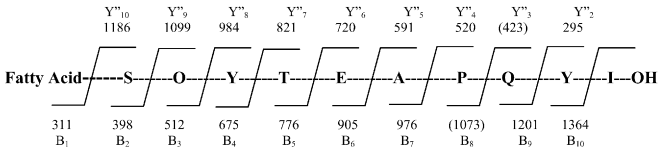
When using product ion mass spectrometry for identification purposes, an exact match of ions and their abundances is not necessarily attainable. For two peptides to be considered similar, a minimum criterion must be that the ions appearing in the spectra of the molecule in study also appear in the spectra of the synthetic standards. In addition, the abundance of ions is also of importance. The abundance of ions is under influence of the chiral sequence and conformation of the molecule [13], so if the chiral sequences are not identical, the ion abundances cannot be expected to be identical either. The greatest effect on the ion abundances would be expected in MS^2 spectra of either the singly or doubly charged species, which involves the whole molecule and less for MS^3 and MS^4 spectra that involves smaller peptide fragments that have fewer conformation states. This trend is indeed observed here for the five peptides under study.

Both electron capture dissociation (ECD) and CID have been used in the study of fragmentation of peptides containing D-amino acid substitutions [14,15]. According to these studies, it is the doubly charged protonated parent ions that exhibit the highest stereo chemical sensitivity [14]. The greatest impact of chiral sensitivity will then be expected for ions of low abundance, because these can be caused to enhance or to disappear from the product ion spectrum.

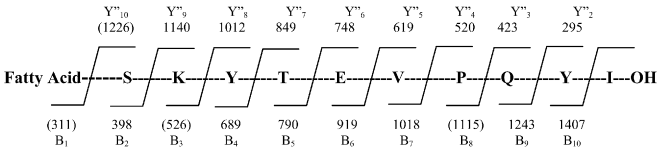
4.1. *Native versus synthetic D1c*

CID of the singly charged protonated molecule shows a relatively good match between synthetic and native peptides (Fig. 2). CID of the doubly charged molecular ion of the native peptide shows the absence of B_2 and B_3 for the suggested N-terminal sequence PQY (Fig. 3). This is in contrast to the spectra of the synthetic analogues, which showes B_2 and B_3. Likewise, evi-

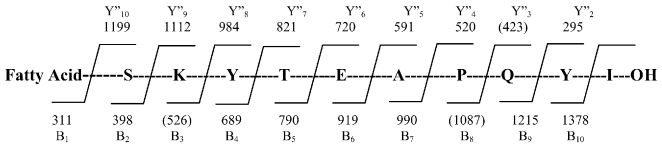
Revised amino acid sequence for linear Maltacin B1a and B2a, M_r=1494.7



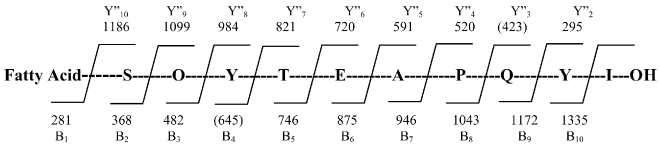
Revised amino acid sequence for linear Maltacin C1b and C2b, M_r=1536.9



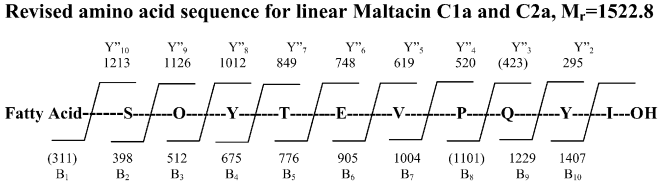
Revised amino acid sequence for linear Maltacin B1b and B2b, M_r=1508.8



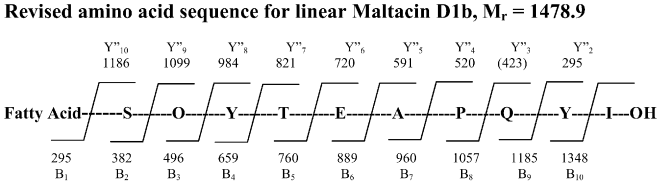
Revised amino acid sequence for linear Maltacin D1a, M_r = 1464.8



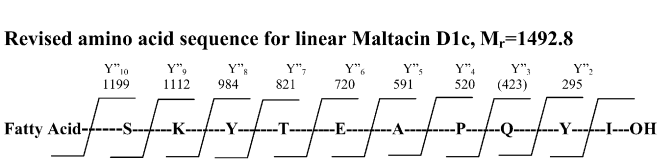
Revised amino acid sequence for linear Maltacin C1a and C2a, M_r=1522.8



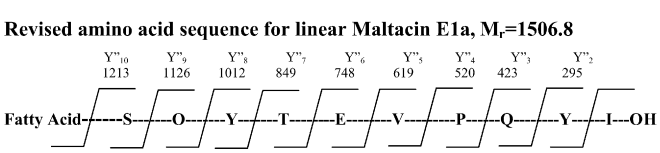
Revised amino acid sequence for linear Maltacin D1b, M_r = 1478.9



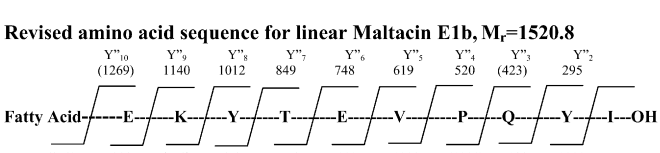
Revised amino acid sequence for linear Maltacin D1c, M_r=1492.8



Revised amino acid sequence for linear Maltacin E1a, M_r=1506.8



Revised amino acid sequence for linear Maltacin E1b, M_r=1520.8



Scheme 2. Revised amino acid sequences of all members of the Maltacin family. Numbers in parentheses shows ions not found in the MS^2 spectra.

dence is lacking for the presence of the N-terminal sequence PQYAdi of native D1c.

4.2. Native versus synthetic E1b

The suggested B_3, Y''_9 and Y''_{10}, are missing in the spectrum of native E1b, ruling out the existence of the sequence Y-Adi-V in the peptide. Apart from that, the match is relatively good for most ions (Figs. 4 and 5).

4.3. The structure of m/z 520 and 226

When it comes to the structure of the fragment at *m/z* 520, CID of this ion and its product ion at *m/z* 226 shows that it is not possible to discern the two tetra-peptide fragments PQYAdi and PQYI. They appear identical (Figs. 6 and 7).

4.4. Native D1c and E1b versus Fengycin IX acid

If the Maltacines have amino acid sequences identical to the Fengycins their C-terminal Y''_n sequence ions will be the same whereas the N-terminal end bearing the fatty acid will cause the B_n ions to shift as this is dependant on the mass of the fatty acid present. The amino acid sequences of Fengycins differ only in position 6 whereas the fatty acids vary. Fengycine IX has alanine in position 6, whereas Fengycin XII has valine in this position [11].

Comparison of the MS^2 spectra of native Maltacine D1c (Figs. 2 and 3) with Fengycin IX acid (Fig. 8) shows that the Y''_n ion series at *m/z* 295 (Y''_2), *m/z* 520 (Y''_4), *m/z* 591 (Y''_5), *m/z* 720 (Y''_6), *m/z* 821 (Y''_7), and *m/z* 984 (Y''_8) matching the sequence YTEAPQYI of Fengycin IX acid, is also found in Maltacin D1c. The expected mass difference for ornitine (residue mass

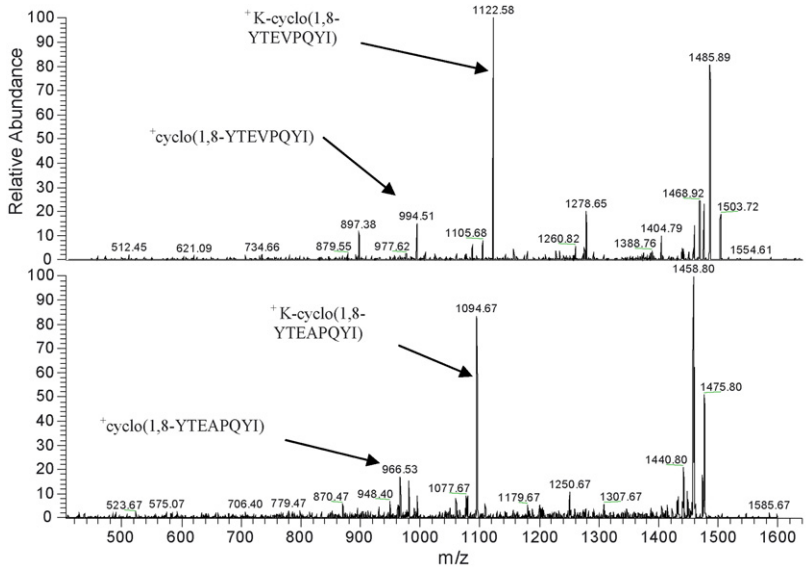


Fig. 9. MS^2 of cyclic Maltacin E1b (top) and D1c (bottom), showing the lacton structure to be between tyrosin[8] and isoleucine[1].

114) from Y''_8–Y''_9 (*m/z* 1098) is not present in the spectrum of Maltacin D1c. Instead the mass difference of lysine (128) is found at *m/z* 1112 (Y''_9) supporting the sequence KYTEAPQYI. The expected mass difference corresponding to glutamic acid is not found either. Instead the Y''_{10} at *m/z* 1199 corresponding to a serine residue is found to be the first amino acid in the sequence (Scheme 2).

Comparison of the MS^2 spectra of native Maltacine E1b (Figs. 4 and 5) with Fengycin IX acid (Fig. 8) shows that the Y″ ions at *m/z* 295 (Y''_2), *m/z* 520 (Y''_4), *m/z* 619 (Y''_5), *m/z* 748 (Y''_6), *m/z* 849 (Y''_7), and *m/z* 1012 (Y''_8) estimated for the expected sequence of Fengycin XII acid having valine in position 6 is also found in Maltacine E1b. The amino acid in position 2 (ornitine) in the Fengycin sequence is not found in Maltacin. Instead the Y''_9 *m/z* 1140 indicate a lysine residue as the second amino acid in the sequence (Scheme 2).

A central question is whether proline occupies an internal or external N-terminal position in the Maltacin peptide. The presence of uninterrupted series of B_n and Y''_n ion series, suggested an unknown residue with nominal mass 131 in position 4 (internal) which after CID appeared C-terminally in the fragment at *m/z* 520 interpreted as B_4 with a sequence PQYAdi$^+$. In this position the Adi-residue has a calculated residue mass 131.0579 that has been problematic to assign to a meaningful structure.

MS^3 of *m/z* 520 gives *m/z* 389 that corresponds to an accurate mass loss of 131.094. This has previously been determined by ESI-ICR-MS and ESI-QTOF-MS [5]. If *m/z* 520 is interpreted as Y''_4 (^+H_3N-Pro-Gln-Tyr-Ile-OH) coming from an internal position in the peptide, loss of 131.094, indicates the loss of isoleucine from the C-terminal position and not any unknown (Adi). The conclusion is that proline has an internal position as in Fengycin. This leads to the conclusion that the primary sequences of Maltacin from amino acid nos. 8–10 is the same as in Fengycin and that the peptide also contains 10 amino acids. No further evidence for more amino acids in the molecule is found. The big difference in chromatographic retention time between synthetic D1c/E1b (8.4 min/8.7 min) and native Maltacin [1] and Fengycin IX acid (both ca. 16 min), indicate that the Maltacines most probably contain a hitherto undetected fatty acid. Further support for the presence of a long chain alkyl group is found with the absorption band at 720 cm^{-1} in the IR-spectrum of Maltacin [1,16].

The internal position of proline could not be judged from the MS^3 and MS^4 experiments, which showed that both, internal and external positions were equally probable.

The mass loss of 131.094 from *m/z* 520, identifying the tetra peptide to be PQYI, and not PQYAdi, has been found for all the members of the Maltacin family and has thus consequences for interpretation of the product ion spectra of these. Scheme 2 shows the revised amino acid sequences for all the Maltacines.

The cyclic structure of the Maltacines is unaffected by the revision. This is shown by the MS^2 spectra of cyclic D1c and E1b, which shows the ions $^+$K-cyclo[1,8-YTEV(A)PQYI] and $^+$cyclo[1,8-YTEV(A)PQYI] of the two peptides (Fig. 9). See also Scheme 2.

5. Conclusion

The product ion spectra of synthetic Fengycin IX acid facilitated interpretation the *m/z* 520 ion appearing in the MS^2 spectra of Maltacines. Together with MS^3 and MS^4 spectra of *m/z* 520 and 226, respectively, and accurate determination of a mass loss of 131.095 Da from *m/z* 520, revealed the tetra peptide to be PQYI (which represent the C-terminal end of the molecule) and disclosed an internal position for proline, not at the N-terminus as first proposed. Evidence for the presence of a fatty acid in Maltacin is also found. The new revised amino acid sequences for the linear Maltacines are close to the sequence of Fengycin IX acid, but they differ in the last two N-terminal amino acids. New linear sequences for the Maltacines have been proposed.

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