Directed Biosynthesis of Peptaibol Antibiotics in Two *Trichoderma* Strains

II. Structure Elucidation

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α-Aminoisobutyric acid-directed biosynthesis in two *Trichoderma* strains has been shown to lead to the simplification of the natural peptaibol microheterogeneous mixtures and to the production of new analogues. Hence, two new peptides originating from *T. harzianum*, trichorzin PA_U 4 and harzianin PC_U 4, were isolated by HPLC. Their sequences were determined by positive liquid secondary-ion mass spectrometry (LSI MS). Trichorzin PA_U 4 and harzianin PC_U 4 are 18- and 14-residue peptaibols, respectively, both containing a high proportion of α-aminoisobutyric acid (Aib). LSI MS performed with lithium cationized peptides, allowed to assign the relative position of leucine/isoleucine isomeric residues, even without the use of tandem mass spectrometry.

In the preceding paper[1], we have described the effect of amino acid supplies on the peptaibol biosynthesis by two *Trichoderma* strains, *T. harzianum* and *T. longibrachiatum*. From the first strain, we have isolated two original peptides, trichorzin PA_U 4 and harzianin PC_U 4. These two peptides are almost exclusively synthesized by *T. harzianum* when grown on an Aib-supplemented fermentation medium, instead of the complex microheterogeneous mixtures obtained on the normal medium. Arising from a nonribosomal biosynthetic pathway,[2] peptaibols are linear peptides with α,α-dialkylated amino acids, such as α-aminoisobutyric acid (Aib, U), exhibiting an *N*-terminal acylated residue and a *C*-terminal amino alcohol. Based on their chain length and chemical characteristics, they are classified into three subclasses: the long-sequence peptaibols (18~20 residues)[3~5], the short-sequence peptaibols (11~16 residues)[5~8] and the lipopeptaibols (7 or 11 residues and an *N*-terminal lipid chain).[9,10]

In the present paper we report on the sequence determination by liquid secondary-ion mass spectrometry (LSI MS) of trichorzin PA_U 4 and harzianin PC_U 4 (Fig. 1), which belong to the long-sequence and the short-sequence peptaibol subclasses, respectively.

Results and Discussion

General Characteristics

HPLC analyses on a Supelcosil LC-18 column of the total acidic hydrolyzates, after derivatization of the given amino acids as phenylthiocarbamyl amino acids, led to the amino acid composition.[11] The results indicated harzianin PC_U 4 to contain Aib (5), Asx (1), Ile (1), Leu (2), Pro (3) and Ser (1) and PA_U 4 to be composed of Aib (8), Ala (1), Glx (2), Gly (1), Leu (2), Pro (1), Ser (1) and Val (1). Nevertheless, this technique did not

Fig. 1. Sequences of trichorzin PA_U 4 and harzianin PC_U 4 resulting from Aib-directed biosynthesis by *T. harzianum*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
PA_U 4	Ac	Aib	Ser	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln Trpol
PC_U 4	Ac	Aib	Asn	Leu	Aib	Pro	Ser	Ile	Aib	Pro	Aib	Leu	Aib	Pro	Valol			

Aib, U: α-aminoisobutyric acid, Trpol, Wol: tryptophanol; Valol, Vol: valinol.

allow to characterize the amino alcohol residues. They were assigned together with the absolute configuration of the constitutive residues by gas chromatography on a Chirasil-L-Val capillary column of the total acidic hydrolyzates after derivatization of the amino acids as *N*-trifluoroacetylisopropyl esters, and comparison with standards.[4,8] That indicated the presence of Valol and Trpol in PC_U 4 and PA_U 4, respectively, and the chirality of all the amino acids and amino alcohols to be L.

The presence of sharp singlets at ~2 ppm in the ^1H NMR spectra and the absence of reaction with ninhydrin, indicating no free NH_2-terminal group, suggested an acetylated *N*-terminal amino acid for harzianin PC_U 4 and trichorzin PA_U 4, as previously observed for other trichorzins and harzianins.[8,12] Moreover, the two L-Glx in PA_U 4 and the L-Asx in PC_U 4 were assigned to L-Gln and L-Asn respectively, from the absence of an acid function in the peptides.

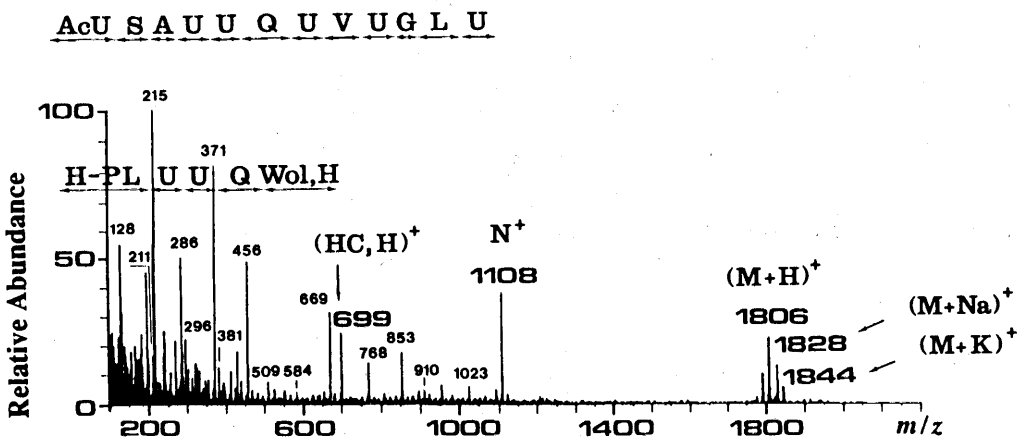
Positive LSI Mass Spectrometry

Sequences were determined by LSI mass spectrometry under two different experimental conditions, in the absence and in the presence of Li^+ ions added to the matrix. In the absence of Li^+ ions, analysis of continuous series of b_n acylium ions generated from peptaibol fragmentation leads to sequence determination, but difficulties arise from the presence of an Aib-Pro tertiary amide link frequently observed in these peptides.[13,14] Indeed, this amide bond generally undergoes a preferential cleavage generating a b_n type *N*-terminal acylium ion N^+ and a y_n type *C*-terminal ammonium ion $(HC, H)^+$. Both ions undergo subsequent fragmentations involving the superimposition of two independent b_n type ion series at lower masses. The first one, originating from the N^+ acylium ion, describes the *N*-terminal sequence, whilst the second one, initiated by the $(HC, H)^+$ ammonium ion, defines the *C*-terminal sequence. The more the peptides contain Aib-Pro motives, the more complex the spectra are.[8]

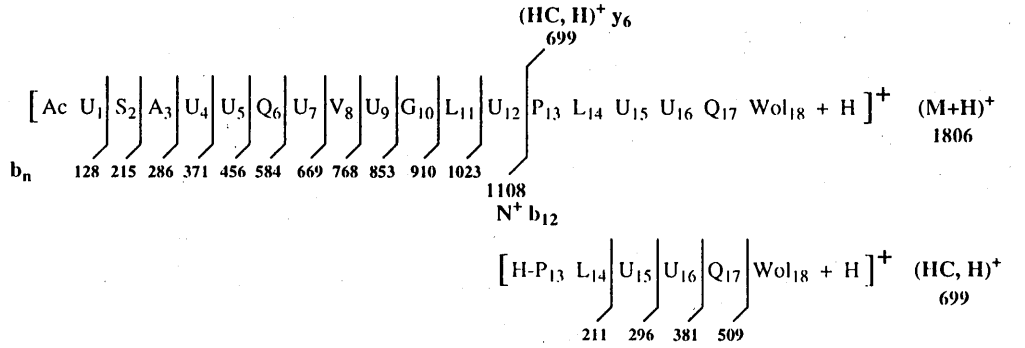
The LSI mass spectrum of PA_U 4 showed the $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$ molecular ion species at *m/z* 1806, 1828 and 1844 respectively, from which a molecular weight of 1805 Da was deduced, leading to the molecular formula $C_{85}H_{139}N_{21}O_{22}$ (Fig. 2-A). Moreover, the

Fig. 2.

A: LSI mass spectrum of trichorzin PA_U 4 exhibiting the main fragment ions formed in the preferential cleavage at the Aib-Pro bond, leading to the complementary N^+ and $(HC, H)^+$ ions.



B: Mass fragmentation pattern.



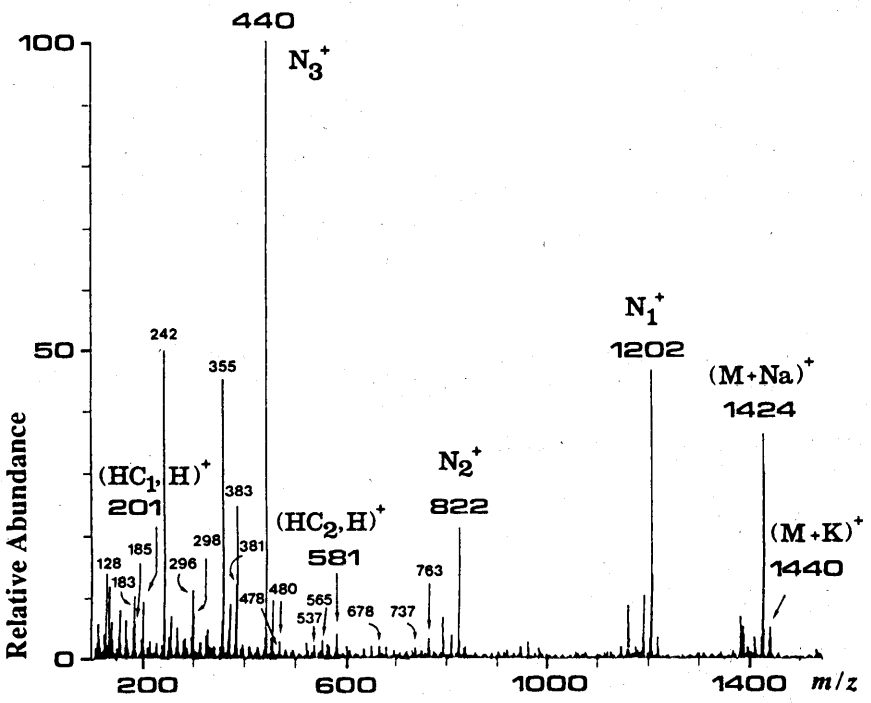
spectrum exhibited intense N^+ and $(HC, H)^+$ complementary ions at *m/z* 1108 and 699 respectively. As described above, subsequent fragmentations of these ions (Fig. 2-B) allowed the whole sequence to be specified, taking into account the amino acid composition previously determined.

Similarly, the LSI mass spectrum of PC_U 4 exhibited $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$ ions at *m/z* 1402, 1424 and 1440 respectively, indicating a molecular weight of 1401 Da and the molecular formula $C_{67}H_{115}N_{15}O_{17}$ (Fig. 3-A). Furthermore, three intense ions at *m/z* 1202, 822 and 440, suggested the formation of three b_n type *N*-terminal acylium ions, noted N_1^+, N_2^+ and N_3^+ which indicated the cleavage of three Aib-Pro amide bonds. The N_1^+ and N_2^+ ions were accompanied by their complementary *C*-terminal ions $(HC_1, H)^+$ and $(HC_2, H)^+$ at *m/z* 201 and *m/z* 581, respectively. These ions underwent subsequent fragmentations (Fig. 3-B), allowing the whole sequence to be specified.

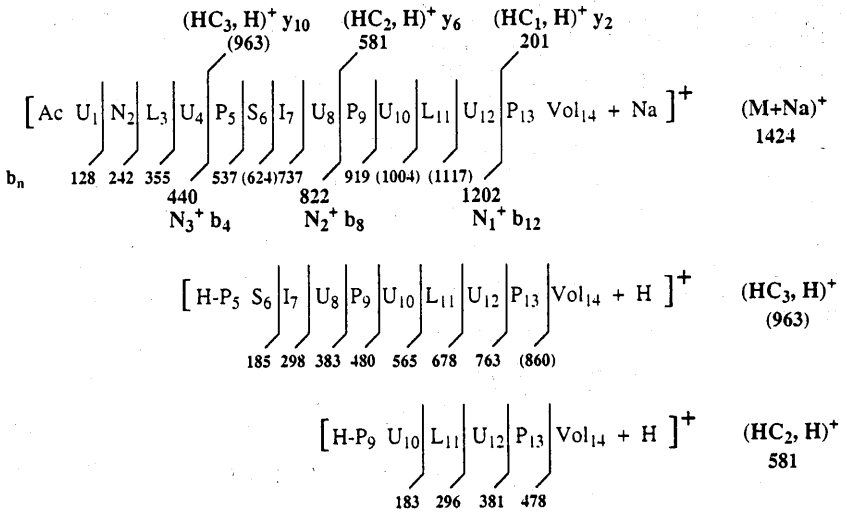
Unlike LSI mass spectra realized in standard condi-

Fig. 3.

A: LSI mass spectrum of harzianin PC_U 4 showing the main fragment ions formed in the three Aib-Pro bond cleavages.



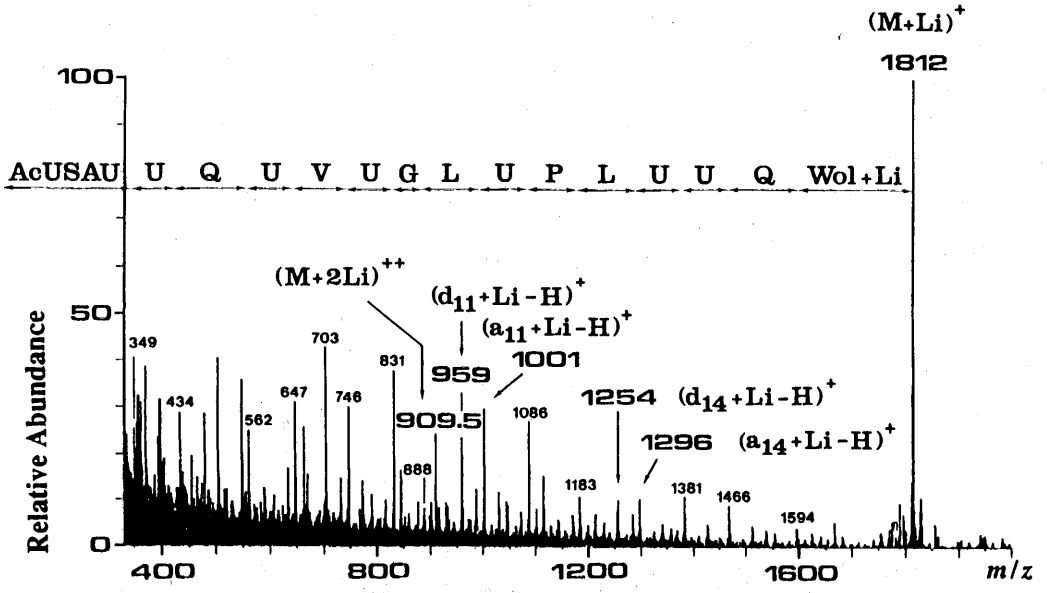
B: Mass fragmentation pattern.



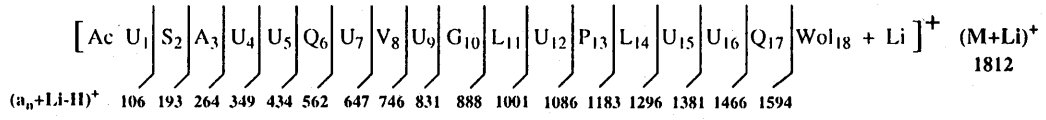
Ions in brackets were not observed.

Fig. 4.

A: LSI mass spectrum of trichorzin PA_U 4 under lithium cationisation showing mainly $(a_n+Li-H)^+$ and $(d_n+Li-H)^+$ sequence-specific ions.



B: Mass fragmentation pattern.



tions and characterized by superimposed acylium ion series, the mass spectra performed under lithium cationization reveal a single characteristic series of lithiated $(a_n+Li-H)^+$ ions leading to the sequencing of the studied peptide.[15] When the amino acid side chain may undergo a cleavage of the β-γ bond, these ions are accompanied by $(d_n+Li-H)^+$ ions, which allow in particular the identification and localisation of the isomeric residues Leu/Ile. Indeed, a $(d_n+Li-H)^+$ ion results from the loss of a radical from the *C*-terminal amino acid side chain of an $(a_n+Li)^+$ ion; the mass difference between a $(d_n+Li-H)^+$ and the respective $(a_n+Li-H)^+$ ion is characteristic of the residue (28 Da for isoleucine and 42 Da for leucine).[15,16] LSI mass spectra of PA_U 4 (Fig. 4-A) and PC_U 4 (Fig. 5-A) exhibited $(M+Li)^+$ adduct ions at *m/z* 1812 and 1408 respectively, confirming their molecular weights and molecular formulae. The presence of the complete series of $(a_n+Li-H)^+$ iminium ions afforded the sequences (Fig. 4-B, 5-B). The sequence of PA_U 4 which does not contain isomeric residues was unambiguously specified, whereas the relative position of one Ile and two Leu at positions 3, 7 and 11 in the sequence of PC_U 4 remained undetermined. Concerning PC_U 4, a 42-Da difference was observed for the ion couples $(a_3+Li-H)^+/(d_3+Li-H)^+$ and $(a_{11}+Li-H)^+/(d_{11}+Li-H)^+$ and a 28-Da difference for the $(a_7+Li-H)^+/(d_7+Li-H)^+$ couple, assigning leucines at positions 3 and 11, and isoleucine at position 7 (Fig. 5-A). In the case of PA_U 4, 42-Da differences were observed for the $(a_{11}+Li-H)^+/(d_{11}+Li-H)^+$ and $(a_{14}+Li-H)^+/(d_{14}+Li-H)^+$ couples (Fig. 4-A), confirming the presence of leucines at positions 11 and 14 and the absence of isoleucine in the sequence.

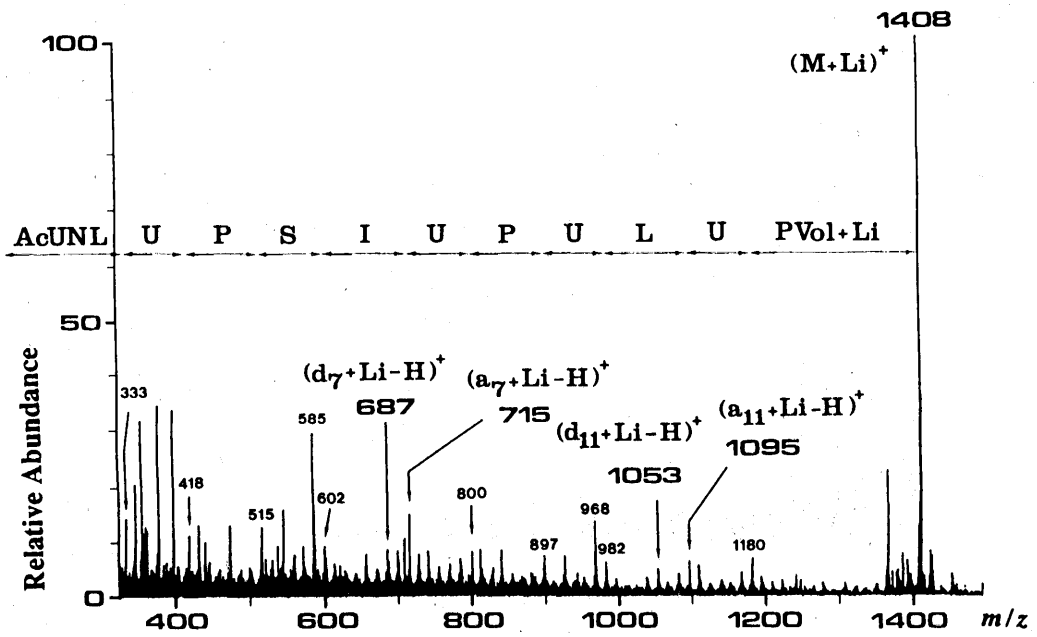
Materials and Methods

Amino Acid Analysis

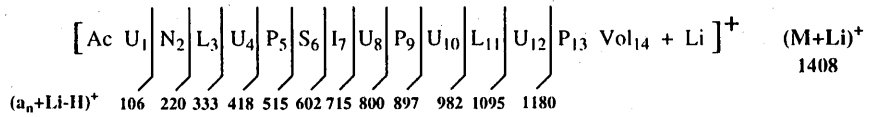
The peptides were hydrolyzed in standard conditions (1 mg, 6N HCl, 110°C, Ar, 24 hours). Amino acid composition was obtained by analysis of the phenylthiocarbamyl derivatives (PTC), as previously described.[11] They were separated on a LKB-Pharmacia HPLC system equipped with a Supelcosil LC-18 column (3 μ,

Fig. 5.

A: LSI mass spectrum of harzianin PC_U 4 under lithium cationisation showing mainly $(a_n+Li-H)^+$ and $(d_n+Li-H)^+$ sequence-specific ions.



B: Mass fragmentation pattern.



150 × 4.6 mm) and detected at 254 nm. The mobile phase consisted of: A, aqueous sodium acetate buffer (0.14 M, adjusted to pH 5.00 with acetic acid); B, water-acetonitrile (40 : 60). The following gradient was used: from 8% B to 40% B in 30 minutes, 80% B at 45 minutes, at a flow rate of 700 μl/minute and an oven temperature of 40°C. Retention times (minute): Aib 31.4, Ala 10.2, Asp 5.0, Glu 5.8, Gly 6.9, Ile 27.0, Leu 27.6, Pro 11.0, Ser 6.2 and Val 20.5.

Amino acid absolute configurations were determined by GC analysis. Hydrolysis was followed by derivatization of the given amino acids and amino alcohol, as previously described.[4,8] Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standards. For Trpol analysis, a special hydrolysis procedure was necessary to prevent its decomposition, using 3 N mercaptoethanesulfonic acid instead of 6 N HCl.[12] The GC analyses were realized on a Hewlett Packard serie II 5890 gas chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.) with He (525 mmHg) as carrier gas and a temperature programme: 50 ~ 130°C, 3°C/minute; 130 ~ 190°C, 10°C/minute. Retention times (minute) (separation factor $\alpha_{L/D}$ for the D,L enantiomers): Aib 5.3, L-Ala 7.8 ($\alpha=1.13$), L-Asp 19.8 ($\alpha=1.02$), L-Glu 25.5 ($\alpha=1.03$), Gly 10.3, L-Ile 12.3 ($\alpha=1.10$), L-Leu 14.8 ($\alpha=1.11$), D,L-Pro 12.6, L-Ser 14.2 ($\alpha=1.07$), L-Val 10.3 ($\alpha=1.14$) and L-Valol 19.3 ($\alpha=0.96$). A different temperature programme was used for the separation of Gly and L-Val and of the proline D,L enantiomers: plateau at 50°C for 15 minutes; 50 ~ 190°C, 10°C/minute; Gly 18.9, L-Val 18.4 ($\alpha=1.14$) and L-Pro 20.1 ($\alpha=1.01$). Similarly, a special programme temperature was used for Trpol analysis: 130 ~ 190°C, 10°C/minute; plateau at 190°C for 20 minute; L-Trpol 31.6 ($\alpha=0.99$).

LSI Mass Spectrometry

Positive LSI mass spectra were recorded on a ZAB2-SEQ (VG Analytical, Manchester, UK) mass spec-

trometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. The peptide methanolic solution was mixed into 3-nitrobenzyl alcohol as matrix or into a matrix consisting of a satured solution of lithium chloride in 3-nitrobenzyl alcohol, in order to realize LSI mass spectrometry experiment on the lithium cationized peptide. The resolution was 2000.

LSI mass fragmentations of PA_U 4 in the absence of Li^+ ions, *m/z* (relative abundance): 1844 (5, $(M+K)^+$), 1828 (12, $(M+Na)^+$), 1806 (22, $(M+H)^+$), fragment ions: 1108 (38), 1023 (5), 910 (3), 853 (17), 768 (14), 699 (24), 669 (31), 584 (3), 509 (7), 456 (48), 381 (12), 371 (81), 296 (22), 286 (50), 215 (100), 211 (17) and 128 (55). LSI mass fragmentations of PA_U 4 under lithium cationization, *m/z* (relative abundance): 1812 (100, $(M+Li)^+$), 909.5 (25, $(M+2Li)^{++}$), fragment ions: 1594 (3), 1466 (10), 1381 (12), 1296 (10), 1254 (9), 1183 (10), 1086 (26), 1001 (30), 959 (33), 888 (9), 831 (38), 746 (30), 703 (43, $(y6+Li-3H)^+$), 647 (31), 562 (25), 434 (28), 349 (25), 264 (33), 193 (36) and 106 (50).

LSI mass fragmentations of PC_U 4 in the absence of Li^+ ions, *m/z* (relative abundance): 1440 (5, $(M+K)^+$), 1424 (36, $(M+Na)^+$), 1402 (1, $(M+H)^+$), fragment ions: 1202 (46), 919 (1), 822 (21), 763 (3), 737 (1), 678 (2), 581 (4), 565 (2), 537 (2), 480 (2), 478 (1), 440 (100), 383 (24), 381 (12), 355 (45), 298 (7), 296 (11), 242 (50), 201 (9), 185 (6), 183 (10) and 128 (14). LSI mass fragmentations of PC_U 4 under lithium cationization, *m/z* (relative abundance): 1408 (100, $(M+Li)^+$), fragment ions: 1180 (5), 1095 (6), 1053 (5), 982 (6), 968 (15, $(y10+Li-3H)^+$), 897 (5), 800 (8), 715 (15), 687 (6), 602 (7), 585 (30, $(y6+Li-3H)^+$), 515 (8), 418 (11), 333 (14), 291 (13), 220 (15), 205 (18, $(y2+Li-3H)^+$) and 106 (17).

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