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Spermidine as a potential biosynthetic precursor to the 1,5-diazabicyclo[4:3:0]nonene residue in the efrapeptins

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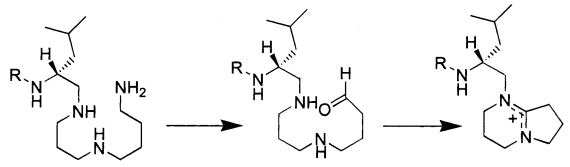
Abstract: Efrapeptins are a group of microheterogeneous polypeptide antibiotics produced by the fungus *Tolypocladium niveum*, which are potent inhibitors of mitochondrial F_1-ATPase. Efrapeptins contain an unusual 1,5-diazabicyclo[4:3:0]nonene (DBN) residue at the C-terminus. This study is driven by the hypothesis that the DBN residue could, in principle, arise by oxidative cyclization of a spermidine moiety. Electrospray ionization mass spectrometry of the peptide antibiotics 'elvapeptins' from *T. niveum* establishes the presence of a C-terminal spermidine residue. Conversion of elvapeptins to efrapeptins by CuCl/pyridine demonstrates the transformation of the spermidine residue to the 1,5-diazabicyclo[4:3:0]nonene system by oxidative cyclization.

Efrapeptins (1–3) (Fig. 1) are a group of microheterogeneous polypeptide antibiotics produced by the fungus *Tolypocladium niveum*, which are potent inhibitors of mitochondrial F_1-ATPase (4–6). The efrapeptins are related to the alamethicin family of peptide antibiotics (7–9), possessing a high number of α-aminoisobutyryl (Aib) residues (10). The major difference between the efrapeptins and the peptaibols (11) is that the former contain an unusual 1,5-diazabicyclo[4:3:0]-nonene (DBN) residue at the C-terminus (2,3), whereas the latter possess a C-terminal aminoalcohol (8). Relatively little is known about the biosynthetic origin of the DBN moiety of the efrapeptins. In an early study, Bullough *et al.* (12) reported the isolation of another microheterogeneous mixture of basic polypeptides from the cultures of *T. niveum*, which differ in mass from the efrapeptins by 20 Da and possess the same peptide component as efrapeptins. Our studies were driven by the hypothesis

C. Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Aib-X (1606)
D. Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X (1620)
E. Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X (1634)
F. Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X (1648)
G. Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X (1662)

Figure 1. Sequence of efrapeptins C-G[2,3]. Their corresponding molecular masses are indicated in parentheses. The underlined residues indicate the sites of replacement leading to the microheterogeneity. Aib, α-aminoisobutyric acid; Iva, *S*-isovaline; Pip, L-pipecolic acid; β-Ala, β-alanine, X=condensation product of L-leucinol and 1,5-diazabicyclo[4 : 3 : 0]nonene.



R = Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Aib

Figure 2. Plausible biosynthetic route to 1,5-diazabicyclo[4:3:0]nonene from spermidine.

that the DBN residue could, in principle, arise by oxidative cyclization of a spermidine moiety (Fig. 2). In this report we demonstrate by mass spectrometry that the elvapeptins produced by the fungal cultures contain a C-terminal spermidine and that conversion of elvapeptins to efrapeptins can be achieved by oxidative cyclization in the presence of CuCl and pyridine.

Materials and Methods

Isolation and purification of efrapeptins

The fungus *T. niveum* (*Beauveria nivea*) obtained from ATCC (USA) was maintained by subculturing on agar slopes containing an oatmeal medium. The slants were grown for 3 days at 25°C and then stored at 4°C. The oatmeal medium used for the maintenance of the fungus is as follows: 6.00% oatmeal (baby), 0.25% yeast extract, 0.10% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% $FeSO_4{\cdot}7H_2O$ and 1.30% agar. The fungus was fermented for 26 days to obtain efrapeptins. The medium used for fermentation was 2.5% glucose, 1.0% meat peptone, 0.4% tryptone and 0.5% $MgSO_4{\cdot}7H_2O$. The seed culture (200 mL) was grown for 2 days in the fermentation medium and then subsequently inoculated into 3.2 L of the sane medium. After fermentation period, the medium was spun at 90×***g*** for 45 min in a Sorvall RC-5B refrigerated superspeed centrifuge and the supernatant was extracted with chloroform. The solvent was evaporated under reduced pressure and the crude material was purified on a reverse-phase C_4 column. Elvapeptins were isolated after 5 days of fermentation and separated from efrapeptins by extracting the supernatant at pH 11 and used without further purification.

Oxidation of elvapeptins to efrapeptins and acetylation of elvapeptins

The chloroform extract obtained after 5–7 days of fermentation of the culture medium was rich in a heterogeneous mixture of elvapeptins. The crude mixture obtained after solvent evaporation was stirred with CuCl in pyridine at room temperature, and the oxidation reaction was monitored using electrospray ionization mass spectrometry (ESI-MS). A small portion of the reaction mixture was diluted with water, extracted with chloroform and the extract was successively washed with dilute HCl and water. The solvent was evaporated under reduced pressure and the crude product was analyzed by ESI-MS without purification. Acetylation of elvapeptins was carried out by stirring the elvapeptins in a 1 : 1 mixture of acetic anhydride and pyridine at room temperature overnight and the product was analyzed by ESI-MS.

Mass spectrometry

All electrospray spectra were recorded in the positive ion detection mode on a Hewlett Packard 1100 MSD model electrospray mass spectrometer equipped with a single quadrupole and a conventional electrospray source. Distilled methanol was used as the running solvent. The solvent flow rate was 30 μL/min. Approximately 100–200 pmol of sample was injected each time for analysis. The source temperature was kept at 300°C. Ions were extracted with an orifice potential of 4000 V applied at the nebulizer tip. Ionization was pneumatically assisted using a constant flow of pure N_2 gas at 10 L/min using a Whatman nitrogen generator. The same gas was used to maintain the nebulizer pressure at 10 bar. The nozzle skimmer potential was kept at 20 V. The mass spectrometer was calibrated using five *m/z* ions supplied by the manufacturer across the mass range 118–2500 *m/z*. The chromatographic peak width for operation was 0.18 min. The cycle time was 1.90 s/cycle and the time filter was kept on. Data were acquired in the scan mode for 2 min and averaged over the complete

chromatogram to obtain the mass spectrum. The charge states were determined using the isotopic distribution of the peaks.

Results and Discussion

Figure 3 shows an electrospray ionisation mass spectrum (ESI-MS) of a mixture of efrapeptins isolated from fungal cultures (13). As many as seven peptide components differing in mass from their neighbors by 14 Da can be seen. The microheterogeneity arises due to the low fidelity of nonribosomal polypeptide synthesis (14,15), resulting in the incorporation of Gly, Ala, Aib or Iva residues at specific positions, leading to sequences which differ in mass from the homologous neighbors by 14 Da. While five efrapeptins (C–G) have been previously identified (3), two additional components have been observed in this study. Figure 4A shows an electrospray mass spectrum of elvapeptin mixture. The peaks (M^+) at 1620 and 1634 Da correspond to a small amount of contaminating efrapeptins. The elvapeptin masses detected are (MH^+) 1641, 1655 and 1669 Da. Two features of the elvapeptins mass spectrum merit special mention. (i) For elvapeptins MH^+ peaks are observed, in contrast to the efrapeptins in which only M^+ peaks are observed, because the DBN group exists as a positively charged quarternary salt. (ii) The mass spectrum exhibits peaks at 1653 and 1667, which differ by 2 Da from that expected for elvapeptins. Presuming a C-terminus spermidine residue, it is possible that the presence of oxidized products (Fig. 2) could lead to species which differ in mass by 2 Da. Schiff base formation by intramolecular cyclization could lead to species differing by 1 Da. The apparent complexity of the mass spectra of the isolated elvapeptins is a consequence of microheterogeneity, the presence of peaks due to partially oxidized species superimposed on the isotopic peaks of the parent peptides.

The ESI mass spectrum of the acetylated elvapeptins showed clearly that three acetyl groups (ΔM=126 Da) are added to the peptide components of the elvapeptin mixture (1641+126=1767 and [M+Na] at 1789, 1655+126=1781 and [M+Na] at 1803, 1669+126=1795 and [M+Na] at 1817). The observation of three sites for acetylation is fully consistent with the presence of spermidine as the C-terminal residue in elvapeptins.

The conversion of primary amines to aldehydes has been readily accomplished using CuCl/pyridine (16,17). We therefore attempted the *in vitro* conversion of elvapeptins to efrapeptins. Figure 4B shows the mass spectrum obtained after 48 h of oxidation. Clearly at the end of 48 h, transformation is almost complete and only efrapeptins are detectable (peaks at 1606, 1620, 1634 and 1648). These results strongly suggest that elvapeptins are biosynthetically related to the efrapeptins and that the C-terminal DBN residue is derived from spermidine. Several instances already exist for spermidine conjugated natural products

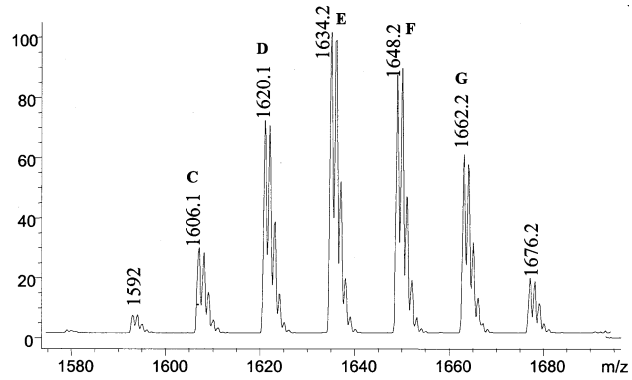


Figure 3. ESI mass spectrum of a heterogeneous mixture of efrapeptins.

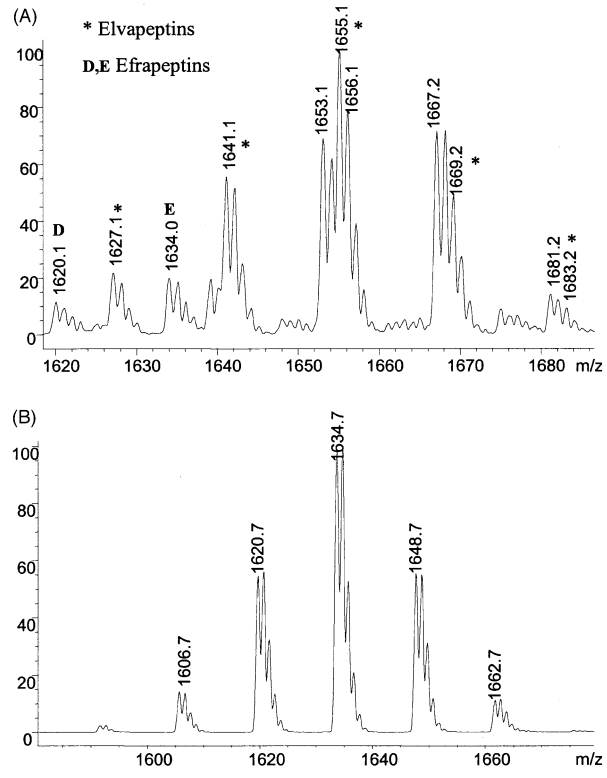


Figure 4. ESI mass spectra of a heterogeneous mixture of elvapeptins *(A)* and elvapeptins after 48 h of oxidation by CuCl/pyridine *(B)*.

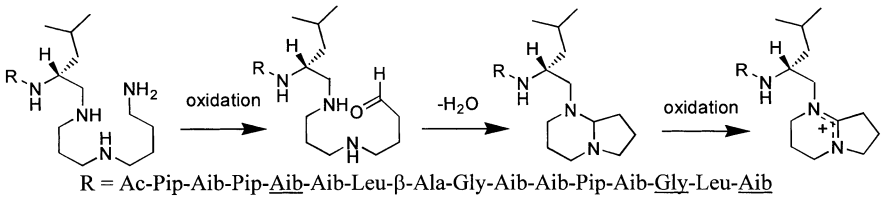


Figure 5. Proposed mechanism for the *in vitro* conversion of elvapeptins to efrapeptins.

inclusive of steroids like squalamine (18) and antibiotics like bleomycin-A_5 and edeine-A_1 (19). Precedence for conversion of spermidine to 1,5-diazabicyclo[4:3:0]nonane exists. Weaver *et al.* (20) used cells of *Neisseria perflava* grown in spermine rich medium to successfully oxidize spermidine. Later, after several years, the oxidation product so obtained (both from enzymatic and chemical oxidation) was established to be 1,5-diazabicyclo[4:3:0]nonane (21).

The experiments reported here unambiguously confirm the chemical and biosynthetic relationship between elvapeptins and efrapeptins and provide firm support for the assignment of the C-terminal residue of elvapeptins as spermidine. A plausible mechanistic route for the conversion of spermidine to 1,5-diazabicyclo[4:3:0]nonene is shown in Fig. 5.

It must be stressed that although condensation of the initially formed aldehyde yields 1,5-diazabicyclononane, further hydrogen abstraction (oxidation) is necessary to yield the 1,5-diazabicyclo[4:3:0]nonene moiety of the efrapeptins. Under the conditions used further oxidation is undoubtedly possible.

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