Structure Analysis of Unsaturated Polymyxin E Components Based on High Performance Liquid Chromatography – Quadrupole/ Time of Flight Tandem Mass Spectrometry and Photochemical Reaction

Hanzhi Zhang[1, 2], Zhenhua Tian[1], Hao Liu[2]

[1] Abiochem Biotechnology Co., Ltd, Shanghai 200003, China

[2] Department of Antibiotics and Microorganisms, Shanghai Institute for Food and Drug Control, Shanghai 201203, China

Corresponding Author

Hanzhi Zhang, Abiochem Biotechnology Co., Ltd, Shanghai, 1299 Ziyue Road, Minhang District, Shanghai 200003, China. E-mail: zhanghanzhi@abiochem.com, zzzw-123@163.com, Tel: +86 021-61453909, Fax: +86 021-61453909.

Hanzhi Zhang, ORCID id: 0000-0003-3672-7503, Email: zhanghanzhi@abiochem.com

Hao Liu: ORCID id: 0000-0001-6235-0514, Email: liuhao1968@hotmail.com

Abstract ***Background*:** Polymyxin E (PME), which is a complex of cationic cyclic lipodecapeptides, is used to treat multidrug-resistant gram-negative bacterial infections. Besides the main components PME1 and PME2, polymyxin containing unsaturated

fatty acyl (FA) group with lower contents is hardly to determine the structure without chromatographic preparations and NMR.

Introduction**:** The peptide sequences of PME components has been carried out based on high performance liquid chromatography-quadrupole / time-of-flight mass spectrometry (HPLC-Q/TOF-MS). However, the components with double bond on the FA, such as 2’, 3’-dehydro PME1, were difficult to be determined or easily misjudged by MS/MS. The transformation of such unsaturated components to be epoxidized components or di-hydroxylated components can promote the acquisition of more fragmentation ions in the MS/MS, so as to assist in judging the position of double bonds on FA.

Methods: In this paper, the PME mixtures were dissolved in an equal proportion of 20% ACN aqueous solution and 2-acetylpyridine. The above PME solution was transferred to a quartz cuvette and irradiated with the ultraviolet lamp at 254 nm for 8h. The dehydro PME components were converted to be epoxy PMEs and dihydroxy PMEs. A fragmentation pathway of epoxidized components or di-hydroxylated components based on Q/TOF-MS/MS was proposed for the first time.

Results: According to the characteristic ions of epoxidized components and di-hydroxylated components, 2’, 3’-epoxy PME1/E2 and 2’, 3’-dihydroxy PME1/E2 were confirmed. It can be inferred that the double bond is located at the 2’, 3’-position of FA.

Conclusion: The structure of unsaturated PME component with double bond on the FA is elucidated by HPLC-Q/TOF-MS combined with photochemical reaction. This strategy is applicable to other lipopeptides containing unsaturated FA chain.

Keywords: 2’, 3’-dehydro PME; 2’, 3’-epoxy PME; 2’, 3’-dihydroxy PME; HPLC-Q/TOF-MS; Photochemical reaction

51 ## 1. INTRODUCTION

52 Antibiotic resistance has become a significant threat to human health across the world
53 [1]. Currently, the polymyxins like polymyxin E (PME, also known as Colistin) are
54 reused in the clinical treatment and viewed as the last resort for the available
55 therapeutic option against multidrug resistant Gram-negative bacterial infections [2-3].
56 Furthermore, some new polymyxin analogues were designed and showed good
57 antibacterial effect [4-5]. However, polymyxin E is a mixture of cyclic
58 lipodecapeptides with similar and relatively complex structures. It is necessary to be
59 aware of the structures of each component, especially the unknown one [6-9]. The
60 general structures of polymyxin E components are composed of a heptapeptide ring
61 and a side-chain consisting of a tripeptide with a fatty acyl (FA) residue on the
62 *N*-terminus. The structures of main components like PME1 and PME2 (shown in Fig.
63 1) were clearly, they differ with each other on the FA as the former owns a
64 6'-methyloctanoic acid (6'-MOA, R_1) and the latter with a 6'-methylheptanoic acid
65 (6'-MPA, R_2). The amino acid at position 7 of PME1 and PME2 is leucine (Leu),
66 which is generally changed to be isoleucine (Ile) to form the PME1-I and PME2-I.
67 This substitution of amino acid (Leu to Ile) is common in other components to judge
68 the isomers of the known compounds.

69 The differences of the components in amino acid or FA (branched or straight
70 chain) make their structural elucidation more complicated. Many attempts have been
71 made to analyze and characterize the components in commercially available PME
72 complex. European Pharmacopoeia (Ph. Eur.) 10.0 adopts the HPLC-UV method
73 developed by Orwa et al. based on the nonvolatile liquid phase contained sodium
74 sulphate solution and acetonitrile to analyze the components and related substances in
75 PME [10]. Classical amino acid and FA analysis combined with partial hydrolysis or
76 enzymatic hydrolysis and Edman degradation after purification of the main
77 component were able to elucidate the peptide sequences [11-13]. In further, the

78 on-line HPLC-fast atom bombardment (FAB) and electrospray ionization (ESI) mass
79 spectrometer (MS) was available to characterize the structures of PMEs [6-8, 14]. The
80 nuclear magnetic resonance (NMR) was also used to identify the structures of PME,
81 where the FA and the isomers of amino acids cannot be confirmed by MS [7].

82 In our previous studies, HPLC-Q/TOF-MS/MS is used as a powerful tool for
83 structural elucidation of components in polymyxin B and PME [9, 15-16]. A vinyl
84 polymyxin B1 named 2’, 3’-dehydro PMB1 was first characterized by
85 chromatographic purification, high resolution MS and NMR [16]. The similar
86 component with unsaturated FA was also found in polymyxin E to be 2’, 3’-dehydro
87 PME1 (shown in Fig. 1, the FA is R_3) and is also controlled in the monograph of Ph.
88 Eur. 10.0. Besides the dehydro PMB1 and PME1, other unsaturated components like
89 dehydro PMB2 and PME2 were also found. But those components with lower
90 contents (<0.1%) are hard to purify and the structures were easily to be determined
91 wrongly without NMR [9, 15]. Xia et al developed a series of Paterǹo-Büchi (PB)
92 reactions with acetone to judge the position of double bond in lipids [17-18]. It has
93 provided a resolution to elucidate the unsaturated polymyxins by photochemical
94 reaction without chromatographic preparations. Although the oxetane products of
95 unsaturated PME1 reacted with acetone were detected in the MS, the ring opening ions
96 due to the reverse reaction in the MS/MS were not found. The double bond of
97 unsaturated PME1 was misjudged to be at position 7’ and 8’ based on the fragmentation
98 ions contained oxetane groups [15].

99 In this study, the double bond positions of unsaturated PME1 and PME2 were
100 elucidated by photochemical reaction combined with HPLC-Q/TOF-MS/MS. The
101 epoxidized and di-hydroxylated products of unsaturated PME1 and PME2 (shown in
102 Fig. 1, the FA is R_4), which were named as 2’, 3’-epoxy PME1/E2, 2’, 3’-dihydroxy
103 PME1/E2, were easily generated by UV irradiation at 254 nm. The characteristic ions

104 of epoxidized and di-hydroxylated products were analyzed by MS/MS, which proved
105 that the double bond was located at the 2’, 3’ position of FA chain.

106 2. MATERIALS AND METHODS

107 2.1. Chemicals and Reagents

108 Polymyxin E sulphate for injection (batch No. 190501) was provided by Shanghai
109 SPH New Asia Pharmaceutical co., LTD. HPLC grade acetonitrile was bought from
110 Merck co., LTD. HPLC grade trifluoroacetic acid (TFA), formic acid, 2-acetylpyridine
111 and acetone were purchased from Sigma reagent co., LTD. Purified water by Milli Q
112 plus purification system from Millipore (Bradford, MA) was used during the
113 experimental studies.

114 2.2. Instrumentation

115 HPLC-Q/TOF-MS/MS analysis was carried out on an Agilent 1260 HPLC and a 6550
116 Q/TOF mass spectrometer (Agilent technologies, CA, USA) equipped with a DualJet
117 ESI source. Agilent Masshunter workstation was used to control the instrument and
118 obtain the data. The ultraviolet analyzer (Shanghai Jingke co., LTD, Shanghai, China)
119 was applied for photochemical reaction.

120 2.3. The photochemical reaction

121 The PME for injection were dissolved with 20% ACN aqueous solution to produce a
122 final concentration of about 2 mg mL^{-1}. Besides, about 3.2 mg of PME was dissolved in
123 an equal proportion of 20% ACN aqueous solution and acetone or 2-acetylpyridine to
124 be 2 mg mL^{-1}. All of the above PME solutions were transferred to a quartz cuvette and
125 irradiated with the ultraviolet lamp at 254 nm for 8h. Finally, the photochemical
126 reaction solutions were filtered through a 0.45 μm (Jinteng, Tianjin, China) nylon
127 membrane before HPLC-Q/TOF-MS analysis.

128 2.4. HPLC-Q/TOF-MS Method

129 Structural characterization of PMEs and the photochemical products were carried out
130 on the Agilent 1260 HPLC-6550 Q/TOF-MS with a DualJet ESI source based on an

131 established method [15]. In brief, the Diamonsil Plus C_{18} column (5 μm, 4.6 mm × 250
132 mm) was kept at 35°C. A volatile mixture mobile phase containing 0.01 mol L^{-1} TFA -
133 ACN (95 : 5, *v* : *v*, mobile phase A) and 0.1% formic acid- ACN (mobile phase B), the
134 ratio of mobile phase B was 21%(*v* : *v*), was used for separation. The flow rate was
135 operated at 1 mL min^{-1} and a third of the flow was split into the MS. The injection
136 volume was 20 μL.

137 The PMEs were analyzed in the positive ion mode by applying a voltage of 3.5
138 kV to the ESI needle. For MS/MS investigation, doubly charged polymyxin ions were
139 selected as the precursor ions and fragmented at 20 eV and 30 eV. The mass ranges
140 were set at *m/z* 500-1700 Da for MS and *m/z* 50-1700 Da for MS/MS with the
141 acquisition rate at 4 spectra/s and 2 spectra/s, respectively. The internal reference ion of
142 fluorinated phosphazine HP-921 with *m/z* 922.0098 ($C_{18}H_{19}O_6N_3P_3F_{24}^+$) was scanned
143 during the acquisition process.

144 **3. RESULTS AND DISCUSSION**

145 Eleven known components such as PME4, PME2-Val, PME6, PME2-I, PME2, PME3,
146 PME1-Nva, PME1-I, 2', 3'-dehydro PME1, PME1 and PME1-7MOA are described in
147 Ph. Eur 10.0. The structures of homologous PME components are slightly different in
148 FA and amino acid residues. The sequences of PME1, PME2 and other known
149 components have been studied thoroughly, so the high resolution TOF mass data and
150 the diagnostic ions could be used as references for the structure elucidation of
151 unknown related substances. Fragmentation pathways for PME were summarized by
152 Govaerts et al. [8] with two series of characteristic ions and Zhang et al. with three
153 series in our lab [15]. These structural strategies were able to analyze the peptide
154 sequences, but failed to determine the position of the double bond on the unsaturated
155 FA residue. The total ion chromatography (TIC) with peaks of PME1, PME2, 2',
156 3'-dehydro PME1 and 2', 3'-dehydro PME2 of commercial PME was shown in
157 supporting information (SI) Fig. S1. The MS of 2', 3'-dehydro PME2 with *m/z*

158 577.3727 ($[M+2H]^{2+}$) and 2’, 3’-dehydro PME1 with *m/z* 584.3802 ($[M+2H]^{2+}$) were
159 shown in SI Fig. S2A and S2B, respectively. Even though the oxetane products of
160 unsaturated PME1 with acetone are obtained by PB reaction, the structure of
161 unsaturated PME1 was still misjudged due to the less characteristic ions. As in the
162 previous report, we mistakenly inferred 2’, 3’-dehydro PME1 as 7’, 8’-dehydro PME1
163 [15].

164 In this study, it has been focused on the structures of the components contained
165 unsaturated FA residue, such as 2’, 3’-dehydro PME1 and 2’, 3’-dehydro PME2. The
166 position of the double bond was elucidated by epoxidized and di-hydroxylated
167 products generated from photochemical reactions combined with
168 HPLC-Q/TOF-MS/MS.

169 **3.1. Structure elucidation of 2’, 3’-epoxy PME1 and 2’, 3’-dihydroxy PME1**

170 The PMEs dissolved in 20% acetonitrile without 2-acetylpyridine or acetone did not
171 get any oxidative products. The molecular weight of the oxetane product formed by
172 the reaction of unsaturated PME with acetone is 58 Da higher than that of unsaturated
173 PME [16]. In the initial assumption of the experiment, 2-acetylpyridine was used as
174 the carbonyl reagent of PB reaction instead of acetone to react with the unsaturated
175 PME. The oxetane products with positive charge were supposed to fragment easily
176 during the MS/MS procedure. However, the expecting oxetane product with the
177 molecular weight of 121.14 Da more than that of the unsaturated PME were not
178 detected. Instead, the 2’, 3’-epoxy PME1 and 2’, 3’-dihydroxy PME1 were obtained,
179 which were synthesized from 2’, 3’-dehydro PME1 under photochemical reaction’s
180 condition, shown in Fig. 2. It is speculated that epoxidized compounds are obtained
181 by adding an oxygen atom to the α, β-unsaturated carbonyl FA through radical
182 reaction (the reaction mechanism is uncertain), and then H_2O attacks the epoxy group
183 to form dihydroxy compound. In further, the stereoscopic configuration of epoxidized

184 and di-hydroxylated products, which could not be determined by MS, are not
185 indicated in the following structure analysis.

186 The TIC of the photochemical products was shown in Fig. 3. There were two
187 components in peak 2. The quasi-molecular ions of one of the components (the MS
188 shown in Fig. 4A) with *m/z* 592.3789 ($[M + 2H]^{2+}$), 1183.7493 ($[M + H]^{+}$) and
189 1205.7304 ($[M + Na]^{+}$) were 16 Da higher than the *m/z* 1167.7494 ($[M + H]^{+}$) of 2',
190 3'-dehydro PME1 (SI Fig. S2B). It means that an oxygen atom is added to 2',
191 3'-dehydro PME1. This component was referred to be 2', 3'-epoxy PME1. For
192 MS/MS (Fig. 4B) investigation, the doubly protonated ion (*m/z* 592.3789) which was
193 the most abundant ion was chosen as the precursor ion with the optimized collision
194 voltage of 20 eV. The structure analysis strategy for PME has been established
195 including three series of characteristic ions. The formation and characterization of the
196 Series I and Series III ions with peaks displayed in SI Fig. S3 were shown in SI Fig.
197 S4. These two series ions were almost the same as the ions as PME1 [16]. The Series I
198 product ions are formed by losses of the FA moiety together with the α,
199 γ-diaminobutyric acid (Dab) to obtain an ion with *m/z* 929.5910, and subsequent
200 losing one of the Dabs in the cyclo-peptide (probably the Dab at position 6) to gain
201 ion with *m/z* 829.5278, then breaking down the adjacent amino acid residues to obtain
202 the ions of the oligopeptide. The Series III ions come into being as a result of firstly
203 lost of ring amino acid (position 6, 7 and 8), then lost FA, Dab and threonine (Thr,
204 position 2) residue of the linear part to obtain a series of peptide ions like *m/z* 603.3551,
205 402.2446, 302.1822, 202.1193 and 101.0710.

206 The Series II ions (Fig. 4B) such as *m/z* 857.5254, 757.4549, 456.2896, 255.1714,
207 127.1132 were 16 Da higher than the Series II ions of 2', 3'-dehydro PME1, proved
208 that the vinyl group changed to be the epoxidized group. Fig. 5 showed a proposed
209 fragmentation pathway in the form of the above ions' structures of 2', 3'-epoxy
210 PME1.

211 The quasi-molecular ion (Fig. 6A) of the component in peak 3 was *m/z*
212 1201.7550 ($[M + H]^+$), together with the doubly protonated ion of *m/z* 601.3842 ($[M +$
213 $2H]^{2+}$) and the metal adduct ion of *m/z* 1223.7380 ($[M + Na]^+$). The molecular weight
214 of this component was 34 Da higher than the *m/z* of 2', 3'-dehydro PME1, which
215 means two hydroxyls were added to 2', 3'-dehydro PME1. This component was
216 referred to be 2', 3'-dihydroxy PME1. The MS/MS of the ion *m/z* 601.3842 was
217 obtained and Series II ions were marked in Fig. 6B. A proposed fragmentation
218 pathway of 2', 3'- dihydro PME1 was shown in Fig. 7. The product ions of Series II
219 are formed by losses of three ring amino acids (*D*-Leu + *L*- Leu + *L*- Dab, position 6,
220 7 and 8) and subsequent losses of other amino acid residues to obtain ion with *m/z*
221 875.5329, 775.4679, 474.2947 and 273.1841. There are two dehydration modes for
222 the di-hydroxylated FA moiety. The OH group at position 3' or 2' was dehydrated with
223 the adjacent H to form the enol ion (a) or (b) with *m/z* 155.1071. Both of the ions (a)
224 and (b) were able to isomerize into more stable carbonyl ion. The (b) ion can be
225 further broken into ion *m/z* 113.0958 at the collision voltage of 30 eV, the
226 fragmentation ions with more detailed information including *m/z*, abundance and
227 relative abundance (%) were shown in SI Table S1.
228 The characteristic ions of 2', 3'-epoxy PME1 and 2', 3'-dihydroxy PME1 were
229 able to reverse-prove the position of double bond in FA moiety of the unsaturated
230 PME. Assuming that the double bond is located at position 7', it is difficult to obtain
231 the above characteristic ions containing epoxy, dihydroxy and carbonyl groups. Based
232 on the structural analysis strategy, 2', 3'-epoxy PME2 and 2', 3'-dihydroxy PME2
233 were detected and characterized.

234 **3.2. Structure elucidation of 2', 3'-epoxy PME2 and 2', 3'-dihydroxy PME2**

235 The 2', 3'-epoxy PME2 and 2', 3'-dihydroxy PME2 were transformed from 2',
236 3'-dehydro PME2 (peak a in SI Fig. S1) under the same photochemical reaction. The
237 quasi-molecular ions of the other component in peak 2 (Fig. 3) with *m/z* 585.3713 ([M

238 + 2H]$^{2+}$), 1169.7352 ([M + H]$^{+}$) and 1191.7155 ([M + Na]$^{+}$) were 16 Da (Fig. 4A)
239 higher than the *m/z* of 2', 3'-dehydro PME2 with *m/z* 577.3727 ([M+2H]$^{2+}$, SI Fig.
240 S2A). This component was referred to be 2', 3'-epoxy PME2. Besides the diagnostic
241 Series II ions of *m/z* 843.4996, 743.4402, 442.2669, 241.1557 and113.0968 which
242 were 14 Da less than the related ions of 2', 3'-epoxy PME1, the ions of (epoxidized
243 FA + *L*-Dab + *L*-Thr) with *m/z* 342.2028 and the epoxidized FA with *m/z* 141.0915
244 were also obtained obviously (Fig. 8A). The proposed structures of the characteristic
245 ions were shown in Fig. 9.

246 The component of peak 1 with *m/z* 1187.7428 ([M + H]$^{+}$), 594.3759 ([M + 2H]$^{2+}$)
247 and 1209.7231 ([M + Na]$^{+}$) was referred as 2', 3'-dihydroxy PME2, which was 34 Da
248 higher than the *m/z* of 2', 3'-dehydro PME2 and 14 Da less than that of 2',
249 3'-dihydroxy PME1. The MS/MS of the ion *m/z* 594.3759 was obtained and Series II
250 ions were marked in Fig 8B. The proposed fragmentation pathway of 2', 3'-
251 dihydroxy PME2 refers to that of 2', 3'- dihydroxy PME1. The Series II product ions
252 of 2', 3'-dihydroxy PME2 were *m/z* 861.5121, 761.4581, 460.2754, 259.1653 and
253 141.0938, which were 14 Da less than those of 2', 3'-dihydroxy PME1.

254 Through the above experiments, we revised the previously reported structure of
255 unsaturated PME to be 2', 3'-dehydro PME1/2 instead of 7', 8'-dehydro PME1/2 [16].

256 **4. CONCLUSION**

257 In this study, the unsaturated PME with double bond on the FA is converted to be
258 epoxidized and di-hydroxylated products. The structures of the oxidative products are
259 elucidated by the comprehensive structure analysis strategy based on
260 HPLC-Q/TOF-MS/MS. Two pairs of epoxidized and di-hydroxylated products named
261 as 2', 3'-epoxy PME1/E2 and 2', 3'-dihydroxy PME1/E2, which were synthesized
262 from 2', 3'-dehydro PME1/E2, were reported. The characteristic ions of epoxidized
263 and di-hydroxylated products were analyzed by MS/MS, which proved that the double
264 bond was located at the 2', 3' position of FA chain. It provides a convenient method to

determine the position of the double bond without purification and NMR for other unsaturated polypeptide antibiotics, such as polymyxin B.

AUTHOR CONTRIBUTIONS

Hanzhi Zhang: Conceptualization, Investigation, Methodology, Writing – original draft, Project administration

Zhenhua Tian: Methodology, Writing – review & editing

Hao Liu: Supervision

Hongwu Wang: Investigation

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No human or animal were used in this study.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

Science and Technology Commission of Shanghai Municipality (China, Grant number: 20142202200).

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interests.

ACKNOWLEDGEMENT

287 We are grateful for financial support from Research Foundation of Science and
288 Technology Commission of Shanghai Municipality (China, Grant number:
289 20142202200).

290 **SUPPLEMENTARY MATERIAL**

291 Available on publisher's website.

292

293

294 **REFERENCES**

295 1. Hofer, U. The cost of antimicrobial resistance. *Nat. Rev. Microbiol.,* **2019**, *17*, 3.
296 https://doi.org/10.1038/s41579-018-0125-x
297 2. Li, J.; Nation, R.L.; Turnidge, J.D.; Milne, R.W.; Coulthard, K.; Rayner, C.R.;
298 Paterson, D.L. Colistin: the re-emerging antibiotic for multidrug-resistant
299 gram-negative bacterial infections. *Lancet. Infect. Dis.,* **2006**, *6*(9), 589-601.
300 https://doi.org/10.1016/S1473-3099(06)70580-1
301 3. Velkov, T.; Roberts, K.D.; Nation, R.L.; Thompson, P.E.; Li, J. Pharmacology of
302 polymyxins: new insights into an 'old' class of antibiotics. *Future Microbiol.*,
303 **2013**, *8*(6), 711-724.
304 https://doi: 10.2217/fmb.13.39
305 4. Rabanal, F.; Cajal, Y. Recent advances and perspectives in the design and
306 development of polymyxins. *Nat. Prod. Rep.,* **2017**, *34*(17), 886-908.
307 https://doi.org/10.1039/C7NP00023E
308 5. Cui, A.L.; Hu, X.X.; Gao, Y.; Wang, X.K.; Nie, T.Y.; Chen, Y.; He, Q.Y.; Guo,
309 H.F.; Jiang, J.D.; You, X.F.; Li, Z.R. Synthesis and Bioactivity investigation of
310 the individual components of cyclic lipopeptide antibiotics. *J. Med. Chem.,* **2018**,
311 *61*(5), 1845−1857.
312 https://doi.org/10.1021/acs.jmedchem.7b01367

313 6. Bossche, L.V.D.; Schepdael, A.V.; Chopra, S.; Hoogmartens, J.; Adams, E.
314 Identification of impurities in polymyxin B and colistin bulk sample using liquid
315 chromatography coupled to mass spectrometry. *Talanta*, **2011**, *83*(5),1521-1529.
316 https://doi.org/10.1016/j.talanta.2010.11.044
317 7. Orwa, J.; Govaerts, C.; Busson, R.; Roets, E.; Schepdael, A.V.; Hoogmartens, J.
318 Isolation and structural characterization of colistin components. *J. Antibiot.,* **2001**,
319 *54*(7), 595-599.
320 https://doi: 10.7164/antibiotics.54.595
321 8. Govaerts, C.; Orwa, J.; Schepdael, A.V.; Roets, E.; Hoogmartens, J. Liquid
322 chromatography-ion trap tandem mass spectrometry for the characterization of
323 polypeptide antibiotics of the colistin series in commercial samples. *J.*
324 *Chromatogr. A.,* **2002**, *976*(1-2), 65-78.
325 https://doi.org/10.1016/S0021-9673(02)00375-8
326 9. Zhang, H.Z.; Qin, F.; Liu, H. Identification of unknown impurities in polymyxin
327 B sulfate by HPLC-MS/MS. *Chin. Pharm. J.,* **2018**, *53*(11), 918-924.
328 https://doi: 10.11669/cpj.2018.11.013
329 10. Orwa, J.; Gerven, A.V.; Roets, E.; Hoogmartens, J. Development and validation
330 of a liquid chromatography method for analysis of colistin sulphate.
331 *Chromatographia,* **2000**, *51*(4), 433-436.
332 https://doi.org/10.1007/BF02490480
333 11. Vogler, K.; Studer, R.O. The chemistry of polymyxin antibiotics. *Experientia*,
334 1966, *22*(6), 345-354.
335 https://doi.org/10.1007%2FBF01901127
336 12. Chihara, S.; Tobita, T.; Yahata, M.; Ito, A.; Koyama, Y. Enzymatic degradation of
337 colistin isolation and identification of α-N-acyl α, γ-diaminobutyric acid and
338 colistin nonapeptide. *Agr. Biol. Chem*., **1972**, *37*(11), 2455-2463.
339 https://doi.org/10.1080/00021369.1973.10861030

340 13. Zhang, H.Z.; Li, F.; Dun, J.L.; Sun, N.; Liu, H.; Chen, G.L. Combination of
341 derivatization-HPLC-MS and enzymatic hydrolysis-Edman degradation for amino
342 sequence and configuration of polymyxin B components. *Chromatographia,*
343 2021, *84*(11), 1057-1064.
344 https://doi.org/10.1007/s10337-021-04091-2
345 14. Ikai, Y.; Oka, H.; Hayakama, J.; Kawamura, N.; Harada, K.; Suzuki, M.;
346 Nakazawa, H.; Ito, Y. Isolation of colistin A and B using high-speed
347 countercurrent chromatography. *J. Liq. Chromatogr. Rel. Technol.,* **1998**, *21*(1-2),
348 143-155.
349 https://doi.org/10.1080/10826079808001943
350 15. Zhang, H.Z.; Sun, N.; Qin, F.; Luo, W.Y.; Zhao, J.D.; Wen, H.L.; Qiu, Y.; Liu,
351 H.; Li, M. Structure analysis of components in polymyxin E sulfate by high
352 performance liquid chromatography-quadrupole/time of flight mass spectrometry.
353 *Chin. Pharm. J.,* **2021**, *56*(1), 1861-1870.
354 https://doi: 10.11669/cpj.2021.01.009
355 16. Zhang, H.Z.; Sun, N.; Liu, X.F.; Qin, F.; Luo, W.Y.; Huang, Z.H.; Bian, X.C.;
356 Ding, J.G.; Liu, H. Structure analysis of vinyl polymyxin B1 and preliminary
357 study on the antibacterial activity. *World Notes on Antibiotics,* **2020**, *41*(6),
358 475-481.
359 http://www.worldnotes.cn/CN/Y2020/V41/I6/475
360 17. Cai, W.B.; Cheng, S.M.; Yang, J.;, Fen, J.X.; Zhang, W.P.; Li, Z.S.; Chen, Q.H.;
361 Xia, Y.; Ouyang, Z.; Ma, X.X. Large-scale lipid analysis with C=C location and
362 *sn*-position isomer resolving power. *Nat. Commun.* **2020**, *11*(1), 375.
363 https://doi.org/10.1038/s41467-019-14180-4
364 18. Ma, X.X.; Chong, L.;, Tian, R.; Shi, R.; Hu, T.Y.; Ouyang, Z.; Xia, Y.
365 Identification and quantitation of lipid C=C location isomers: a shotgun lipidomics

366 approach enabled by photochemical reaction. *Proc. Natl. Acad .Sci. U.S.A.,* **2016**,
367 *113*(10), 2573-2578.
368 https://doi.org/10.1073/pnas.1523356113