

Biosynthesis and in vitro enzymatic synthesis of the isoleucine conjugate of 12-oxo-phytodienoic acid from the isoleucine conjugate of α -linolenic acid

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1 Title

2 Biosynthesis and *in vitro* enzymatic synthesis of the isoleucine conjugate of 12-oxo-
3 phytodienoic acid from the isoleucine conjugate of α -linolenic acid.

4

5 Authors

6 Akira Uchiyama^a, Takaomi Yaguchi^a, Hiroyuki Nakagawa^b, Kento Sasaki^a, Naoshige
7 Kuwata^a, Hideyuki Matsuura^a, and Kosaku Takahashi^{a†}

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9 Affiliations

10 ^aDivision of Fundamental Agrosience Research, Research Faculty of Agriculture,
11 Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060-8589, Japan

12

13 ^bNational Agriculture and Food Research Organization (NARO), Food Research Institute,
14 2-1-12 Kannon-dai, Tsukuba-shi, Ibaraki 305-8642, Japan

15

16 †To whom correspondence should be addressed.

17 Tel: +81-11-706-3349; Fax: +81-11-706-2505; E-mail: kosaku@chem.agr.hokudai.ac.jp

18

19 Abbreviations

20 AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1;
21 12,13-EOT, allene oxide; GC-MS, gas chromatography-mass spectrometry; 13-HPOT, 13-
22 hydroperoxy octadecatrienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine;
23 JAR1, jasmonic acid-resistant 1; JAZ, jasmonate-zim domain; LC-MS/MS, liquid
24 chromatography-tandem mass spectrometry; LA-Ile, isoleucine conjugate of α -linolenic
25 acid; OPC-8:0, 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid; OPDA-Ile,
26 isoleucine conjugate of OPDA; OPR, 12-oxo-phytodienoic acid reductase; SCF, skp-cullin-
27 F box.

28

29 Abstract

30 The isoleucine conjugate of 12-oxo-phytodienoic acid (OPDA-Ile), a new member of the
31 jasmonate family, was recently identified in *Arabidopsis thaliana* and might be a signaling molecule
32 in plants. However, the biosynthesis and function of OPDA-Ile remains elusive. This study reports an
33 *in vitro* enzymatic method for synthesizing OPDA-Ile, which is catalyzed by reactions of lipoxygenase
34 (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) using isoleucine conjugates of
35 α -linolenic acid (LA-Ile) as the substrate. *A. thaliana* fed LA-Ile exhibited a marked increase in the
36 OPDA-Ile concentration. LA-Ile was also detected in *A. thaliana*. Furthermore, stable isotope labelled
37 LA-Ile was incorporated into OPDA-Ile. Thus, OPDA-Ile is biosynthesized via the cyclization of LA-
38 Ile in *A. thaliana*.

39

40 Keywords

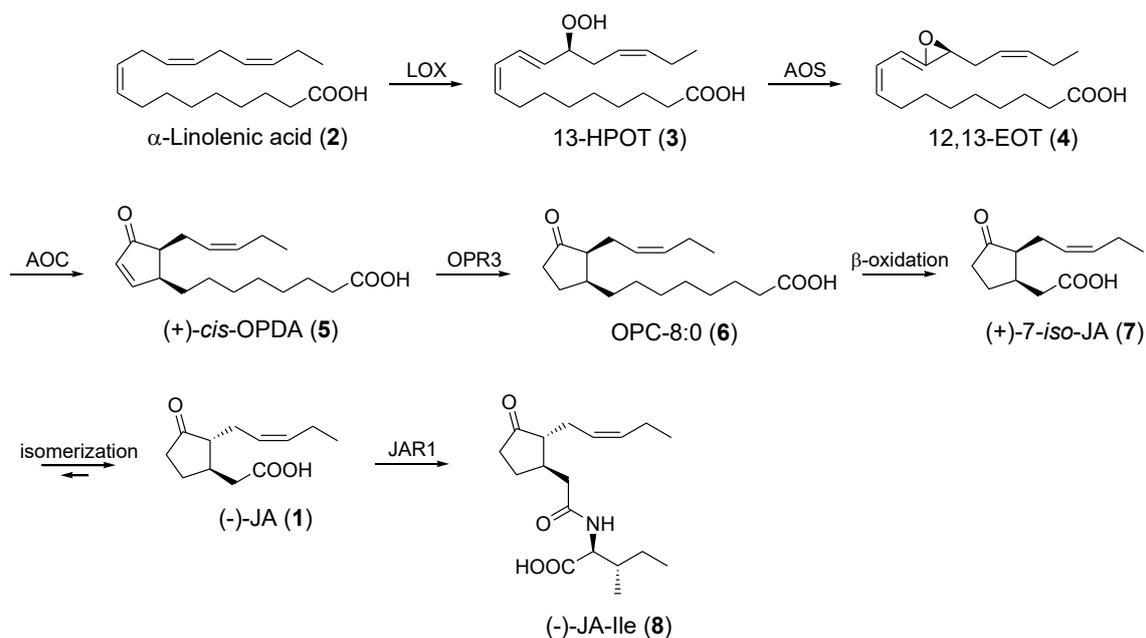
41 *Arabidopsis thaliana*, jasmonates, LA-Ile, OPDA-Ile, 12-oxo-phytodienoic acid.

42

43 Plants have a wide variety of physiological responses that allow them to adapt to adverse
44 environmental conditions that negatively affect their growth and development. Jasmonic acid (JA, **1**)
45 plays important roles in stress responses and development in plants. JA (**1**) functions as a signaling
46 molecule in numerous plant physiological processes related to development and defense responses.¹
47 Most enzymes that participate in JA (**1**) biosynthesis have been successfully characterized. JA (**1**) has
48 been shown to be a signaling molecule in both flowering plants and a model lycophyte, *Selaginella*
49 *moellendorffii*.² JA (**1**) is a ubiquitous phytohormone detected in vascular plant species.

50 The JA (**1**) biosynthetic pathway begins with the lipase-mediated release of α -linolenic acid (**2**)
51 from the membrane lipids of chloroplasts (Fig. 1).¹ In chloroplasts, lipoxygenase (LOX) oxidizes α -
52 linolenic acid (**2**) into 13(*S*)-hydroperoxy octadecatrienoic acid (13-HPOT, **3**). 13-HPOT (**3**) is
53 metabolized by allene oxide synthase (AOS) into an unstable allene oxide (12,13-EOT, **4**), which is
54 cyclized by allene oxide cyclase (AOC) into *cis*-(+)-12-oxo-phytodienoic acid (OPDA, **5**). The AOC
55 reaction provides two side chain configurations in the naturally occurring jasmonate structure.
56 Reduction of the 10,11-double bond in OPDA (**5**) by OPDA reductase 3 (OPR3) then yields 3-oxo-2-

57 (2-*cis*-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0, **6**). Three β -oxidation steps convert OPC-8:0
 58 (**6**) into (+)-7-*iso*-JA (**7**), which is naturally isomerized to (-)-JA (**1**). JA (**1**) is converted to the
 59 isoleucine conjugate of JA (JA-Ile, **8**) by JAR1. JA-Ile (**8**) is considered a versatile signaling compound
 60 in the JA signaling pathway.^{1,3} JA-Ile (**8**) binds to its receptor, coronatine insensitive 1 (COI1), and
 61 then mediates the binding of the JAZ protein to the COI1-JA-Ile unit of the skp-cullin-F box (SCF)
 62 complex, resulting in degradation by the 26S proteasome and the subsequent induction of COI1-
 63 dependent JA responses.^{4,6} OPDA (**5**) is not only an intermediate in the JA biosynthetic pathway but
 64 also exerts individual JA (**1**)-independent biological functions.⁷⁻⁹ OPDA (**5**) binds cyclophilin 20-3,
 65 leading to enhanced redox capability in *Arabidopsis thaliana*.¹⁰ In contrast, OPDA (**5**), but not JA (**1**),
 66 is present in the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens*, with functions
 67 in defense and development.¹¹⁻¹³ However, the detailed mechanism of the OPDA signaling system
 68 remains unknown.



69

70 Fig. 1. Octadecanoid pathway.

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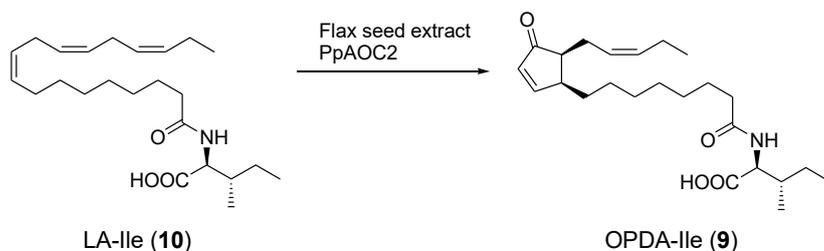
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73 OPDA-Ile (**9**), a new member of the jasmonate family, was recently identified in *A. thaliana*.¹⁴

74 Moreover, OPDA-Ile (**9**) induces the expression of the *ZAT10* gene, which encodes a salt tolerance

75 zinc finger protein, and the *GRX480* gene, which encodes a GLUTAREDOXIN.^{15,16} Based on these
76 findings, OPDA-Ile (**9**) may function as a signaling molecule in plants. The OPDA-Ile (**9**) biosynthetic
77 mechanism has not yet been determined, whereas the *A. thaliana jar1* mutant, which lacks the *jar1*
78 gene encoding a protein that catalyzes the conjugation of JA (**1**) with Ile, produces OPDA-Ile (**9**).¹⁶
79 Thus, the OPDA-Ile (**9**) biosynthetic pathway, which is independent of JAR1, is proposed to be present
80 in *A. thaliana*.

81 The biological functions of OPDA-Ile (**9**) remain elusive. An efficient method for synthesizing
82 OPDA-Ile (**9**) should be developed to investigate the detailed biological activities of this compound.
83 OPDA-Ile (**9**) was previously produced via the chemical conjugation of Ile and OPDA (**5**) under
84 alkaline conditions.^{16,17} The stereochemistry of the two side chains of OPDA (**5**) is easily converted
85 from the *cis*-form to *trans*-form under alkaline conditions; therefore, the previously reported method
86 for synthesizing OPDA-Ile (**9**) is not necessarily optimal. For OPDA (**5**) biosynthesis, reactions with
87 LOX, AOS and AOC occur on the unsaturated alkyl chains of α -linolenic acid (**2**), 13-HPOT (**3**), and
88 12,13-EOT (**4**), respectively.¹⁸⁻²⁰ Analysis of the crystal structures of AOS and AOC suggests that
89 unsaturated alkyl chains of 13-HPOT (**3**) and 12,13-EOT (**4**) are present in the active sites of the
90 corresponding enzymes.^{18,19}



92 Fig. 2. *In vitro* enzymatic synthesis of OPDA-Ile (**9**). LA-Ile (**10**) was incubated in the reaction mixture
93 [50 mM Tris-HCl (pH 8.0), flax seed extract, PpAOC2] at 25 °C for 1 hours.

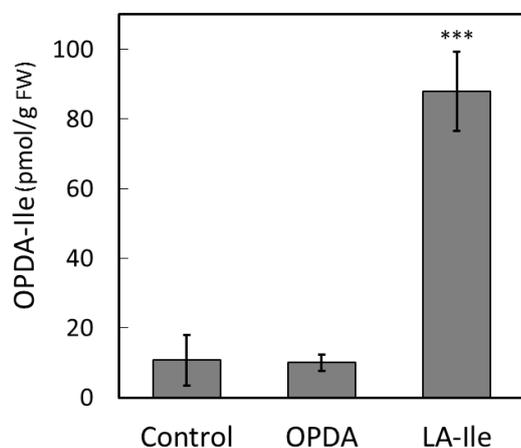
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96 We attempted the *in vitro* cyclization of LA-Ile (**10**) to produce OPDA-Ile (**9**) by performing
97 continuous reactions with LOX, AOS, and AOC according to the method for *in vitro* stereoselective
98 OPDA (**5**) synthesis (Fig. 2).²¹ The mixture used for the *in vitro* synthesis of OPDA-Ile (**10**) contained
99 flaxseed extract, recombinant PpAOC2 derived from the model moss *Physcomitrella patens*, and LA-

100 Ile (**10**) and was incubated at 25 °C for 1 hour. As a result, 11 mg of OPDA-Ile (**9**) was successfully
101 synthesized from 30 mg of LA-Ile (**10**) with a 35% yield (Supplemental data). Analysis of the AOS
102 crystal structure suggests that a lysine residue of AOS near the substrate interacts with the carboxyl
103 group of 13-HPOT (**3**), thereby playing an important role in its binding.¹⁸ While the carboxyl group in
104 linolenic acid (**2**) is replaced by an amide bond in LA-Ile (**10**), a lysine residue near the substrate of
105 AOS may interact with the oxygen of the amide bond in a possible LOX product of LA-Ile (**10**). The
106 alkyl chain of Ile moiety derived from LA-Ile (**10**) must not interfere with binding to LOX, AOS, or
107 AOC. Therefore, the cyclization of LA-Ile (**10**) into OPDA-Ile (**9**) is found to have occurred.
108 Additionally, the *in vitro* enzymatic synthesis of OPDA-Ile (**9**) was conducted under mild conditions
109 and efficiently yielded OPDA-Ile (**9**). Considering the mechanisms of the LOX, AOS, and AOC
110 reactions, the method reported in this study could be applied to the synthesis of other amino acid
111 conjugates of OPDA.

112 The biosynthetic mechanism of OPDA-Ile (**9**) was not revealed until recently. Two possibilities
113 exist for the OPDA-Ile (**9**) biosynthetic pathway. One possibility is that OPDA-Ile (**9**) is synthesized
114 by a protein that conjugates OPDA (**5**) and Ile. In the case of JA-Ile (**8**), a GH3 protein, JAR1
115 conjugates JA (**1**) and Ile.¹ A protein from the GH3 protein family is predicted to catalyze the
116 conjugation of OPDA (**5**) and Ile. The other possibility is that OPDA-Ile (**9**) is biosynthesized by three
117 continuous reactions with LOX, AOS, and AOC with LA-Ile (**10**) as the substrate, similar to the *in*
118 *vitro* enzymatic synthesis of OPDA-Ile (**9**). In a previous study, the marginal conversion of OPDA into
119 OPDA-Ile was observed in WS.¹⁵ The *in vitro* enzymatic synthesis of OPDA-Ile (**9**) in this study
120 supports the hypothesis that OPDA-Ile (**9**) is biosynthesized from LA-Ile (**10**) via LOX-, AOS-, and
121 AOC-mediated reactions in plants. *A. thaliana* plants grown for 30 days under short-day conditions
122 were treated with 100 μM LA-Ile (**10**) or OPDA (**5**), and the accumulation of OPDA-Ile (**9**) was
123 analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).
124 Based on the analytical data, the application of LA-Ile (**10**) significantly increased the OPDA-Ile (**9**)
125 concentration in *A. thaliana*. The amount of OPDA-Ile (**9**) in plants treated with LA-Ile (**10**) was
126 greater than that in untreated plants (Fig. 3). In contrast, the OPDA (**5**) treatment did not increase the
127 OPDA-Ile (**9**) concentration (Fig. 3). These results suggested that OPDA-Ile (**9**) was biosynthesized

128 from LA-Ile (**10**) but not OPDA (**5**).



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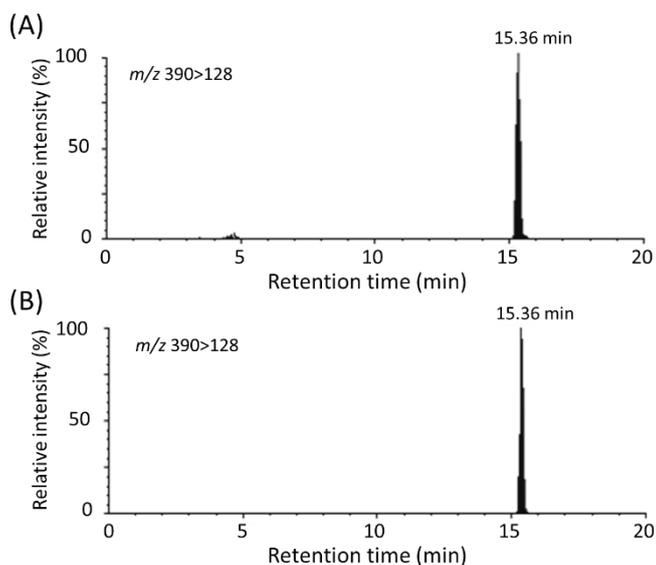
130 Fig. 3. UPLC-MS/MS analysis of OPDA-Ile (**9**) in *A. thaliana* treated with OPDA (**5**) or LA-Ile (**10**).
131 Plants were treated with either 100 μ M LA-Ile (**10**) or OPDA (**5**). OPDA-Ile (**9**) was analyzed by
132 UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak at m/z 130.00 $[M-H]^-$
133 derived from the peak at m/z 404.28 $[M-H]^-$. Each value is represented by the mean \pm SD of five
134 independent biological replicates. Student's *t*-test, *** $p < 0.001$.

135

136

137 As described above, OPDA-Ile (**9**) was postulated to be converted from LA-Ile (**10**); however, LA-
138 Ile (**10**) has not been reported as a natural product. *A. thaliana* was analyzed by liquid chromatography-
139 tandem mass spectrometry (LC-MS/MS) to determine the presence of LA-Ile (**10**). The analytical data
140 revealed a predominant peak derived from LA-Ile (**10**) in the chromatogram of an *A. thaliana* extract
141 (Fig. 4). The retention time of the peak was the same as the peak for the LA-Ile (**10**) standard. Thus,
142 LA-Ile (10 pmol/g FW, **10**) is present in *A. thaliana*. To our knowledge, this report represents the first
143 evidence identifying LA-Ile (**10**) as a natural product.

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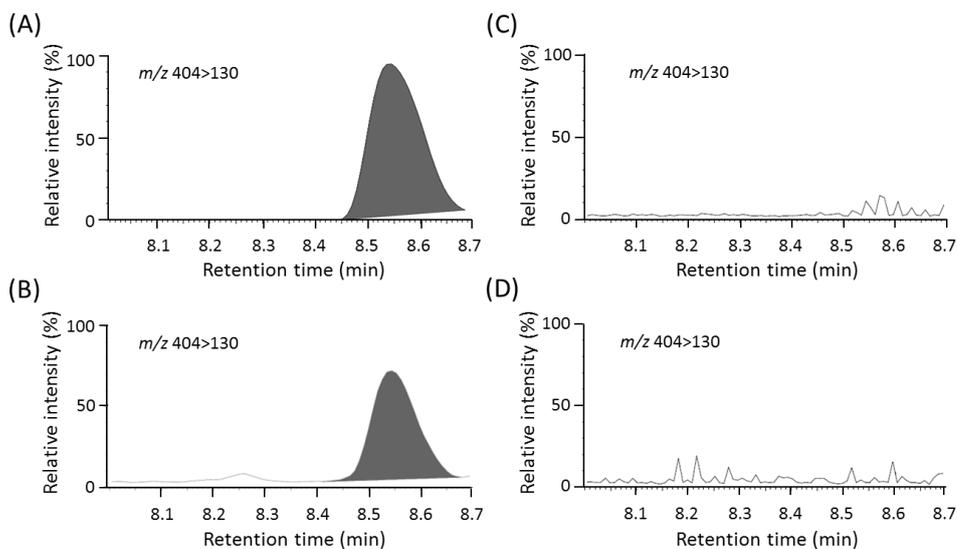
146 Fig. 4. Analysis of LA-Ile (**10**) in *A. thaliana*. LA-Ile (**10**) was analyzed by LC-MS/MS. The MRM
 147 mode was used to analyze a specific fragment peak at m/z 128.0 $[M-H]^-$ derived from the peak at m/z
 148 390.3 $[M-H]^-$. (A): standard; (B): plant extract.

149

150

151 To examine whether OPDA-Ile (**9**) was synthesized by an *A. thaliana* protein extract, a reaction
 152 mixture comprising LA-Ile (**10**) and an *A. thaliana* protein extract was incubated for 1 hour, and then
 153 the reaction mixture was analyzed for the presence of OPDA-Ile (**9**) by UPLC-MS/MS. The peak
 154 derived from OPDA-Ile (**9**) appeared clearly in the reaction mixture of LA-Ile (**10**) and the protein
 155 extract (Fig. 5). In contrast, no clear peak derived from OPDA-Ile (**10**) was detected in the protein
 156 extract lacking LA-Ile (**10**) or in the buffer used to generate the protein extract supplemented with LA-
 157 Ile (**10**) (Fig. 5). *Arabidopsis* protein extract was shown to exhibit sequential LOX, AOS, and AOC
 158 enzymatic activities to convert LA-Ile (**10**) into OPDA-Ile (**9**).

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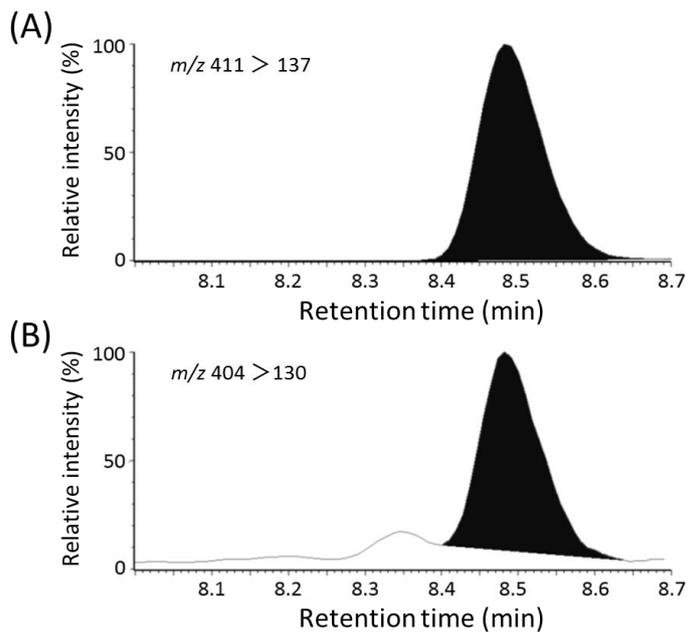
161 Fig. 5. *In vitro* synthesis of OPDA-Ile (**9**) by protein extracts from *A. thaliana*. LA-Ile (**10**) was
 162 incubated with a protein extract prepared from *A. thaliana* at 25 °C for 1 hour, and the mixture was
 163 then analyzed for OPDA-Ile (**9**) by UPLC-MS/MS. The MRM mode was used to analyze a specific
 164 fragment peak at m/z 130.00 $[M-H]^-$ derived from the peak at m/z 404.28 $[M-H]^-$. (A): standard
 165 OPDA-Ile (**9**); (B): LA-Ile (**10**) in protein extract; (C): protein extract without added LA-Ile (**10**); (D):
 166 LA-Ile (**10**) in the buffer used for protein extraction.

167

168

169 Next, we investigated whether stable-isotope-labelled LA-Ile (**9**) was incorporated into OPDA-Ile
 170 (**9**). LA- $[^{13}C_6,^{15}N]$ Ile was fed to *A. thaliana*, and OPDA- $[^{13}C_6,^{15}N]$ Ile in *A. thaliana* was then analyzed
 171 by UPLC-MS/MS. The analytical data showed that the peak derived from OPDA- $[^{13}C_6,^{15}N]$ Ile (m/z
 172 411>137) clearly appeared, and the retention time of OPDA- $[^{13}C_6,^{15}N]$ Ile was in accordance with that
 173 of non-labelled OPDA-Ile (m/z 414>130) (Fig. 5). Accordingly, OPDA-Ile (**9**) is biosynthesized via
 174 cyclization of the LA-Ile (**10**) substrate in *A. thaliana*.

175



176

177 Fig. 5. Incorporation of LA-[¹³C₆,¹⁵N]Ile into OPDA-[¹³C₆,¹⁵N]Ile into *A. thaliana*. Plants were treated
 178 with or without 100 μM LA-[¹³C₆,¹⁵N]Ile, and the resulting mixture was analyzed for OPDA-
 179 [¹³C₆,¹⁵N]Ile by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak of
 180 OPDA-[¹³C₆,¹⁵N]Ile at *m/z* 137.00 [M-H]⁻ derived from the peak at *m/z* 411.28 [M-H]⁻ and a specific
 181 fragment peak of OPDA-Ile at *m/z* 130.00 [M-H]⁻ derived from the peak at *m/z* 404.28 [M-H]⁻. (A);
 182 extract of plant treated with LA-[¹³C₆,¹⁵N]Ile; (B); extract of control plant.

183

184

185 Based on the data described above, OPDA-Ile (**9**) is biosynthesized in *A. thaliana* via LOX-, AOS-,
 186 and AOC-mediated reactions, which participate in the octadecanoid pathway, using LA-Ile (**10**) as the
 187 substrate. This result is supported by previous studies showing that the *jar1* mutant still produces
 188 OPDA-Ile (**9**), that the *aos* mutant does not produce OPDA-Ile and that marginal conversion of OPDA
 189 into OPDA-Ile occurs in *A. thaliana*.^{14,15} Because the three proteins, LOX, AOS and AOC, are
 190 localized in chloroplasts, OPDA-Ile (**9**) is predicted to be located in chloroplasts. Additionally,
 191 arabidopsides, monogalactosyl glycerol lipids containing OPDA,²⁰ are likely synthesized by a
 192 combination of LOX-, AOS-, and AOC-mediated reactions using monogalactosyldiacylglycerol as the
 193 substrate.²² The previously reported data also support the results obtained in this study. Thus, the

194 present study suggests that an α -linolenic acid-related compound with a modified carboxylic acid can
195 become a substrate in the octadecanoid pathway. It is possible that a variety of OPDA-related
196 compounds are biosynthesized through the octadecanoid pathway in plants.

197 As described above, LA-Ile (**10**) is a synthetic precursor of OPDA-Ile (**9**). LA-Ile (**10**) synthesis is
198 also a crucial step in OPDA-Ile (**9**) biosynthesis; however, the conjugation mechanism of α -linolenic
199 acid (**2**) and Ile in *A. thaliana* remains unclear. As GH3 proteins conjugate amino acids and plant
200 hormones, such as indole-3-acetic acid and JA (**1**),²³ a member of the GH3 protein family likely plays
201 an important role in OPDA-Ile (**10**) biosynthesis. The identification of an enzyme that catalyzes the
202 conjugation of α -linolenic acid (**2**) and Ile is required to elucidate the total biosynthetic pathway for
203 OPDA-Ile (**9**).

204

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207 data for the synthetic compounds. This study was financially supported by Hokkaido University.

208

209 Supplementary data

210 The supplementary data associated with this article can be found in the online version.

211

212 References

213 1. C. Wasternack, M. Hause, *Ann. Bot.* 2013, 111, 1021–1058.

214

215 2. P. Pratiwi, G. Tanaka, T. Takahashi, X. Xie, K. Yoneyama, H. Matsuura, K. Takahashi, *Plant Cell*
216 *Physiol.*, 2017, 58, 789–801.

217

218 3. P.E. Staswick, I. Tiryaki, *Plant Cell*, 2014, 16, 2117–2127.

219

220 4. S. Fonseca, A. Chini, M. Hamberg, B. Adie, A. Porzel, R. Kramell, O. Miersch, C. Wasternack, R.

221 Solano, *Nat. Chem. Biol.*, 2009, 5, 344–350.

222

223 5. J. Yan, C. Zhang, M. Gu, Z. Bai, W. Zhang, T. Qi, Z. Cheng, W. Peng, H. Luo, F. Nan, Z. Wang, D.
224 Xie, *Plant Cell*, 2009, 21, 2220–2236.

225

226 6. L.B. Sheard, X. Tan, H. Mao, J. Withers, G. Ben-Nissan, T.R. Hinds, T. Kobayashi, F.F. Hsu, M.
227 Sharon, J. Browse, S.Y. He, J. Rizo, G.A. Howe, N. Zheng, *Nature*, 2010, 468, 400–405.

228

229 7. N. Taki, Y. Sasaki-Sekimoto, T. Obayashi, A. Kikuta, K. Kobayashi, T. Ainai, K. Yagi, N. Sakurai,
230 H. Suzuki, T. Masuda, K. Takamiya, D. Shibata, Y. Kobayashi, H. Ohta, *Plant Physiol.*, 2005, 139,
231 1268–1283.

232

233 8. C. Böttcher, S. Pollmann, *FEBS J.*, 2009, 276, 4693–4704.

234

235 9. S. Goetz, A. Hellwege, I. Stenzel, C. Kutter, V. Hauptmann, S. Forner, B. McCaig, G. Hause, O.
236 Miersch, C. Wasternack, B. Hause, *Plant Physiol.*, 2012, 158, 1715–1727.

237

238 10. S.W. Park, W. Li, A. Viehhauser, B. He, S. Kim, A.K. Nilsson, M.X. Andersson, J.D. Kittle, M.M.
239 Ambavaram, S. Luan, A.R. Esker, D. Tholl, D. Cimini, M. Ellerström, G. Coaker, T.K. Mitchell, A.
240 Pereira, K.J. Dietz, C.B. Lawrence, *Proc. Natl. Acad. Sci. USA*, 2013, 110, 9559–9564.

241

242 11. M. Stumpe, C. Göbel, B. Faltin, A.K. Beike, B. Hause, K. Himmelsbach, J. Bode, R. Kramell, C.
243 Wasternack, W. Frank, R. Reski, I. Feussner, *New Phytol.*, 2010, 188, 740–749.

244

245 12. I. Ponce De León, E.A. Schmelz, C. Gaggero, A. Castro, A. Álvarez, M. Montesano, *Mol. Plant*
246 *Pathol.*, 2012, 13, 960–974.

247

248 13. Y. Yamamoto, J. Ohshika, T. Takahashi, K. Ishizaki, T. Kohchi, H. Matusuura, K. Takahashi,
249 *Phytochemistry*, 2015, 116, 48–56.

250

251 14. Floková K, Feussner K, Herrfurth C, Miersch O, Mik V, Tarkowská D, Strnad M, Feussner I,
252 Wasternack C, Novák O. A previously undescribed jasmonate compound in flowering *Arabidopsis*
253 *thaliana* - The identification of *cis*-(+)-OPDA-Ile. *Phytochemistry* 2016;122:230–237.

254

255 15. Arnold MD, Gruber C, Floková K, Miersch O, Strnad M, Novák O, Wasternack C, Hause B. The
256 recently identified isoleucine conjugate of *cis*-12-oxo-phytodienoic acid is partially active in *cis*-12-
257 oxo-phytodienoic acid-specific gene expression of *Arabidopsis thaliana*. *PLoS One*
258 2016;11:e0162829.

259

260 16. Wasternack C, Hause B. OPDA-Ile - a new JA-Ile-independent signal? *Plant Signal Behav*
261 2016;11: e1253646.

262

263 17. R. Karamell, J. Schmidt, G. Schneder, G. Sembdner, K. Schreiber, *Tetrahedron*, 1998, 44, 5791–
264 5807.

265

266 18. E. Hofmann, P. Zerbe, F. Schaller, *Plant Cell*, 2006, 18, 3201–3217.

267

268 19. L. Li, Z. Chang, Z. Pan, Z.Q. Fu, X. Wang, *Proc. Nat.l Acad. Sci. USA* 2008, 105, 13883–13888.

269

270 20. S. Schaller, A. Stintzi, *Phytochemistry*, 2009, 70, 1532–1538.

271

272 21. A. Kajiwara, T. Abe, T. Hashimoto, H. Matsuura, K. Takahashi, *Biosci. Biotechnol. Biochem.*, 2012,
273 76, 2325–2328.

274

275 22. A.K. Nilsson, P. Fahlberg, M. Ellerström, M.X. Andersson, *FEBS lett.*, 2012, 586, 2483–2487.

276

277 23. C.S. Westfall, A.M. Muehler, J.M. Jez, *J. Biol. Chem.*, 2013, 288, 19304-19311.

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279 Supplemental data

280 1. General methods

281 The ^1H - and ^{13}C -NMR spectra were recorded on a Jeol EX-270 NMR spectrometer (Jeol, Tokyo,
282 Japan). ^1H -NMR chemical shifts are referenced to the residual CDCl_3 solvent peak at $\delta 7.24$ ppm. ^{13}C -
283 NMR chemical shifts are referenced to the residual CDCl_3 solvent peak at $\delta 77.0$ ppm. Field
284 desorption-high resolution mass spectra (FD-HR-MS) were recorded on a JEOL JMS T100GCV mass
285 spectrometer (Jeol, Tokyo, Japan). Specific rotation values were measured on a JASCO DIP-310
286 polarimeter (Jasco Corporation, Tokyo, Japan).

287

288 2. LA-Ile (**10**) synthesis

289 α -Linolenic acid (**2**, 0.88 mmol, 245 mg) was dissolved in tetrahydrofuran (11 ml) with
290 trimethylamine (0.98 mmol, 0.14 ml). Chloroformic acid ethyl ester (0.1 mmol, 0.1 ml) was added
291 while the mixture was stirred at -10 °C. After the resulting solution was stirred for 20 min, a 0.3 M
292 aqueous NaOH solution (6.9 ml) containing Ile (1.77 mmol, 232 mg) was added to the solution and
293 stirred for an additional 25 min at room temperature. After evaporation to remove the solvent, the
294 obtained residue was cooled at 0 °C, and poured into 1 M HCl, extracted with ethyl acetate, and dried
295 over Mg_2SO_4 . The extract was evaporated and purified by SiO_2 gel column chromatography (Kanto
296 Chemical, Tokyo, Japan), which was developed with a mixed solvent of acetic acid/ethyl acetate/*n*-
297 hexane (1/30/70, v/v). LA-Ile (**10**) was obtained as a colorless oil (293.2 mg, 85%). FD-HR-MS: found
298 m/z 392.3150 $[\text{M}+\text{H}]^+$; calculated m/z 392.3165 for $\text{C}_{24}\text{H}_{42}\text{NO}_3$; $[\alpha]^{25}_{\text{D}} +19.8$ (c 0.3, CHCl_3); ^1H -NMR
299 (CDCl_3 , 270 MHz) δ : 11.03 (s, 1H), 6.44 (d, $J = 8.6$ Hz, 1H), 5.38-5.21 (m, 6H), 4.59 (dd, $J = 4.6, 8.6$
300 Hz, 1H), 2.77-2.73 (m, 4H), 2.22 (t, $J = 8.1$ Hz, 2H), 2.08-1.85 (m, 6H), 1.60-1.38 (m, 3H), 1.22-1.07
301 (m, 8H), 0.97-0.81 (m, 9H).

302

303 3. LA- $^{13}\text{C}_6,^{15}\text{N}$ Ile synthesis

304 Instead of Ile, $^{13}\text{C}_6,^{15}\text{N}$ Ile was conjugated to α -linolenic acid. The reaction was carried out
305 according to the method described in the previous section.

306

307 4. OPDA- $^{13}\text{C}_6$, ^{15}N]Ile synthesis

308 Instead of LA-Ile (**10**), LA- $^{13}\text{C}_6$, ^{15}N]Ile was cyclized to afford OPDA- $^{13}\text{C}_6$, ^{15}N]Ile according to
309 the method described in the previous section.

310

311 4. *In vitro* enzymatic synthesis of OPDA-Ile (**9**)

312 OPDA-Ile (**9**) was synthesized *in vitro* using LA-Ile (**10**) as the substrate, according to the method
313 used for the *in vitro* synthesis of OPDA (**5**) (Kajiwara et al., 2012). The reaction mixture used for the
314 *in vitro* synthesis of OPDA-Ile (**9**) contained a flaxseed extract, recombinant PpAOC2 and LA-Ile (**10**).
315 An acetone powder of the flaxseed extract (625 mg) was extracted with 5 ml of 50 mM Tris-HCl buffer
316 (pH 8.0, 20 mM NaCl) containing 500 μg of recombinant PpAOC2 and then centrifuged at 21,500 \times
317 g for 30 min at 4 $^\circ\text{C}$. The prepared enzyme solution was incubated with 30 mg of LA-Ile (**10**) and
318 stirred under an oxygen atmosphere for 3 hours at room temperature. The reaction mixture was
319 extracted with ethyl acetate. After the extract was evaporated, the resulting residue was purified by
320 SiO_2 gel column chromatography, which was developed with a mixed solvent of acetic acid/ethyl
321 acetate/*n*-hexane (1/30/70, v/v) to obtain 11 mg of OPDA-Ile (**9**). $[\alpha]^{25}_{\text{D}} +42.3$ (*c* 0.6, CHCl_3). FD-
322 HR-MS: found *m/z* 404.2804 [M-H]; calculated *m/z* 404.2801 for $\text{C}_{24}\text{H}_{38}\text{NO}_4$. $^1\text{H-NMR}$ (CDCl_3 , 270
323 MHz) δ : 7.74-7.70 (dd, *J* = 5.8, 2.6 Hz, 1H), 6.20-6.12 (dd, *J* = 3.1, 1.8 Hz, 1H), 6.08-5.98 (d, *J* = 8.3
324 Hz, 1H), 5.48-5.28 (m, 2H), 4.64-4.54 (dd, *J* = 5.6, 4.8 Hz, 1H), 3.03-2.88 (m, 1H), 2.55-2.46 (m, 1H),
325 2.46-2.36 (m, 1H), 2.26-2.17 (t, *J* = 7.4 Hz, 2H), 2.15-2.07 (m, 1H), 2.06-1.96 (m, 2H), 1.96-1.84 (m,
326 1H), 1.77-1.65 (m, 1H), 1.64-1.54 (m, 2H), 1.53-1.40 (m, 1H), 1.34-1.24 (m, 6H), 1.23-1.09 (m, 2H),
327 1.05-0.72 (m, 11H). $^{13}\text{C-NMR}$ (CDCl_3 , 67.5 MHz) δ : 208.8, 172.4, 171.1, 165.0, 130.5, 129.8, 124.3,
328 53.8, 47.4, 41.8, 35.1, 34.0, 28.1, 27.0, 26.6, 26.5, 25.0, 23.1, 22.6, 21.2, 18.2, 12.7, 11.4, 9.0.

329

330 5. Plants and chemical treatments

331 *A. thaliana* (Col-0) was grown on soil under short day conditions (10 hours of light/14 hours of
332 dark) at 25 $^\circ\text{C}$ for 30 days under a white fluorescent light. Plants were sprayed with 100 μM OPDA
333 and LA-Ile once per day for 3 days. For the feeding experiment with LA- $^{13}\text{C}_6$, ^{15}N]Ile, *A. thaliana* was

334 harvested at 12 hours after spraying 100 μ M LA-[¹³C₆,¹⁵N]Ile. OPDA was synthesized according to
335 the method reported by Kajiwara et al. (2012).

336

337 6. Analysis of OPDA-Ile (9)

338 *A. thaliana* plants were grown on Jiffy-7 (Sakata Seed Corporation, Yokohama, Japan) for 5 weeks
339 at 22 °C under a white fluorescent light with 10 h/14 h photoperiods (short-day conditions). Samples
340 were prepared according to the method developed by Floková et al. (Phytochemistry. 2016; 122: 230–
341 237). UPLC was performed using an ACQUITY UPLC system (Waters Corporation, Milford, MA,
342 USA) equipped with a binary solvent manager and a sample manager. MS/MS was subsequently
343 performed using a Micromass Quattro Premier tandem quadrupole MS (Waters Corporation, Milford,
344 MA, USA). The UPLC/MS system was controlled by Micromass MassLynx 4.0 (Waters Corporation,
345 Milford, MA, USA). The UPLC conditions were described previously (Sato et al., Plant Cell Physiol.
346 2011; 52: 509-517). The MS parameters for the detection of OPDA-Ile were set according to the
347 method described by Floková et al. (Phytochemistry. 2016; 122: 230–237). OPDA-[¹³C₆,¹⁵N]Ile was
348 used as a standard for quantitative analysis.

349

350 7. Analysis of LA-Ile (10)

351 *A. thaliana* plants were grown according to the method described in the previous section. Plants
352 (500 mg) were then extracted with 10 ml of an 80% aqueous MeOH solution. The resulting extract
353 was applied onto a C18 solid phase extraction cartridge (Bond Elut, 6 ml, Agilent Technologies, CA,
354 USA) that had been equilibrated with 80% aqueous MeOH. After the cartridge was washed with 6 ml
355 of 80% MeOH, LA-Ile (10) was eluted with 6 ml of MeOH. The eluate was evaporated, and the
356 obtained residue was dissolved in 500 μ l of 80% aqueous MeOH for analysis. The detection and
357 quantification of LA-Ile (10) were performed using a 4000Q TRAP LC-MS/MS system (Sciex,
358 Framingham, MA, USA) equipped with an electrospray ionization (ESI) source (turbo V) and 1290
359 Infinity HPLC system (Agilent, Santa Clara, CA, USA). Chromatographic separation was
360 performed at 40 °C on a TSK-gel ODS-100V column (150 mm \times 2 mm inner diameter (i.d.), 5 μ m)
361 (Tosoh Corporation, Tokyo, Japan). Eluents were composed of water/formic acid (99.9/0.1, v/v)

362 (eluent A) and methanol/formic acid (99.9/0.1, v/v) (eluent B). Elution was conducted at a flow rate
363 of 0.20 ml/min with the following linear gradient: 0–3 min, 50% B; 3–18 min, 50–97% B; 18–22 min,
364 97% B; 22–22.1 min, 97–50% B; and 22.1–29 min, 50% B. The injection volume was 10 μ l. MS data
365 were acquired in multiple reaction monitoring (MRM) mode. The conditions of the interface were as
366 follows: ion spray voltage, –4500 V; source temperature, 450 °C; curtain gas pressure, 10 psi;
367 nebulizing gas pressure, 70 psi; and turbo gas pressure, 80 psi. The parameters used for the mass
368 spectrometry of LA-Ile (**10**) are listed in Supplemental Table S1. Analyst 1.6.2 software was used for
369 data acquisition and processing. LA-[¹³C₆,¹⁵N]Ile was used as a standard for quantitative analysis. The
370 values given are the mean \pm SD of five independent biological replicates.

371

372 8. *In vitro* synthesis of OPDA-Ile (**9**) by an *A. thaliana* protein extract

373 Plants (1.0 g) were ground in liquid nitrogen and extracted with 10 ml of 100 mM sodium
374 phosphate buffer (pH 7.8). The residue was removed by centrifugation at 20,000 \times g for 15 min,
375 and the supernatant was then used as a protein extract to synthesize OPDA-Ile (**9**). One milliliter of
376 the protein extract supplemented with 1 mM LA-Ile (**10**) was incubated at 25 °C for 1 hour. The pH
377 of the reaction solution was adjusted to approximately 3, and the solution was next extracted with an
378 equal volume of ethyl acetate and then evaporated. The resulting residue was dissolved in 200 μ l of
379 80% aqueous MeOH, and OPDA-Ile (**9**) was analyzed by UPLC-MS/MS according to the method
380 described above. A protein extract without added LA-Ile (**10**) and 100 mM sodium phosphate buffer
381 (pH 7.8) supplemented with LA-Ile (**10**) were used as controls.

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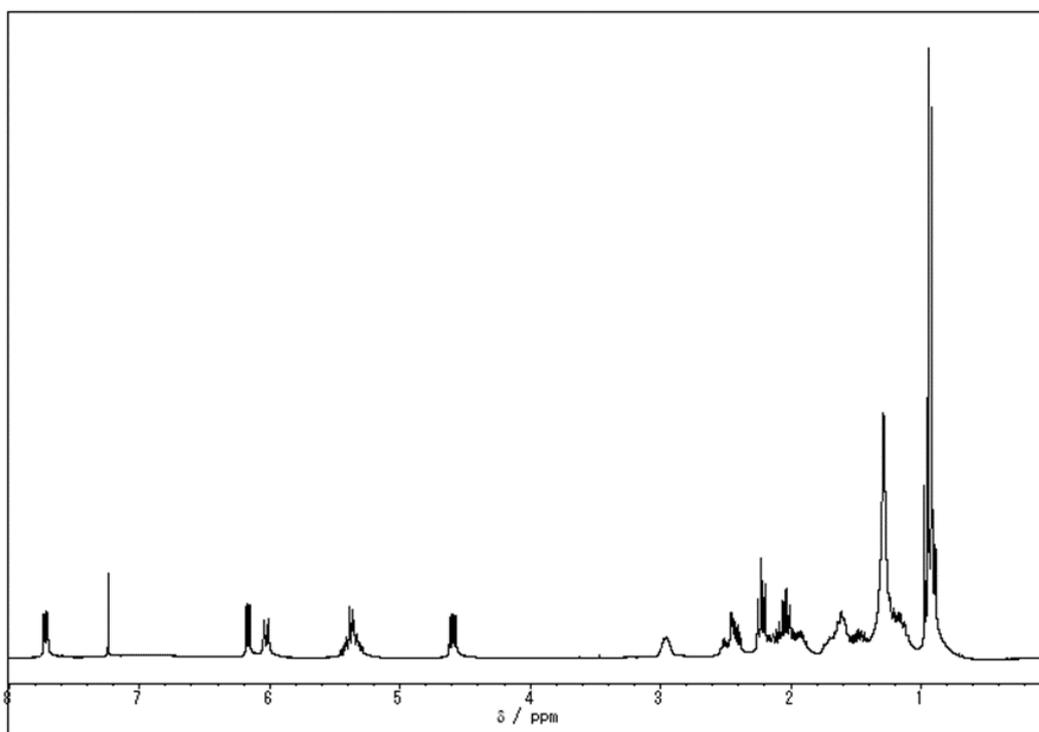
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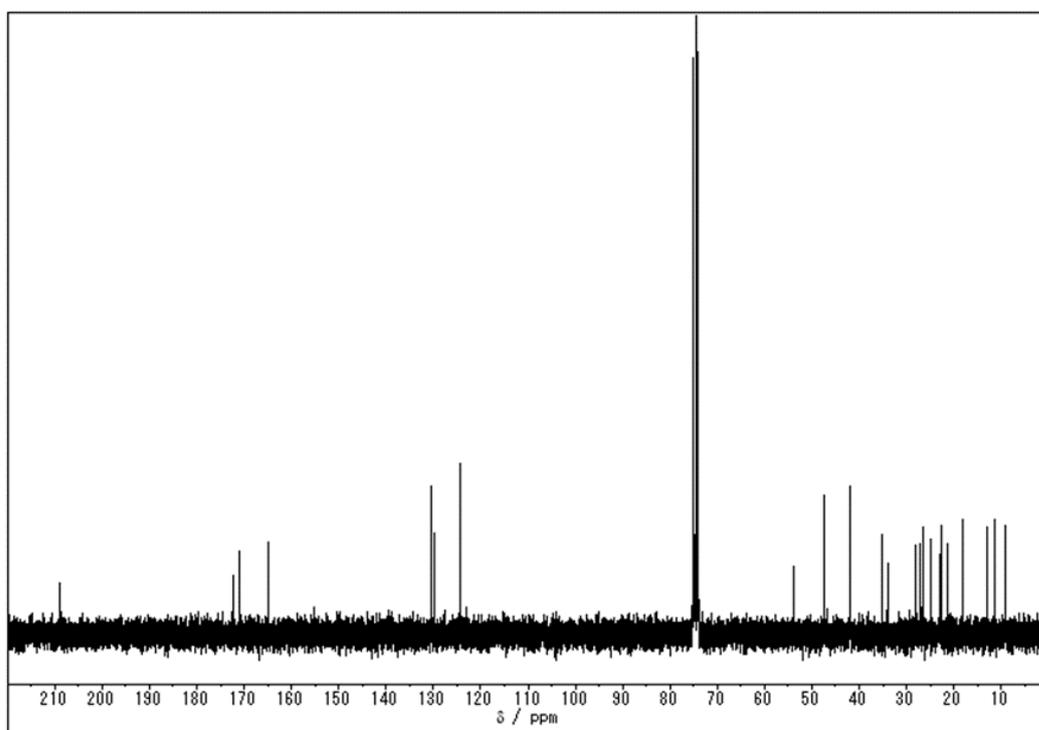
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392 Supplemental Fig. S1. ¹H-NMR spectrum of OPDA-Ile (**9**) (270 MHz, CDCl₃).

393



394

395 Supplemental Fig. S2. ¹³C-NMR spectrum of OPDA-Ile (**9**) (67.5 MHz, CDCl₃).

396

397 Supplemental Table S1. Optimized MS parameters for the analysis of LA-Ile.

Compound	Scan mode	MRM transition	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision exit potential (V)
LA-Ile	–	390.3/128.0	-80	-10	-34	-19

398