Biosynthesis of (2-nitroethyl)benzene and (Z)- and (E)-(2-nitroethenyl)benzenes from (Z)- and (E)-phenylacetaldoximes and phenylacetonitrile; defense allomone of Eutrichodesmus elegans and Eutrichodesmus armatus (Polydesmida: Haplodesmidae)

Yasumasa Kuwahara,1,3 Yayoi Ichiki,1,3,* Masashi Morita,1,3,# Tsutomu Tanabe4 and Yasuhisa Asano2,3,6

1 Asano Active Enzyme Molecular Project, JST, ERATO, Kyoto Branch, Kyoto, Kyoto 602–0841, Japan
2 Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939–0398, Japan
3 Asano Active Enzyme Molecular Project, JST, ERATO, 5180 Kurokawa, Imizu, Toyama 939–0398, Japan
4 Faculty of Education, Kumamoto University, Kumamoto, Kumamoto 860–8555, Japan
5 Asano Active Enzyme Molecular Project, JST, ERATO, Kyoto Branch, Kyoto, Kyoto 602–0841, Japan
6 Asano Active Enzyme Molecular Project, JST, ERATO, 5180 Kurokawa, Imizu, Toyama 939–0398, Japan

(Received December 28, 2017; Accepted April 25, 2018)

The defense allomones of two haplodesmid millipedes, Eutrichodesmus elegans and E. armatus (Polydesmida: Haplodesmidae), are known as a mixture of the following three nitro compounds: (2-nitroethyl)benzene and (Z)- and (E)-(2-nitroethenyl)benzenes. Administrations of a mixture of [3H]labeled (Z)- and (E)-phenylacetaldoximes and of [4H]labeled phenylacetonitrile as precursors resulted in the same production of three [3H]-labeled nitro compounds, [2H]-nitroethyl][2,3,4,5,6-H5]benzene and [(Z)- and (E)-2′-nitroethyl][2,3,4,5,6-H5]benzenes, in both species. Oxime administration at an appropriate dose resulted in the production of three nitro compounds with similar natural ratios more effectively than nitrile administration. Conversion from oximes to nitrile and vice versa was evidenced during administration. Occurrences of three precursors (Z- and E-oximes and nitrile) were detected sporadically in millipede extracts by selected ion chromatography.

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Keywords: Eutrichodesmus elegans, Eutrichodesmus armatus, [2H]-nitroethyl][2,3,4,5,6-H5]benzene, [(Z)- and (E)-2′-nitroethyl]-[2,3,4,5,6-H5]benzenes, biosynthesis, millipede allomone, (Z)- and (E)-phenylacetaldoximes.

Electronic supplemental material: The online version of this article contains supplemental information (Supplementary material), which is available at http://www.jstage.jst.go.jp/browse/jpestics/

Introduction

The two haplodesmid millipedes, Eutrichodesmus elegans (Polydesmida: Haplodesmidae) and E. armatus (Polydesmida: Haplodesmidae), are both white endemic species (body length ca. 6 mm and 5 mm, respectively) in Japan, commonly possessing three nitro compounds, (2-nitroethyl)benzene (1), (Z)- and (E)-(2-nitroethenyl)benzenes (2 and 3), as their defense allomone.1,2,3 Natural occurrences of 2 and 3 have been hitherto unknown except in the present two species. In plants, compound 1 is widely distributed not only as an essential oil component in Demnetitia tripetal (Magnoliales, Annonaceae),5,6 Aniba cannabina (H.B.K.) Mez (Laurales, Lauraceae), and Ocotea pretiosa (Nees) Mez (Laurales, Lauraceae),5 but also as a flavor component in many kinds of flowers.6 The nitro compounds detected as unusual volatiles from Solanum lycopersicum (Solanaceae, Solanaceae) tomato fruits7 have been identified as one of the major flower scents in the Japanese loquat Eriobotrya japonica (Rosales, Rosaceae),7 Compound 1 smells sweet and has recently been identified as one of the major flower scents in the Japanese loquat Eriobotrya japonica (Rosales, Rosaceae).7 The nitro compounds detected as unusual volatiles from Solanum lycopersicum (Solanaceae, Solanaceae) tomato fruits7 have been identified as one of the major flower scents in the Japanese loquat Eriobotrya japonica (Rosales, Rosaceae).7 In higher plants, (Z)- and (E)-phenylacetaldoxime (F and G) are produced from 1-phenylalanine,11 and the radioactive 1 has been confirmed in plants after application of 14C-labeled F and G.10 The generation of [(E)-2-nitroethenyl][α,β,2,3,4,5,6-H5]-benzene ([4H]-3, 2.8%) has been demonstrated as a mixture with 3 after the administration of α,β,β,2,3,4,5,6-H5-1-phenylalanine for 5 days from adult E. elegans via gas chromatography-mass spectrometry (GC/MS) analysis. This indicates that 1-phenylala-
nine is a common precursor not only of $3^{1}$ but also of the other well-known and widely distributed allomones (=mandelonitrile and its related compounds) of polydesmid millipedes. The other related species, $E$. armatus (Miyosi) (Polydesmida: Haplodesmidae), also contains a mixture of 1, 2, and 3. $^{2}$ However, the biogenetic pathway from L-phenylalanine to 3, as well as biogenetic relationships among the three nitro compounds (1, 2, and 3), remains obscure.

In the present study, the biosynthesis of these millipede allomones was examined by feeding (Z)- and (E)-2,3,4,5,6-$^{2}$H$_{5}$-phenylacetaldoximes (H$_{2}$-F and -G, equilibrium mixture) and 2,3,4,5,6-$^{2}$H$_{5}$-phenylacetonitrile (H$_{2}$-E), which were prepared from 2,3,4,5,6-$^{2}$H$_{5}$-bromobenzene by three- or four-step reactions (Grignard reaction, oxidation, oxime preparation, and subsequent dehydration). After administration of these compounds to millipedes and subsequent incubation overnight, the resulting $^{2}$H$_{5}$-labeled products in the millipedes were examined via selected ion chromatography (SIC) using GC/MS. From $^{2}$H$_{5}$-F and -G and $^{2}$H$_{5}$-E-administered millipedes, three 2,3,4,5,6-$^{2}$H$_{5}$-isomers ($^{2}$H$_{5}$-1, $^{2}$H$_{5}$-2, and $^{2}$H$_{5}$-3) were obtained with 1, 2, and 3, respectively. The presence of natural F, G, and E was also supposed based on isotope dilution phenomena in the $^{2}$H$_{5}$-F and -G peaks and $^{2}$H$_{5}$-E after incubation, and their natural presence along with E was accessed via SIC. Then precursors and biogenetic sequences of the millipede allomones were elucidated based on these findings.

**Materials and Methods**

1. **Millipede species**

*Eutrichodesmus elegans* (Fig. 1A) was collected from leaf litter at Nashinoki Shrine (N 35.024°, E 135.767°), Kamigyoku, Kyoto, and at Yukusyouji-ike Park (N 36.700°, E 137.084°) in Toyama Prefecture (Fig. 1A). *E. armatus* (formerly *Thelodesmus armatus*, reassigned)$^{13}$ (Fig. 1B) was collected in Kyoto (as mentioned above) and Family Park (N 36.692°, E 137.146°) in Toyama. Both species were kept in the laboratory by feeding litter obtained from the collected site under an ambient photoperiod and humid conditions at 20°C. Adults were mainly used without discrimination of the sexes, along with juveniles, at each indicated stadium (growth stage). The stadium of each juvenile was recognizable by two criteria, $^{14}$1) the number of body segments and 2) the number of leg pairs under a binocular microscope (Shimadzu Co., Ltd., STZ-168).

2. **Hexane extraction of millipedes**

Hexane (40 μL or each indicated volume) was added to a millipede kept in a glass tube (conical-bottomed micro-insert, 28.96 mm in length, φ5.73 mm). After 3 min, the hexane layer (4 μL or indicated volume) was removed using a 10 μL micro syringe and subjected to GC/MS analysis.

3. “**Forced feeding**” incubation$^{15}$ of aqueous (Z)- and (E)-2-$^{2}$H$_{5}$-phenylacetaldoxime (H$_{2}$-F and -G) and of $^{2}$H$_{5}$-phenylacetonitrile (H$_{5}$-E) against adult millipedes *E. elegans* and *E. armatus*

Adult millipedes *E. elegans* (without discrimination of sex) were separately introduced upside down into each glass tube (as mentioned above) containing the following doses of $^{2}$H$_{5}$-F and -G (purity 99.3%, equilibrium mixture) as the substrate in aqueous solution (total volume 10 μL): 5 μg, 3 μg, 2 μg, and 1.5 μg, and the control. Most millipedes could not turn around because of the narrow diameter of the tube and were kept submerged with the substrate (forced feeding, Fig. 1C). Then hexane (40 μL or indicated volume) was added to the tube as mentioned above (millipede drowned or barely survived after being kept 24 hr at room temperature), and after 3 min, the hexane extract layer (4 μL) was subjected to GC/MS analysis.

Similarly, oximes ($^{2}$H$_{5}$-F and -G; purity 99.3%) and nitrile ($^{2}$H$_{5}$-E; purity 99.5%) of each 1.5 μg/10 μL were prepared and administered to *E. elegans* and *E. armatus* to compare feasibility as a substrate.

4. **Preparation of compounds**

Preparation methods of (Z)- and (E)-2,3,4,5,6-$^{2}$H$_{5}$-phenylacetaldoximes (H$_{2}$-F and -G, equilibrium mixture) and 2,3,4,5,6-$^{2}$H$_{5}$-phenylacetonitrile (H$_{5}$-E) are shown in electronic supplement material.

5. **Chemical analysis**

GC/MS was performed using an Agilent Technologies 6890N Network GC System, $^{16}$ which was coupled with a 5975 Inert XL Mass Selective Detector operated at 70 eV, using an HP-5MS capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness; Agilent Technologies), with He as the carrier gas at a flow rate of 1.00 ml/min in the splitless mode at a temperature programmed to change from 60°C (2 min) to 290°C at a rate of 10°C/min and then to hold for 5 min. GC and GC/MS signals were acquired and processed with a Chemstation (Hewlett-Packard) installed with an MS database (Wiley275 library, Hewlett-Packard).

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**Fig. 1. Pictures of millipedes and force-feeding administration.** (A) Adult and stadium II nymph of *E. elegans* (Miyosi). (B) Female adult of *E. armatus* (Miyosi) constructing an egg chamber. The white part of the center is the egg cluster. (C) Force-feeding administration to adult *E. elegans*.**
Table 1. Gas chromatographic and mass spectral data of compounds from extracts and incubation results of millipede E. elegans and E. armatus

| Compound identified as | Standard obtained (%) | Mass spectrometric fragmentation (m/z) | Retention Time (min) |
|------------------------|-----------------------|---------------------------------------|----------------------|
| Compound               | Index | Peak no. | Index (RI) | Time |
| Benzaldehyde           | com. (97%) | 106 (M⁺, 105), 104 (96), 77 (90), 51 (32) | 957 | 5.61 |
| 2H5-Benzaldehyde       | synthesis | 111 (M⁺, 95), 110 (100), 82 (78), 54 (27) | 956 | 5.59 |
| Benzyl alcohol         | com. (97%) | 113 (M⁺, 100), 84 (63), 63 (64), 54 (17) | 1048 | 6.72 |
| 2H5-Benzyl alcohol     | synthesis | 120 (M⁺, 22), 91 (100), 65 (16), 51 (4) | 1046 | 6.69 |
| Phenylacetaldehyde     | synthesis | 125 (M⁺, 24), 96 (100), 69 (8), 68 (8) | 1062 | 6.89 |
| Phenethyl alcohol      | synthesis | 127 (M⁺, 30), 109 (2), 96 (100), 83 (3), 68 (8), 54 (3) | 1148 | 7.93 |
| Phenacetonitrile       | com. (93%) | 117 (M⁺, 90), 77 (6), 63 (9), 51 (8) | 1188 | 8.38 |
| 2H5-Phenylacetonitrile | synthesis | 122 (M⁺, 100), 94 (27), 82 (3), 65 (5), 54 (7) | 1182 | 8.35 |
| 2H5-(Z)-Phenylacetaldoxime | synthesis (94%) | 145 (M⁺, 35), 117 (88), 91 (100), 65 (21) | 1322 | 10.05 |
| 2H5-(E)-Phenylacetaldoxime | synthesis (96%) | 140 (M⁺, 27), 120 (100), 96 (99) | 1318 | 10.00 |
| 2H5-Phenylacetonitrile | synthesis | 117 (M⁺, 90), 117 (4), 104 (100), 91 (11), 77 (25), 63 (4), 51 (10) | 1346 | 10.34 |
| 2H5-(2-Nitroethyl)benene | synthesis (94%) | 149 (M⁺, 23), 132 (32), 119 (9), 102 (71), 91 (66), 77 (100), 65 (25), 51 (40) | 1381 | 10.76 |
| 2H5-(2-Nitroethenyl)benene | synthesis | 145 (M⁺, 35), 137 (39), 124 (31), 108 (62), 96 (100), 81 (73), 71 (26), 51 (41) | 1382 | 10.73 |
| 2H5-(Z)-2-Nitroethenylbenzene | synthesis (95%) | 140 (M⁺, 27), 120 (100), 96 (99) | 1399 | 11.11 |
| 2H5-(E)-2-Nitroethenylbenzene | synthesis (91%) | 149 (M⁺, 69), 132 (18), 119 (4), 102 (78), 91 (63), 77 (100), 65 (19), 51 (33) | 1504 | 12.26 |
| 2H5-(2-Nitroethenyl)benzene | synthesis | 154 (M⁺, 99), 137 (21), 124 (13), 107 (100), 96 (81), 81 (77), 71 (25), 54 (34) | 1501 | 12.22 |

6. Detection and assessment of compounds via SIC using GC/MS

The M⁺ ion, the base ion, and several diagnostic ions were employed to detect and identify the target compound in GC/MS analysis, considering the m/z +5 increments of each 2H5-labeled compound (Table 1). The contents of natural component and its 2H5-labeled isomer were accessed via SIC, using the base ion of the natural compound and that of 2H5-labeled as each monitor ions. After the integration of each SIC profile on all GC peaks, the 2H5-labeled isomer content for each components was evaluated as a percentage.

In the present study, each pair of base ions was selected as follows: 2H5-1 and 1 (m/z 109 and m/z 104, respectively), 2H5-2 and 2 (m/z 96 and m/z 77, respectively), and 2H5-3 and 3 (m/z 107 and m/z 77, respectively) (see Table 1, Fig. 3). Natural abundances of E: 2H5-E, F: 2H5-F, and G: 2H5-G were similarly assessed using the following pairs of base ions as monitors: m/z 117:122, m/z 91:96, and m/z 117:122, respectively.

7. Chemicals

2,3,4,5,6-2H5-Bromobenzene was obtained from Cambridge Isotope Laboratories, Inc (Tewksbury, MA). The following six compounds and materials were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan): benzaldehyde (A), hydroxylamine (aq. 50%), phenethanol alcohol (D), Wako Chemical Co., dichloromethane, and hexane. Benzyl alcohol (B), Florisil, and magnesium turnings were obtained from Nacalai Tesque, Inc., (Kyoto, Japan); 2-chloroethanol was obtained from Kanto Chemical Co., Inc., (Tokyo, Japan); and phenylacetonitrile (E), pyridinium chlorochromate, and diaethyl ether (super dehydrated and reagent grade) were obtained from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). The following compounds were prepared as described: Phenylacetaldehyde (C) was prepared from phenylacetaldehyde dimethyl acetal (Tokyo Chemical Industry Co., Ltd.) by acid hydrolysis. A mixture of (Z)- and (E)-phenylacetaldoxime [F (53%) and G (47%)] was prepared from C and hydroxylamine (aq. 50%). (2-Nitroethyl)benzene (1) was prepared from 2’-bromoethylbenzene (Tokyo Chemical Industry Co., Ltd.) by refluxing successively in acetone (Wako Pure Chemical Industries, Ltd.) with KI (Nacalai Tesque Inc.) and then with sodium nitrite (Tokyo Chemical Industry Co., Ltd.). (Z)- and (E)- (2-Nitroethyl)benzene (2 and 3) were prepared as described.1)
Results

1. Relationships between administered $^2$H$_5$-F and -G quantities and GC profiles

Among five profiles of $^2$H$_5$-F and -G administration [equilibrium mixture (5 $\mu$g, 3 $\mu$g, 2 $\mu$g and 1.5 $\mu$g), and the control] to each adult E. elegans, peak 3 (=compound 3) was the major component in all profiles, while peak 1 (=compound 1) increased proportionally to applied substrate doses, and peak 2 (=compound 2) decreased inversely (Fig. 2). The relative abundance of peaks A–G (=compound A–G) decreased proportionally to the applied substrate doses. The 1.5 $\mu$g dose of $^2$H$_5$-F and -G indicated almost the same profile as that of the control and appeared to be the most appropriate condition physiologically. In the profile, excess amounts of $^2$H$_5$-F and -G were not detected, and all millipedes survived the 24 hr incubation. On the other hand, all millipedes incubated with substrates at 5 $\mu$g, 3 $\mu$g, and 2 $\mu$g drowned during the 24 hr incubation.

All peaks (A–G and 1–3) were composed of a mixture of the natural compounds and their $^2$H$_5$-isomers. As shown in Fig. 3 and Table 1, $^2$H$_5$-labeled isomers appeared at ca. 0.02–0.05 min sooner $t_k$ (dotted line) than those of natural compounds (solid line). Most mass fragments appeared with an $m/z$ 5 larger than that of corresponding fragments from the natural compound (Table 1), but base ion pairs were not always found as $m/z + 5$ differences, such as A, B, 2, and 3.

After detection and integration of all peaks by SIC, the $^2$H$_5$-labeled isomer content (%) in each peak was assessable, such as *51.6% for $^2$H$_5$-1, *5.9% for $^2$H$_5$-2, and *5.5% for $^2$H$_5$-3, as demonstrated in Fig. 3 (a typical profile of 2 $\mu$g administration on peaks 1–3; *data are not identical to those averaged as in Table 2, b). Likewise, all other peaks, including A–G in all profiles (Fig. 2), are assessed and summarized in Table 2, b.

2. Results of $^2$H$_5$-F and -G incubation with E. elegans

In the case of the 5 $\mu$g administration, peak A (1.2%, Table 2, a) was concluded to be a mixture of $^2$H$_5$-A [57.5% in Table 2, b], monitored by the base ion at $m/z$ 110 ($t_k$ 5.59 min in Table 1)] and A [natural non-labeled, monitored by the base and molecular ion at $m/z$ 106, $t_k$ 5.61 min, as in Table 1)]. Likewise, peak B (1.9%) was a mixture of $^2$H$_5$-B (55.3%) and B. The relative peak area (%) and $^2$H$_5$-isomer content (%) in all other peaks and those of all other substrate applications were similarly obtained and are summarized in Table 2, a and b.

It may be noteworthy to mention that the $^2$H$_5$-labeled isomer content (Table 2, b) in all peaks (A–G and 1–3) at all substrate applications (5 $\mu$g, 3 $\mu$g, 2 $\mu$g, and 1.5 $\mu$g) did not reach the originally applied purities [(E)-$^2$H$_5$-F, 99.3%; (Z)-$^2$H$_5$-G, 99.3%]. This means that all peaks were accompanied by each non-labeled natural isomer (content %; 99.3–b). Peaks E, F, and G showed almost the same high $^2$H$_5$-labeled isomer content, especially at 5 $\mu$g and 3 $\mu$g applications of more than 98% (Table 2, b). This indicated that nitrile (E) was derived directly from oximes (F and G) by dehydration, which is indicative of the interchangeable nature between nitrile (E) and oximes (F and G).

3. Effect of substrate decreases on the $^2$H$_5$ incorporation rate in E. elegans

As indicated in Table 2, all peaks (A–E and 1–3) were composed of pairs of non-labeled compounds and corresponding $^2$H$_5$-
isomers. Even the substrate peaks (F and G) were presumably contaminated with the natural isomers.

The $^3$H$_2$ incorporation rates were assessed for all compounds (A–G and 1–3) and the results are shown in Table 2a, b. Most compounds (A-F and 1) increased from the 5µg to the 3µg administration (the reason remains obscure) and then markedly decreased from 3 to 1.5µg. On the other hand, the rate of compound 2 remained almost unchanged (4.7%, 3.1%, 4.0%, and 4.5%), and that of compound 3 increased gradually from 1.1 to 2.4%, then 2.7%, and finally to 4.3%, inversely against decreasing $^2$H$_5$-F and -G doses.

The lower four-line groups (a×b/µg) of Table 2 indicated relative amounts of generated $^2$H$_5$-labeled isomers per 1µg $^2$H$_5$-F and -G application. The peak areas ratios [1:2:3 as 0.3%:1.9%:97.8% (re-calculated from 0.3%:1.8%:97.1%, based on A–G and 1–3 at 1.5µg $^2$H$_5$-F and -G doses in Table 2a)] were reproduced by the generated $^2$H$_5$-labeled isomer ratios [$^2$H$_5$-1:2H$_5$-2:2H$_5$-3 as 0.5% (1.5%):1.9% (5.4%):97.6% (278.4%)] (Table 2a, a×b/µg, at 1.5µg). Those ratios (%) were identical to results summarized in Table 4 and to adults reported. 

4. Comparison of $^2$H$_5$-E and $^2$H$_5$-F and -G incubation on E. elegans and E. armatus

$^2$H$_5$-E [1.5µg/µL] and $^2$H$_5$-F and -G [1.5µg/µL, equilibrium mixture] were administered to E. elegans and E. armatus to compare feasibility as a substrate (Table 3 and Fig. 4). In E. elegans, $^2$H$_5$-F and -G (72.1% of the substrate) were changed to a mixture of $^2$H$_5$-E (17.7, a×b in Table 3), $^2$H$_5$-1 (51.9), $^2$H$_5$-2 (2.6), and $^2$H$_5$-3 (215.0), with unchanged (27.9%, corresponded to $^2$H$_5$-F (54.2) and $^2$H$_5$-G (56.7). Likewise, $^2$H$_5$-E (4.3% of the substrate) to a mixture of $^2$H$_5$-F (19.3), $^2$H$_5$-G (30.4), $^2$H$_5$-1 (42.4), $^2$H$_5$-2 (n.d.), and $^2$H$_5$-3 (1.7), with un-reacted substrate (95.7%, corresponded to $^2$H$_5$-E (2109.0).

In E. armatus, $^2$H$_5$-F and G (48.4% of the substrate) were used to produce a mixture of $^2$H$_5$-E (357.2), $^2$H$_5$-1 (314.8), $^2$H$_5$-2 (196.2), and $^2$H$_5$-3 (49.3), with unchanged (51.6%) as $^2$H$_5$-F (552.3) and $^2$H$_5$-G (427.2). Similarly, $^2$H$_5$-E (9.5%) was used to produce a mixture of $^2$H$_5$-F (10.2), $^2$H$_5$-G (6.5), $^2$H$_5$-1 (165.4), $^2$H$_5$-2 (0.3), and $^2$H$_5$-3 (14.3), with unchanged (90%).

As a result, F and G were concluded to be the better precursors to generate a mixture of 1, 2, and 3 than E in both species (Fig. 4). In the case of E. elegans, the ratio (1:2:3) of relative production rates (a×b) of $^2$H$_5$ isomers after $^2$H$_5$-F and -G administration was observed to be 51.9 (19.2%):2.6 (1.0%):215.0 (79.8%). Although the ratio indicated the same trend, it is not identical to those of peak ratios (0.3%:1.9%:97.8%) nor $^2$H$_5$-labeled ratios (0.5%:1.9%:97.6%) summarized in Table 2a, but also to those of reported. 

A similar trend was at least reproduced like the case of $^2$H$_5$ administration in Table 2a: 15.1 (10.7%), 2: 3.8 (2.7%), and 3: 122.4 (86.6%). In the case of E. armatus, the ratios (a×b) [1:2:3 = 314.8 (56.2%):196.2 (35.0%):49.3 (8.8%)] showed a large discrepancy with the natural one reported. This might be attributable to inappropriate incubation conditions, where relatively large abundances (51.6%) of F and G were left unchanged, as mentioned above.

The compound (E) was also metabolized into 1, 2, and 3, but incorporation appeared to be sporadic and less effective, as mentioned above. The deuterium content in E (originally >99.5%),
Table 3. Comparison of $^{2}$H$_{5}$-phenylacetonitrile ($^{2}$H$_{5}$-E) and (E)- and (Z)-$^{2}$H$_{5}$-phenylacetaldoxime ($^{2}$H$_{5}$-F and -G) incubation against two species of Eutrichodesmus.

| Monitor ions by MS | E  | F  | G  | 1  | 2  | 3  |
|-------------------|----|----|----|----|----|----|
| Phenylacetonitrile (122, 117) | Phenylnitrobenzene (109, 104) | (Z)-Phenylnitrobenzene (96, 77) | (E)-Phenylnitrobenzene (96, 77) |
| 2H$_{5}$-incubate | $^{2}$H$_{5}$-incubate | $^{2}$H$_{5}$-incubate | $^{2}$H$_{5}$-incubate |
| a: peak area (%) | 1.1 | 0.6 | 0.7 | 8.8 | 12.4 | 0.7 | 89.6 | 13.6 |
| b: 2H$_{5}$-isomer content (%) | 16.1 | 20.4 | 90.3 | 81.0 | 5.9 | 6.2 | 3.7 | 2.4 | 2.2 |
| a×b: production rate | 17.7 | 54.2 | 56.7 | 51.9 | 2.6 | 215.0 |
| a: peak area (%) | 22.2 | 11.0 | 5.0 | 0.3 | 0.5 | 0.7 | 35.3 | 20.6 | n.d. |
| b: 2H$_{5}$-isomer content (%) | 95.0 | 2.7 | 98.6 | 54.3 | 60.7 | 12.6 | n.d. | tr ($=0.04$) |
| a×b: production rate | 2109.0 | 19.3 | 30.4 | 42.4 | n.d. | 1.7 |
| 2H$_{5}$-incubate | 3.9 | 6.3 | 5.7 | 9.5 | 4.7 | 8.0 | 17.2 | 28.7 | 13.7 | 22.5 | 19.1 | 54.8 | 40.9 |
| a: peak area (%) | 91.6 | 5.4 | 96.9 | 1.6 | 90.9 | 8.5 | 18.3 | 11.7 | 14.3 | 30.4 | 196.2 | 49.3 |
| b: 2H$_{5}$-isomer content (%) | 357.2 | 552.3 | 427.2 | 314.8 | 196.2 | 196.2 | 14.3 |
| a×b: production rate | 1871.8 | 10.2 | 6.5 | 165.4 | 0.3 | 14.3 |

$^{a}$ $n=1$, $^{b}$ $n=2$, $^{c}$ $n=3$. tr; $<0.1$. Mean ± STD.

Fig. 4. Proposed biosynthetic pathway of (2-nitroethyl)benzene (1) and (Z)- and (E)-(2-nitroethenyl)benzenes (2 and 3) with incubation results of $^{2}$H$_{5}$-F and -G and $^{2}$H$_{5}$-E against E. elegans (in bold letter) and E. armatus (in underlined light letter). Left bold letter: $^{2}$H$_{5}$-F and -G incubation result for E. elegans; right bold letter: $^{2}$H$_{5}$-E incubation result for E. elegans; left underlined light letter: $^{2}$H$_{5}$-F and -G incubation result for E. armatus; right underlined light letter: $^{2}$H$_{5}$-E incubation result for E. armatus.

found as 95.0% in E. elegans and 98.0% in E. armatus, was diluted to a mixture of F and G (38.6% and 60.7%, respectively, in E. elegans) and (51.0% and 32.6%, respectively, in E. armatus), while the total production rate of $^{2}$H$_{5}$ compounds (1, 2, and 3) was less than half in E. armatus and much less in E. elegans than that from F and G. A larger amount of E remained unchanged.
Table 4. Relative abundances of three nitro-compounds, and MS counts of three biogenetic precursors

| Species | Abundance (% by RIC) | MS counts* by the selected ion chromatography |
|---------|----------------------|---------------------------------------------|
|         | (2-Nitroethyl)-benzene | (Z)-(2-Nitroethenyl)-(E)-(2-Nitroethenyl)-benzene | Phenylacetonitrile | (Z)-Phenyl-acetaldoxime | (E)-Phenyl-acetaldoxime |
| Stadium | Ext. vol/Inj. vol | 1 | 2 | 3 | E | F | G |
|---------|-----------------|----|----|----|----|----|----|
| E. elegans |                  |    |    |    |    |    |    |
| I†      | 5 µL (n=7)      | 2.6±3.0 | 0.1±0.1 | 97.3±3.0 | 1988 (n=1) |
| II      | 10 µL/4 µL (n=6) | 0.8±0.5 | 0.1±0.1 | 99.1±0.5 | 1169±151 (n=2) |
| III     | 20 µL/1 µL (n=4) | 0.7±0.8 | 0.3±0.2 | 99.0±0.8 | 4673±459 (n=2) |
| IV      | 20 µL/1 µL (n=8) | 0.4±0.6 | 4.9±6.0 | 94.7±5.4 | 3845±631 (n=6) |
| V       | 50 µL/1 µL (n=5) | 0.2±0.1 | 1.0±0.8 | 98.8±0.8 | 6100±4932 (n=4) |
| VI      | 200 µL/1 µL (n=4) | 0.1±0.1 | 1.2±0.3 | 98.7±0.3 | 5135±1783 (n=5) |
| ♀       |                  | 0.4±0.1 | 99.5±0.1 | 531±228 (n=2) |
| E. armatus |                  |    |    |    |    |    |    |
| III     | 10 µL/1 µL (n=5) | 0.5±0.6 | 8.3±5.4 | 91.2±5.1 | 3731±736 (n=4) |
| IV      | 20 µL/1 µL (n=6) | 0.2±0.1 | 25.0±16.8 | 74.8±16.8 | 3102±1691 (n=4) |
| V       | 40 µL/1 µL (n=6) | 0.1±0.1 | 40.0±5.2 | 59.9±5.2 | 5197±1359 (n=2) |
| VI      | 40 µL/1 µL (n=5) | 0.1±0.1 | 35.7±9.7 | 64.2±9.8 | 4571±1190 (n=4) |
| ♀       | 50 µL/1 µL (n=4) | 1.2±1.2 | 26.5±3.2 | 73.5±3.2 | 1528 (n=1) |
| ♀       | 50 µL/1 µL (n=4) | 1.2±1.2 | 17.4±2.4 | 82.6±2.4 | 2550 (n=1) |

Mean ± STD; t; <0.1%. *Stayed in egg chamber without feeding. †Stayed overnight in egg chamber with feeding. *Counts by each injected volume.

(95.7% in E. elegans and 90.5% in E. armatus) than the corresponding amounts of F and G, as mentioned above.

The interchangeable nature from E to F and G and vice versa, as mentioned above, seemed to differ between species (Table 3, Fig. 4): in E. elegans from E to F and G (from 40.7 to 49.7 = 42.4:1) and F and G to E (from 110.9 to 17.7 = 6.3:1) and in E. armatus from E to F and G (from 1871.8 to 16.7 = 112.1:1) and F and G to E (from 979.5 to 357.2 = 2.7:1). These facts indicated that the ease of interchange seemed to differ in both species and directions.

5. Direct assessments of E, F, and G in millipedes, together with 1, 2, and 3

Relative abundances of natural E, F, and G were assessed in both millipede extracts via SIC (Table 4). Natural ratios among 1, 2, and 3 are also listed and are the same as reported.17) Compound E was distributed in all observed stadia, though distribution was sporadic. Compounds F and G were observed occasionally and always in lower amounts than E, indicating a feasibility difference between F and G for allomone production.

Discussion

A haplodesmid millipede E. elegans (Miyosi) is known to use a mixture of 2 and 3 as its defense allomone.1) The presence of 1 has been known in E. armatus2) and recently re-identified in E. elegans as a new discovery by observing allomone composition along with ontogenetic development.17) By this sequential observation of two species, 1 (as a minor component) and 3 (as a major one) have been detected from the beginning of ontogenetic development, with a decreasing trend of 1 to a trace in adults and increasing trends of 3 (up to 80% in E. armatus and to 99% in E. elegans). Accumulation of compound 2 has become recognizable from the foraging nymph at the later stadium I to adults in a species-specific manner with a convex curve17) and reached to the maximum (less than 5% in E. elegans and up to 40.0% in E. armatus, Table 4).

1-Phenylalanine has been known as the precursor of 3 in E. elegans,1) and F and G have been transformed successfully into 1 (up to 18%, data not shown), like the case of higher plants.12) No reliable generation of 2 and 3 has, however, been attained in any trial of incubations using bodies, degutted bodies, or macerated tissues of the species.

In the present study, generation of $^2$H$_5$F, $^3$H$_2$-2, and $^3$H$_2$-3 by E. elegans has been successfully demonstrated using a “force-feeding” method15) of $^2$H$_5$F and -G in ideal conditions haphazardly found. The peak area ratios (1:2:3) at 1.5 µg $^2$H$_2$-F and -G administration (Table 2, a) were identical to those of the generated $^2$H$_5$F isomer ratios in each peak ($^2$H$_5$-1:$^2$H$_5$-2:$^2$H$_5$-3 in Table 2, a×b/µg) and to those results in Table 4 and adult reported.17) From the experiment, the following facts were also noticed: 1) desaturation step(s) from 1 to 3 (or 2) might be rate determined and (or) controlled by feedback regulation; 2) F and G seemed to be convertible to E, and vice versa, during incubation. Although $^2$H$_5$-E was a less effective substrate against both species than F and G, all three nitro compounds (1, 2, and 3) contained corresponding $^2$H$_5$ isomers. The rate of conversion seemed to differ between species, as did the direction. As summarized in Fig. 4, F and G were demonstrated as precursors of
Desaturation to generate Z geometry is known in the fatty-acyl CoA-related desaturation process. The generation of Z was observable as a minor component in E. elegans (less than 5% in Table 4), while up to 40.0% was generated in E. armatus. Similar large Z-isomer production [16%=(13.7/ (17.2+13.7+54.8)×100%=16%)] in E. armatus was reproduced in 2H5-, F, and G administration (Table 3). This large accumulation of 2 is puzzling, because it is chemically difficult to keep Z geometry under conjugation with aromatic π electrons. Actually, chemical preparation is a mixture of 2 and 3 with a ratio of 1:56. Therefore, there should be no difference conceivable between two species on each ratios of 2 and 3, but actually different. There might be a species-specific mechanism by which E. armatus keeps an unusual amount (up to 40.0%) of Z after Z desaturation from 1. If so, then 3 might be produced from 1 by Z desaturation to 2 and subsequent isomerization to 3. At present, however, there is no concrete evidence to deny the possibility of the other pathway from 1 to 3 and then to 2.

In conclusion, F and G were identified as the precursors of 1 in both species, as reported in plants, and then 2 and 3 are produced by desaturation from 1. No rational evidence was obtained by the present study, to conclude whether geometric isomerization from 2 to 3 or regio-selective desaturation from 1 to 3, in addition to biological observation reported. Based on our results and the reported production of 1 in a plant, the other two nitro compounds (2-methyl-1-nitropropane and 3-methyl-1-nitrobutane) detectable in tomatoes are also considered to be produced by the oxidation process of 2-methylpropanol oxime and 3-methylbutanal oxime, derived originally from valine and leucine, respectively. The present oxidation step to 1 in both millipedes was not accompanied by hydrogen peroxide production, and no color reaction to Trinder reagent was observed.

Elucidation of the desaturation process from compounds 1 to 2 or (and) 3 may be the next step necessary to develop and understand this bioactive molecule, which originated as a natural product.

Acknowledgements

This work was supported by JST ERATO Asano Active Enzyme Molecule Project (Grant Number JPMJER1102), Japan. We would like to thank Dr. Kimiyasu Isobe of our project for his critical reading of this manuscript.

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