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1 **Characterization of a trifunctional fatty acid desaturase from**  
2 **oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast**  
3 **expression system**

4

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15

1           A  $\omega$ 3-fatty acid desaturase gene (*maw3*) which is involved in biosynthesis of  
2 *n*-3 polyunsaturated fatty acids (PUFAs) was previously isolated from *Mortierella*  
3 *alpina* 1S-4. In this report, we investigated the products of MAW3 catalyzing  
4 reaction with endogenous and exogenous fatty acids in the yeast transformant.  
5 Two unusual fatty acids *de novo* synthesized in the yeast transformant expressing  
6 *maw3* gene were identified as *n*-4 hexadecadienoic acid (16:2<sup>9*cis*,12*cis*</sup>) and *n*-1  
7 hexadecatrienoic acid (16:3<sup>9*cis*,12*cis*,15</sup>) by GC-MS and <sup>1</sup>H-NMR analyses. In  
8 addition to the desaturation activity at the  $\omega$ 3-position for 18- and 20-carbon  
9 PUFAs, MAW3 in the yeast transformant inserted a double bond at  $\Delta$ 12-position of  
10 endogenous palmitoleic acid (16:1<sup>9*cis*</sup>) and further at  $\Delta$ 15-position of the resulting  
11 16:2<sup>9*cis*,12*cis*</sup> to result in the formation of 16:3<sup>9*cis*,12*cis*,15</sup> leading to a bifunctional  
12  $\Delta$ 12/ $\Delta$ 15-desaturase for 16-carbon fatty acids. Moreover, we evaluated the  
13 activity of MAW3 in the yeast transformant under different temperatures. The  
14 MAW3 did not have desaturation activities in *M. alpina* 1S-4 at 28°C but it had in  
15 the yeast transformant for various fatty acids. The MAW3 was demonstrated to  
16 be a trifunctional  $\Delta$ 12/ $\Delta$ 15/ $\omega$ 3-desaturase, exhibiting  $\Delta$ 12-desaturation for 16:1<sup>9*cis*</sup>,  
17  $\Delta$ 15-desaturation for 16- and 18-carbon fatty acids that had a preexisting  
18 *cis*-double bond at  $\Delta$ 12 position, and  $\omega$ 3-desaturation for 20-carbon fatty acids

1 **having that at  $\Delta 14$ -position. It is the first report that the fatty acid desaturase**  
2 **(MAW3) is shown to have  $\Delta 12$ - and  $\Delta 15$ -desaturation activities for a 16-carbon**  
3 **fatty acid, in addition to its major function,  $\omega 3$ -desaturation activity.**

4

1 Biosynthetic pathways of fatty acids have been determined in many species by  
2 identification of their genes encoding fatty acid desaturases (1-4). An oleaginous  
3 filamentous fungus, *Mortierella alpina* 1S-4, has been shown to contain  $\Delta$ 5-,  $\Delta$ 6-,  $\Delta$ 9-,  
4  $\Delta$ 12-, and  $\omega$ 3-fatty acid desaturases and four kinds of elongases involved in  
5 polyunsaturated fatty acids (PUFAs) biosynthesis (5). In *M. alpina* 1S-4,  $\Delta$ 5-,  $\Delta$ 6-,  $\Delta$ 9-,  
6 and  $\Delta$ 12-desaturases exhibit their desaturation activity at a cultural temperature of 28°C,  
7 whereas  $\omega$ 3-fatty acid desaturase exhibits its activity only below a cultural temperature  
8 of 20°C.

9 *M. alpina* 1S-4 is capable of producing triacylglycerols rich in arachidonic acid  
10 (ARA; 20:4<sup>5cis,8cis,11cis,14cis</sup>) with the amount ranging of 3-20 g/L of culture broth and  
11 30-70% in the total fatty acid composition. On the other hand, this fungus  
12 accumulates a small amount of eicosapentaenoic acid (EPA; 20:5<sup>5cis,8cis,11cis,14cis,17cis</sup>)  
13 with 0.3 g/L of culture broth and 10% in total fatty acids below a cultural temperature of  
14 20°C (5). We had previously reported isolation of the gene (*maw3*) encoding an  
15  $\omega$ 3-desaturase, involved in biosynthesis of *n*-3 PUFAs, from *M. alpina* 1S-4, and have  
16 confirmed that the gene product (MAW3) converts *n*-6 PUFAs into *n*-3 PUFAs (6).  
17 MAW3 possesses three histidine rich-cluster motifs containing eight histidine residues  
18 that are proposed to form an active-site domain and two hydrophobic

1 membrane-spanning regions in its deduced amino acid sequence (6,7). Similar to  $\Delta 12$ -  
2 and  $\omega 3$ -desaturases found in other organisms, MAW3 has no cytochrome  $b_5$  motif in its  
3 structure (6,8,9). Overexpression of *maw3* gene in *M. alpina* 1S-4 through  
4 *Agrobacterium tumefaciens*-mediated transformation led to an increase in EPA  
5 production contributing to 40% of total fatty acid content (10).

6 Fatty acid desaturases are known to have five specificities: chemoselectivity  
7 (desaturation or hydroxylation), regioselectivity (double bond position),  
8 stereoselectivity (*cis* or *trans*), chain length specificity, and acyl carrier specificity  
9 (acyl-CoA, acyl-lipid, or acyl-ACP substrate) (11). Three types of regioselectivities  
10 have been defined (11). The  $\Delta x$ -desaturases such as  $\Delta 9$ -desaturases insert a first  
11 double bond at x carbon atom position away from the carboxyl end. On the other hand,  
12 the  $\omega y$ -desaturases such as  $\omega 3$ -desaturases insert a double bond at position y from the  
13 methyl end. The  $v + z$ -desaturases require a preexisting double bond as a mark (v) and  
14 catalyze the formation of a new double bond z carbon atoms further along the acyl chain,  
15 either toward the methyl end (*i.e.*  $\Delta 12$ -desaturase) or toward the carboxyl end (*i.e.*  
16  $\Delta 6$ -desaturase). The aforementioned types of regioselectivity are explained for  
17 bifunctional fungal  $\Delta 12/\omega 3$ -desaturases from *Fusarium moniliforme* (12) and  
18 *Aspergillus nidulans* (13). These bifunctional enzymes desaturate oleic acid (OA;

1 18:1<sup>9cis</sup>) at  $\Delta$ 12-position to form linoleic acid (LA; 18:2<sup>9cis,12cis</sup>) and further at the  
2  $\Delta$ 15-position to form  $\alpha$ -linolenic acid (ALA; 18:3<sup>9cis,12cis,15cis</sup>). Additionally, these  
3 desaturases also convert *n*-6 PUFAs: LA,  $\gamma$ -linolenic acid (GLA; 18:3<sup>6cis,9cis,12cis</sup>),  
4 dihomono- $\gamma$ -linolenic acid (DGLA; 20:3<sup>8cis,11cis,14cis</sup>), and ARA to their corresponding *n*-3  
5 PUFAs with preference of the 18-carbon PUFAs. Other bifunctional  
6  $\Delta$ 12/ $\Delta$ 15-desaturases that catalyze the synthesis of unusual *n*-1 hexadecatrienoic acid  
7 (16:3<sup>9cis,12cis,15</sup>) have been reported in the free-living soil protozoon *Acanthamoeba*  
8 *castellanii* (14), phytopathogenic fungus *Claviceps purpurea* (15), basidiomycete  
9 *Coprinus cinereus* (16), and nematode *Caenorhabditis elegans* (17).

10 In this study, we attempted to characterize the MAW3 using the yeast  
11 *Saccharomyces cerevisiae* expression system. Two unusual fatty acids synthesized *de*  
12 *novo* in *maw3*-expressing yeast transformant were identified by GC-MS and NMR.  
13 The MAW3 in yeast transformant inserted a double bond at  $\Delta$ 12-position and further at  
14  $\Delta$ 15-position for endogenous 16-carbon fatty acids as well as at  $\omega$ 3-position of  
15 exogenous 18- and 20-carbon PUFAs. The *M. alpina* 1S-4  $\omega$ 3-desaturase was found to  
16 be a trifunctional  $\Delta$ 12/ $\Delta$ 15/ $\omega$ 3-desaturase that mainly acts as  $\Delta$ 15- and  $\omega$ 3-desaturase,  
17 and as a  $\Delta$ 12-desaturase for 18- and 20-carbon PUFAs in addition to 16-carbon fatty  
18 acids. The finding is different from other bifunctional desaturases so far reported



1 (12-17).

2

## MATERIALS AND METHODS

### Strains, plasmid, growth media, and culture conditions

The *maw3* gene from *M. alpina* 1S-4 (18) was registered in the DDBJ database under the accession number AB182163 (6). *S. cerevisiae* EH1315 ( $\alpha$  *trp1*) was used as the recipient strain in transformation experiments (19). Plasmid pYE22m, used as a shuttle vector, carried the ampicillin resistance gene (for selection in *Escherichia coli*) and *TRP1* (for tryptophan prototrophy selection in *S. cerevisiae* EH1315), with an expression of a target gene controlled under the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and the GAP terminator from *S. cerevisiae*.

*M. alpina* 1S-4 strain was cultured in GY medium (containing 20 g/L of glucose, 10 g/L of yeast extract, pH 6.0) for 5 days at 28°C. Yeast strains were cultured in a yeast synthetic complete medium (SC medium; containing 20 g/L of glucose, 5 g/L of ammonium sulfate, 1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate, 60 mg/L of isoleucine, 60 mg/L of leucine, 60 mg/L of phenylalanine, 50 mg/L of threonine, 40 mg/L of lysine, 30 mg/L of tyrosine, 20 mg/L of adenine, 20 mg/L of arginine, 20 mg/L of uracil, 20 mg/L of histidine, and 10 mg/L of methionine) for 2 days at 15°C (or at 28°C).

**maw3 expression in yeast** The full-length *maw3* cDNA was amplified by PCR in

1 a total reaction volume of 50  $\mu$ l sample containing  $< 0.2 \mu$ g of plasmid pYMAW3 (6) as  
2 template DNA, 0.5  $\mu$ l of *PrimeSTAR HS* DNA polymerase (Takara Bio, Shiga, Japan),  
3 10  $\mu$ l of  $5 \times$  *PrimeSTAR* buffer ( $Mg^{2+}$  plus), 200  $\mu$ M of each dNTP and 200 pM of each  
4 primer:  $\omega$ 3-1F (5'-ATGGCCCCCCTCACGTTGTCGACGAACA-3') containing an  
5 ATG start site (underline) and  $\omega$ 3-3R  
6 (5'-TTAATGCTTGTAAAAACACTACATCTCC-3') containing a TAA stop site  
7 (underline). PCR amplification was performed as follows: initial denaturation at 94°C  
8 for 2 min, followed by 25 cycles of 98°C for 10 sec, 55°C for 15 sec, and 72°C for 2  
9 min. The resultant 1212-bp PCR fragment was digested with *EcoRI* and treated with  
10 DNA Blunting Kit (Takara Bio) for blunt-end ligation into the yeast expression vector  
11 pYE22m to result in the construction of a plasmid, designated as pYE22MAW3, which  
12 was further transformed into the *S. cerevisiae* EH1315 strain by means of the lithium  
13 acetate-mediated transformation protocol (20). Transformants was selected for  
14 tryptophan (Trp) auxotrophy on SC (-Trp) medium.

15 The transformants were grown at 28°C or 15°C with shaking for 48 h in SC  
16 medium. To test for the substrate specificities of MAW3 in yeast, various free fatty  
17 acids (FFAs) were added to the culture broth at 0.1 mM concentration after 24 h of  
18 cultivation. The yeast transformant with an empty vector was used as the control

1 strain.

2 **Fatty acid analysis** Total fatty acid compositions of the yeast transformants were  
3 analyzed as described by Sakuradani et al. (6). Yeast cells after cultivation were  
4 harvested by centrifugation and dried at 120°C for 1.5 h. The dried cells were directly  
5 transmethylated with 10% methanolic HCl at 55°C for 1.5 h. The resultant fatty acid  
6 methyl esters (FAMES) were extracted with *n*-hexane, concentrated and analyzed by gas  
7 chromatography (GC; Shimadzu, Kyoto, Japan). The conversion rate of substrate into  
8 the product was calculated as the enzymatic activity of the desaturase [conversion rate  
9 (%) = 100 × product/(product + substrate)].

10 Total lipids were extracted from wet yeast cells using water/chloroform/methanol  
11 (2/2.5/2.5, v/v/v) according to the Bligh-Dyer method (21). The extracted lipids were  
12 saponified to obtain their FFAs using 0.5 N KOH in 90% MeOH. The unidentified  
13 FFAs were separated by HPLC with a LC-20A system (Shimadzu). A Develosil  
14 C30-UG-5 column (4.6 × 250 mm, Nomura chemical, Aichi, Japan) was used for FFA  
15 separation by HPLC with 80% (v/v) acetonitrile aqueous solution as a mobile phase at a  
16 flow rate of 1.0 ml/min. The column oven temperature was maintained at 35°C, and  
17 the absorbance was measured by UV/VIS detector at 205 nm. The mixture of FFAs  
18 separated by HPLC were transmethylated with methanol/benzene/1% diazomethane

1 (4/6/1, v/v/v) for 30 min at room temperature for preparation of FAMES. Through the  
2 Discovery Ag-Ion SPE column (SUPELCO, Bellefonte, PA, USA), most of FAMES in  
3 the UK2 fraction except for the UK2 were removed with acetone and the UK2 was  
4 eluted with acetonitrile. The 4,4-Dimethyloxazoline (DMOX) derivatives of the UK1  
5 and UK2 obtained through the HPLC purification was prepared and analyzed by  
6 GC-MS as described by Yu et al. (22). A GC-MS QP2010 (Shimadzu) with GC-2010  
7 gas chromatograph was used for MS analysis. The SPB<sup>TM-1</sup> column (0.25 mm I.D. ×  
8 30 m, SUPELCO) was used for separation of DMOX derivatives. Initial column  
9 temperature was set at 180°C and was raised to 300°C later at a rate of 5°C/min. The  
10 apparatus provides the electron impact mode at 70 eV with a source temperature of  
11 250°C. The injection temperature was 250°C. The <sup>1</sup>H-NMR and DQF-COSY  
12 experiments were performed with a Bruker Advance 500 (500 MHz). Samples were  
13 dissolved in chloroform-*d* (Sigma, St. Louis, MO, USA). Chemical shifts were  
14 assigned relative to the solvent signal.

15

16

## RESULTS

17

18 **Functional analysis of the maw3 gene product in the yeast transformant To**

1 characterize the *maw3* gene product from *M. alpina* 1S-4 by using a yeast expression  
2 system, the yeast transformant with pYE22MAW3 containing the *maw3* cDNA was  
3 obtained. The yeast transformants were grown in SC medium, and their total fatty acid  
4 compositions were analyzed by GC (Fig. 1A and 1B). The *maw3*-expressing yeast  
5 transformant accumulated two unusual fatty acids, named UK1 and UK2 respectively,  
6 which were not detected in the control strain. The UK1 was expected to be 16:2<sup>9cis,12cis</sup>,  
7 because the retention time of the UK1 was consistent with that of an authentic standard  
8 (Fig. 1C).

9 Each FFA of UK1 and UK2 was purified by HPLC equipped with a Develosil  
10 C30-UG-5 column, followed by the further purification through Discovery Ag-Ion SPE  
11 column for UK2. The positions of the double bonds in UK1 and UK2 were  
12 determined by GC-MS analysis of their DMOX derivatives (Fig. 2). MS analysis of  
13 UK1 and UK2 DMOX derivatives in Fig. 2 revealed molecular ions of  $m/z$  305 and 303,  
14 and the gaps of 26 atomic mass unit (amu) between  $m/z$  196 and 222, and  $m/z$  236 and  
15 262, indicating double bond at the  $\Delta$ 9- and  $\Delta$ 12-positions in their structures, respectively.  
16 The gap of 41 amu between  $m/z$  262 and 303 in the mass spectrum of UK2 (Fig. 2B)  
17 suggested a double bond at the  $\Delta$ 14- or  $\Delta$ 15-position. To determine a position of a  
18 double bond at methyl terminal of UK2 completely, the FAME of UK2 showing 95%

1 purity on GC chromatogram was applied for  $^1\text{H-NMR}$  analysis (Fig. 3) and DQF-COSY  
2 analysis (FIGURE S1).  $^1\text{H-NMR}$   $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ) spectral data of UK2: 5.82 (1H, *tt*,  
3  $-\text{CH}_2-\text{CH}=\text{CH}_2$ , H-15), 5.38 (4H, *m*,  $-\text{CH}_2-\text{CH}=\text{CH}-$ , H-9, H-10, H-12, H-13), 5.02 (2H,  
4 *d*,  $J= 10.1, 17.0$  Hz,  $-\text{CH}_2-\text{CH}=\text{CH}_2$ , H-16), 3.67 (3H, *s*,  $-\text{C}(=\text{O})-\text{O}-\text{CH}_3$ ), 2.83 (2H, *dd*,  
5  $J= 6.07, 6.07$  Hz,  $=\text{CH}-\text{CH}_2-\text{CH}=\text{}$ , H-14), 2.79 (2H, *dd*,  $J= 6.07, 6.07$  Hz,  
6  $=\text{CH}-\text{CH}_2-\text{CH}=\text{}$ , H-11), 2.30 (2H, *t*,  $J= 7.28$  Hz,  $-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-$ , H-2), 2.05 (2H, *dt*,  
7  $J= 6.78, 6.78$  Hz,  $-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$ , H-8), 1.679 (2H, *tt*,  $J= 7.33, 7.41$  Hz,  
8  $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ , H-3), and 1.31 (8H, *m*,  $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ , H-4, H-5, H-6, H-7). The  
9 absorption peaks at  $\delta$  5.82 ( $-\text{CH}_2-\text{CH}=\text{CH}_2$ , H-15) and 5.02 ( $-\text{CH}_2-\text{CH}=\text{CH}_2$ , H-16) in  
10 Fig. 3 revealed a double bond at the  $\Delta 15$ -position in the structure of UK2. From these  
11 analyses, UK1 and UK2 were identified as  $16:2^{9\text{cis},12\text{cis}}$  and  $16:3^{9\text{cis},12\text{cis},15}$ , respectively.

12 As shown in Table 1, the fatty acid composition of the *maw3*-expressing yeast  
13 transformant was different from that of the control strain. The  $16:2^{9\text{cis},12\text{cis}}$  and  
14  $16:3^{9\text{cis},12\text{cis},15}$  comprised 0.8% and 4.0% of the total fatty acid content, respectively,  
15 which were not detected in the control strain. The percentage of  $16:1^{9\text{cis}}$  was decreased  
16 slightly in the yeast transformant, compared to that in the control strain. Neither LA  
17 nor ALA was detected in both yeast strains, suggesting that MAW3 in the transformant  
18 prefers a 16-carbon fatty acid like  $16:1^{9\text{cis}}$  to an 18-carbon fatty acid like OA.

**Catalytic properties of MAW3 gene product analyzed in the transformant** To investigate the substrate preference of MAW3, the yeast transformants were grown in SC medium containing 0.1 mM of exogenous FFA substrates such as 16:2<sup>9cis,12cis</sup>, 18:1<sup>9cis</sup> (OA), crepenynic acid (9cis-octadecen-12-ynoic acid; 18:1<sup>9cis,12yn</sup>), octadecadienoic acid isomers [18:2<sup>6cis,9cis</sup>, 18:2<sup>9cis,12cis</sup> (LA), 18:2<sup>9cis,11trans</sup>, 18:2<sup>9trans,12cis</sup>, 18:2<sup>9trans,12trans</sup>, and 18:2<sup>10trans,12cis</sup>], 18:3<sup>6cis,9cis,12cis</sup> (GLA), 20:2<sup>9cis,12cis</sup>, 20:3<sup>8cis,11cis,14cis</sup> (DGLA), and 20:4<sup>5cis,8cis,11cis,14cis</sup> (ARA). Conversions of the substrates by the *maw3*-expressing yeast transformant were carried out during cultivation at 15°C or 28°C (Table 2). *M. alpina* 1S-4 has no ω3-desaturation activity at 28°C cultivation, whereas the yeast transformant exhibited the activity for the substrates even at 28°C. The MAW3 in the yeast transformant desaturated the following PUFAs: 16:2<sup>9cis,12cis</sup>, 18:2<sup>9cis,12cis</sup> (LA), 18:3<sup>6cis,9cis,12cis</sup> (GLA), 20:3<sup>8cis,11cis,14cis</sup> (DGLA), and 20:4<sup>5cis,8cis,11cis,14cis</sup> (ARA) to 16:3<sup>9cis,12cis,15</sup>, 18:3<sup>9cis,12cis,15cis</sup> (ALA), stearidonic acid (SDA; 18:4<sup>6cis,9cis,12cis,15cis</sup>), eicosatetraenoic acid (ETA; 20:4<sup>8cis,11cis,14cis,17cis</sup>), and 20:5<sup>5cis,8cis,11cis,14cis,17cis</sup> (EPA). Putative Δ15-desaturation products of 18:2<sup>9cis,12yn,15cis</sup> (9cis,15cis-octadecadien-12-ynoic acid), 18:3<sup>9trans,12cis,15cis</sup>, and 18:3<sup>10trans,12cis,15cis</sup> from 18:1<sup>9cis,12yn</sup>, 18:2<sup>9trans,12cis</sup>, 18:2<sup>10trans,12cis</sup>, respectively, were also observed on GC chromatograms. The *n*-6 PUFAs (LA, GLA, DGLA, and ARA) were more effectively



1 converted into *n*-3 PUFAs (ALA, SDA, ETA, and EPA) at a low temperature (15°C)  
2 cultivation of *M. alpina* 1S-4 than at 28°C temperature. In addition, conversion rates  
3 for 16:2<sup>9cis,12cis</sup> and crepenynic acid were observed at similar or higher levels at 28°C as  
4 compared to the ones at 15°C. Neither Δ15-desaturation nor ω3-desaturation products  
5 were detected for 20:2<sup>9cis,12cis</sup> in the yeast transformant.

6 The Δ12-desaturation catalyzed by MAW3 was observed only for 16:1<sup>9cis</sup>, and  
7 not for 18:1<sup>9cis</sup> and 18:2<sup>6cis,9cis</sup>. The high conversion rate with more than 60% was  
8 shown for 16:2<sup>9cis,12cis</sup> to 16:3<sup>9cis,12cis,15</sup> by Δ15-desaturation of MAW3 in the yeast  
9 transformant. Furthermore, the ω3-desaturation of MAW3 was observed for 18- and  
10 20-carbon PUFAs having a preexisting 12*cis*-double bond or 12-triple bond and a  
11 preexisting 14*cis*-double bond, respectively.

12

13

## DISCUSSION

14

15 The fungus *M. alpina* 1S-4 not only produces a great number of *n*-6 PUFAs rich  
16 in ARA, but also accumulates *n*-3 PUFAs such as EPA below a cultural temperature of  
17 20°C (23). The *n*-3 PUFAs in *M. alpina* 1S-4 *in vivo* are expected to be synthesized  
18 from *n*-6 PUFAs through ω3-desaturation. The *maw3* gene was previously isolated

1 and characterized as to its function of *n*-6 PUFAs conversion to *n*-3 PUFAs in *M. alpina*  
2 1S-4 (6).

3 In this study, the *maw3*-expressing yeast transformant was found to accumulate  
4 two newly synthesized fatty acids, 16:2<sup>9cis,12cis</sup> and 16:3<sup>9cis,12cis,15</sup>. Thus far, we had no  
5 idea that the MAW3 had  $\Delta$ 12-desaturase activity as an alternative function. The  
6 accumulation of 16:2<sup>9cis,12cis</sup> and 16:3<sup>9cis,12cis,15</sup> suggested that the yeast transformant had  
7 both  $\Delta$ 12-desaturation activity with a conversion rate of 10.2% (calculated as  $100 \times$   
8  $[16:2 + 16:3]/[16:1 + 16:2 + 16:3]$ ) for endogenous 16:1<sup>9cis</sup>, and  $\Delta$ 15-desaturation one at  
9  $\omega$ 1-position, not  $\omega$ 3, with a conversion rate of 83.0% (calculated as  $100 \times [16:3]/[16:2 +$   
10  $16:3]$ ) for the resultant 16:2<sup>9cis,12cis</sup>. Once the 16:2<sup>9cis,12cis</sup> was formed from 16:1<sup>9cis</sup> by  
11  $\Delta$ 12-desaturation, 16:2<sup>9cis,12cis</sup> was demonstrated to be rapidly converted to  
12 16:3<sup>9cis,12cis,15</sup>.

13 The analysis of substrate specificities (Table 2) showed that the MAW3 not only  
14 had  $\omega$ 3-desaturation activity for 18- and 20-carbon PUFAs, but also demonstrated  
15 bifunctional  $\Delta$ 12/ $\Delta$ 15-desaturation activities for 16-carbon fatty acids. The  $\Delta$ 12-,  $\Delta$ 15-,  
16 or  $\omega$ 3-desaturation activity of MAW3 requires a preexisting double bond at  $\Delta$ 9-,  $\Delta$ 12-,  
17 or  $\omega$ 6-position, respectively. Hence, MAW3 is classified as *v* + 3 desaturase (*v*  
18 indicating the position of the preexisting double bond) which inserted a new double

1 bond 3 carbon atoms further along the acyl chain toward the methyl end.

2 Compared to other bifunctional  $\Delta 12/\Delta 15$ -desaturases [amoeba *A. castellanii* (14);

3 basidiomycete *C. cinereus* (16); nematode *C. elegans* (17)], MAW3 in the yeast

4 transformant exhibited  $\Delta 12$ -desaturation for 16:1<sup>9cis</sup>, not 18:1<sup>9cis</sup>, and in addition to that,

5 also exhibited  $\Delta 15$ -desaturation for 16:2<sup>9cis,12cis</sup> with a high conversion rate (83.0%).

6 The previously reported bifunctional  $\Delta 12/\Delta 15$ -desaturases mainly act as a

7  $\Delta 12$ -desaturase, except for the fungal  $\omega 3$ -desaturases described by Damude et al. (12)

8 and Hoffmann et al. (13), however do not possess  $\Delta 12$ -desaturation activity for the

9 16-carbon fatty acid. The ratios of  $\omega 3/\omega 6$  fatty acids (calculated as [16:3 + 18:3]/[16:2

10 + 18:2]) in yeast transformant were calculated as follows: 5.0 [MAW3, described here],

11 21 [ $\omega 3$ -desaturase from *F. moniliforme* (12)], 0.0080 [ $\Delta 12$ -desaturase from *F.*

12 *moniliforme* (12)], 2.1 [ $\omega 3$ -desaturase from *A. nidulans* (13)], 0.48 [ $\Delta 12$ -desaturase

13 from *A. castellanii* (14)], 0.042 [ $\Delta 12$ -desaturase from *C. cinereus* (16)], and 0.090

14 [ $\Delta 12$ -desaturase from *C. elegans* (17)] respectively based on the information derived

15 from each report. The bifunctional  $\Delta 12/\Delta 15$ -desaturases from *F. moniliforme* and that

16 has a high ratio of 21, mainly work as a  $\omega 3$ -desaturase, which prefers the 18-carbon

17 PUFAs as substrates and not the 16-carbon fatty acids. On the other hand, the

18 bifunctional  $\Delta 12/\Delta 15$ -desaturase from *C. purpurea* (15) introduces a double bond for

1 both 16- and 18-carbon fatty acids, demonstrating increased activity of  $\Delta 12$ -desaturation  
2 as compared to  $\Delta 15$ -desaturation.

3 On investigation of substrate specificities, the characteristic feature of MAW3  
4 was shown to have both the  $\Delta 12/\Delta 15$ -desaturation activity for the 16-carbon fatty acids  
5 and  $\omega 3$ -desaturation activity for 18- and 20-carbon PUFAs, indicating its substrate  
6 specificities that differed from the other bifunctional  $\Delta 12/\Delta 15$ -desaturases (12-17). It  
7 is of interest to note that, the *maw3*-expressing yeast transformant exhibited the  
8  $\omega 3$ -desaturation activity on cultivation at 28°C, whereas *M. alpina* 1S-4 had no such  
9  $\omega 3$ -deaturation activity under similar culture conditions. This may imply the  
10 possibility of some regulation in the translation process and/or localization of MAW3 in  
11 *M. alpina* 1S-4 *in vivo*. The localization of MAW3 might be detected in cells of *M.*  
12 *alpina* 1S-4 by means of labeling techniques. We expect that the elucidation of the  
13 mechanism for the difference in MAW3 activity (depending on the culture temperature)  
14 may contribute to the accumulation of basic information on membrane-bound  
15 desaturases and to efficient production of n-3 PUFAs by *M. alpina* breeding.

16

17

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18

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4

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- 8

## Figure legends

1

2

3 **FIG. 1.** GC chromatograms of FAMES prepared from total lipids of the control strain  
4 (pYE22m) (A), the *maw3*-expressing yeast transformant (pYE22MAW3) (B), and the  
5 FAMES of 16:2<sup>9cis,12cis</sup> (C).

6

7 **FIG. 2.** Mass spectra of DMOX derivatives of (A) UK1 and (B) UK2 obtained from the  
8 yeast transformant (pYE22MAW3). The specific mass fragments of authentic  
9 16:2<sup>9cis,12cis</sup> were *m/z* 113, 126, 140, 154, 168, 182, 196, 208, 222, 236, 248, 262, 276,  
10 276, 290, and 305.

11

12 **FIG. 3.** <sup>1</sup>H-NMR analysis of the UK2 methyl ester.

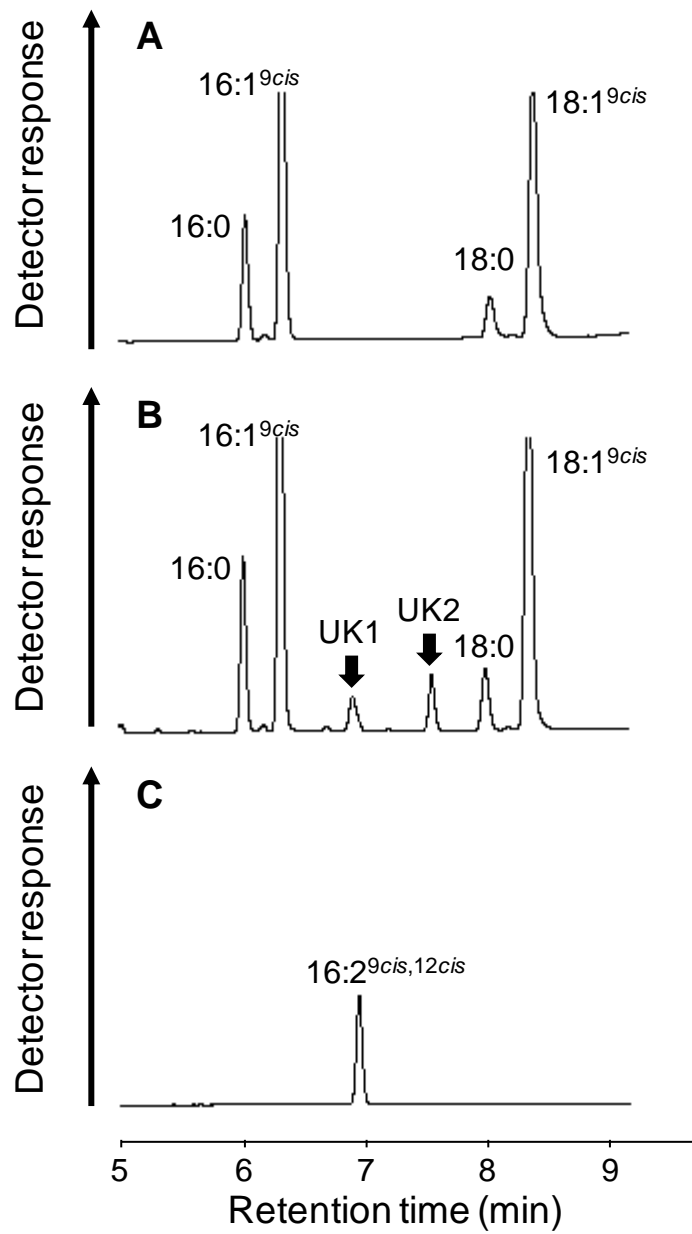


FIG.1. Kikukawa et al.

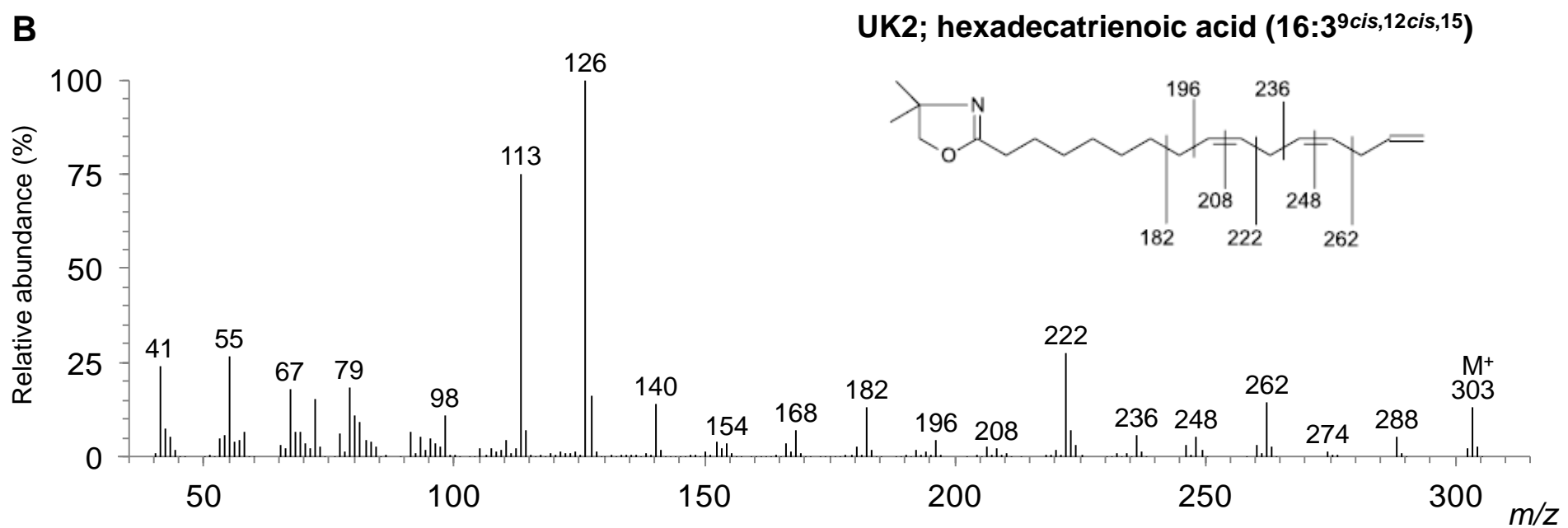
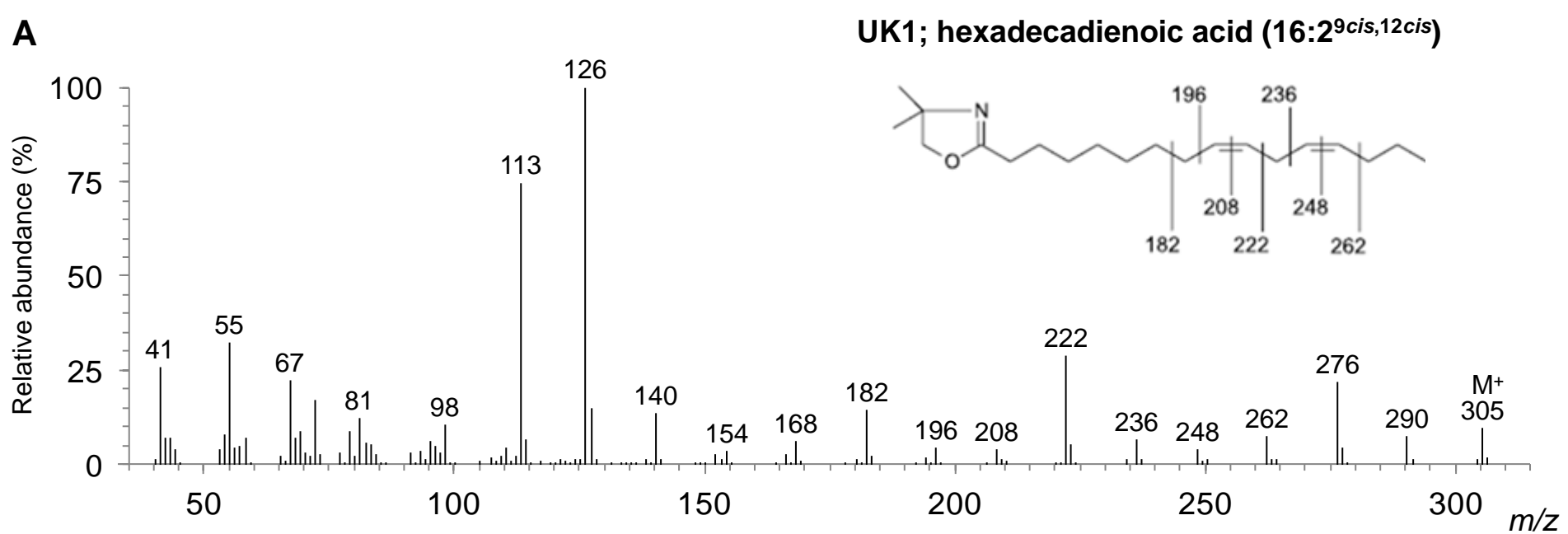


FIG.2. Kikukawa et al.

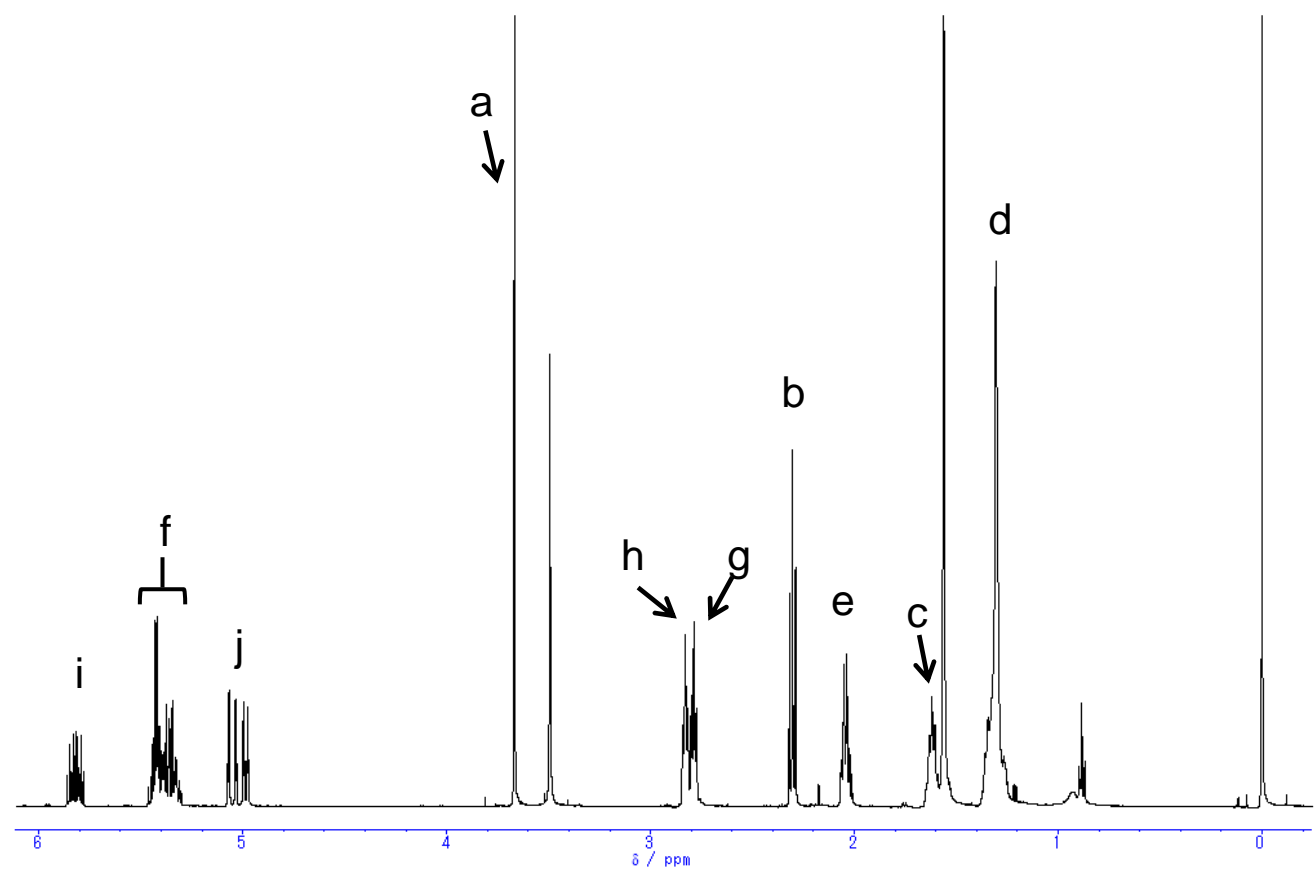
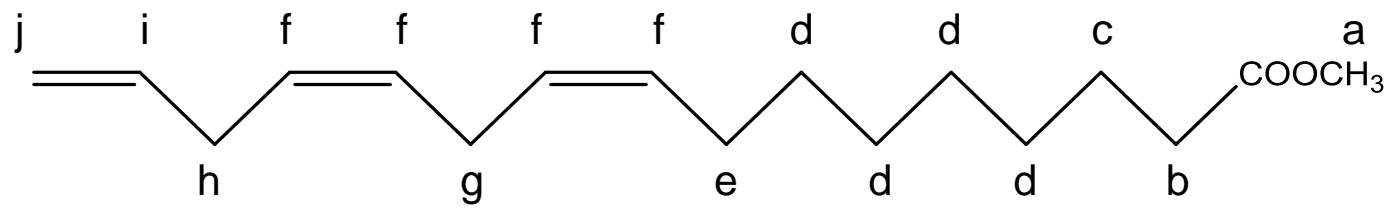


FIG. 3. Kikukawa et al.

1 **Table 1.** Fatty acid compositions of the yeast transformants

2

3	Fatty acid	Fatty acid composition (%)	
4		Control strain	<i>maw3</i> transformant
5		(pYE22m)	(pYE22MAW3)
6	16:0	12.4	11.9
7	16:1 <sup>9cis</sup>	45.4	40.2
8	16:2 <sup>9cis,12cis</sup>	N.D. <sup>a</sup>	0.8
9	16:3 <sup>9cis,12cis,15</sup>	N.D.	4.0
10	18:0	5.7	5.2
11	18:1 <sup>9cis</sup>	34.6	32.8

12 <sup>a</sup> N.D., not detected

13

1 **Table 2.** Conversion of various fatty acids by the *maw3*-expressing yeast  
 2 transformant<sup>a</sup>

3	4 Substrate	5 Product	6 Conversion rate (%) <sup>b</sup>	
			7 15°C	8 28°C
9	Endogenous fatty acid			
10	16:1 <sup>9cis</sup>	16:2 <sup>9cis,12cis</sup>	10.2 ± 0.2	N.D. <sup>c</sup>
11	16:2 <sup>9cis,12cis</sup>	16:3 <sup>9cis,12cis,15</sup>	83.0 ± 0.5	N.D.
12	Exogenous fatty acid			
13	16:2 <sup>9cis,12cis</sup>	16:3 <sup>9cis,12cis,15</sup>	68.8 ± 0.6	60.3 ± 0.6
14	18:1 <sup>9cis</sup> (OA)	N.D.	-	-
15	18:1 <sup>9cis,12yn</sup>	18:2 <sup>9cis,12yn,15cis</sup> d	11.0 ± 0.4	19.5 ± 1.5
16	18:2 <sup>6cis,9cis</sup>	N.D.	-	-
17	18:2 <sup>9cis,12cis</sup> (LA)	18:3 <sup>9cis,12cis,15cis</sup> (ALA)	65.2 ± 0.6	24.4 ± 0.4
18	18:2 <sup>9cis,11trans</sup>	N.D.	-	-
19	18:2 <sup>9trans,12cis</sup>	18:3 <sup>9trans,12cis,15cis</sup> d	63.8 ± 0.2	40.0 ± 3.7
20	18:2 <sup>9trans,12trans</sup>	N.D.	-	-
21	18:2 <sup>10trans,12cis</sup>	18:3 <sup>10trans,12cis,15cis</sup> d	41.3 ± 0.2	18.1 ± 0.5

1	18:3 <sup>6cis,9cis,12cis</sup> (GLA)	18:4 <sup>6cis,9cis,12cis,15cis</sup> (SDA)	74.8 ± 0.1	32.6 ± 0.7
2	20:2 <sup>9cis,12cis</sup>	N.D.	-	-
3	20:3 <sup>8cis,11cis,14cis</sup> (DGLA)	20:4 <sup>8cis,11cis,14cis,17cis</sup> (ETA)	57.7 ± 1.1	6.7 ± 0.3
4	20:4 <sup>5cis,8cis,11cis,14cis</sup> (ARA)	20:5 <sup>5cis,8cis,11cis,14cis,17cis</sup> (EPA)	52.5 ± 2.6	8.7 ± 0.7

---

5 <sup>a</sup> All exogenous free fatty acid substrates were added at 0.1 mM. All the values are for

6 three independent samples (mean ± SD)

7 <sup>b</sup> Conversion rate (%) = 100 × [product]/[product + substrate]

8 <sup>c</sup> N.D., not detected

9 <sup>d</sup> Putative product



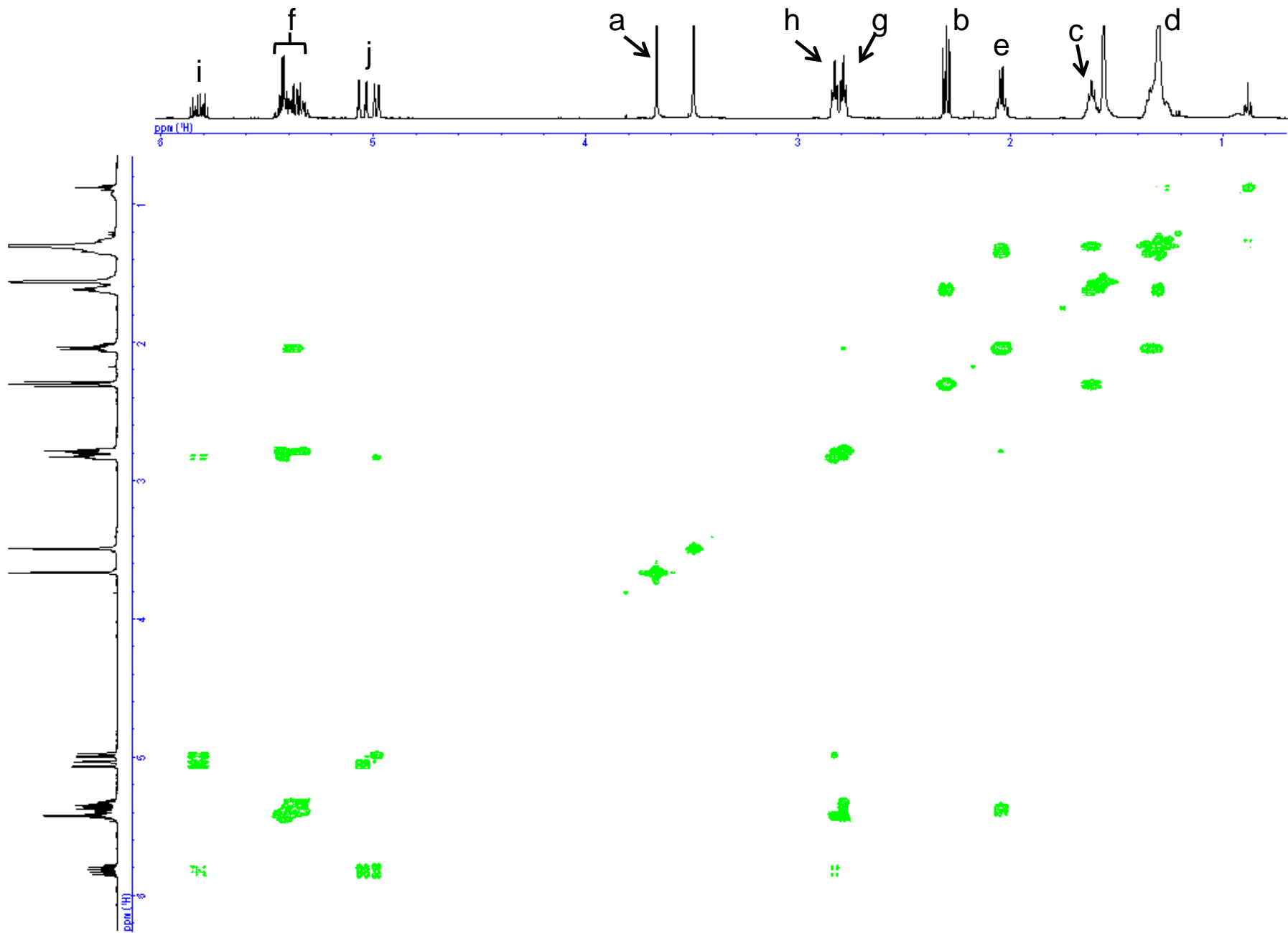


FIGURE S1. DQF-COSY analysis of the UK2 methyl ester.