

REVIEW (58th Award for Prominent Studies, JOCS)

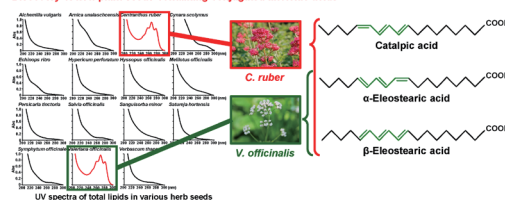
Discovery and Synthesis of Conjugated Fatty Acids from Natural Products

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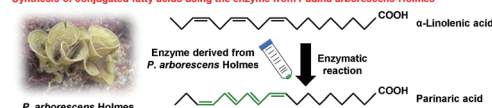
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Abstract: Conjugated fatty acids are a promising ingredient for cancer prevention and treatment. Conjugated fatty acids are minor fatty acids that are rarely found in nature, although a wide variety of structures are known. In recent years, studies have been conducted to screen natural products containing conjugated fatty acids and to synthesize conjugated fatty acids using enzymes derived from natural products. As a result, it was found that the seed oils of *Centranthus ruber* and *Valeriana officinalis*, which belong to the Valerianaceae family, contain conjugated linolenic acid, which has a conjugated triene structure in the molecule. Furthermore, it was found that parinaric acid, a conjugated tetraenoic fatty acid, can be synthesized by adding α -linolenic acid to enzymes extracted from the brown alga *Padina arborescens* Holmes. These research results are expected to be useful in securing conjugated fatty acids in quantities that can withstand practical application. Recent studies have reported that the cytotoxic effect of conjugated fatty acids is due to a programmed cell death called "ferroptosis". Many anticancer drugs exhibit anticancer activity through DNA modification, cell cycle arrest, angiogenesis inhibition, and epidermal growth factor receptor inhibition. Conjugated fatty acids, however, induce cell death through a mechanism distinct from these mechanisms and are therefore expected to be effective against cancers resistant to currently used anticancer drugs. The results of these studies will help to promote research on the use of conjugated fatty acids to overcome intractable cancers in the future.

Discovery of new plant seeds containing conjugated linolenic acids



Synthesis of conjugated fatty acids using the enzyme from *Padina arborescens* Holmes



Key words: *Centranthus ruber*, conjugated fatty acid, *Padina arborescens* Holmes, parinaric acid, *Valeriana officinalis*

1 Introduction

Cancer is a major cause of death worldwide, making research into its prevention and treatment is crucial¹. Ensuring healthy aging without disease is vital not only for maintaining individual quality of life but also for supporting social and economic progress. Therefore, ingredients that are expected to have preventive or therapeutic effects on cancer are actively studied²⁻⁴.

One example of a natural product with potential anticancer effects is conjugated fatty acids. Most naturally occurring polyunsaturated fatty acids have a structure with a methylene group intervening between cis-type double bonds, whereas conjugated fatty acids contain a conjugated

double bond in their structure. Conjugated linoleic acid (CLA) is among the most recognized conjugated fatty acids. CLAs are geometric and positional isomers of linoleic acid, characterized by their conjugated diene structure, and are found in foods such as beef, milk, and other dairy products^{5, 6}. In 1985, Pariza et al. found antimutagenic properties in hamburger extract, and the antimutagen was shown to be CLA in 1987^{5, 7}. Since the cancer-preventive properties of CLAs were first identified, conjugated fatty acids have been extensively studied and have demonstrated various beneficial physiological activities, such as anti-obesity, immunomodulatory, and bone-forming effects, alongside their antitumor capabilities^{5, 8-11}. However, the

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amount of CLA present in dietary foods is likely inadequate to trigger these physiological effects¹². Furthermore, the naturally occurring CLA isomers exhibit relatively weak physiological activity¹³. Thus, it is important to identify conjugated fatty acids that are more active than CLAs. Conjugated linolenic acids (CLNs) exhibit stronger antitumor effects compared to CLAs¹³. CLNs are conjugated trienoic fatty acids that serve as geometric and positional isomers of α -linolenic acid (α -LnA; 9Z,12Z,15Z-18:3; Fig. 1). CLNs are reported to exert cytotoxic effects by inducing lipid peroxidation in human colon cancer cells (DLD-1)^{13,14}.

Though conjugated fatty acids are expected to be beneficial for the prevention and treatment of cancer there are very few reports of natural products containing conjugated fatty acids. Since conjugated fatty acids are thought to have great potential for the prevention and treatment of cancer, the discovery of new natural products containing conjugated fatty acids will contribute to the improvement of people's health and the prevention of disease. Therefore, this review presents information about plant seeds containing CLNs, the synthesis of conjugated fatty acids using natural product-derived enzymes, and findings on the anti-cancer mechanism of conjugated fatty acids.

2 Plant Seeds Containing CLN

A variety of CLNs exist in nature (Fig. 1). Pomegranate seed oil contains punicic acid (9Z,11E,13Z-18:3), while catalpa seed oil contains catalpic acid (CPA; 9E,11E,13Z-18:3). Karela seed oil and tung oil are sources of α -eleostearic acid (α -ESA; 9Z,11E,13E-18:3) and β -eleostearic acid (β -ESA; 9E,11E,13E-18:3). Jacaranda seed oil contains jacaric acid (8Z,10E,12Z-18:3), and calendula seed oil provides both α -calendic acid (8E,10E,12Z-18:3) and β -calendic acid (8E,10E,12E-18:3). These are very interesting research subjects because the carcinocidal effects are different on the isomer type¹⁴. However, only a limited number of plants are known to have CLNs in their seed oils^{14,15}. Thus, the discovery and study of new plants containing CLNs in their seeds would be advantageous for cancer prevention. A recent study screened various medicinal and edible herbs for CLNs-containing seeds and found that several species in the Valerianaceae family contained CLNs¹⁶. A simple method for detecting the presence of CLN in seed oils is to measure the UV spectra of total lipids from seed oils. If the fatty acid has a conjugated triene structure in its structure, three absorption peaks around 270 nm are observed¹⁵. In the study measuring the UV spectra of various herb seed oils, three absorption peaks were observed around 270 nm in the seed oils of *Centranthus ruber* and *Valeriana officinalis* (Fig. 2). A useful method to confirm the presence or absence of conjugated fatty acids for lipids in detail is the comparison

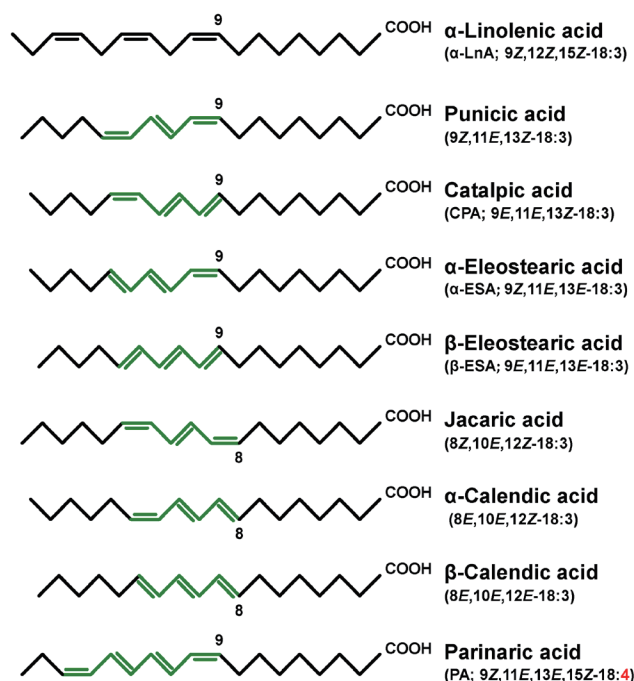


Fig. 1 Chemical structures of α -linolenic acid (α -LnA) and various conjugated fatty acids.

of retention times by gas chromatography (GC) analysis and fragmentation analysis by GC-MS analysis^{17–19}. When fatty acids are analyzed by GC, it is common practice to methyl esterify the carboxylic acid portion of fatty acids¹⁷. However, when estimating the structures of fatty acids that are similar to each other, such as CLNs, the fragments obtained from methyl esters cannot identify the exact location of unsaturated bonds. Therefore, the double bond positions of fatty acids can be estimated by dimethylloxazoline (DMOX) derivatization of fatty acids^{16,18,19}.

For example, in previous reports analyzing the fatty acid composition of *Centranthus ruber* or *Valeriana officinalis* seed oils, the results of the GC analysis of the seed oils were compared to the retention times of conjugated fatty acid samples. To confirm that the obtained peak corresponds to α -ESA in the peaks where the retention time matched that of the α -ESA standard, the position of the double bond was determined by analyzing the GC-EI/MS spectra of the DMOX derivatives of the fatty acids. These analyses confirmed the presence of α -ESA in these seed oils (Fig. 3). The GC-EI/MS spectrum of the DMOX derivative of the compound showed a molecular ion with an m/z ratio of 331, along with fragments at m/z 196 and 274, resulting from allylic cleavage of the fatty acid chain. The mass spectrum also showed differences of 12 mass units between the fragments at m/z 196 and 208, 222 and 234, and 248 and 260, suggesting the presence of conjugated double bonds at carbon positions 9, 11, and 13. Since all other fatty acids with double bonds at these positions

(whose structures are described in Fig. 1) had retention times different from that of α -ESA (data not shown), the peak in question was identified as α -ESA. Similarly, CLNs other than α -ESA can be identified by analysis of DMOX derivatives. Finally, α -ESA, β -ESA and CPA were present in the seed oils of *Centranthus ruber*, and α -ESA and β -ESA were also found to be present in the seed oil of *Valeriana officinalis*¹⁶.

As shown in Table 1, α -ESA and β -ESA were present in the seed oils of *Momordica charantia*, *Centranthus*

ruber, and *Valeriana officinalis*¹⁶. For reference, the analysis of *Momordica charantia* seed oil, in which the presence of conjugated fatty acids is already known, is also presented (Table 1). In terms of fatty acid composition, *Centranthus ruber* seed oil contained 79.1 mg α -ESA/g lipids, 19.2 mg CPA/g lipids, and 237.1 mg β -ESA/g lipids, while *Valeriana officinalis* seed oil contained 225.0 mg α -ESA/g lipids and 7.1 mg of β -ESA/g lipids¹⁶. Notably, the β -ESA content in *Centranthus ruber* seed oil was much higher than in the other seed oils¹⁵. Therefore, *Centran-*

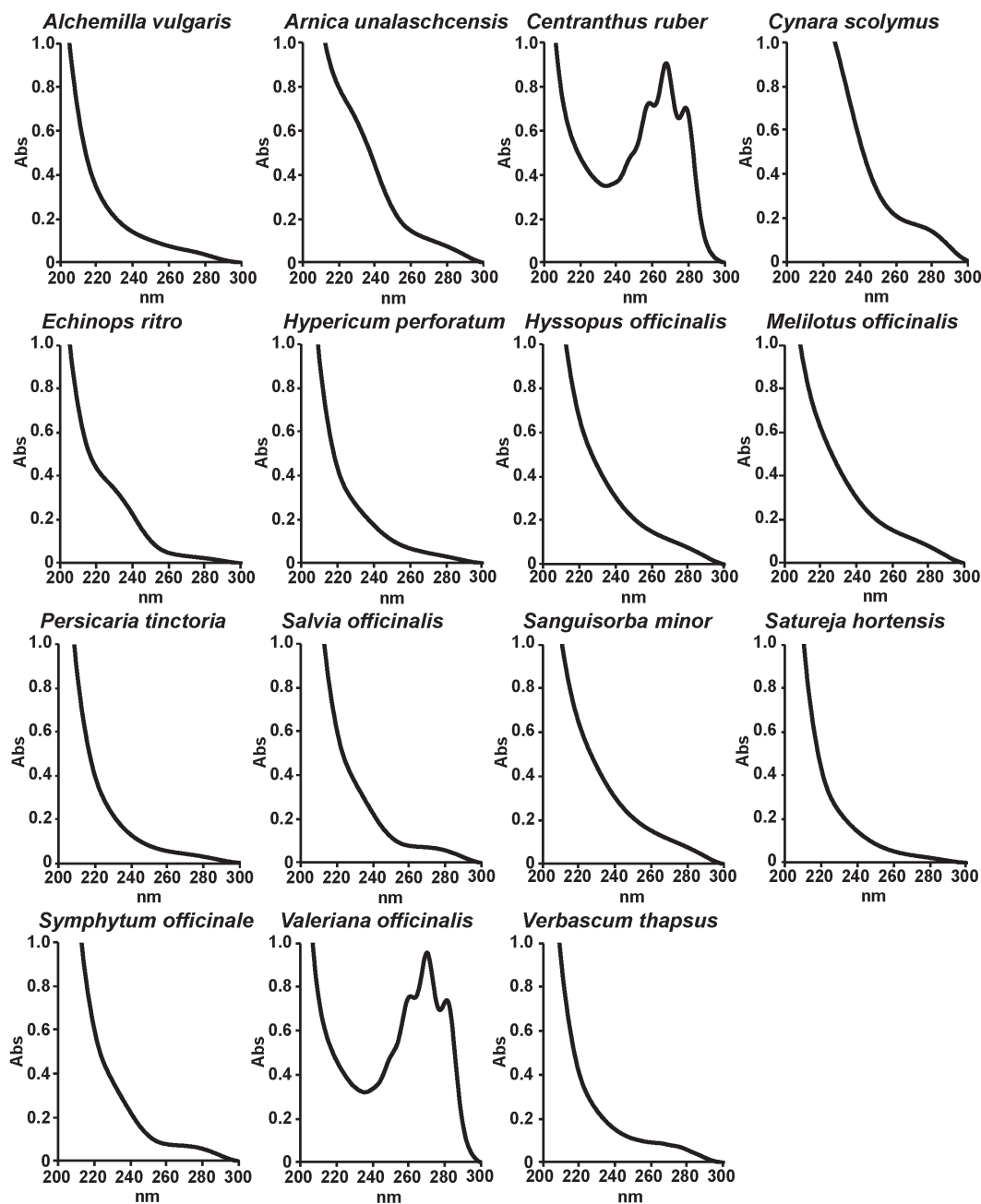


Fig. 2 UV spectra of total lipids in various herb seeds. *Centranthus ruber* seed oil and *Valeriana officinalis* seed oil have characteristic absorption of the conjugated triene structure around 270 nm.

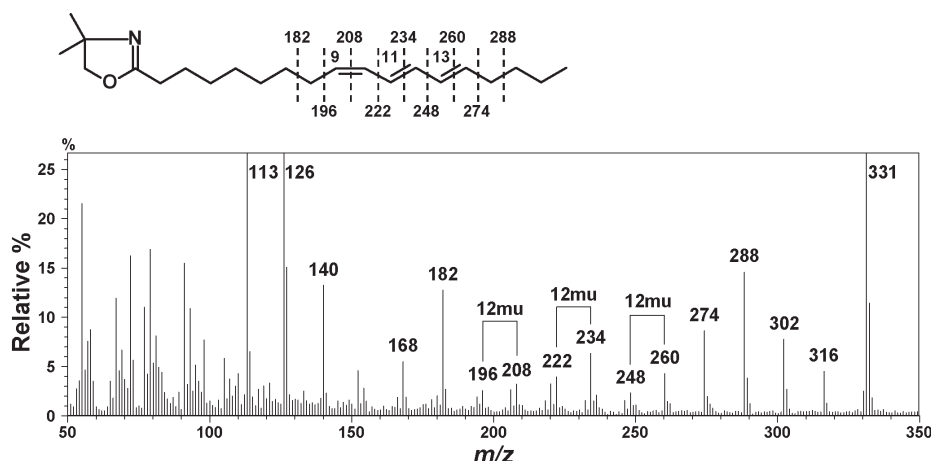


Fig. 3 GC-EI/MS spectrum of DMOX derivatives of compound (determined as α -ESA).

Table 1 Lipid contents and fatty acid compositions of seeds obtained from *Centranthus ruber*, *Valeriana officinalis*, and *Momordica charantia*.

	<i>C. ruber</i>	<i>V. officinalis</i>	<i>M. charantia</i>
total lipid contents (% of the wet weight)			
	31.6 $\pm$ 1.5	27.5 $\pm$ 1.2	41.5 $\pm$ 0.5
fatty acids (mg/g lipids)			
C16:0	49.6 $\pm$ 1.3	42.1 $\pm$ 2.8	21.6 $\pm$ 1.5
C18:0	33.8 $\pm$ 1.0	15.9 $\pm$ 0.7	379.6 $\pm$ 27.3
C18:1	54.2 $\pm$ 0.7	72.5 $\pm$ 5.9	25.8 $\pm$ 9.1
C18:2	377.5 $\pm$ 6.8	512.1 $\pm$ 34.5	43.0 $\pm$ 11.9
C18:3	N.D.	6.9 $\pm$ 0.4	N.D.
α -ESA	79.1 $\pm$ 1.9	225.0 $\pm$ 15.1	418.1 $\pm$ 65.2
CPA	19.2 $\pm$ 0.8	N.D.	N.D.
β -ESA	237.1 $\pm$ 6.2	7.1 $\pm$ 0.8	1.4 $\pm$ 0.4
Total CLN	335.4 $\pm$ 8.2	232.1 $\pm$ 15.5	419.5 $\pm$ 65.6
others	6.9 $\pm$ 1.0	18.4 $\pm$ 1.5	36.1 $\pm$ 10.3

α -ESA; α -eleostearic acid, CPA; catalpic acid, β -ESA; β -eleostearic acid, n = 3, mean $\pm$ SD, N.D.; not detected.

thus ruber seed oil may be very useful as a research material in obtaining large amounts of β -ESA at low cost. *Momordica charantia* seed oil is rich in stearic acid (C18:0) as well as CLNs. In contrast, *Centranthus ruber* and *Valeriana officinalis* seed oils are rich in linoleic acid (C18:2) in addition to CLNs and are likely to have lower melting points than *Momordica charantia* seed oil¹⁶⁾. Thus, although the CLNs content of *Centranthus ruber* and *Valeriana officinalis* seed oils is lower than that of *Momordica charantia*, they may be less likely to solidify in the human body and may be more readily absorbed in the

small intestine^{20, 21)}. The absorption efficiencies of the CLNs from the seed oils of *Centranthus ruber*, *Valeriana officinalis*, and *Momordica charantia* warrant further investigation.

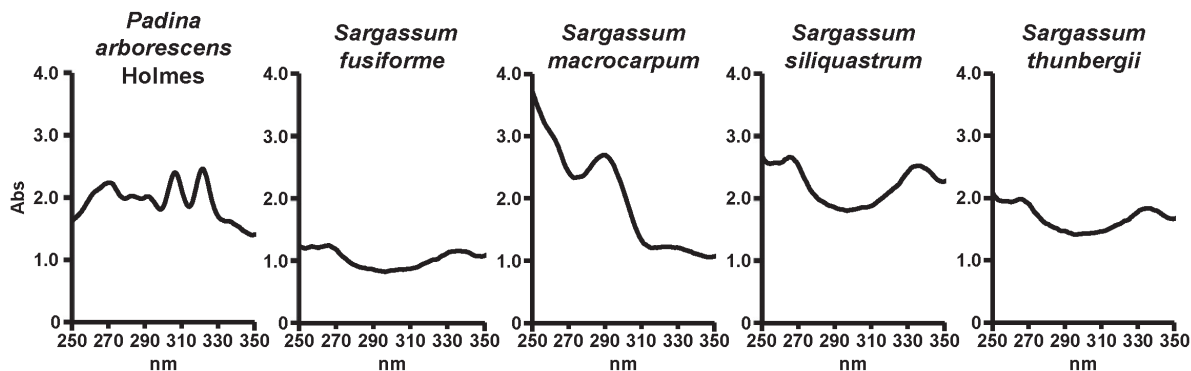
3 Synthesis of Conjugated Fatty Acids Using Enzymes from Seaweeds

Seaweeds contain lipids in trace amounts, and polyunsaturated fatty acids are present. In addition, minor fatty acids are present in certain seaweeds, such as conjugated eicosapentaenoic acid and conjugated docosaheptaenoic acid^{22, 23)}. Additionally, *Ptilota filicina*, a species belonging to the Rhodophyta group, has been reported to contain conjugated trienoic eicosapentaenoic acid²²⁾.

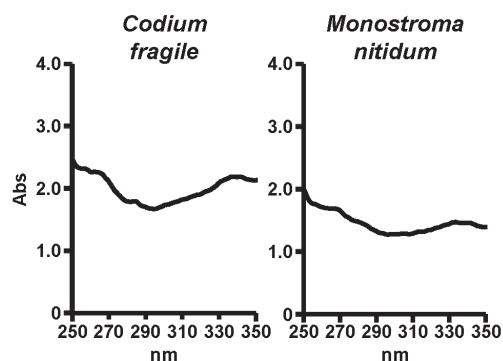
In a recent study, lipid analysis of various species of seaweeds from the seas around Japan was conducted to identify new natural products containing conjugated fatty acids²⁴⁾. As shown in Fig. 4, the absorption maxima of the lipids extracted from *Padina arborescens* Holmes were consistent with the absorption maxima of conjugated tetraenoic fatty acids²³⁾ at 291, 307, and 322 nm, indicating the presence of conjugated tetraenoic fatty acids in *Padina arborescens* Holmes.

However, lipids in seaweeds are generally not very abundant, and the conjugated fatty acids in *Padina arborescens* Holmes are expected to be negligible. Thus, extracting conjugated tetraenoic fatty acids from *Padina arborescens* Holmes may not be feasible for practical applications. Previous studies have reported that *Ptilota filicina* (Rhodophyta), *Lithothamnion corallioides* (Rhodophyta) and *Anadyomene stellata* (Chlorophyta) contain enzymes capable of converting non-conjugated polyunsaturated fatty acids into conjugated fatty acids^{23, 25, 26)}. The enzyme in *Padina arborescens* Holmes was likewise shown to convert nonconjugated polyunsaturated fatty

Phaeophyta



Chlorophyta



Rhodophyta

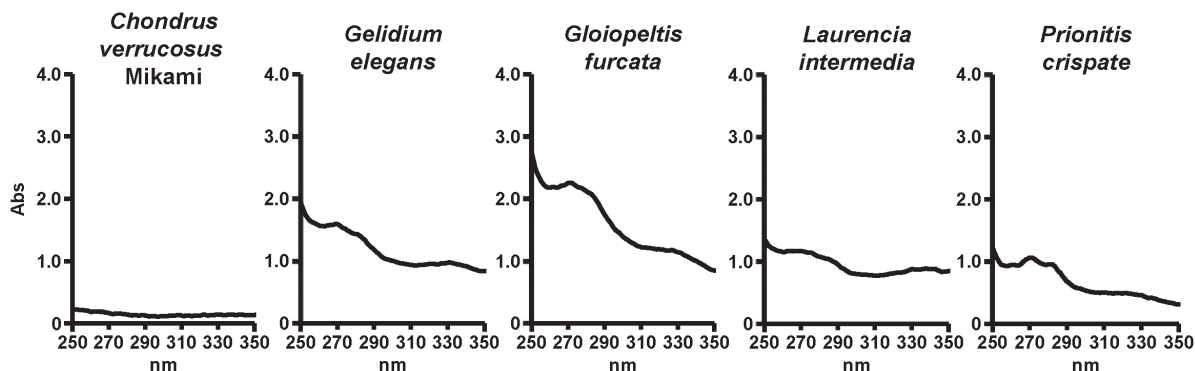


Fig. 4 UV spectra of total lipids in different seaweed species. Absorption maxima characteristic of conjugated tetraene structures have been observed in *Padina arborescens* Holmes.

acids to conjugated fatty acids²⁴⁾. Specifically, parinaric acid (PA, Fig. 1), a conjugated tetraene fatty acid, was synthesized by adding α -LnA as a substrate to a crude enzyme solution extracted from *Padina arborescens* Holmes (Fig. 5).

As mentioned above, CLNs with a conjugated triene structure are well known as a conjugated fatty acid with anticancer activity^{14, 27)}. On the other hand, few studies

have explored the cancer-preventive effects of conjugated tetraenoic fatty acids. However, since PA has been reported to exert cytotoxic effects^{28, 29)}, conjugated tetraenoic fatty acids are also anticipated to be valuable for cancer prevention and treatment. Thus, identifying various conjugated tetraenoic fatty acids is crucial for future research.

Conjugated tetraenoic fatty acids, which have been previously reported to be synthesized using seaweeds other

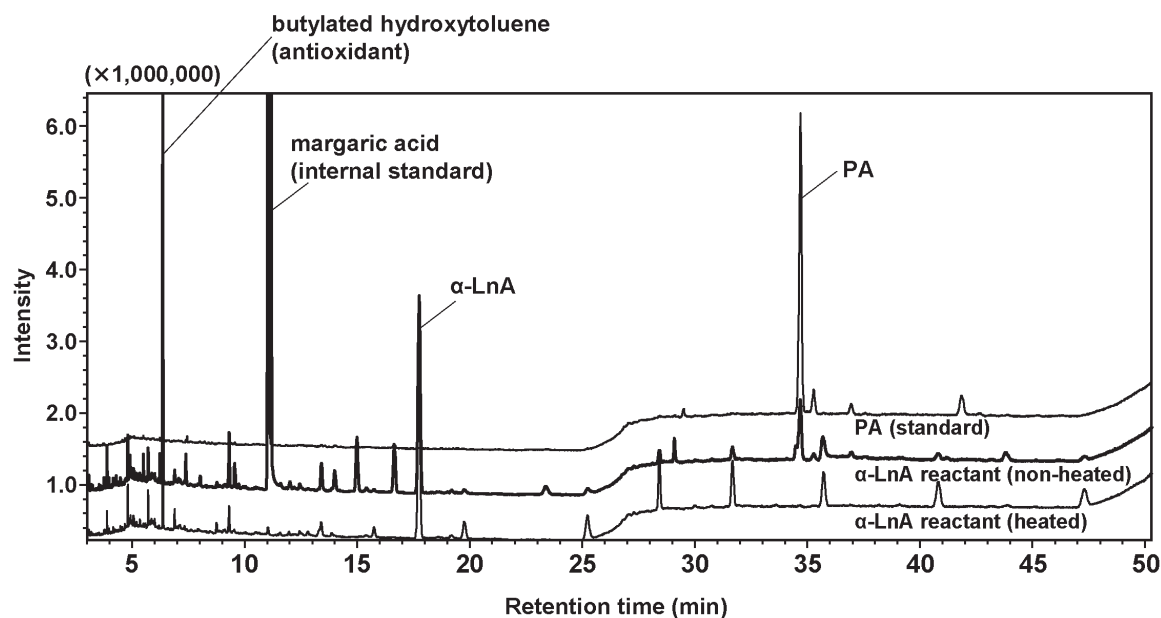


Fig. 5 Gas chromatography chromatograms of parinaric acid (PA: standard) and α -linolenic acid (α -LnA) reactants. α -LnA reactants were obtained from a lipid fraction following incubation with a *Padina arborescens* chloroplast fraction with or without pre-heating. α -LnA reactant (non-heated): α -LnA reactant obtained from α -LnA incubated with a chloroplast fraction without pre-heating. α -LnA reactant (heated): α -LnA reactant obtained from α -LnA incubated with the pre-heated chloroplast fraction (95°C, 10 min).

than *Padina arborescens* Holmes, are thought to be different from PA^{23, 25}, suggesting that the enzyme involved in this study is different from those previously reported. The structure of conjugated tetraenoic fatty acids in *Padina arborescens* Holmes has not been identified because the content is too low. However, it is possible that conjugated tetraenoic fatty acids other than PA are synthesized using other polyunsaturated fatty acids besides α -LnA as substrates. In the future, it may be possible to discover conjugated fatty acids with stronger cytotoxic effects. This could be achieved by testing whether enzymes from *Padina arborescens* Holmes can synthesize fatty acids distinct from PA using other polyunsaturated fatty acids as substrates. Additionally, it is essential to investigate whether similar enzymes are present in other seaweeds. Such discoveries could enable the synthesis of new or lesser-known conjugated fatty acids using enzymes from these seaweeds.

Certain seaweeds produce several types of polyunsaturated fatty acid isomerases, which are used to synthesize conjugated polyene fatty acids. These isomerases can synthesize many types of conjugated fatty acids, and gaining a better understanding of these enzymes could significantly advance research on the antitumor effects of conjugated fatty acids. Conjugated fatty acids are reportedly synthesized through enzymatic reactions in the seeds of terrestrial plants^{30, 31}. A method for the mass production of a target enzyme through the transfection of microorganisms using an enzyme gene has been previously reported^{32, 33}. Since

the polyenoic fatty acid isomerase from *Ptilota filicina* has reportedly been expressed in *Arabidopsis* through cDNA cloning technology³⁴, it may be possible to synthesize conjugated fatty acids in large quantities via genetic engineering for practical applications. In future research on the synthesis of conjugated tetraene fatty acids, purifying the enzyme from conjugated fatty acid-containing seaweeds such as *Padina arborescens* Holmes will be a key step. If its nucleic acid and amino acid sequences can also be determined, large-scale synthesis of conjugated tetraene fatty acids may become feasible. This advancement is expected to contribute to practical applications.

4 Anticancer Effect of Conjugated Fatty Acid

Previous reports have shown that CLA inhibits cancer cell proliferation and CLN induces cell death against cancer cells¹³. Furthermore, it has been reported that oral administration of CLN inhibits tumor growth in tumor-induced mice^{13, 14}. The mechanism by which conjugated fatty acids induce cell death has not been clearly understood. Recently, however, it has been reported that CLN induces a necrosis-like programmed cell death, called ferroptosis, in cancer cells, attracting much attention^{35, 36}. Ferroptosis is considered to be cell death induced by the lipid peroxidation reaction of cell membranes, which is triggered by the action of reactive oxygen species and iron ions in the cell^{37, 38}.

This mechanism is quite different from many currently used anticancer drugs. For example, platinum-containing drugs like cisplatin, a typical anticancer drug, induce apoptosis by forming cross-links in cellular DNA strands and inhibiting DNA replication^{39–41}. In addition, vincristine induces cell death by arresting the cell cycle^{42, 43}, and bevacizumab exerts its anticancer effects by inhibiting angiogenesis⁴⁴. Cetuximab also exhibits anticancer activity by binding to the epidermal growth factor receptor^{45, 46}. In contrast, CLN induces ferroptosis by targeting the cell membrane^{35, 36}. Further studies are needed to investigate the resistance of various cancer cells to ferroptosis. However, it is anticipated that even apoptosis-resistant cancer cells may not exhibit resistance to ferroptosis induced by conjugated fatty acids. Consequently, conjugated fatty acids could potentially induce cell death in cancers that are resistant to conventional anticancer drugs. Previous studies have reported that ferroptosis-sensitive malignant breast cancer cells (MDA-MB-231) are more susceptible to cell death induced by conjugated fatty acids than breast cancer cells with relatively low ferroptosis sensitivity (MCF-7)⁴⁷. Therefore, conjugated fatty acids may be expected to be effective against cancer cells that have been difficult to treat. Future research is needed on this point.

5 Conclusion

Conjugated fatty acids are fatty acids with an intramolecular conjugated double bond and are attracting attention for their anticancer effects. Although conjugated fatty acids are known to have a wide variety of structures, they are minor fatty acids that are rarely found in nature. In a recent study, by screening plant seeds, seed oils of *Centranthus ruber* and *Valeriana officinalis* belonging to the Valerianaceae family were found to contain CLN. Furthermore, it was found that PA, a conjugated tetraenoic fatty acid, can be synthesized by adding α -LnA to enzymes extracted from the brown alga *Padina arborescens* Holmes. This method is expected to enable verification of the anticancer effects through the synthesis of various conjugated fatty acids, which have been difficult to obtain from natural products so far. Furthermore, if mass synthesis becomes possible, practical application of this method is also in sight. Recent study has reported that the cytotoxic effect of conjugated fatty acids is due to ferroptosis. Therefore, conjugated fatty acids are expected to be effective against apoptosis-resistant cancers. The results of these studies will help to promote research on the use of conjugated fatty acids to overcome intractable cancers in the future.

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