Technical

# Lipids in Chinese Medicine. Characterization of all *cis* 5,11,14,17-eicosatetraenoic Acid in *Biota orientalis* Seed Oil and a Study of Oxo/Furanoid Esters Derived from *Biota oil*

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**The fatty acid composition of *Biota orientalis* seed oil consists of palmitic (5.1%), stearic (3.4%), oleic (15.3%), linoleic (25.6%), linolenic (34.7%), C20:3(11c,14c,17c) 4.9%, and C20:4(5c,11c,14c,17c) 10.5%. The unsaturated fatty esters derived from *Biota* oil were epoxidized and subsequently treated with NaI-PrI-DMSO. Chromatographic separation of the complex product mixture revealed the presence of C18-oxo, C18-furanoid, and C18- and C20-oxo-furanoid esters. Epoxidation of a pure sample of C20:4(5c,11c,14c,17c) followed by NaI-PrI-DMSO treatment gave a mixture of C20-dioxo-furanoid esters. The positions of the oxo and furanoid groups in the various derivatives were determined by GC/MS analysis.**

A systematic study has been launched to assess the fatty acid composition of seeds used in Chinese medicine. Instead of taking a taxonomical approach to this investigation, our choice of seeds rests on the therapeutic effect claimed. Hence our selection of *Biota orientalis* seed, which is reported to alleviate problems related to the male reproductive system, countering infertility, regulating nocturnal emission of semen and other male sex-related problems (1).

In an effort to explore the chemical behavior of *Biota oil*, the unsaturated methyl esters were converted to the epoxy derivatives and subsequently transformed to a mixture of the corresponding methyl oxo, furanoid and oxo-furanoid derivatives by using propyl iodide, sodium iodide and dimethylsulfoxide (2). Analysis of the various products provided an insight into the chemical transformations taking place during these reactions.

## EXPERIMENTAL

*Materials and methods. Biota orientalis* seeds were purchased from herb shops in Guangzhou, China. The crude oil (35%) was extracted with petroleum ether (60–80 C), and the ordinary oil constants, i.e. saponification value, iodine value, refractive index and specific gravity, were determined according to AOAC methods (3). The crude oil sample was saponified with methanolic KOH (5%) by refluxing it for 30 min. The unsaponifiable matter was removed by petroleum ether extraction. The fatty acids were isolated by diethyl ether extraction of the sulfuric acidified aqueous phase. The ethereal extract was washed with water and dried over anhydrous sodium sulfate. The total fatty acids were methylated by treatment with 2% sulfuric acid in absolute methanol.

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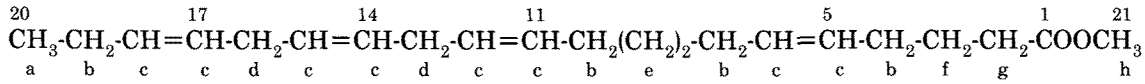
*Analysis of fatty acid methyl esters by TLC and GLC.* The total fatty acid methyl esters were analyzed on either a Varian 2400 or Hewlett Packard 5970 gas chromatograph, both equipped with flame ionization detectors. The separation of fatty acid methyl esters was conducted on a 10-m SE-30 glass microbore column (0.53 mm i.d., 2.65 micron film thickness) or on a 2-m SP-2300 stainless steel column (1.3 mm i.d., containing 10% SP-2300 on Chromosorb W), and nitrogen (20–30 ml/min) was used as the carrier gas at an isothermal column temperature of 190 C. External standards of methyl myristate, palmitate and stearate were used as reference compounds and the equivalent chain length (ECL) values calculated accordingly for each component.

Fractions separated by preparative silver ion TLC (silica, 0.5-mm thick layers, 15% $AgNO_3$, petroleum ether:diethyl ether, 7:3, v/v) were reanalyzed by GLC. Polyunsaturated fatty acid methyl esters were partially reduced with hydrazine (4), and the monoenoic acid methyl esters isolated by silver ion TLC. The monoene fractions were subjected to von Rudloff's oxidation procedure (5), and the mono and diacid fragments were characterized by GLC (after methylation with $BF_3$-methanol complex) to determine the positions of the ethylenic linkages. The presence of any *trans*-ethylenic bonds was ruled out as no absorption was found at 965 $cm^{-1}$ in the infrared spectra of the various unsaturated fatty acid methyl ester fractions by TLC separation. GC/MS analysis was conducted on a Hewlett Packard 5970 GC fitted with a mass selective detector and a 30-m capillary OV-101 column. Nuclear magnetic resonance spectroscopic analyses were performed on a JEOL FX90 (90 MHz) instrument.

TABLE 1

**Analytical Data of *Biota orientalis* Seed Oil**

| | |
|---|---|
| Seed oil | |
| Iodine value (Wijs) | 166.5 |
| Saponification value | 173.2 |
| Peroxide value | 4.5 meq/kg |
| Refractive index | 1.4752 |
| Specific gravity | 0.9184 |
| Fatty acid composition | % |
| Palmitic (16:0) | 5.1 |
| Stearic (18:0) | 3.4 |
| Oleic (18:1) | 15.3 |
| Linoleic (18:2) | 25.6 |
| Linolenic (18:3) | 34.7 |
| Eicosatrienoic (20:3) | 4.9 |
| Eicosatetraenoic (20:4) | 10.5 |
| Trace of 20:0 and 20:2 | 0.5 |




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $^1$H NMR (δ): | a: | 0.97 | d: | 2.81 | g: | 2.23 | | |
| | b: | 2.04 | e: | 1.3 | h: | 3.66 | | |
| | c: | 5.36 | f: | 1.67 | | | | |
| $^{13}$C NMR (ppm): | C-1: | 174.01 | C-6: | 130.94 | C-11: | 130.13 | C-16: | 25.57 |
| | C-2: | 33.48 | C-7: | 27.20 | C-12: | 127.15 | C-17: | 128.56 |
| | C-3: | 24.97 | C-8: | 29.36 | C-13: | 25.68 | C-18: | 131.97 |
| | C-4: | 26.60 | C-9: | 29.36 | C-14: | 128.34 | C-19: | 20.59 |
| | C-5: | 127.85 | C-10: | 27.20 | C-15: | 128.34 | C-20: | 14.25 |
| | | | | | | | C-21: | 51.41 |

STRUCTURE 1.

*Epoxidation of methyl esters of* Biota *seed oil.* A mixture of methyl esters (2.1 g), m-chloroperbenzoic acid (3.1 g) and dichloromethane (50 ml) was stirred for eight hr at room temperature. Saturated sodium sulfite (30 ml) was added to remove excess peracid. The dichloromethane layer was isolated, washed with water (50 ml), sodium bicarbonate solution (10%, 30 ml) and sodium chloride solution (10%, 30 ml) and dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure gave the crude epoxy ester mixture (1.95 g).

*Reaction of epoxy esters with propyl iodide, sodium iodide and dimethylsulfoxide.* A mixture of epoxy fatty esters (0.34 g), propyl iodide (2.6 g), sodium iodide (0.32 g) and dimethylsulfoxide (50 ml) was heated at 100 C for five hr under nitrogen. The reaction mixture was cooled and sodium thiosulfate (10%, 20 ml) was added to remove free iodine. Water (100 ml) was then added and the reaction mixture extracted with diethyl ether (2 × 40 ml). The organic extract was washed with water (50 ml) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give a mixture of the crude oxo and oxo-furanoid esters (0.19 g).

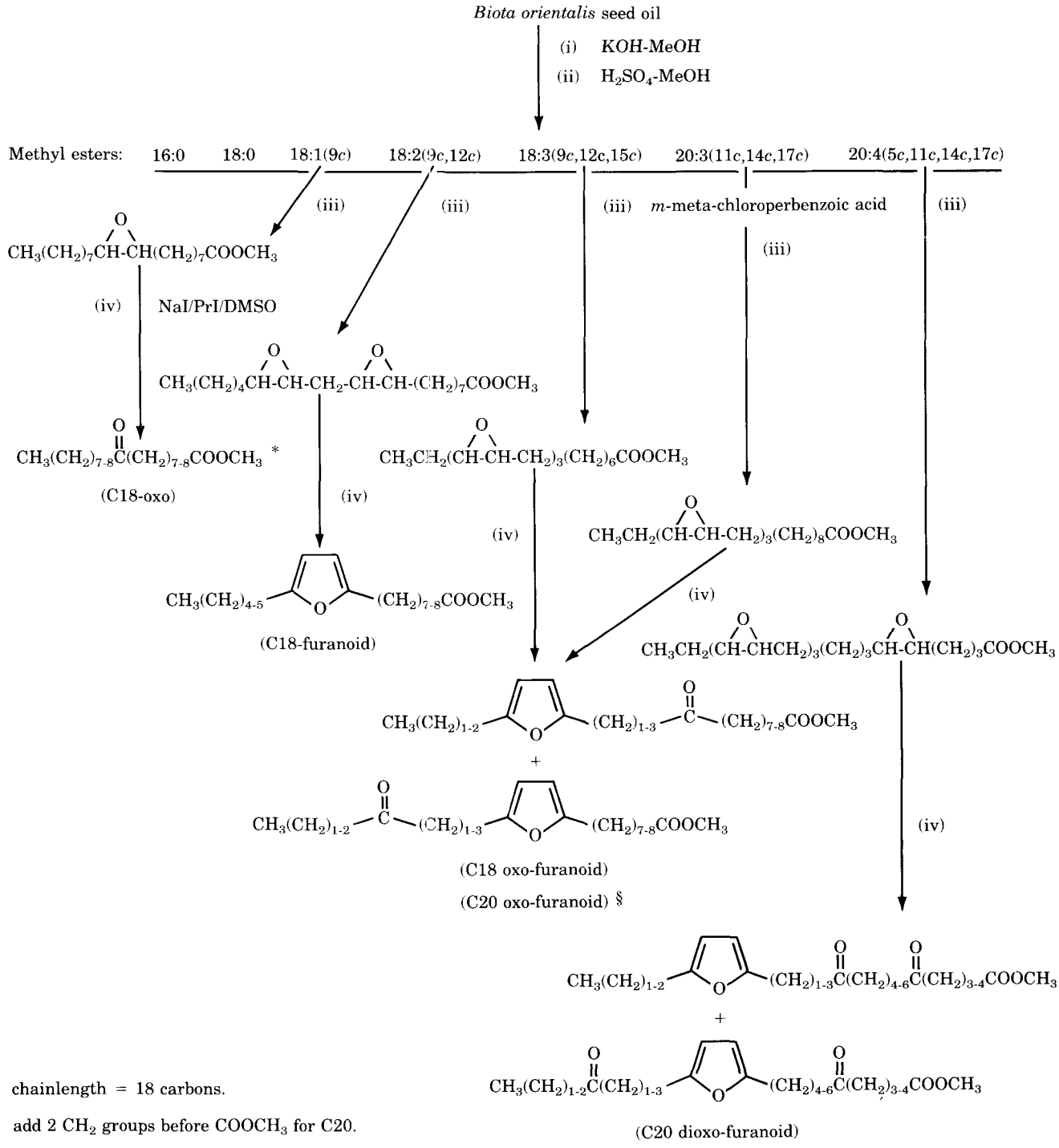
## RESULTS AND DISCUSSION

*Fatty acid composition.* The analytical values of the seed oil (*Biota orientalis*) are summarized in Table 1. The results show the presence of relatively large quantities of C20:3(11c,14c,17c), 4.9%, and C20:4(5c,11c,-14c,17c), 10.5%. The structure of the C20:4 fatty acid was confirmed by NMR analysis, as shown in Structure 1.

The natural occurrences of the same C20:3 and C20:4 fatty acids were reported in the seed oils of *Ephedra foliata, E. campylopoda, Caltha palustris* and in some gymnosperms (6–9). There exists no report regarding the possibility of C20:4(5c,11c,14c,17c) being used by the animal system as an intermediate during the biosynthesis of prostaglandins. However, it has been well established that animal systems are capable of desaturating unsaturated fatty acids, viz conversion of linoleic acid via γ-linolenic acid to arachidonic acid (10). The therapeutic effects observed by Chinese herbalists may be related to C20:4, which could serve as a precursor for prostaglandin production through a single desaturation at the $\Delta^8$ position.

*Epoxidation and furan formation from* Biota *fatty esters.* The anticipated reactions involving unsaturated fatty esters of the *Biota* oil are presented in Scheme 1. Epoxidation of the ethylenic fatty esters with m-chloroperbenzoic acid was readily achieved as indicated by the disappearance of ethylenic proton signals in the NMR spectrum of the crude product. GLC analysis on the SE-30 stationary phase of the epoxy fatty ester mixture gave peaks corresponding to the saturated fatty esters (16:0 and 18:0), methyl 9,10-epoxystearate (ECL = 19.45), 9,10;12,13-diepoxystearate (ECL = 20.6–20.9) and 9,10;12,13;15,16-triepoxystearate (ECL = 22.5), but no peak for the anticipated C20-tetraepoxy fatty ester (Calc. ECL = 26.0) was observed. Efforts to obtain evidence for the presence of this epoxy derivative in the mixture failed after several attempts. It appeared that methyl tetraepoxyeicosanoate was rather labile and readily decomposed.

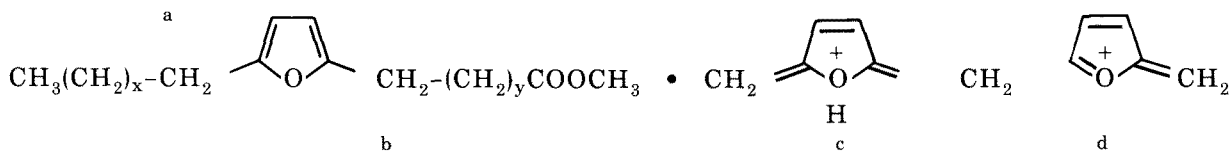
Initial GLC analysis of the complex reaction product on SE-30 stationary after treatment of the epoxy derivatives with a mixture of propyl iodide, sodium iodide and dimethyl sulfoxide gave four major components (peaks A–D) with ECL values: peak A = 16.0 (15%), B = 18.2 (31.5%), C = 19.7 (14.7%) and D = 20.5 (28.9%) and three minor components (total 9.9%). Preparative TLC on silica furnished three bands (I–III). The least polar band (I) consisted of two major components corresponding to peaks A and B. The mass spectral analysis of compound A and a forerunning component of peak B (separated on a 30-m OV-101 capillary column, ECL = 18.0) were identified as methyl palmitate and stearate, respectively. The major component in peak B was identified as a mixture of two C18 furanoid positional isomers, viz. methyl 9,12-epoxy-9,11- and methyl 10,13-epoxy-10,12-octadecadienoate derived from methyl linoleate. The mass spectral fragmentation pattern of this furanoid ester mixture was as shown in Scheme 2 [m/z (fragment, intensity)].




* chainlength = 18 carbons.

§ add 2 $CH_2$ groups before $COOCH_3$ for C20.

**SCHEME 1. Anticipated reactions involving unsaturated fatty esters of *Biota orientalis* seed oil (epoxidation, furan formation).**



(x = 4 and y = 6 or x = 3 and y = 7)

308(M, 14.2), 277(M-31, 8.9), 251(a, 7.5 where y = 7), 237(a, 7.1 where y = 6), 165(b, 60.2 where x = 4), 151(b, 91.8 where x = 3), 95(c, 100), 81(d, 42.3).

**SCHEME 2.**

The proton NMR spectrum of TLC band I confirmed the presence of the furan nucleus where the furan protons appeared at 5.7δ.

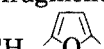
TLC band II furnished two components on GLC analysis corresponding to component C and one minor peak with ECL = 20.0. The infrared spectrum of this fraction indicated the presence of an additional carbonyl group (at 1720 $cm^{-1}$) to that of the ester carbonyl group (at 1745 $cm^{-1}$). Component C gave similar ECL values by GLC analysis on polar and nonpolar columns with methyl 12-oxostearate as reference standard. The mass spectral fragmentation of the derived methyl oxostearates was suggestive of the presence of a mixture of methyl 9- and 10-oxostearate, which were derived from methyl oleate (11). However, the mass spectral analysis of the minor component (ECL = 20.0) furnished no adequate clues regarding the nature of the structure of this component.

Band III of the TLC separation was composed of component D (ECL = 20.5). The proton NMR analysis revealed a complex spectrum, confirming the presence of the furan nucleus (5.8 δ). Signals appearing at 2.4 and 2.9 δ inferred to $CH_2$ protons α to carbonyl groups and between carbonyl and the furan nucleus, respectively. The infrared spectrum showed carbonyl stretching vibrations at 1720 and 1745 $cm^{-1}$. The mass spectral fragmentation pattern gave basic fragments for the furan system, viz m/z = 95 (c, 22.6) and 81 (d, 26.3). In addition, a host of relatively high intensity fragments were observed:

m/z = 185 (15.4), 173 (11.7), 169 (17.2), 158 (18.3), 155 (55.1), 141 (26.3), 115 (17.1), 101 (25.7), 87 (64.0) and 55 (100).

From the fragmentation pattern it was difficult to assess the composition of the eight possible methyl C18 oxo-furanoid esters which could be derived from methyl linolenate. The ECL value of component D agreed with the calculated ECL value for a methyl C18 oxo-furanoid fatty ester.

A pure sample of C20:4(5c,11c,14c,17c) was isolated by silver ion chromatography from the methyl esters of *Biota* oil. This ester was epoxidized with m-chloroperbenzoic acid, and the GLC analysis on SE-30 of the product gave a single peak with an ECL = 26.1, which corresponded to the calculated value for a C20-tetraepoxy methyl ester. The NMR spectrum confirmed the presence of epoxy groups (3.0 δ, 8H equivalent).

This tetraepoxy derivative was treated with propyl iodide, sodium iodide and dimethylsulfoxide to give a mixture of methyl dioxo-furanoid esters (20.4% yield based on C20:4). The GLC analysis furnished a broad peak with an ECL range of 23.0-24.0 on SE-30. The proton NMR spectrum indicated the presence of the furan nucleus (at 5.7δ) and also the signals associated with the methylene protons α to the oxo group and between the oxo and furan system appearing at 2.4 and 2.9δ, respectively. The infrared spectrum gave furan C-H stretching vibration at 3100 and carbonyl stretchings at 1745 and 1720 $cm^{-1}$. The GC/MS analysis of the peak between 23.0-24.0 ECL gave a complex fragmentation pattern. However, when GC/MS "cuts" were made of the broad peak (at ECL corresponding to 23.2, 23.5,23.7, 23.8), the fragmentation patterns of all subfractions were very similar. Each analysis gave two characteristic fragments, viz. m/z = 364 (M, 11-20) and 122 ($^+O{\equiv}CCH_2$  $CH_2$, 70-100). It appeared from the mass spectral study that the broad peak consisted of a mixture of similar compounds with the same molecular ion. The results also inferred that the cyclization of the methylene-interrupted tetraepoxy intermediate was not specific and gave no preferred C20 dioxo-furanoid derivative.

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