A Cytotoxic Principle of *Tamarindus indica,* Di-*n*-butyl Malate and the Structure-Activity Relationship of Its Analogues

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Di-*n*-butyl Malate, *Tamarindus indica,* Cytotoxic Compound, Sea Urchin Eggs, Structure-Activity Relationship

Bioassay-guided fractionation of the methanolic extract of *Tamarindus indica* fruits led to the isolation of L-(–)-di-*n*-butyl malate which exhibited a pronounced cytotoxic activity against sea urchin embryo cells. In order to study structure-activity relationships, close-structure relatives of di-*n*-butyl malate were synthesized using D-(+)- and L-(–)-malic acid as starting materials, and their cytotoxic activities were examined for the sea urchin embryo assay. L-(–)-Di-*n*-pentyl malate was the most effective inhibitor to the development of the fertilized eggs. Significant inhibitory activity was not seen in the esters of D-(–)-isomer.

Introduction

Mitotic events which are tightly linked to the cell cycle in eukaryote have a great significance for cell biologists. This has prompted us to begin a systematic survey of new effective inhibitors which can react with cytoskeletal components and arrest living, dividing cells. Using a unique, fast and easy-handling bioassay system with sea urchin eggs, we have reported previously on the isolation and the mode of action of active compounds obtained from microbial origins (Kobayashi *et al.*, 1988, 1989, and 1994). In another study, known antineoplastic agents such as aphidicolin, taxol, maytansine and so on were evaluated their efficacy for antimitotic effects on sea urchin egg development (Sato *et al.*, 1989). We extended our screening program to search for potential naturally occurring cytotoxic constituents from tropical plants using the sea urchin bioassay system and found that the *n*-butanol extract from the ripe pulp of *T. indica* (leguminosae) fruits exhibited a pronounced cytotoxic activity against sea urchin egg development. *T. indica* commonly known as tamarind has been widely used in Malaysia for various purposes. In the food industry, it has been used as a food additive. The fruits have also been reported as expectorant and anti-inflammatory agents (Burkill, 1966). In the present paper, we report the isolation and the structural elucidation of an active constituent together with the structure-activity relationship studies of its analogues.

Reprint requests to Prof. A. Kobayashi.

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Results and Discussion

Isolation and structural elucidation of an active compound

The BuOH layer from the MeOH extract of the ripe pulp of *T. indica* fruits, exhibited a cytotoxic activity (MIC: minimum inhibitory concentration, 125 μg/ml) by preventing the first division of fertilized *Hemicentrotus pulcherrimus* embryo cells. An active principle (**1**) was obtained in a pure oil by repeated column chromatography under guidance of the bioassay with sea urchin embryo cells. Compound **1** was characterized as a single purple spot on silica gel TLC (*Rf* 0.40; hexane-EtOAc, 8:2) by heating after spraying with vanillin-sulfuric acid. Its HR-FAB-MS data established a molecular formula of $C_{12}H_{22}O_5$ ($[M+H]^+$: calcd. 247.1545; found 247.1538). The ^1H-^1H COSY spectrum together with the ^{13}C-NMR spectrum indicated the pres-

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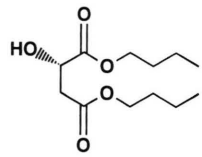


Fig. 1. Chemical structure of **1;** L-(−)-di-*n*-butyl malate.

ence of two buthoxy groups (δ 0.91, 2 x 3H; δ 1.3–1.6, 4 x 2H; δ 4.09, 2H; δ 4.17 and 4.20, 1H each). The ^{1}H-NMR spectrum of **1** also displayed a deuterium-exchangeable signal at δ 3.19 which was assignable to a hydroxyl. The presence of hydroxyl group in **1** was also confirmed by acetylation (δ 2.11, 3H, s). A pair of double doublet (δ 2.76, J=16.4, 6.1; δ 2.83, J=16.4, 4.5, 1H each) were rationally assignable to methylene protons next to a carbonyl. A 1H double double doublet (J=4.5, 5.4, 6.1 Hz) at δ 4.46 was assigned to a carbinol methine. Two sp^2 carbon signals at δ 170.54 and 173.47 in the ^{13}C-NMR together with the IR spectrum (1739 cm^{-1}) indicated that **1** has two ester groups. This observation allowed us to identify **1** to be di-*n*-butyl malate. Judging from the negative optical rotation, the stereochemistry of the chiral carbon must be L-form. On the basis of these spectral data, **1** was identified as L-(−)-di-*n*-butyl malate (Fig. 1). Compound **1** was found to be identical with the synthetic L-(−)-di-*n*-butyl malate, in all spectroscopic features ($[\alpha]_D$, IR, MS, ^{1}H-NMR) and also in the cytotoxic activity toward sea urchin eggs.

Biological Activities of **1**

Compound **1** was subjected to various biological tests such as a cytotoxicity test with sea urchin eggs, an antimicrobial test with *Escherichia coli* and *Bacillus subtilis* and a spore germination test with *Aspergillus candidus* and *Cladosporium herbarum*.

Table I indicates the minimum inhibitory concentration (MIC, μg/ml) of **1** in the 3 biological tests mentioned above. Compound **1** prevented the first cleavage of fertilized *Hemicentrotus pulcherrimus* eggs at the MIC of 8 μg/ml. When the incubation period was extended to 24 h, the embryo development was interfered with at the morula or blastula stage in the concentrations up to 1 μg/ml. However, no morphological damage was seen in these embryo stage. These inhibitory pattern is close to that of typical RNA synthesis inhibitors such as actinomycin, rifamycin and 8-azaguanine which cause gastrulation failure (Bamberger *et al.*, 1963; Gross, 1967). Some attempt directed to clarify the operation mode of **1** is currently underway. Compound **1,** however was not active against *E. coli* and *B. subtilis* but showed a significant antifungal activity against *A. candidus* (MIC: 63 μg/ml).

Table I. Biological activities of **1**[a].

Test organism	MIC [μg/ml]	
Sea urchin (eggs)	1 cell stage 8.0	Blastula stage 1.0
Escherichia coli[b]	1000	
Bacillus subtilis[b]	1000	
Aspergillus candidus[c]	63	
Cladosporium herbarum[c]	250	

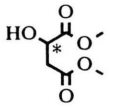
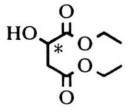
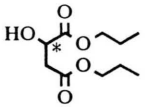
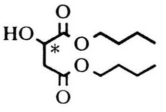
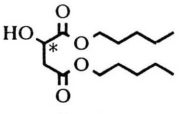
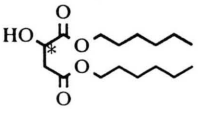
[a] Method for each test and the meaning of minimum inhibitory concentration were described in the experimental section; [b] dilution method; [c] spore germination test.

Structure-activity relationship studies of **1**

I. Alteration of the length of alkoxyl moieties

In order to study the structure-activity relationship, a series of structurally related compounds of di-*n*-butyl malate were synthesized using D-(+)- and L-(−)-malic acid as starting materials. The cytotoxic activities of various di-alkyl malates against fertilized sea urchin eggs are shown in Table II. Di-*n*-butyl (**12**), di-*n*-pentyl (**15**) and di-*n*-hexyl (**18**) esters of the L-(−)- isomer exhibited marked cytotoxic activities. Among these, **15** was the most effective and inhibited the first cleavage of the fertilized sea urchin eggs (MIC: 3 μg/ml). This indicates that the chain length:C5 is most optimal as alcohol moieties to exert the cytotoxicity. The results also suggest that the alkoxyl moieties, consisting of L-(−)-malic acid and *n*-alcohols with 4 to 6 carbons play an important role in exerting the cytotoxicity. The corresponding alcohols were not active but malic acids were weakly active. (data not shown) The D-(+)- isomers of the di-alkyl malates were completely inactive. The strict discrimination in biological activity between the enantiomers was seen in this assay system and

Table II. Alteration of the length of alkoxyl moieties in **1** and cytotoxic activities of compounds against fertilized sea urchin eggs.

Compound		Sea urchin embryo test MIC [μg/ml]
	Dimethyl malate	
	(2) D-(+)-	>1000
	(3) L-(−)-	125
	(4) DL-	500
	Diethyl malate	
	(5) D-(+)-	>1000
	(6) L-(−)-	250
	(7) DL-	500
	Di-*n*-propyl malate	
	(8) D-(+)-	>1000
	(9) L-(−)-	63
	(10) DL-	125
	Di-*n*-butyl malate	
	(11) D-(+)-	1000
	(12) L-(−)-	8.0
	(13) DL-	31
	Di-*n*-pentyl malate	
	(14) D-(+)-	1000
	(15) L-(−)-	3.0
	(16) DL-	6.0
	Di-*n*-hexyl malate	
	(17) D-(+)-	1000
	(18) L(−)-	16
	(19) DL-	125

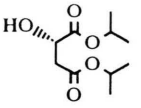
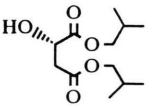
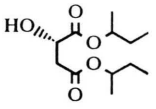
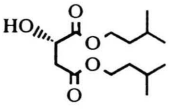
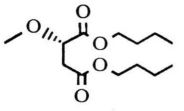
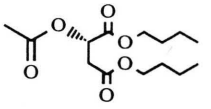
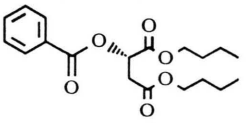
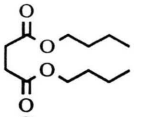
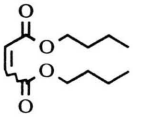
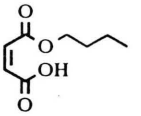
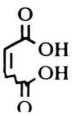
[a] The synthesis for each compound was described in the experimental section; [b] number in parentheses indicates entry number of compounds; * chiral center.

such observation is uncommon in these simple compounds. In the present study, mixtures of the two enantiomers (racemates) were two-fold less active than the L-(−)- analogues (Table II). These findings are consistent with a general understanding that when one optical enantiomorph is more biologically active than the other, antagonism between them is rarely found. This is because the space-relationship required for adsorption on the receptor is the very one altered by passing from D-(+)- to L-(−)-forms or *vice versa.*

II. Alteration of straight alkyl chains with the branched

Esters synthesized from L-(−)-malic acid and branched alcohols such as isopropyl, isobutyl, *sec*-butyl and isopentyl alcohols were also examined (Table III). However, these compounds showed weaker activities than **1,** suggesting that altering

Table III. Modification of straight alkyl chain and hydroxyl group in **1** and the cytotoxic activities of compounds against fertilized sea urchin eggs.

Compound		MIC (μg/ml)
20:		500
21:		125
22:		500
23:		32
24:		64
25:		32
26:		4.0
27:		500
28: *cis*		63
29: *trans*		500
30: *cis*		1000
Maleic acid		125
Fumaric acid		500

the steric factor along the alkyl chains contributes to the decrease of the cytotoxicity. The introduction of branched elements into the alkoxyl chains adds bulkiness to the molecule, and the steric factors affect the penetration of the compound into the membrane. Therefore, such branched molecules might be hard to be uptaken into the eggs.

The relationship between molecular structure and the chemical efficacy of derivatives of **1** was also analyzed on the basis of the partition coefficients (data not shown). We concluded that **15** must be an optimal structure with respect to steric factors and also has a preferable hydrophobicity.

III. Contribution of the OH group of malic acid moiety

The hydroxyl group of the malic acid moiety in **1** was subjected to methylation, acetylation and benzoylation. Introduction of benzoyl moiety enhanced the cytotoxicity by 2-times (**26**, Table III). However, 2-methoxy and 2-acetoxy-*n*-dibutyl malates (**24** and **25,** respectively) caused 4 and 3-fold decrease in the activity, respectively. The results suggest that the introduction of aromatic elements with high electron density may increase the activity. Removal of the hydroxyl substantially decreased the activity by 6 times (**27**), suggesting that the presence of an OH group is preferable to exert the cytotoxic activity.

Flexibility of the alkoxyl portions was restricted in the derivatives of fumaric acid and maleic acid since these acids possess a double bond. It was found that, the *cis* analogue (**28**) was three times more active than the *trans* form (**29**). However, **28** was three times less active than **1** (Table III). It was also found that, removal of one of the *n*-butyls from **28** lost the activity (**30**). This could suggest that the presence of two alkyl moieties is necessary to exert the higher activity and this fact could be common in all of these analogues.

IV. Antimicrobial activities of the analogues

The two most cytotoxic analogues (**15**, **26**) of **1,** were also subjected to antibacterial and antifungal assays. Compound **15,** having longer alkoxyl chains by one methylene units than **1** possessed a moderate antimicrobial activity and its antibacterial activity was 2-fold higher than **1** (Table IV). However, **26** was not active in the antibacterial test but showed a weak activity against *A. candidus* and *C. herbarum*. This suggests that **26** showed more specific biological activities compared with those of **1** and **15.** The esterification of an hydroxyl of **1** with a benzoyl group failed to increase the antifungal activity and the alteration of the butyl chains with pentyls brought about different significance in exerting antimicrobial activities.

Table IV. Cytotoxic and antimicrobial activities of **1** and its analogues.

Test Organism	MIC [μg/ml] **1**	**15**	**26**
Sea urchin (eggs)	8.0	3.0	4.0
E. coli	1000	250	>1000
B. subtils	1000	125	>1000
A. candidus	63	125	500
C. herbarum	250	125	500

Di-*n*-butyl malate (**1**) and its analogues displayed a variety of biological activities. These findings indicate that malic acid as well as maleic acid could be an important building entity for the synthesis of the derivatives which may exert unique biological activities as insecticides, antimicrobial agents and so on.

In addition, due to the relative simplicity of its chemical structure and its biological potency, **1** could be used as a model compound in the study of the biological specificity and its mode of action. The systematic evaluation of the derivatives of this type of compounds for the biological activities could contribute to further understanding of the binding properties of the receptors.

Experimental

General experimental procedure

^{1}H-NMR and ^{13}C-NMR were recorded with a Varian VXR-500 instrument, using deuterized solvents as internal standards. Mass spectra were recorded with a JEOL JMS-SX102A instrument, and optical rotations were measured with a Jasco DIP-360. A phase-contrast microscope (NIKON DIAPHOT-TMD) was used for the sea urchin embryo cell cytotoxic assay.

Plant material

The ripe pulp together with the whole seeds (*T. indica*) were purchased from a local market in Kuala Lumpur, Malaysia.

Chemicals

D-(+)-, L-(−)- , DL-Malic acids, acetyl chloride and alcohols were purchased from Nacalai Tesque Inc., Kyoto. Fumaric acid and maleic acid were obtained from Ishizu Pharmaceutical Co., Ltd., Osaka. Sephadex LH-20 (Pharmacia) and silica (Wakogel C-100 and C-200, C-300, Wako Pure Chemical Industries, Ltd, Osaka) were used for column chromatography. Merck Kieselgel $60F_{254}$ was used for thin layer chromatography. Di-*n*-butyl succinate (**27**), maleic acid di-*n*-butyl ester (**28**), maleic acid mono-*n*-butyl ester (**30**) were purchased from Tokyo Kasei Kogyo Co., Ltd, Tokyo.

Extraction and isolation

Ripe pulp together with the whole seeds (without homogenization, 1 kg) were soaked in 2 liters of methanol for 5 days. The MeOH extract was dissolved in water and partitioned with EtOAc. The EtOAc layer was concentrated *in vacuo* to give a dark brownish oil (2.88 g). This residue showed a weak cytotoxic activity and its MIC value was 500 μg/ml. The H_2O layer was further extracted with BuOH to give a brownish oil (10.22 g) after removal of the solvent. This residue was active against sea urchin embryos (MIC: 125 μg/ml).

Separation of the active residue from the BuOH layer was achieved by silica gel column chromatography, using toluene-acetone as the eluent. The active fraction (20% acetone eluates: 171 mg) was then subjected to Sephadex LH-20 chromatography (glass column, φ 1.6 cm x 62 cm) and eluted with MeOH. The most active fraction (55 mg) was further chromatographed on a silica gel (C 300; steel column, φ 0.6 cm x 90 cm) eluted with *n*-hexane-EtOAc (6:4) to give a pure compound (**1;** 16 mg): colorless oil; $[\alpha]_D^{25}$ = -9.8° (*c* =1.0, acetone); FAB-MS *m/z* 247 $[M+H]^+$; HR-FAB-MS *m/z* : $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found 247.1538; IR υ max. (KBr) cm^{-1}: 3500 (br.) 2962, 2935, 2875, 1739, 1470, 1447, 1385; ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.91 (6H, t, *J*=7.4 Hz), 1.35 (2H, sxt, *J*=14.9, 7.5 Hz), 1.36 (2H, sxt, *J*=14.9, 7.5 Hz), 1.61 (4H, m, overlapped), 2.76 (1H, dd, *J*=16.4, 6.1 Hz), 2.83 (1H, dd, *J*=16.4, 4.5 Hz), 3.19 (1H, d, *J*=5.4 Hz, D_2O exchangeable), 4.09 (2H, t, *J*=6.7 Hz), 4.17 (1H, dt, *J*=10.7, 6.7 Hz), 4.20 (1H, dt, *J*= 10.7, 6.7 Hz), 4.46 (1H, ddd, *J*=16.4, 5.4, 4.5 Hz; D_2O treatment, 1H, t, *J*=5.2 Hz);

^{13}C-NMR δ (125 MHz, $CDCl_3$): 13.62, 13.65, 19.01, 19.07, 30.50, 30.56, 38.74, 64.90, 65.90, 67.30, 170.50 and, 173.40.

Compound **1** (1.8 mg) was acetylated with a mixture of Ac_2O (0.5 ml) and pyridine (1 ml) for 12 h at 25 °C. The solvent was removed in a stream of N_2 after adding cold MeOH and toluene to give acetylated **1** (1.56 mg, 74 %); EI-MS *m/z* 288 (M^+) and 246 (M^+-Ac); ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.92 (6H, t, *J*=7.4 Hz), 1.35 (2H, sxt, *J*=15.0, 7.5 Hz), 1.36 (2H, sxt, *J*=15.0, 7.5 Hz), 1.61 (4H, m), 2.11 (3H, s), 2.75 (1H, dd, *J*= 16.4, 6.2), 2.83 (1H, dd, *J*= 16.4, 6.2), 4.10 (2H, t, *J*= 6.6), 4.15 (1H, t, *J*=6.6 Hz), 4.16 (1H, t, *J*=6.6 Hz), 5.44 (1H, t, *J*= 6.3 Hz).

Assay of cytotoxic activity

Sexually matured sea urchin (*Hemicentrotus pulcherrimus*) were collected during the breeding season (January-March) from the coastal water near Ushimado Marine Laboratory in Okayama Prefecture. The eggs and sperm were obtained by KCl-shedding. Eggs were agitated and left for 3 minutes. The eggs near the surface and the bottom were removed by decantation and suction. This process promised better fertilization and synchronous development of the eggs. The cytotoxic activities of the plant extract and various synthetic samples were evaluated by adding a small number of fertilized eggs (*ca.* 100) of *H. pulcherrimus* to a serially diluted sample solutions, and morphological changes of the embryos were observed in the manner described previously (Kobayashi *et al.*, 1989).

Antimicrobial assay

The antimicrobial assay method described previously was adopted (Kobayashi *et al.*, 1994). In this assay, the MIC values against bacteria (*E. coli* and *B. subtilis*) and fungi (*A. candidus* and *C. herbarum)* were determined by the 2-fold serial

dilution method. As for controls, tetracyclin inhibited the growth of *B. subtilis*, and *E. coli* at MICs of 0.8 μg/ml and 1.6 μg/ml, respectively. In the antifungal test, cycloheximide suppressed the spore germination of *A. candidus* and *C. herbarum* at MICs of 6.3 μg/ml and 12.5 μg/ml, respectively.

Synthesis of Di-alkyl malates

General procedure of esterification

The esterification was performed in HCl-alcoholic solution, prepared with acetyl chloride (Riegel *et al.*, 1955). Since all of the reactions performed are essentially same, one of the reactions was described as a typical example.

D-(+)-*Dimethyl malate* (**2**). To a cooled solution of 200 mg (1.5 mmole) of D-(+)-malic acid in 5 ml toluene is added carefully a cooled solution of acetyl chloride (0.3 ml) in 20 ml dried methanol. The solution was stirred and allowed to stand overnight at room temperature. The solution was concentrated to dryness *in vacuo*, and the residue was taken up with ethyl acetate (25 ml), washed with saturated sodium bicarbonate solution (2 x 10 ml) and water (2 x 10 ml) and then dried over Na_2SO_4. The solvent was removed *in vacuo*, and the product was purified by chromatography on silica gel (Wakogel C-200; *n*-hexane-EtOAc, 70:30) to give **2** (141 mg, 58%). $[\alpha]_D^{25}$ = +8.0° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2958, 2908, 2856, 1741, 1479, 1441, 1369. ^{1}H-NMR δ (200 MHz, $CDCl_3$): 2.76 (1H, dd, *J*=16.2, 5.6 Hz), 2.85 (1H, dd, *J*=16.2, 5.6 Hz), 3.23 (1H, d, *J*=5.3 Hz), 3.70 (3H, s), 3.78 (3H, s), 4.48 (1H, dd, *J*=5.6, 5.3 Hz); FAB-MS *m/z*: 163 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_6H_{11}O_5$, 163.0606; found, 163.0627.

L-(−)-*Dimethyl malate* (**3**). From 200 mg of L-(−)-malic acid and 20 ml methanol; as a colorless oil. (154 mg, 63%). $[\alpha]_D^{25}$ = −8.0° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2924, 2874, 1736, 1459, 1438, 1320. ^{1}H-NMR δ (200 MHz, $CDCl_3$): 2.76 (1H, dd, *J*=16.3, 5.2 Hz), 2.85 (1H, dd, *J*=16.3, 4.4 Hz), 3.17 (1H, d, *J*=4.0 Hz), 3.71 (3H, s), 3.79 (3H, s), 4.48 (1H, ddd, *J*=5.2, 4.4, 4.0 Hz); FAB-MS *m/z*: 163 $[M+H]^+$. HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_6H_{11}O_5$, 163.0606; found, 163.0607.

DL-*Dimethyl malate* (**4**). From 200 mg of DL-malic acid and 20 ml methanol; as a colorless oil. (170 mg, 70%). IR υ max (KBr) cm^{-1}: 3500 (br.), 2958, 2908, 2856, 1741, 1479, 1441, 1369; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 2.77 (1H, dd, *J*=16.4, 5.5 Hz), 2.86 (1H, dd, *J*=16.4, 4.4 Hz), 3.17 (1H, d, *J*=4.9 Hz), 3.70 (3H, s), 3.80 (3H, s), 4.48 (1H, ddd, *J*=5.5, 4.9, 4.4 Hz); FAB-MS *m/z*: 163 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_6H_{11}O_5$, 163.0606; found, 163.0628.

D-(+)-*Diethyl malate* (**5**). From 200 mg of D-(+)-malic acid and 20 ml ethanol; as a colorless oil. (220 mg, 78%). $[\alpha]_D^{25}$ = +7.8° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3490 (br.), 2923, 2871, 1740, 1478, 1442, 1368; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 1.23 (3H, t, *J*=7.1 Hz), 1.26 (3H, t, *J*=7.1 Hz), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.86 (1H, dd, *J*=16.4, 4.6 Hz), 3.23 (1H, d, *J*=5.4 Hz), 4.14 (2H, q, *J*=7.11), 4.24 (2H, q, *J*=7.11), 4.45 (1H, ddd, *J*=5.8, 5.4, 4.6 Hz); FAB-MS *m/z*: 191 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_8H_{15}O_5$, 191.0919; found, 191.0917.

L-(−)-*Diethyl malate* (**6**). From 200 mg of L-(−)-malic acid and 20 ml ethanol; as a colorless oil. (171 mg, 60%). $[\alpha]_D^{25}$ = -7.8° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3492 (br.), 2984, 2940, 2877, 1738, 1464, 1447, 1374 ; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 1.23 (3H, t, *J*=7.1 Hz), 1.26 (3H, t, *J*=7.1 Hz), 2.73 (1H, dd, *J*=16.4, 5.8 Hz), 2.86 (1H, dd, *J*=16.4, 4.7 Hz), 3.25 (1H, s, br.), 4.13 (2H, q, *J*= 7.1 Hz), 4.23 (2H, q, *J*=7.1 Hz), 4.45 (1H, dd, *J*= 5.8, 4.7 Hz); FAB-MS *m/z*: 191 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_8H_{15}O_5$, 191.0919; found 191.0937.

DL-*Diethyl malate* (**7**). From 200 mg of DL-malic acid and 20 ml ethanol as a colorless oil (177 mg, 62%). IR υ max (KBr) cm^{-1}: 3492 (br.), 2985, 2940, 2909, 1740, 1465, 1445, 1374; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 1.23 (3H, t, *J*=7.1 Hz), 1.27 (3H, t, *J*=7.1 Hz), 2.73 (1H, dd, *J*=16.4, 5.8 Hz), 2.82 (1H, dd, *J*=16.4, 4.7 Hz), 3.22 (1H, d, *J*=5.4 Hz), 4.14 (2H, q, *J*=7.1 Hz), 4.24 (2H, q, *J*=7.1 Hz), 4.45 (1H, ddd, *J*=5.8, 5.4, 4.7 Hz); FAB-MS *m/z*: 191 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_8H_{15}O_5$, 191.0919; found, 191. 0899.

D-(+)-*Di-n-propyl malate* (**8**). From 200 mg of D-(+)-malic acid and 20 ml *n*-propanol; as a colorless oil (246 mg, 75%).

$[\alpha]_D^{25}$ = +9.6° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2970, 2925, 2858, 1740, 1463, 1441, 1378; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.93 (6H, t, *J*=7.4 Hz), 1.62 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8

Hz), 2.85 (1H, dd, *J*=16.4, 4.7 Hz), 3.21 (1H, d, *J*= 5.4 Hz), 4.05 (2H, t, *J*=6.7 Hz), 4.14 (1H, t, *J*=6.7 Hz), 4.15 (1H, t, *J*=6.7 Hz), 4.45 (1H, ddd, *J*=5.8, 5.4, 4.7 Hz); FAB-MS *m/z*: 219 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{10}H_{19}O_5$, 219.1232; found, 219.1245.

L-(−)-*Di-n-propyl malate* (**9**). From 200 mg of L-(−)-malic acid and 20 ml *n*-propanol; as a colorless oil (256 mg, 78%).

$[\alpha]_D^{25}$ = -9.6° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3486 (br.), 2956, 2922, 2856, 1738, 1458, 1425, 1374; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.91 (6H, t, *J*=7.4 Hz), 1.63 (4H, m), 2.74 (1H, dd, *J*=16.4, 5.8 Hz), 2.84 (1H, dd, *J*=16.4, 4.7 Hz), 3.25 (1H, d, *J*= 5.4 Hz), 4.01 (2H, t, *J*=6.7 Hz), 4.13 (1H, t, *J*=6.7 Hz), 4.14 (1H, t, *J*=6.7 Hz), 4.46 (1H, ddd, *J*=5.8, 5.4, 4.7 Hz); FAB-MS *m/z*: 219 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{10}H_{19}O_5$, 219.1232; found, 219.1257.

DL-*Di-n-propyl malate* (**10**). From 200 mg of DL-malic acid and 20 ml methanol; as a colorless oil. (266 mg, 81%). IR υ max (KBr) cm^{-1}: 3500 (br.), 2970, 2941, 2898, 1739, 1463, 1449, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.91 (6H, t, *J*=7.4 Hz), 1.63 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.85 (1H, dd, *J*=16.4, 4.6 Hz), 3.23 (1H, d, *J*=5.3 Hz), 4.05 (2H, t, *J*=6.7 Hz), 4.14 (1H, t, *J*=6.7 Hz), 4.15 (1H, t, *J*=6.7 Hz), 4.47 (1H, ddd, *J*=5.8, 5.3, 4.6 Hz); FAB-MS *m/z*: 219 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{10}H_{19}O_5$, 219.1232; found, 219.1253.

D-(+)-*Di-n-butyl malate* (**11**). From 200 mg of D-(+)-malic acid and 20 ml *n*-butanol; as a colorless oil (296 mg, 80%). $[\alpha]_D^{25}$ = +9.8° (*c*=1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2962, 2936, 2875, 1740, 1464, 1448, 1381; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.91 (6H, t, *J*=7.1 Hz), 1.35 (4H, sxt, *J*=15.0, 7.5 Hz), 1.61 (4H, m), 2.75 (1H, dd, *J*= 16.4, 6.1 Hz), 2.85 (1H, dd, *J*=16.4, 4.6 Hz), 3.19 (1H, d, *J*=5.4 Hz), 4.09 (2H, t, *J*=6.7 Hz), 4.18 (1H, t, *J*=6.7 Hz), 4.19 (1H, t, *J*=6.7 Hz), 4.47 (1H, ddd, *J*=6.1, 5.4, 4.6 Hz); FAB-MS *m/z*: 247 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found, 247.1536.

L-(−)-*Di-n-butyl malate* (**12**). From 200 mg of L-(−)-malic acid and 20 ml *n*-BuOH; as a colorless oil (274 mg, 74%). $[\alpha]_D^{25}$ = -9.8° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2962, 2936, 2875, 1740, 1464, 1448, 1381; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.91 (6H, t, *J*=7.3 Hz), 1.35 (4H, sxt, *J*= 15.0, 7.5 Hz), 1.60 (4H, m), 2.76 (1H, dd, *J*=16.4, 6.1 Hz), 2.83 (1H, dd, *J*=16.4, 4.5 Hz), 3.20 (1H, d, *J*=5.4 Hz), 4.09 (2H, t, *J*=6.6 Hz), 4.18 (1H, t, *J*= 6.7 Hz), 4.19 (1H, t, *J*=6.7 Hz), 4.45 (1H, ddd, *J*= 6.1, 5.4, 4.5 Hz); FAB-MS *m/z*: 247 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found 247.1530.

DL-*Di-n-butyl malate* (**13**). From 200 mg of DL-malic acid and 20 ml *n*-BuOH; as a colorless oil (256 mg, 69%). IR ᵞ max (KBr) cm^{-1}: 3485 (br.), 2959, 2924, 2878, 1739, 1458, 1422, 1384; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.90 (6H, t, *J*=7.1 Hz), 1.35 (4H, sxt, *J*=15.0, 7.5 Hz), 1.60 (4H, m), 2.75 (1H, dd, *J*=16.4, 6.1 Hz), 2.84 (1H, dd, *J*=16.4, 4.5 Hz), 3.21 (1H, d, *J*=5.4 Hz), 4.09 (2H, t, *J*=6.6 Hz), 4.18 (1H, t, *J*=6.6 Hz), 4.19 (1H, t, *J*=6.5 Hz), 4.46 (1H, ddd, *J*=6.1, 5.4, 4.5 Hz); FAB-MS *m/z*: 247 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found, 247.1551.

D-(+)-*Di-n-pentyl malate* (**14**). From 200 mg of D-(+)-malic acid and 20 ml *n*-pentanol; as a colorless oil (321 mg, 78%). $[\alpha]_D^{25}$ = +9.2° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2959, 2932, 2872, 1740, 1464, 1442, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.88 (6H, t, *J*=6.3 Hz), 1.28 (8H, m), 1.63 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.85 (1H, dd, *J*=16.4, 4.5 Hz), 3.18 (1H, d, *J*=5.4 Hz), 4.09 (2H, t, *J*=6.7 Hz), 4.18 (2H, t, *J*=6.8 Hz), 4.46 (1H, ddd, *J*=5.8, 5.4, 4.5 Hz); FAB-MS *m/z*: 275 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{14}H_{27}O_5$, 275.1858; found, 275.1842.

L-(−)-*Di-n-pentyl malate* (**15**). From 200 mg of L-(−)-malic acid and 20 ml *n*-pentanol; as a colorless oil (288 mg, 70%). $[\alpha]_D^{25}$ = -9.2° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2959, 2934, 2872, 1740, 1465, 1448, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.87 (6H, t, *J*=6.3 Hz), 1.29 (8H, m), 1.62 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.83 (1H, dd, *J*=16.4, 4.6 Hz), 3.22 (1H, d, *J*=5.4 Hz), 4.07 (2H, t, *J*=6.7 Hz), 4.17 (2H, t, *J*=6.6 Hz), 4.45 (1H, ddd *J*=5.8, 5.4, 4.6 Hz); FAB-MS *m/z*: 275 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{14}H_{27}O_5$, 275.1842; found, 275. 1842.

DL-*Di-n-pentyl malate* (**16**). From 200 mg of DL-malic acid and 20 ml *n*-pentanol; as a colorless oil (342 mg, 83%).

IR υ max (KBr) cm^{-1}: 3494 (br.), 2959, 2934, 2863, 1740, 1465, 1442, 1382; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.87 (6H, t, *J*=6.6 Hz), 1.31 (8H, m), 1.62 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz),

2.85 (1H, dd, *J*=16.4, 4.7 Hz), 3.21 (1H, d, *J*=5.4 Hz), 4.08 (2H, t, *J*=6.7 Hz), 4.17 (2H, t, *J*=6.8 Hz), 4.46 (1H, ddd, *J*=5.8, 5.4, 4.7 Hz); FAB-MS *m/z*: 275 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{14}H_{27}O_5$, 275.1858; found, 275.1867.

D-(+)-*Di-n-hexyl malate* (**17**). From 200 mg of D-(+)-malic acid and 20 ml *n*-hexanol; as a colorless oil. (322 mg, 71%).

$[\alpha]_D^{25}$ = +10.8° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2958, 2931, 2875, 1740, 1463, 1447, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.87 (6H, t, *J*=7.0 Hz), 1.27 (12H, m), 1.61 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.83 (1H, dd, *J*=16.4, 4.7 Hz), 3.17 (1H, d, *J*=5.3 Hz), 4.08 (2H, t, *J*=6.7 Hz), 4.18 (2H, t, *J*=6.8 Hz), 4.46 (1H, ddd, *J*=5.8, 5.3, 4.7 Hz); FAB-MS *m/z*: 303 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{16}H_{31}O_5$, 303.2171; found, 303.2185.

L-(−)-*Di-n-hexyl malate* (**18**). From 200 mg of L-(−)-malic acid and 20 ml *n*-hexanol; (332 mg, 73%) as a colorless oil.

$[\alpha]_D^{25}$ = -10.8° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2958, 2932, 2860, 1740, 1463, 1442, 1379; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.87 (6H, t, *J*=6.5 Hz), 1.27 (12H, m), 1.60 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.9 Hz), 2.83 (1H, dd, *J*=16.4, 4.7 Hz), 3.18 (1H, d, *J*=5.3 Hz), 4.05 (2H, t, *J*=6.7 Hz), 4.14 (1H, t, *J*=6.6 Hz), 4.15 (2H, t, *J*=6.8 Hz), 4.47 (1H, ddd, *J*=5.9, 5.3, 4.7 Hz), FAB-MS *m/z* 303 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{16}H_{31}O_5$, 303.2171; found, 303.2151.

DL-*Di-n-hexyl malate* (**19**). From 200 mg of DL-malic acid and 20 ml *n*-hexanol; as a colorless oil (309 mg, 68%). IR υ max (KBr) cm^{-1}: 3494 (br.), 2958, 2932, 2860, 1740, 1464, 1436, 1379; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.87 (6H, t, *J*=6.5 Hz), 1.29 (12H, m), 1.63 (4H, m), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.84 (1H, dd, *J*=16.4, 4.7 Hz), 3.18 (1H, d, *J*=5.4 Hz), 4.08 (2H, t, *J*=6.7 Hz), 4.18 (2H, t, *J*=6.8 Hz), 4.46 (1H, ddd, *J*=5.8, 5.4, 4.7 Hz); FAB-MS *m/z*: 303 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{16}H_{31}O_5$, 303.2171; found, 303.2179.

Using the same method, compounds **20**-**23** were prepared from L-(−)-malic acid (200 mg) and isopropanol, isobutanol, **sec**-butanol and isopentanol (20 ml each), respectively.

L-(−)-*Diisopropyl malate* (**20**). Obtained as a colorless oil (183 mg, 56%). $[\alpha]_D^{25}$ = -3.4° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2983, 2940, 2881, 1738, 1467, 1455, 1376; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 1.23 (6H, d, *J*=6.3 Hz), 1.24 (3H, d, *J*=6.2 Hz), 1.30 (3H, d, *J*=6.2 Hz), 2.75 (1H, dd, *J*=16.4, 5.8 Hz), 2.83 (1H, dd, *J*=16.4, 4.8 Hz), 3.23 (1H, d, *J*=5.5 Hz), 4.40 (1H, ddd, *J*=5.8, 5.5, 4.8 Hz), 5.04 (1H, spt, *J*=6.4 Hz), 5.04 (1H, spt, *J*=6.4 Hz); FAB-MS *m/z*: 219 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{10}H_{19}O_5$, 219.1232; found, 219.1237.

L-(−)-*Diisobutyl malate* (**21**). Obtained as a colorless oil (236 mg, 64%). $[\alpha]_D^{25}$ = -9.2° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2963, 2915, 2896, 1740, 1465, 1442, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.9 (6H, d, *J*=6.7 Hz), 1.0 (6H, d, *J*=6.7 Hz),1.90 (2H, m), 2.76 (1H, dd, *J*=16.4, 5.8 Hz), 2.87 (1H, dd, *J*=16.4, 4.7 Hz), 3.21 (1H, d, *J*=5.5 Hz), 3.88 (2H, d, *J*=6.7 Hz), 3.93 (1H, dd, *J*=10.3, 6.5 Hz), 4.10 (1H, dd, *J*=10.3, 6.7 Hz), 4.48 (1H, ddd, *J*=5.8, 5.5, 4.7 Hz); FAB-MS *m/z*: 247 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found, 247.1519.

L-(−)-*Di-sec-butyl malate* (**22**). Obtained as a colorless oil (192 mg, 52 %). $[\alpha]_D^{25}$ = -10.4° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2974, 2939, 2898, 1740, 1464, 1447, 1380; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.80 (6H, d, *J*=6.8 Hz), 0.90 (6H, d, *J*=6.8 Hz), 1.50 (4H, m), 2.75 (1H, dd, *J*=16.4, 6.3 Hz), 2.84 (1H, dd, *J*=16.4, 4.6 Hz), 3.23 (1H, d, *J*=5.5 Hz), 4.42 (1H, ddd, *J*=5.6.3, 5.5, 4.6 Hz), 4.87 (2H, m); FAB-MS *m/z*: 247 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{23}O_5$, 247.1545; found, 247.1537.

L-(−)-*Diisopentyl malate* (**23**). Obtained as a colorless oil (271 mg, 66%). $[\alpha]_D^{25}$ = -26° (*c* =1, MeOH); IR υ max (KBr) cm^{-1}: 3493 (br.), 2959, 2930, 2873, 1740, 1464, 1443, 1381; ^{1}H-NMR δ (200 MHz, $CDCl_3$): 0.89 (12H, d, *J*=6.5 Hz), 1.59 (6H, m), 2.75 (1H, dd, *J*=16.4, 6.1 Hz), 2.84 (1H, dd, *J*=16.4, 4.7 Hz), 3.18 (1H, d, *J*=4.0 Hz), 4.12 (2H, t, *J*=6.8 Hz), 4.21 (2H, d, *J*=6.7 Hz), 4.45 (1H, ddd, *J*=6.1, 4.7, 4.0 Hz); FAB-MS *m/z*: 275 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{14}H_{27}O_5$, 275.1858; found, 275.1872.

Methylation of L-*(−)-di-n-butyl malate* (**12**) *with silver oxide-methyl iodide reagent*. The similar procedure by Ferguson and Haines (1969) was adopted. L-(−)-Di-*n*-butyl malate (**12,** 50 mg) was stirred with methyl iodide (1 ml) and silver oxide (0.01 g) for 18 h at room temperature and then the mixture was heated under reflux for 6 h. Diethyl ether (10 ml) was added and the silver resi-

dues were filtered off. The product was purified by a silica gel column (Wakogel C-200; hexane-AcOEt, 80:20) to give compound **24** (25 mg, 47%). $[\alpha]_D^{25}$ = -33.0° (*c*=0.4, MeOH); IR υ max (KBr) cm^{-1}: 3494 (br.), 2961, 2936, 2875, 1741, 1464, 1448, 1380, 1278, 1066; ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.93 (3H, t, *J*=7.3 Hz), 0.94 (3H, t, *J*=7.3 Hz), 1.35 (2H, sxt, *J*=15.0, 7.63 Hz), 1.36 (2H, sxt, *J*=15.0, 7.63 Hz), 1.62 (4H, m), 2.76 (1H, dd, *J*=16.2, 8.0 Hz), 2.83 (1H, dd, *J*=16.2, 5.0 Hz), 3.45 (3H, s), 4.11 (1H, t, *J*=6.7 Hz), 4.17 (4H, m); FAB-MS *m/z*: 261 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{13}H_{25}O_5$, 261.1702; found, 261.1700.

Acetylation of L-*(−)-di-n-butyl malate* (**12**) with acetic anhydride-pyridine reagent. L-(−)-Di-*n*-butyl malate(**12,** 56 mg) was dissolved in anhydrous pyridine (0.4 ml) in a sealed vial and acetic anhydride (0.2 ml) was added. The mixture was left at room temperature for 20 h and worked up in the usual manner to give an oily compound which was purified by a silica gel column (Wakogel C-200; hexane-EtOAc, 80:20) to give compound **25** (57 mg, 97%). $[\alpha]_D^{25}$ = -24.4° (*c*=1, MeOH); IR υ max (KBr) cm^{-1}: 2976, 2936, 2876, 1749, 1462, 1447, 1373; ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.92 (6H, t, *J*=7.3 Hz), 1.36 (2H, sxt, *J*=15.0, 7,5 Hz), 1.37 (2H, sxt, *J*=15.0, 7,5 Hz), 1.60 (2H, tt, *J*=13.8, 7.02 Hz), 1.62 (2H, tt, *J*=14.0, 7.33 Hz), 2.12 (3H, s), 2.86 (2H, d, *J*=6.1 Hz), 4.11 (2H, t, *J*= 6.4 Hz), 4.15 (1H, dt, *J*=6.7, 3.3 Hz), 4.16 (1H, dt, *J*=6.7, 3.3 Hz), 5.45 (1H, t, *J*= 6.1 Hz); FAB-MS *m/z*: 289 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{14}H_{25}O_6$, 289.1651; found, 289.1644.

Benzoylation of L-*(−)-di-n-butyl malate* (**12**) *with benzoyl chloride-pyridine reagent.* L-(−)-Di-*n*-butyl malate (**12,** 9.5 mg) was dissolved in anhydrous pyridine (0.1 ml) in a sealed vial and benzoyl chloride (0.05 ml) was added. The reaction mixture was worked up in the usual manner to give an oily compound which was purified by a silica gel column (Wakogel C-200; hexane-EtOAc, 90:10) to give compound **26** (9.9 mg, 73%). $[\alpha]_D^{25}$ = -28° (*c*=0.1, MeOH); IR υ max (KBr) cm^{-1}: 3048, 2961, 2934, 2875, 1741, 1464, 1444, 1380, 1278; ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.87 (3H, t, *J*=7.3 Hz), 0.91 (3H, t, *J*=7.3 Hz), 1.35 (2H, sxt, *J*= 15.0, 7.5 Hz), 1.36 (2H, sxt, *J*=15.0, 7.5 Hz), 1.60 (4H, m), 3.01 (1H, d, *J*=5.2 Hz), 3.02 (1H, d, *J*=7.3 Hz), 4.13 (2H, d, *J*=6.7 Hz), 4.18 (2H, dt, *J*=6.7 Hz), 5.70 (1H, dd, *J*=7.0, 5.2 Hz), 7.43 (2H, dd, *J*= 15.0, 7.3 Hz), 7.57 (1H, t, *J*=7.3 Hz), 8.05 (2H, d, *J*=8.2 Hz); FAB-MS *m/z*: 351 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{19}H_{27}O_6$, 351.1808; found, 351.1806.

Fumaric acid di-n-butyl ester (**29**). The same procedure as that for preparing **2** was adopted. Fumaric acid (200 mg) was converted into **29** (a colorless oil, 290 mg, 74%). IR υ max (KBr) cm^{-1}: 2962, 2937, 2875, 1731, 1675, 1463, 1448, 1378; ^{1}H-NMR δ (500 MHz, $CDCl_3$): 0.95 (6H, t, *J*=7.33Hz), 1.41 (4H, sxt, *J*=15.0, 7.3 Hz), 1.70 (4H, tt, *J*=6.7 Hz), 4.20 (4H, t, *J*=6.7 Hz), 6.80 (2H, s); FAB-MS *m/z*: 229 $[M+H]^+$; HR-FAB-MS *m/z*: $[M+H]^+$: calcd. for $C_{12}H_{21}O_4$, 229.1440; found, 229.1441.

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