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Synthesis and Biological Activities of Acetylenic Fatty Acids and Their Esters

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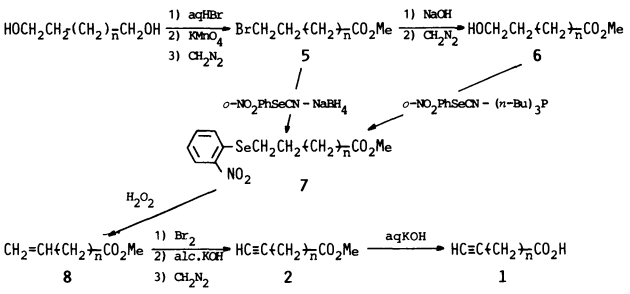
Eight terminal acetylenic fatty acids from 5-hexynoic to 12-tridecynoic acids and their methyl, ethyl, and isopropyl esters were synthesized via *o*-nitrophenyl selenides. These acids and esters all showed inhibitory effect on lettuce seed germination and seedling growth. The effect was dependent on the number of carbon atoms in acetylenic chain. 8-Nonynoic acid showed potent activity, and its methyl ester was the most effective among the test compounds. In addition, the acids showed antifungal activity which increased as the number of carbon atoms increased.

There have been some attempts to relate the chemical structure of fatty acids to inhibition of seed and pollen germination. In a series of straight-chain saturated monocarboxylic acids, Raynolds showed that the inhibitory activity on lettuce seed germination increased with the number of carbon atoms of the acids and that the main structure-activity correlation was lipophilic in nature.[1,2] Orito et al. studied the effects of a variety of monocarboxylic acids with and without hydroxy substituent at the 2- or 3-positions on pollen germination in *Camellia sinensis*.[3] They showed that the intensity of inhibition was related not only to the carbon number of the acid but also to the degree of unsubstitution of the carbon chain. We recently reported the isolation of 8-nonynoic (**1d**) and 9-decynoic (**1e**) acids and their methyl esters (**2d**) and (**2e**) from the stem bark of *Hibiscus rosa-sinensis*.[4] These metabolites were found to be inhibitors of the germination of lettuce seeds. The acid (**1d**) showed higher activity than **1e**, and the esters (**2d**) and (**2e**) were more active than the corresponding free acids. Thereupon, considerable attention was devoted to the correlation of inhibitory activity with number of carbon atoms in terminal acetylenic fatty acids and esters.

In this research, eight acids from 5-hexynoic (**1a**) to 12-tridecynoic (**1h**) acids and their methyl (**2a—h**), ethyl (**3a—h**) and isopropyl (**4a—h**) esters were prepared via ω-(*o*-nitrophenylseleno) esters and their effects on lettuce seed germination and seedling growth were studied. Further, the antimicrobial properties of the acids (**1a—h**) were examined, since acetylenic fatty acids 7-octynoic (**1c**), 8-nonynoic (**1d**), 10-undecynoic (**1f**), and 11-dodecynoic (**1g**) acids have been described to inhibit the growth of some fungi.[5-8]

Results and Discussion

Synthesis. Seven acids from 5-hexynoic (**1a**) to 12-tridecynoic (**1h**) acids were prepared from the corresponding terminal diols via the *o*-nitrophenyl selenides (Scheme 1), except that **1f** was prepared from commercially available 10-undecenoic acid. Primary alkyl phenyl selenides obtained from bromides or alcohols were shown to produce terminal olefins in good yield,[9,10] which were easily converted into acetylenes by a typical method. From the terminal diols (n=3—10), 1,6-hexanediol to 1,13-tridecanediol, except 1,11-undecanediol, the corresponding bromo esters (**5**) were prepared in three steps in ca. 35% yield by monobromination with hydrobromic acid followed by oxidation and methylation. Treatment of the esters (**5**) with *o*-nitrophenyl selenocyanate in the presence of sodium borohydride under nitrogen in ethanol at room temperature[9] gave *o*-nitrophenyl selenides (**7**) in 75—80% yields. The structures of **7** were comfirmed by elemental analysis, ^{1}H NMR and mass spectra (Table 1). In their ^{1}H NMR spectra, the methylene protons attached to selenium atom appeared at about δ 2.9. The EI-MS of each selenide displayed a $(M+1)^+$ ion and a characteristic M−201 peak which was apparently due to the fission of $-CH_2-Se-$ bond. It also contained to more prominent peaks indicating further successive loss of methanol and carbon monoxide. On the other hand, the alcohols (**4d**) and (**4e**), obtained by hydrolysis of the bromo esters (**5d**) and (**5e**), were also converted into the selenides (**7d**)



Scheme 1.

Table 1. Yields and Physicochemical Properties of Phenyl Selenides **7**

Compound	Yield %	Mp θ_m/°C	^{1}H-NMR δ ppm(Hz) PhSe-C$\underline{H}_2$-	Ms *m/z*(rel. int.)	Formula	Analysis Found(Calcd) C (%)	H (%)	N (%)
7a	68	25.0—26.0	2.95 (t, J=7.2)	69(100), 97(97) 129(15), 331(19)	$C_{13}H_{17}NO_4Se$	47.22 (47.13)	5.17 (5.14)	4.22 (4.06)
7b	73	33.0—33.5	2.88 (t, J=7.5)	83(100), 111(81) 143(20), 345(26)	$C_{14}H_{19}NO_4Se$	48.96 (48.70)	5.56 (5.51)	4.01 (4.06)
7c	75	37.5—38.0	2.88 (t, J=7.5)	97(80), 125(100) 157(15), 359(34)	$C_{15}H_{21}NO_4Se$	50.39 (50.14)	5.90 (5.85)	3.91 (3.90)
7d	79	61.5—62.5	2.90 (t, J=7.3)	111(15), 139(93) 171(12), 373(40)	$C_{16}H_{23}NO_4Se$	51.65 (51.61)	6.23 (6.23)	3.75 (3.76)
7e	76	44.0—44.5	2.96 (t, J=7.5)	125(11), 153(58) 185(11), 387(45)	$C_{17}H_{25}NO_4Se$	52.77 (52.89)	6.61 (6.53)	3.65 (3.63)
7g	75	52.0—53.0	2.92 (t, J=7.5)	153(9), 181(20) 213(10), 415(50)	$C_{19}H_{29}NO_4Se$	54.98 (54.94)	7.08 (6.99)	3.35 (3.37)
7h	75	57.0—57.5	2.90 (t, J=7.3)	167(9), 195(19) 227(10), 529(53)	$C_{20}H_{31}NO_4Se$	55.88 (55.94)	7.24 (7.23)	3.13 (3.26)

Table 2. Inhibitory Effect of Acetylenic Fatty Acids and Ester Derivatives on Lettuce Seed Germination

		Number of carbon atom in acid	Concentration/mM[a)] Acid **1**	Me ester **2**	Et ester **3**	Isopropyl ester **4**
5-Hexynoic	**a**	6	0.99	3.2	2.9	6.5
6-Heptynoic	**b**	7	0.92	2.8	2.5	6.0
7-Octynoic	**c**	8	0.78	0.49	2.3	5.5
8-Nonynoic	**d**	9	0.13	0.030	0.41	0.77
9-Decynoic	**e**	10	0.24	0.055	1.00	0.83
10-Undecynoic	**f**	11	0.41	0.38	1.43	4.5
11-Dodecynoic	**g**	12	0.44	0.90	1.08	4.2
12-Tridecynoic	**h**	13	0.48	0.98	1.19	4.0

a) Concentrations at which percentage germination of the seeds at 25 °C is 50% of the control value.

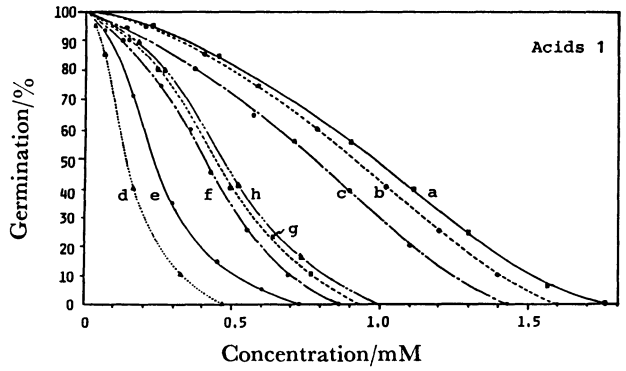


Fig. 1. Relationship between the concentration of the acid (**1a**—**h**) and the percentage of seed germination.

and (**7e**) in 73 and 78% yields, respectively, by using *o*-nitrophenyl selenocyanate with tributylphosphine in tetrahydrofuran at room temperature.[10] Decomposition of these selenides (**7**) by 30% hydrogen peroxide produced the corresponding olefins (**8**) in ca. 85% yields.

The synthesis was completed by converting **8** into the corresponding acetylenes (**1**), by bromination with bromine and subsequent dehydrobromination with ethanolic potassium hydroxide under reflux, in ca. 80% yields. In this simple method, the formation of allene compounds was minimal. The purification of these (**1a**—**h**) acids was done by column chromatography after methylation into **2a**—**h**. The ethyl (**3a**—**h**) and isopropyl (**4a**—**h**) esters were obtained from **1** by treatment with concentrated sulfuric acid under reflux in absolute ethanol and 2-propanol, respectively.

Activity on Seed Germination. To assess the effect of the variation in number of carbon atoms and ester substituents on the acetylenic fatty acids and their derivatives, the inhibitory activity of eight synthetic acids (**1a**—**h**) and their methyl (**2a**—**h**), ethyl (**3a**—**h**), and isopropyl (**4a**—**h**) esters on lettuce seed germina-

Table 3. Inhibitory Effect of Acetylenic Fatty Acids and Ester Derivatives on Hypocotyl Growth of Lettuce Seedlings

Compd	Concentration[a] mM	Compd	Concentration[a] mM
1b	1.2	**2d**	0.089
1d	0.13	**3d**	0.16
1f	0.55	**4d**	0.38
1h	0.35		

a) Concentrations at which hypocotyl is 50% of the control value.

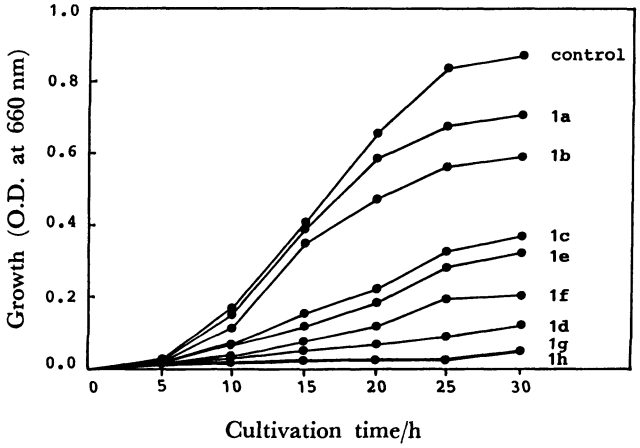


Fig. 2. Inhibitory effect of the acid (**1a**—**h**) on growth of *Hansenula anomala*.

tion were compared. Figure 1 presents the relationship between the concentration of the acids and the percentage of germination. The millimolar concentration producing 50% inhibition of germination is obtained from each curve. The inhibitory activity of all test compounds at a given concentration is shown in Table 2.

Among the acids tested, 8-nonynoic acid (**1d**) was the most potent and showed 50% inhibition at 0.13 mM (1 M=1 mol dm^{-3}). The activity of **1d** was much greater than that of nonanoic and 8-nonenoic acids so far reported.[1,4] Thus, it is apparent that the introduction of a terminal acetylenic group in the fatty acids increase inhibitory activity. As with the case of the acids, the activity of each set of methyl, ethyl and isopropyl esters was dependent on the number of carbon atoms in acetylenic chain with maximum activity at carbon number 9. The ester moiety also affected activity, a bulky one causing a decrease in activity. These results suggest that the lipophilicity-hydrophilicity balance of the compound is a fundamental requirement for the expression of marked inhibitory activity. In general, free acids showed greater activity than the corresponding esters. However, naturally occurring methyl 8-nonynoate (**2d**) and methyl 9-decynoate (**2e**) showed greater activity than the free acids (**1d**) and (**1e**). Their activities are comparable to those of well-known inhibitors, 3-indoleacetic acid[2] and abscisic acid.[11]

Activity on Seedling Growth. Four acids and three esters were selected among the synthetic compounds based on the above results of germination test and their activity tested on the hypocotyl growth in lettuce seedlings. As shown in Table 3, the acids showed similar inhibitory activity to the germination test, In which 8-nonynoic acid (**1d**) exhibited greater activity than the other acids. Its methyl ester (**2d**) was the most effective. It is interesting to note that these terminal acetylene compounds are also observed to inhibit a photosynthetic path.[12]

Antimicrobial Activity. The acids (**1g**) and (**1h**) showed weak and broad antifungal activity. The minimum inhibitory concentrations against almost all yeasts and filamentous fungi tested were 20—200 μg ml^{-1} (data not shown). Bacteria were insensitive to both **1g** and **1h**. The other acids (**1a**—**f**) were little antifungal. However, they inhibited in some degree, the growth of *Hansenula anomala* as shown in Fig. 2. In general, the inhibitory activity increased as the number of carbon atoms in the acids increased. Interestingly, **1d** was exceptionally active.

The structural significance of 8-nonynoic acid and its ester derivatives on the biological activity in microorganisms and plants remains to be elucidated.

$HC{\equiv}C{-}(CH_2{-})_n{-}CO_2R$

	n	R		*n*	R		*n*	R		*n*	R
1a:	3	H	**2a**:	3	Me	**3a**:	3	Et	**4a**:	3	*i*-Pr
1b:	4	H	**2b**:	4	Me	**3b**:	4	Et	**4b**:	4	*i*-Pr
1c:	5	H	**2c**:	5	Me	**3c**:	5	Et	**4c**:	5	*i*-Pr
1d:	6	H	**2d**:	6	Me	**3d**:	6	Et	**4d**:	6	*i*-Pr
1e:	7	H	**2e**:	7	Me	**3e**:	7	Et	**4e**:	7	*i*-Pr
1f:	8	H	**2f**:	8	Me	**3f**:	8	Et	**4f**:	8	*i*-Pr
1g:	9	H	**2g**:	9	Me	**3g**:	9	Et	**4g**:	9	*i*-Pr
1h:	10	H	**2h**:	10	Me	**3h**:	10	Et	**4h**:	10	*i*-Pr

Experimental

Mps are uncorrected. UV spectra were measured in methanol (MeOH) with a Shimadzu UV-210A spectrometer and IR spectra recorded on a Shimadzu IR-408 spectrometer. ^{1}H NMR spectra in chloroform-*d* with TMS as internal standard (δ=0) were measured at 60 (JEOL MH-60), 100 (JEOL MH-100), and 360 MHz (by Dr. Iwashita at Suntory Institute for Bioorganic Research). EI-Mass spectra were measured with a JEOL JMS-D300 apparatus and FD-mass spectra by Dr. Naoki at Suntory Institute for Bioorganic Research.

Bromo Esters from Terminal Diols. Commercially available terminal diols of 1,6-hexanediol to 1,10-decanediol and 1,12-dodecanediol, and 1,13-tridecanediol obtained from 1,13-tridecanedioic acid by methylation with diazomethane (CH_2N_2) and lithium aluminium hydride (LAH) reduction, were converted to the corresponding bromo esters by the following general procedure. A terminal diol (0.03 mol) was treated in a soln of 48% hydrobromic acid (HBr) (8.7 g) and concd sulfuric acid (H_2SO_4) (1.5 ml) for 3 h under reflux. A crude product extracted with ether was oxidized with potassium permanganate ($KMnO_4$) (2.5 g) in 20% H_2SO_4 (28 ml) at 15—20 °C. After methylation with CH_2N_2, the crude product was chromatographed over silica gel to give the bromo ester (**5**).

Bromo Ester 5. **5a** (oil): Yield 33%; IR (neat) 1740 cm^{-1}; ^{1}H NMR δ=2.36 (2H, t, J=7 Hz), 3.42 (2H, t, J=6.5 Hz), 3.70 (3H, s). **5b** (oil): Yield 33%. **5c** (oil): Yield 35%. **5d** (oil): Yield 36%. **5e** (oil): Yield 37%. **5g** (oil): Yield 37%. **5h** (oil): Yield 39%.

Methyl 9-Hydroxynonanoate (6d) and Methyl 10-Hydroxydecanoate (6e). The bromo esters (**5d**) and (**5e**) were hydrolyzed with 20% aq sodium hydroxide (NaOH) at 50 °C and the products were methylated with CH_2N_2 to give **6d** and **6e** in 93 and 91% yields, respectively. **6d**, mp 56.5—57.5 °C: IR (neat) 3400, 1740 cm^{-1}; ^{1}H NMR δ=2.27 (2H, t, J=7 Hz), 3.33 (2H, t, J=6 Hz), 3.69 (3H, s). **6e**, mp 59.5—60.5 °C: IR (neat) 3350, 1740 cm^{-1}; ^{1}H NMR δ=2.31 (2H, t, J=7 Hz), 3.32 (2H, t, J=6 Hz), 3.67 (3H, s).

Preparation of Selenides 7. 1) **From Bromide.** To a cooled (0 °C) suspension of *o*-nitrophenyl selenocyanate (1.43 g, 6.3 mmol) in abs ethanol (EtOH) (20 ml) under nitrogen (N_2), sodium borohydride ($NaBH_4$) (0.28 g, 7.4 mmol) was added with stirring. A bromo ester (**5**) (5.7 mmol) was added and the soln stirred at room temp for 13 h. After removal of EtOH, the crude product was chromatographed over silica gel to give **7**, a yellow crystalline compound. ii) **From Alcohol.** A soln of **6** (2.4 mmol) in tetrahydrofuran (THF) (8 ml) containing *o*-nitrophenyl selenocyanate (2.9 mmol) under N_2 was treated dropwise with tributylphosphine (2.9 mmol) at room temp. After being stirred for 30 min, the solvent was removed in vacuo. The crude product was purified by chromatography.

***o*-Nitrophenyl Selenides 7.** **7a**, mp 25.0—26.0 °C: IR (Nujol) 1730, 1590, 1560 cm^{-1}; ^{1}H NMR δ=2.35 (2H, t, J=7 Hz), 2.95 (2H, t, J=7.5 Hz), 3.70 (3H, s), 7.37—7.73 (3H, m), 8.47 (1H, d, J=8 Hz). Table 1.

Olefins 8 from Selenides 7. Into a cooled (0 °C) soln of a selenide **7** (3 mmol) in THF (30 ml), 30% aq hydrogen peroxide (H_2O_2) (8 ml) was added dropwise. The ice bath was removed and the soln was stirred for additional 24 h. The reaction mixture was diluted with water (H_2O) and extracted with hexane to give **8**, a very smelly oil.

Terminal Olefins 8. **8a**: Yield 68%; EIMS m/z 128 (M$^+$); IR (neat) 3070, 1740, 1640 cm^{-1}; ^{1}H NMR δ=2.35 (2H, t, J=7 Hz), 3.66 (3H, s), 4.78 (1H, br d, J=8 Hz), 4.88 (1H, br d, J=15 Hz), 5.41—6.11 (1H, m). **8b**: Yield 72%; EIMS m/z 142 (M$^+$). **8c**: Yield 80%; EIMS m/z 156 (M$^+$). **8d**: Yield 85%: EIMS m/z 170 (M$^+$). **8e**: Yield 84%; EIMS m/z 184 (M$^+$). **8g**: Yield 82%; EIMS m/z 198 (M$^+$). **8h**: Yield 80%; EIMS m/z 212 (M$^+$).

Acetylenic Esters 2 from Olefins 8. To a soln of olefin **8** (2 mmol) in chloroform ($CHCl_3$) (10 ml), excess bromine (Br_2) was added and the soln was stirred for 1 h at room temp. After being treated in the usual way, the product was made to react with potassium hydroxide (KOH) (1.7 g) in abs EtOH under reflux for 3 h. After being acidified, the product was extracted with hexane and methylated with CH_2N_2 to give **2**, a smelly oil, which was purified by chromatography over silica gel.

Acetylenic Esters 2. **2a**: Yield 68%; EIMS m/z 126 (M$^+$), 94, 81, 74, 68; IR (neat) 3300, 2100, 1730 cm^{-1}; UV 206 nm (ε 150); ^{1}H NMR δ=1.98 (1H, t, J=2.8 Hz, HC≡C-), 2.17 (2H, dt, J=2.8 and 7.0 Hz, ≡C-CH$_2$-), 2.28 (2H, t, J=7.0 Hz, -CH$_2$CO-), 3.73 (3H, s, -CO$_2$CH$_3$). **2b**: Yield 65%; EIMS m/z 140 (M$^+$), 108, 81, 74, 68. **2c**: Yield 70%; EIMS m/z 156 (M$^+$), 94, 81, 74, 68. **2d**: Yield 75%; EIMS m/z 168 (M$^+$), 137, 94, 81, 74, 68, 54. **2e**: Yield 78%; EIMS m/z 182 (M$^+$), 151, 108, 87, 74, 68, 54. **2f**: Yield 75%; EIMS m/z 196 (M$^+$), 165, 108, 87, 81, 74, 68. **2g**: Yield 73%; EIMS m/z 210 (M$^+$), 179, 108, 87, 81, 74, 68. **2h**: Yield 74%; EIMS m/z 224 (M$^+$), 193, 108, 87, 81, 74, 68.

Acetylenic Acids 1. Methyl ester **2** was gently refluxed in a small amount of 30% aq KOH for 30 min and the soln acidified with dil hydrochloric acid (HCl) to give **1**. **1a** (oil)[13]: UV 206 nm (ε 430); IR (neat) 3300, 3200—2500, 2100, 1705 cm^{-1}. **1b** (oil)[13]: UV 206 nm (ε 450). **1c** (oil): UV 206 nm (ε 450). **1d**, mp 22—23 °C: UV 206 nm (ε 490). **1e**,[14] mp 24.5—25.5 °C: UV 206 nm (ε 510). **1f**,[15] mp 29.5—30.0 °C: UV 206 nm (ε 540). **1g**, mp 33—34 °C: UV 206 nm (ε 780). **1h**, mp 40—41 °C: UV 206 nm (ε 860).

Ethyl and Isopropyl Esters 3 and 4. Ethyl and isopropyl esters **3** and **4** were obtained from the acids (**1**) by treatment with concd H_2SO_4 in abs EtOH and *i*-PrOH under reflux, respectively.

Ethyl Esters 3. **3a** (oil)[13]: Yield 70%; EIMS m/z 140 (M$^+$), 94, 88, 81, 68. UV 206 nm (ε 170); IR ($CHCl_3$) 3300, 2100, 1735 cm^{-1}; ^{1}H NMR δ=1.26 (3H, t, J=7.5 Hz, -CO$_2$CH$_2$CH$_3$), 1.94 (1H, t, J=3 Hz, HC≡C-), 2.24 (2H, dt, J=3 and 7 Hz, ≡C-CH$_2$-), 2.38 (2H, t, J=7 Hz, -CH$_2$-CO-), 4.20 (2H, q, J=7.5 Hz, -CO$_2$CH$_2$CH$_3$). **3b** (oil)[13]: Yield 70%; EIMS m/z 154 (M$^+$), 108, 88, 81, 68; UV 206 nm (ε 170). **3c** (oil): Yield 75%; EIMS m/z 168 (M$^+$), 108, 94, 88, 68; UV 206 nm (ε 204). **3d** (oil): Yield 74%; EIMS m/z 182 (M$^+$), 167, 108, 81, 68; UV 206 nm (ε 270). **3e** (oil)[14]: Yield 74%; EIMS m/z 196 (M$^+$), 108, 88, 81, 68; UV 206 nm (ε 470). **3f** (oil): Yield 72%; EIMS m/z 210 (M$^+$), 94, 88, 81, 68; UV 206 nm (ε 470). **3g** (oil): Yield 75%; EIMS m/z 224 (M$^+$), 94, 88, 81, 68; UV 206 nm (ε 830). **3h** (oil): Yield 72%; EIMS m/z 238 (M$^+$), 108, 88, 81, 68; UV 206 nm (ε 950).

Isopropyl Esters 4. **4a** (oil): Yield 68%; EIMS m/z 154 (M$^+$), 112, 94, 81, 68; UV 205 nm (ε 150); IR ($CHCl_3$) 3300, 2100, 1735 cm^{-1}; ^{1}H NMR δ=1.21 (6H, d, J=6 Hz, -CO$_2$CH-(CH$_3$)$_2$), 1.97 (1H, t, J=3 Hz, HC≡C-), 2.27 (2H, dt, J=3 and 7 Hz, ≡C-CH$_2$-), 2.39 (2H, t, J=7 Hz, -CH$_2$CO-), 4.09 (1H,

sept, J=6 Hz, $-CO_2C\underline{H}Me_2$). **4b** (oil): Yield 69%; EIMS m/z 168 (M^+), 108, 94, 81, 68; UV 205 nm (ε 170). **4c** (oil): Yield 70%; EIMS m/z 182 (M^+), 94, 81, 80, 68; UV 205 nm (ε 190). **4d** (oil): Yield 70%; EIMS m/z 196 (M^+), 102, 94, 81, 68; UV 205 nm (ε 190). **4e** (oil): Yield 72%; EIMS m/z 210 (M^+), 108, 102, 94, 81; UV 205 nm (ε 830). **4f** (oil): Yield 72%; EIMS m/z 224 (M^+), 122, 102, 81, 68; UV 205 nm (ε 1400). **4g** (oil): Yield 71%; EIMS m/z 238 (M^+), 136, 102, 94, 81; UV 205 nm (ε 1700). **4h** (oil): Yield 70%; EIMS m/z 252 (M^+), 150, 102, 81, 68; UV 205 nm (ε 1700).

Seed Germination Test. Twenty seeds of lettuce (*Lactuca sativa*, L., cv. Grand Rapids) were placed on 2 layers of filter paper in a 9-cm Petri dish containing 4 ml of test solution. The dish was incubated at 23 °C in the dark room. After 3 d, the percentage germination was determined. The test was repeated three times and the mean values calculated.

Seedling Growth Test. Lettuce seeds were germinated on filter paper moistened with distilled water for 2 d. Twenty seedlings were selected for size uniformity and planted on 2 layers of filter paper in a 9-cm Petri dish containing 4 ml of test solution. Seedlings were grown for another 3 d at 23 °C in the dark, and then hypocotyl length was measured.

Antimicrobial Test. Test organisms: fungus; *Mucor mucedo* IFO 0136, *Rh. chinensis* IFO 4745, *Asp. niger* ATCC 6275 and *P. crustosum* Thom: Yiest; *S. cerevisiae* IFO 0203, *C. utilis* ATCC 42402, *Schiz. pombe* IFO 0342 and *H. anomala* IFO 0136: bacteria; *S. aureus* NCTC 8530, *B. subtilis* K-49, *E. coli* IFO 3545 and *Ps. aeruginosa* IAM 1007. Minimum inhibitory concentration of test compound was determined by a two-fold dilution method reported previously.[16] Effect on yeast growth was evaluated turbidimetrically as follows. *Hansenula anomala* IFO 0136 was cultured in 5 ml malt wort containing a test compound at 100 μg/ml in a test tube at 25 °C. The growth was periodically examined as the optical density at 660 nm.

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