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Potent Inhibition of Macrophomate Synthase by Reaction Intermediate Analogs

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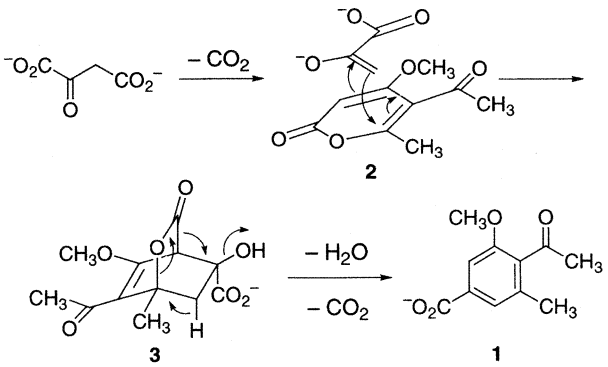
Potent inhibitors for macrophomate synthase, which has recently been found to catalyze a highly unusual five-step chemical transformation, were explored. Among 11 oxalacetate analogs tested, only three analogs had moderate to relatively strong inhibitory activities (I_{50} 1.3–8.1 mM). On the other hand, among 35 bicyclic intermediate analogs synthesized, two diacids were found to be the most potent inhibitors (I_{50} 0.80, 0.84 mM) which had a much higher affinity than that of the natural substrate 2-pyrone. (−)-Enantiomers of the diacids showed 30 times stronger activity (I_{50} 0.34, 0.41 mM) than (+)-ones. The I_{50}/K_m values (0.20, 0.24) showed their potent inhibitions. Competitive inhibitions were observed in two representative inhibitors.

Key words: macrophomate synthase; inhibitors; 2-pyrone; oxalacetate

Macrophomate synthase[1,2] catalyzes an unusual conversion[3,4] from oxalacetate and 2-pyrone **2** to benzoate (macrophomic acid **1**[5]) which is commonly biosynthesized *via* either a shikimate or polyketide pathway (Scheme 1). Recently, we have purified the enzyme from the pathogenic fungus *Macrophoma commelinae* IFO 9570.[1,2] Subsequently, the gene encoding macrophomate synthase from *M. commelinae* has been cloned and overexpressed in *Escherichia coli*.[2] Sequence alignments show that it has no homology to known enzymes reported to date. Macrophomate synthase is a homodimer of a 36-kDa protein[2] and tightly associated with a Mg(II) per monomer.[6] This relatively small enzyme catalyzes an extraordinary five-step transformation involving two decarboxylations, two C–C bond formations, and dehydration. To our knowledge, there is no precedent of an enzyme catalyzing different types of multistep chemical reactions, except dehydroquinate synthase[7] and the mechanistically related deoxy-*scyllo*-inosose synthase.[8,9] In addition, the enzyme has an unique substrate specificity, as it converts various 2-pyrones to benzoates.[10]

The detailed mechanism of the macrophomate synthase catalyzed reaction has been extensively investigated with the enzyme overexpressed.[6] In the absence of 2-pyrone **2**, decarboxylation of oxalacetate was predominant.[6] Incubation of a 2-pyrone analog resulted in the formation of a chiral bicyclic intermediate analog.[6,11] From the kinetic studies and the experiments with ^{2}H-labeled oxalacetate, the reaction mechanism of this complicated transformation was proposed to be the following three chemical steps; 1) decarboxylation of oxalacetate; 2) two C–C bond formations to afford the bicyclic intermediate **3**; 3) decomposition of **3** *via* dehydration-decarboxylation (Scheme 1).[6] Involvement of a Diels-Alder reaction in the second step has been proposed.

The finding that a bicyclic intermediate mimic moderately inhibited the conversion catalyzed by macrophomate synthase[1,12] prompted us to search for more potent inhibitors. In this paper, we report



Scheme 1. The Proposed Mechanism for Macrophomate Synthase Reaction.

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the synthesis of various intermediate analogs, their inhibitory activities and then discussed structural requirements for the inhibition of macrophomate synthase.

Materials and Methods

General experimental details were recently reported.[2,6,10] Macrophomate synthase used in this study was purified from the overproducing strain *Escherichia coli* BL21 harboring the gene which encodes macrophomate synthase from *Macrophoma commelinae* as described earlier.[2]

Assay of macrophomate synthase inhibition. Final concentrations of bicyclic compounds in DMF solution were adjusted to 1.7 or 5.1 mM which corresponded to K_m or $3K_m$ for 2-pyrone **2**, respectively. In the case of oxalacetate analogs, their final concentrations were adjusted to 1.2 or 3.6 mM which corresponded to K_m or $3K_m$ for oxalacetate, respectively. To a solution of macrophomate synthase[2] (85 μl, 0.4 μg, 48.3 U) in 50 mM PIPES (pH 7.0) were added 2-pyrone **2** (5 μl, final concentration 1.7 or 5.1 mM) and bicyclic inhibitors (5 μl). The mixture was preincubated at 30°C for 5 min. Then oxalacetate (5 μl, final concentration 5 mM) was added and the resultant mixture was incubated at 30°C for 10 min. In the cases of oxalacetate analogs, final concentration of 2-pyrone was adjusted to 5 mM and that of oxalacetate was to 1.2 or 3.6 mM. The enzymatic reactions were terminated by adding 2-propanol (100 μl) and an aliquot was directly analyzed by HPLC under the conditions reported.[10] I_{50} is the inhibitor concentration that gives 50% inhibition at substrate concentration equal to K_m or $3K_m$.[13] In the cases obtaining K_i values for **6** and **20b**, concentrations of 2-pyrone were 1.19, 1.56, 2.27, 4.17, and 25.0 mM.

(1S, 4R*, 5R*)-2-Oxo-4, 7, 7-trimethylbicyclo [2,2,2]octane-5-carboxylic acid (5).* To a solution of ester **6** (12 mg, 0.055 mmol) in H_2O-EtOH (1:1, 1 ml) was added KOH (3.1 mg, 0.055 mmol). The resultant mixture was stirred at 40°C for 2 h and concentrated *in vacuo*. To the residue were added water and 1 M HCl and the mixture was extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give **5** (10.8 mg, 93%) as colorless crystallines, mp 146–150°C. IR ν_{max} (neat) cm^{-1}: 2958, 1698, 1460, 1242, 1023, 935. ^{1}H-NMR (270 MHz, CD_3OD) δ: 0.94 (3H, s), 1.03 (3H, s), 1.09 (3H, s), 1.31 (1H, d, J=13 Hz), 1.39 (1H, dd, J=13, 3.3 Hz), 1.81 (1H, dd, J=19 Hz), 1.97 (2H, m), 2.30 (1H, dt, J=11, 3.9 Hz), 2.53 (1H, m), 2.67 (1H, dd, J=19, 3.3 Hz). MS (EI) m/z: 210 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{12}H_{18}O_3$, 210.1256; found 210.1260.

(1S, 2R*, 4S*)-2-Chloro-2-cyano-1-methylbicyclo [2.2.2]octan-5-one (8).* To a solution of 1.5 M LDA (0.733 ml, 1.10 mmol) in THF (7 ml) was added dropwise 3-methylcyclohexanone (0.13 ml, 1.00 mmol) in HMPA (0.1 ml) at −78°C. The mixture was stirred for 30 min and then warmed to 0°C. After addition of chlorotrimethylsilane (0.25 ml, 2.00 mmol), the mixture was stirred at 25°C for 18 h. The reaction mixture was diluted with pentane and then filtered through Celite. The filtrate was distilled under reduced pressure to collect an enol ether (1.3 g, 78%) at 84°C (15 mmHg). To a solution of α-chloroacrylonitlile (0.24 ml, 3.00 mmol) in toluene (10 ml) was added the enol ether (150 mg, 0.82 mmol) prepared above and the mixture was heated under reflux for 12 h. After cooling, the mixture was concentrated *in vacuo* and the resultant residue was chromatographed on a silica gel column (benzene-ethyl acetate, 8:2) to give **8** (78.6 mg, 49%) as colorless crystallines, mp 91°C. IR ν_{max} (neat) cm^{-1}: 2967, 2879, 2241, 1712, 753. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30 (3H, s), 1.86–2.85 (4H, m), 2.16 (1H, d, J=19 Hz), 2.42 (1H, m), 2.58 (1H, d, J=16 Hz), 2.90 (1H, dd, J=19, 3.0 Hz), 2.90 (1H, dd, J=16, 3.0 Hz). MS (EI) m/z: 197 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{10}H_{12}ClNO$, 197.0609; found 197.0612.

(1R, 2RS, 4S*)-2-Bromomethyl-bicyclo[2.2.1] hept-5-ene-2-carboxylic acid (9).* To 2-(bromomethyl)acrylic acid (500 mg, 3.03 mmol) was added dropwise cyclopentadiene (0.38 ml, 4.55 mmol) at 0°C. After warming to ambient temperature, the reaction mixture was stirred for 1 h and then was directly chromatographed on a silica gel column (hexane-ethyl acetate, 6:4) to give **9** (340 mg, 50%) as a white solid, mp 57°C. IR ν_{max} (neat) cm^{-1}: 2984, 1707, 1313, 1252, 943, 723. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.12 (1H, dd, J=1.9, 13.2 Hz), 1.54 (1H, m), 1.62 (2H, s), 2.40 (1H, dd, J=3.9, 13.2 Hz), 2.95 (2H, m), 3.24 (1H, m), 6.26 (1H, m), 6.31 (1H, m). MS (EI) m/z: 230 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_9H_{11}O_2Br$, 229.9942; found, 229.9894.

Methyl 2-(diethylphosphonomethyl)acrylate (16). To a solution of $LiOH \cdot 2H_2O$ (254 mg, 6.06 mmol) in water (13 ml) was added dropwise 2-(bromomethyl)acrylic acid (1.01 g, 6.06 mmol) at 0°C with stirring. The resultant solution was lyophilized to give the lithium salt. To a solution of *t*-BuOK (786 mg, 10.0 mmol) and diethylphosphite (0.94 ml, 10.0 mmol) in DMF (8 ml) was added the lithium salt (400 mg, 2.00 mmol) prepared above at 0°C. The mixture was stirred for 10 min and then diluted with water. The mixture was extracted with ethyl acetate, and the extract was washed with brine and dried over Na_2SO_4. Concentration of the extract gave a residue which was chromatographed on a silica gel column

($CHCl_3$-MeOH, 9:1) to give acid (220 mg, 50%) as an oil.

To a suspension of K_2CO_3 (182 mg, 1.32 mmol) and the acid (200 mg, 1.20 mmol) prepared above in HMPA (0.8 ml) was added CH_3I (0.3 ml, 4.80 mmol). The resultant mixture was stirred for 2 h and then diluted with water. The mixture was extracted with ether and dried over Na_2SO_4. Concentration of the extract gave a residue which was chromatographed on a silica gel column ($CHCl_3$-MeOH, 9:1) to give **16** (109 mg, 38%) as an oil. IR ν_{max} (neat) cm^{-1}: 3448, 2986, 1725, 1634, 1440, 1370, 1246, 1202, 1053, 1026, 969, 836, 773. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.29 (6H, t, J=7.3 Hz), 2.95 (2H, d, J=12 Hz), 3.77 (3H, s), 4.10 (4H, m), 5.86 (1H, d, J=5.9 Hz), 6.35 (1H, d, J=5.9 Hz). MS (EI) m/z: 237 (MH^+). HRMS (EI) m/z (MH^+): calcd. for $C_9H_{18}O_5P$, 238.0901; found, 238.0906.

(1R, 4S*, 7RS) - 7 - Diethylphosphonomethyl - 1-hydroxy-2-oxo-3-oxabicyclo-[2.2.2]oct-5-ene-7-carboxylic acid methyl ester (12).* To a solution of 3-hydroxy-2-pyrone (5 mg, 0.05 mmol) and Et_3N (0.026 ml, 0.18 mmol) in $CHCl_3$ (1 ml) was added **16** (5 mg, 0.05 mmol). The reaction mixture was stirred for 12 h and then diluted with water. The organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 and the combined extracts were washed with brine, dried over Na_2SO_4. Concentration of the extract gave a residue which was chromatographed on a silica gel column (hexane-ethyl acetate, 1:1) to give **12** (17.5 mg, 51%) as an oil. IR ν_{max} (neat) cm^{-1}: 3250, 2985, 1736, 1654, 1438, 1229, 1024, 968. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.29 (6H, t, J=7.26 Hz), 2.09 (2H, d, J=12 Hz), 2.71 (1H, d, J=13.8 Hz), 3.15 (1H, dd, J=13.9, 1.3 Hz), 3.79 (3H, s), 4.05 (4H, m), 5.20 (1H, m), 6.25 (1H, dd, J=8.6, 1.3 Hz), 6.51 (1H, dd, J=8.6, 4.3 Hz). MS (EI) m/z: 303 (M^+). HRMS (EI) m/z (M^+-Et): calcd. for $C_{12}H_{16}O_7P$, 303.0940; found, 303.0967.

(1R, 2S*, 4S*) - 2 - Acetoxy - 1 - methoxybicyclo [2.2.2]oct-5-en-2-carboxylic acid methyl ester (13a) and (1R*, 2R*, 4S*)-2-acetoxy-1-methoxybicyclo [2.2.2]oct-5-en-2-carboxylic acid methyl ester (13b).* A mixture of 1-methoxy-1,4-cyclohexadiene (0.53 ml, 4.54 mmol) and methyl 2-acetoxyacrylate (0.8 ml, 4.45 mmol) was heated at 150°C for 15 h. After cooling, the mixture was directly chromatographed on a silica gel column ($CHCl_3$-ethyl acetate, 7:3) to give a diastereomeric mixture of **13a** and **13b** (300 mg, 29%, 1:1) which were separated by HPLC [Wakosil-5sil (10×250 mm. Wako), UV 240 nm, hexane-ethyl acetate (8:2), flow rate 2 ml/min]. **13a**: colorless crystallines, mp 39°C. IR ν_{max} (neat) cm^{-1}: 2949, 1739, 1435, 1258, 1200, 1108, 1032. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–1.80 (5H, m), 2.03 (3H, s), 2.61 (1H, m), 2.77 (1H, dd, J=15, 2.6 Hz), 3.49 (3H, s), 3.76 (3H, s), 6.37 (2H, m). MS (EI) m/z: 255 (MH^+). HRMS (EI) m/z (M^+): calcd. for $C_{13}H_{18}O_5$, 254.1154; found, 254.1131. **13b**: colorless crystallines, mp 37°C. IR ν_{max} (neat) cm^{-1}: 2949, 1739, 1435, 1258, 1200, 1108, 1032. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–1.80 (4H, m), 2.14 (3H, s), 2.32 (1H, m), 2.61 (1H, m), 2.63 (1H, m), 3.44 (3H, s), 3.69 (3H, s), 6.05 (1H, d, J=8.6 Hz), 6.34 (1H, dd, J=8.6, 7.6 Hz). MS (EI) m/z: 255 (MH^+). HRMS (EI) m/z (M^+): calcd. for $C_{13}H_{18}O_5$, 254.1154; found, 254.1131.

(1R, 2S*, 4S*) - 2 - Hydroxy - 1 - methoxybicyclo [2.2.2]oct-5-en-2-carboxylic acid (14a).* To a solution of ester **13a** (14 mg, 0.055 mmol) in EtOH-H_2O (0.4 ml, 1:1) was added NaOH (30 mg, 0.75 mmol). The mixture was stirred at 40°C for 15 min and then acidified with 1 M HCl to pH 2. The resultant mixture was extracted with ethyl acetate and the extract was dried over Na_2SO_4. Removal of solvent afforded **14a** (10.3 mg, 95%) as colorless needles, mp 42°C. IR ν_{max} (neat) cm^{-1}: 3453, 2944, 1722, 1193, 1112, 1011. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–2.20 (5H, m), 2.41 (1H, br.d, J=13 Hz), 2.69 (1H, m), 3.41 (3H, s), 6.39 (1H, d, J=8.6 Hz), 6.43 (1H, dd, J=9.6, 8.6 Hz). MS (EI) m/z: 200 (MH_2^+). HRMS (EI) m/z (MH_2^+): calcd. for $C_{10}H_{16}O_4$, 200.1049; found, 200.1046.

(1R, 2R*, 4S*) - 2 - Hydroxy - 1 - methoxybicyclo [2.2.2]oct-5-en-2-carboxylic acid (14b).* The title compound **14b** was synthesized from ester **13b** as described above. Colorless needles, mp 53°C. IR ν_{max} (neat) cm^{-1}: 3456, 2941, 1720, 1190, 1116, 1008. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–2.20 (6H, m), 2.66 (1H, m), 3.40 (3H, s), 6.32 (2H, m). MS (EI) m/z: 200 (MH_2^+). HRMS (EI) m/z (MH_2^+): calcd. for $C_{10}H_{16}O_4$, 200.1049; found, 200.1065.

Anhydrides (15a) and (15b). A mixture of 1-methoxy-1,4-cyclohexadiene (3 ml, 25.59 mmol) and itaconic anhydride (2.9 g, 25.59 mmol) was warmed at 60°C for 2 h. After cooling, the reaction mixture was directly chromatographed on a silica gel column (hexane-ethyl acetate, 4:1) to afford **15a** (2.52 g, 44 %) and **15b** (1.10 g, 19%). **15a**: colorless crystallines, mp 79°C. IR ν_{max} (neat) cm^{-1}: 3065, 2954, 2844, 1846, 1776, 1470, 1406, 1373, 1339, 1235, 1111, 1049, 978, 907, 826, 751, 716, 686, 630, 493. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.51 (3H, m), 1.91 (2H, m), 2.34 (1H, d, J=18 Hz), 2.41 (1H, dd, J=14, 2.0 Hz), 2.68 (1H, m), 3.10 (1H, d, J=18 Hz), 3.33 (3H, s), 6.39 (2H, m). MS (EI) m/z: 222 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{12}H_{14}O_4$, 222.0892; found, 222.0895. **15b**: colorless crystallines, mp 58–60°C. IR ν_{max} (neat) cm^{-1}: 2949, 1781, 1459, 1411, 1373, 1244, 1116, 1048, 1021, 979, 934, 915, 894, 827, 753, 703, 684, 652, 624, 587, 560, 538, 494, 412. ^{1}H-NMR (270

MHz, $CDCl_3$) δ: 1.45–1.70 (5H, m), 2.14 (1H, dt, J = 13, 2.5 Hz), 2.70 (1H, m), 2.72 (1H, d, J = 18 Hz), 3.34 (3H, s), 3.35 (1H, d, J = 18 Hz), 6.40 (2H, d, J = 4.0 Hz). MS (EI) m/z: 222 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{12}H_{14}O_4$, 222.0892; found, 222.0881.

Iodolactone (17). To a suspension of **14b** (10 mg, 0.05 mmol) and $NaHCO_3$ (7.56 mg, 0.09 mmol) in water (0.1 ml) and $CHCl_3$ (0.2 ml) was added iodine (12.7 mg, 0.05 mmol) at 0°C. After vigorous stirring for 5 h, the reaction mixture was diluted with 1 M NaOH (0.1 ml) and extracted with CH_2Cl_2. The extract was washed with 10% $Na_2S_2O_3$, brine, successively and dried over Na_2SO_4. Concentration of the extract gave a residue which was chromatographed by preparative TLC ($CHCl_3$-ethyl acetate, 7:3) to give **17** (12 mg, 90%) as colorless needles, mp 139–141°C. IR ν_{max} (neat) cm^{-1}: 3738, 1795, 1719, 1686, 1655, 1177, 1120, 977, 691. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.82–2.40 (6H, m), 2.81 (1H, s), 3.29 (3H, s), 4.42 (1H, m), 4.96 (1H, s). MS (EI) m/z: 325 (MH^+). HRMS (EI) m/z (MH^+): calcd. for $C_{10}H_{14}IO_4$, 324.9937; found, 324.9942.

(1S, 2S*, 4S*)-2-Carboxymethyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (19a) and (1S*, 2R*, 4S*)-2-carboxymethyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (19b).* A mixture of cyclopentadiene (2 ml, 24.24 mmol) and itaconic anhydride (1.5 g, 13.38 mmol) in EtOH (20 ml) was stirred at room temperature for 1.5 h. After concentration *in vacuo*, the residue was chromatographed on silica gel column (hexane-ethyl acetate, 4:1) to afford a mixture of anhydride **18a** and **18b** (647 mg, 27%, 1:1). A portion (10 mg) was separated by preparative TLC (hexane-ethyl acetate, 4:1). **18a**: ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.26 (1H, dd, J = 3.0, 12.1 Hz), 1.48 (1H, m), 2.07 (1H, d, J = 8.9 Hz), 2.45 (1H, dd, J = 3.6, 12.1 Hz), 2.59 (1H, d, J = 19.1 Hz), 2.77 (1H, d, J = 19.1 Hz), 3.03 (1H, br.s), 3.06 (1H, br.s), 6.15 (1H, m), 6.39 (1H, m). **18b**: ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.43 (1H, d, J = 8.9 Hz), 1.68 (1H, br.d, J = 8.9 Hz), 1.82–1.95 (2H, m), 2.94 (1H, br.s), 2.95 (1H, d, J = 18.5 Hz), 3.10 (1H, br.s), 3.15 (1H, d, J = 18.5 Hz), 6.07 (1H, m), 6.40 (1H, m). To a solution of anhydrides **18a** and **18b** (226 mg, 1.269 mmol) in THF-H_2O (2:1, 6 ml) was added trifluoroacetic acid (0.5 ml). The reaction mixture was stirred at 60°C for 2 h and then concentrated *in vacuo* to give a residue which was chromatographed by silica gel column ($CHCl_3$-ethyl acetate, 3:1) to afford **19a** (121 mg, 50%) and **19b** (120 mg, 50%). **19a**: a colorless gum. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2976, 1705, 1652, 1251, 1209, 1103, 1079. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.01 (1H, dd, J = 3.3, 12.5 Hz), 1.42 (1H, d, J = 8.6 Hz), 1.74 (1H, d, J = 8.6 Hz), 2.38 (1H, d, J = 17.5 Hz), 2.55 (1H, dd, J = 3.3, 12.5 Hz), 2.87 (1H, d, J = 17.5 Hz), 2.90 (1H, br.s), 3.07 (1H, br.s), 6.08 (1H, dd, J = 3.3, 5.6 Hz), 6.28 (1H, dd, J = 3.3, 5.3 Hz). MS (FD) m/z: 197 (MH^+). HRMS (FD) m/z (M^+): calcd. for $C_{10}H_{12}O_4$, 196.0735; found, 196.0752. **19b**: a colorless gum. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2982, 1715, 1233, 1209, 1178, 1111. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.51 (2H, s), 1.52 (1H, dd, J = 3.0, 12.5 Hz), 1.99 (1H, d, J = 12.5 Hz), 2.64 (1H, d, J = 16.7 Hz), 2.88 (1H, br.s), 2.91 (1H, br.s), 3.13 (1H, d, J = 16.7 Hz), 6.00 (1H, dd, J = 3.0, 5.6 Hz), 6.24 (1H, dd, J = 3.0, 5.6 Hz). MS (FD) m/z: 196 (M^+). HRMS (FD) m/z (M^+): calcd. for $C_{10}H_{12}O_4$, 196.0735; found, 196.0732.

(1R,2S*,4S*)-2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (20a).* To a solution of anhydride (**15a**) (15 mg, 0.07 mmol) in THF-H_2O (1:1, 1 ml) trifluoroacetic acid (0.5 ml) was added. The reaction mixture was stirred at 45°C for 2 h and then concentrated *in vacuo* to give **20a** (14.7 mg, 88%) as a colorless gum. IR ν_{max} (neat) cm^{-1}: 3423, 2953, 1730, 1280, 1209, 1107, 701. ^{1}H-NMR (270 MHz, CD_3OD) δ: 1.26 (1H, dt, J = 13, 2.6 Hz), 1.40–1.90 (4H, m), 2.14 (1H, d, J = 17 Hz), 2.48 (1H, m), 2.58 (1H, dd, J = 13, 2.0 Hz), 3.24 (1H, d, J = 18 Hz), 3.30 (3H, s), 6.28 (2H, d, J = 3.3 Hz). MS (EI) m/z: 239 (M^+-H). HRMS (EI) m/z (M^+-H): calcd. for $C_{12}H_{15}O_5$, 239.0920; found, 239.0963.

(1R, 2R*, 4S*)-2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (20b).* The title compound **20b** was synthesized from anhydride **15b** as described above. **20a**: a colorless gum. IR ν_{max} (neat) cm^{-1}: 2957, 2362, 2346, 1702, 1655, 1648, 1637, 1560, 1542, 1508, 1459, 1189, 1110. ^{1}H-NMR (270 MHz, CD_3OD) δ: 1.54 (1H, dd, J = 13, 2.0 Hz), 1.70 (4H, m), 2.33 (1H, dt, J = 13, 3.2 Hz), 2.45 (1H, d, J = 16 Hz), 2.49 (1H, m), 3.20 (1H, d, J = 16 Hz), 3.31 (3H, s), 6.26 (2H, d, J = 2.6 Hz). MS (EI) m/z: 239 (M^+-H). HRMS (EI) m/z (M^+-H): calcd. for $C_{12}H_{15}O_5$, 239.0920; found, 239.0943.

2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]octane-2-carboxylic acid (21). To a solution of diacid **20a** (13.4 mg, 0.053 mmol) in MeOH (2 ml) was added 10 % palladium on carbon (3 mg). After being stirred for 3 h under a hydrogen atmosphere, the reaction mixture was filtered through a Celite pad. The filtrate was concentrated *in vacuo* to give a residue which was purified with preparative TLC ($CHCl_3$-MeOH, 9:1) to yield **21** (10.7 mg, 79%) as a white solid, mp 77–79°C. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2951, 1705, 1228, 1197, 1106. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.43–2.00 (10H, m), 2.52 (1H, d, J = 16.5 Hz), 2.70 (1H, d, J = 13.5 Hz), 3.19 (3H, s), 3.23 (1H, d, J = 16.5 Hz), 7.60 (2H, br.s). MS (FD) m/z: 242 (M^+). HRMS (FD) m/z (M^+): calcd. for $C_{12}H_{18}O_5$, 242.1154; found 242.1152.

Iodolactone (22). The title compound **22** was synthesized from ester **20a** as described in the synthesis of iodolactone **18** as colorless needles, mp 184–186°C. IR ν_{max} (neat) cm^{-1}: 2942, 1725, 1244, 1115, 1058, 1040, 940, 883, 770. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.63 (1H, d, J=15.0 Hz), 1.91–2.19 (4H, m), 2.41 (1H, m), 2.52 (1H, d, J=15.8 Hz), 2.76 (1H, dd, J=3.7, 15.0 Hz), 3.26 (3H, s), 3.31 (1H, d, J=15.8 Hz), 4.29 (1H, d, J=3.3 Hz), 5.02 (1H, s). MS (EI) m/z: 320 (M^+-COOH). HRMS (EI) m/z (M^+-COOH): calcd. for $C_{11}H_{14}O_3I$, 320.9988; found, 320.9964.

(1R, 2S*, 4S*) - 2 - Hexylcarbamoylmethyl - 1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (23a).* To a solution of **15a** (10.6 mg, 0.048 mmol) in THF (0.2 ml) was added hexylamine (0.03 ml, 0.23 mmol). After this was stirred for 10 min at ambient temperature, the reaction mixture was diluted with water, acidified with 2 M HCl, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give **23a** (13.7 mg, 88%) as a white solid, mp 140–142°C. IR ν_{max} (KBr) cm^{-1}: 3335, 2933, 1700, 1636, 1558, 1459. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.87 (3H, t, J=6.9 Hz), 1.22–1.55 (6H, m), 1.60–1.85 (5H, m), 2.21 (1H, d, J=14.5 Hz), 2.56 (1H, m), 2.62 (1H, dd, J=13.2, 2.6 Hz), 2.76 (1H, d, J=14.9 Hz), 3.18 (2H, m), 3.42 (3H, s), 5.74 (1H, br.s), 6.21 (1H, d, J=8.9 Hz), 6.35 (1H, dd, J=8.9, 6.3 Hz). MS (EI) m/z: 323 (M^+). HRMS (EI) m/z (MH^+): calcd. for $C_{18}H_{29}NO_4$, 323.2096; found, 323.2112.

(1R, 2R*, 4S*) - 2 - Hexylcarbamoylmethyl - 1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (23b).* The title compound **20b** was synthesized from anhydride **15b** as described above. **23b**: a pale yellow oil. IR ν_{max} (KBr) cm^{-1}: 3300, 2934, 1702, 1638, 1560, 1459. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.87 (3H, t, J=6.9 Hz), 1.23–1.55 (6H, m), 1.60–1.80 (5H, m), 2.44 (1H, dt, J=13.5, 4.0 Hz), 2.52 (1H, d, J=14.9 Hz), 2.59 (1H, m), 2.77 (1H, d, J=14.9 Hz), 3.18 (2H, m), 3.49 (3H, s), 5.05 (1H, quint., J=7.3 Hz), 5.90 (1H, br.s), 6.27 (1H, d, J=8.6 Hz), 6.37 (1H, dd, J=8.6, 6.6 Hz). MS (EI) m/z: 323 (M^+): HRMS (EI) m/z (MH^+): calcd. for $C_{18}H_{29}NO_4$: 323.2096; found: 323.2068.

(1R, 2S*, 4S*) - 2 - Methoxycarbonylmethyl - 1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (24).* To a solution of anhydride **15a** (209 mg, 0.924 mmol) in MeOH (1 ml) was added triethylamine (0.77 ml, 5.524 mmol). After being stirred at ambient temperature for 40 min, the mixture was concentrated *in vacuo*. The resultant residue was chromatographed on a silica gel column (hexane-ethyl acetate, 1:1) to give monoester **24** (237 mg, 99%) as colorless needles, mp 117–119°C. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2950, 1734, 1699, 1652, 1195, 1102, 1025. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–1.50 (2H, m), 1.60–1.90 (3H, m), 2.26 (1H, d, J=15.5 Hz), 2.57 (1H, m), 2.70 (1H, dd, J=13.5, 2.6 Hz), 3.11 (1H, d, J=15.5 Hz), 3.45 (3H, s), 3.62 (3H, s), 6.22 (1H, d, J=8.6 Hz), 6.36 (1H, dd, J=8.6, 6.3 Hz). MS (FD) m/z: 255 (MH^+). HRMS (FD) m/z (M^+): calcd. for $C_{13}H_{18}O_5$, 254.1154; found 254.1169.

(1R, 2S*, 4S*) - 2 - Methoxycarbonylmethyl -1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid methyl ester (25).* The monoester **24** (53.6 mg, 0.211 mmol) was treated with a large excess of ethereal diazomethane. Remaining diazomethane was quenched with acetic acid. The reaction mixture was concentrated *in vacuo* to give dieser **25** (56.5 mg, quantitative) as colorless needles, mp 86–88°C. IR ν_{max} (neat) cm^{-1}: 3046, 2950, 1732, 1207, 1169, 1104, 1063. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.13 (1H, dt, J=13.2, 3.0 Hz), 1.28 (1H, m), 1.40–1.51 (2H, m), 1.80 (1H, m), 2.11 (1H, d, J=17.5 Hz), 2.43 (1H, m), 2.51 (1H, dd, J=13.2, 2.6 Hz), 3.20 (3H, s), 3.28 (1H, d, J=17.5 Hz), 3.49 (3H, s), 3.58 (3H, s), 6.14–6.28 (2H, m). MS (EI) m/z: 268 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{14}H_{20}O_5$, 268.1310; found 268.1285.

(1R,2S*,4S*)-2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid methyl ester (26).* The solution of diester **25** (387 mg, 1.444 mmol) in THF (1 ml) and 1 M NaOH (1 ml) was stirred for 24 h. The mixture was diluted with water, acidified with 2 M HCl, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was chromatographed on a silica gel column (hexane-ethyl acetate, 1:1) to yield monoester **26** (368 mg, quantitative) as colorless needles, mp 128–129°C. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2950, 1732, 1707, 1202, 1209, 1119, 1104. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.28 (1H, dt, J=13.8, 3.6 Hz), 1.39 (1H, m), 1.50–1.70 (2H, m), 1.89 (1H, m), 2.28 (1H, d, J=17.8 Hz), 2.55 (1H, m), 2.58 (1H, dd, J=13.8, 2.6 Hz), 3.32 (3H, s), 3.41 (1H, d, J=17.8 Hz), 3.71 (3H, s), 6.24–6.36 (2H, m). MS (FD) m/z: 255 (MH^+). HRMS (FD) m/z (M^+): calcd. for $C_{13}H_{18}O_5$, 254.1154; found 254.1146.

Compound (27). To a solution of nitroethane (450 mg, 6.00 mmol) and phenyl isocyanate (717 mg, 6.03 mmol) in benzene (6 ml) was added dropwise triethylamine (0.25 ml, 1.79 mmol). The mixture was stirred at room temperature. After 30 min, diester **25** (540 mg, 2.01 mmol) in benzene (3 ml) was added and the resultant mixture was heated under reflux overnight. After filtration, the filtrate was poured into water. The resultant mixture was extracted with ethyl acetate, dried over anhydrous Na_2SO_4 and con-

centrated *in vacuo*. The resultant residue was chromatographed on a silica gel column (hexane-ethyl acetate, 2:1) to give starting material (188 mg, 35%), minor isomer (26 mg, 4%) and **27** (385 mg, 59%) as colorless needles, mp 121–122°C. IR ν_{max} (KBr) cm^{-1}: 2951, 1732, 1638, 1435, 1212, 1111, 1005. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.46 (2H, m), 1.52 (1H, d, J=14.3 Hz), 1.86 (2H, m), 1.89 (3H, s), 2.17 (1H, m), 3.31 (3H, s), 3.32 (1H, m), 3.47 (1H, d, J=17.2 Hz), 3.65 (3H, s), 3.69 (3H, s), 4.67 (1H, dd, J=11.3, 2.0 Hz). MS (EI) m/z: 326 (MH^+). HRMS (EI) m/z (M^+): calcd. for $C_{16}H_{23}NO_6$, 325.1525; found 325.1567.

Compound (28). To a solution of diester **27** (61.3 mg, 0.188 mmol) in THF (1.5 ml) was added 1 M NaOH (2 ml). After being stirred at ambient temperature for 2.5 h, the mixture was diluted with water, acidified with 2 M HCl, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was chromatographed on a silica gel column (hexane-ethyl acetate, 1:1) to afford monoester **28** (58.0 mg, quantitative) as colorless needles, mp 211–213°C. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2948, 1732, 1703, 1651, 1210, 1109. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.40–1.70 (3H, m), 1.80–2.00 (2H, m), 1.91 (3H, s), 2.20 (1H, m), 2.38 (1H, d, J=17.5 Hz), 2.86 (1H, dd, J=14.2, 3.3 Hz), 3.33 (3H, s), 3.34 (1H, m), 3.50 (1H, d, J=17.5 Hz), 3.71 (3H, s), 4.70 (1H, dd, J=11.5, 2.0 Hz). MS (FD) m/z: 312 (MH^+). HRMS (FD) m/z (M^+): calcd. for $C_{15}H_{21}NO_6$, 311.1369; found 311.1366.

Compound (29). To a solution of adduct **27** (96.1 mg, 0.296 mmol) were added aluminum tribromide (434 mg, 5.497 mmol) and tetrahydrothiophene (3 ml, 11.34 mmol) and the mixture was stirred at ambient temperature for 1.5 h. The reaction mixture was quenched with $CHCl_3$ and 1 M NaOH. The aqueous layer was acidified with 2 M HCl and extracted with $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a residue which was chromatographed on a silica gel column (hexane-ethyl acetate, 1:1) to give **29** (62.5 mg, 66%) as a colorless gum. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2951, 1733, 1705, 1651, 1210, 1209, 1110, 1079. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.30–1.80 (2H, m), 1.89 (3H, s), 2.17 (1H, m), 2.30 (1H, m), 2.39 (1H, d, J=15.3 Hz), 2.98 (1H, dd, J=14.2, 3.6 Hz), 3.17 (1H, d, J=15.3 Hz), 3.40 (1H, br.d, J=11.2 Hz), 3.49 (3H, s), 3.65 (3H, s), 4.75 (1H, dd, J=11.5, 2.3 Hz). MS (EI) m/z: 326 (MH^+). HRMS (EI) m/z (M^+): calcd. for $C_{20}H_{25}NO_4$, 325.1525; found 325.1567.

(1S, 2S*, 4S*, 5S*, 6S*)-5-Acetyl-6-hydroxy-1-methoxy-2-methoxycarbonylmethyl-bicyclo[2.2.2]octane-2-carboxylic acid methyl ester (30).* To the solution of adduct **27** (246 mg, 0.758 mmol) in MeOH-H_2O-AcOH (3 ml, 100:10:1) was added 5% rhodium on alumina (10 mg). After being stirred for 24 h under a hydrogen atmosphere, the reaction mixture was filtered through a Celite pad. The resultant filtrate was concentrated *in vacuo* to give a residue, which was chromatographed on a silica gel column (hexane-ethyl acetate, 2:1) to afford **30** (155 mg, 62%) as a colorless gum. IR ν_{max} (neat) cm^{-1}: 3503, 2951, 1733, 1700, 1652, 1212, 1112, 1092. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.29 (1H, br.d, J=14.5 Hz), 1.60–2.10 (5H, m), 2.20 (3H, s), 2.23 (1H, br.s), 2.58 (1H, ddd, J=14.5, 4.0, 2.0 Hz), 2.65 (1H, m), 2.67 (1H, d, J=17.3 Hz), 3.24 (3H, s), 3.30 (1H, d, J=17.3 Hz), 3.62 (3H, s), 3.70 (3H, s), 4.28 (1H, m). MS (FD) m/z: 328 (M^+). HRMS (FD) m/z (M^+): calcd. for $C_{15}H_{20}O_6$, 328.1522; found 328.1541.

(1R, 2S*, 4S*)-5-Acetyl-2-carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid methyl ester (31).* To a solution of diester **30** (12.0 mg, 0.037 mmol) in THF (0.3 ml) was added 1 M NaOH (0.4 ml). After being stirred at ambient temperature for 3 h, the mixture was diluted with water, acidified with 2 M HCl, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was purified with preparative TLC (hexane-ethyl acetate, 1:1) to afford **31** (7.3 mg, 67%) as a colorless gum. IR ν_{max} (neat) cm^{-1}: 3600–2200, 2952, 1731, 1703, 1668, 1271, 1208, 1118, 1038. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.16 (1H, m), 1.31 (1H, m), 1.57 (1H, dt, J=11.9, 4.9 Hz), 1.70 (1H, dt, J=12.5, 3.3 Hz), 1.94 (1H, m), 2.13 (1H, d, J=20.0 Hz), 2.30 (3H, s), 2.62 (1H, dd, J=13.5, 2.6 Hz), 3.21 (1H, m), 3.35 (3H, s), 3.46 (1H, d, J=20.0 Hz), 3.69 (3H, s), 7.11 (1H, br.s). MS (FD) m/z: 296 (M^+). HRMS (FD) m/z (M^+): calcd. for $C_{15}H_{20}O_6$, 296.1260; found 296.1260.

(1S, 4R, 5R, 1'R)-2-Oxo-4, 7, 7-trimethylbicyclo[2,2,2]octane-5-[N-(1'-phenylethyl)]-carboxamide (32a) and (1R, 4S, 5S, 1'R)-2-oxo-4,7,7-trimethylbicyclo[2,2,2]octane-5-[N-(1'-phenylethyl)]-carboxamide (32b). To a solution of carboxylic acid **5** (20 mg, 0.10 mmol), D-(+)-1-phenylethylamine (0.04 ml, 0.30 mmol), Et_3N (0.07 ml, 0.50 mmol), DMAP (12 mg, 0.10 mmol) in CH_2Cl_2 (1 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (23 mg, 0.12 mmol) at 0°C. The mixture was stirred at ambient temperature for 12 h and was diluted with water, then acidified with 1 M HCl, and extracted with CH_2Cl_2. After drying with Na_2SO_4 and concentration, the residue was chromatographed on a silica gel column (hexane-ethyl acetate, 7:3) to give amides **32a** (5 mg, 33%) and **32b** (5 mg, 33%). **32a**: colorless prisms, mp 190–191°C. IR ν_{max} (neat) cm^{-1}: 3304, 2958, 1719, 1636, 1542, 699. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.91 (3H, s),

1.00 (3H, s), 1.04 (3H, s), 1.27 (2H, s), 1.48 (3H, d, J = 6.6 Hz), 1.71 (1H, d, J = 19 Hz), 1.92 (1H, m), 2.09 (2H, m), 2.10 (1H, m), 3.00 (1H, dt, J = 19, 1.1 Hz), 5.10 (1H, quint., J = 6.6 Hz), 5.98 (1H, m), 7.26–7.38 (5H, m). MS (EI) m/z: 314 (MH^+). HRMS (EI) m/z (MH^+): calcd. for $C_{20}H_{28}NO_3$, 314.2042; found, 314.2111. **32b**: colorless prisms, mp 168–170°C. IR ν_{max} (neat) cm^{-1}: 3294, 2959, 1719, 1638, 1542, 1458, 702. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.81 (3H, s), 0.91 (3H, s), 1.07 (3H, s), 1.25 (2H, s), 1.49 (3H, d, J = 7.3 Hz), 1.67 (1H, d, J = 19 Hz), 1.95 (1H, m), 2.05 (2H, m), 2.06 (1H, m), 2.93 (1H, dd, J = 19, 1.3 Hz), 5.11 (1H, quint., J = 7.3 Hz), 5.68 (1H, m), 7.26–7.38 (5H, m). MS (EI) m/z: 314 (MH^+). HRMS (EI) m/z (MH^+): calcd. for $C_{20}H_{28}NO_3$, 314.2042; found, 314.2092.

(1S, 4R, 5R)-2-Oxo-4, 7, 7-trimethylbicyclo[2,2,2]octane-5-carboxylic acid methyl ester (+)-(6). To a solution of amide **32a** (200 mg, 0.55 mmol) was added 6 M HCl (3 ml). The mixture was heated under reflux for 12 h. After cooling, the mixture was extracted with ether. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give acid (+)-**5** which was treated with ethereal diazomethane and concentrated *in vacuo* to afford methyl ester (+)-**6** (102 mg, 74%) as colorless needles, mp 50°C; $[\alpha]_D^{23}$ +98.3° (*c* 1.00, $CHCl_3$).

(1R, 4S, 5S)-2-Oxo-4, 7, 7-trimethylbicyclo[2, 2, 2]octane-5-carboxylic acid methyl ester (−)-(6). The title compound (−)-**6** was synthesized from amide **32b** as described above. (−)-**6**: colorless needles, mp 48°C; $[\alpha]_D^{23}$ −113° (*c* 1.00, $CHCl_3$).

(1R, 2S, 4S, 1′R)-2-(1′-Phenylethyl)carbamoylmethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (33a-1) and (1S, 2R, 4R, 1′R)-2-(1′-phenylethyl)carbamoylmethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (33a-2). To a solution of **15a** (150.0 mg, 0.68 mmol) in THF (1.5 ml) was added D-(+)-phenethylamine (0.2 ml, 1.54 mmol). After being stirred for 40 min at ambient temperature, the mixture was diluted with water, acidified with 2 M HCl, and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was purified with preparative TLC (ethyl acetate, 100%) to give a mixture of **33a-1** and **33a-2** (234 mg, 98%). Portion (50 mg) of the mixture was further purified by HPLC (Inertsil ODS, 6×250 mm, MeOH-H_2O containing 0.2% AcOH, 60:40, flow rate 1 ml/min, UV 254 nm) to give amide **33a-1** (24.5 mg) and **33a-2** (21.8 mg). **33a-1**: a colorless gum. IR ν_{max} (neat) cm^{-1}: 3367, 2953, 1702, 1672, 1523, 1458, 700. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.46 (3H, d, J = 6.9 Hz), 1.60–1.85 (5H, m), 2.22 (1H, d, J = 14.5 Hz), 2.56 (1H, m), 2.65 (1H, dd, J = 13.2, 2.6 Hz), 2.74 (1H, d, J = 14.5 Hz), 3.42 (3H, s), 5.06 (1H, quint., J = 7.3 Hz), 5.89 (1H, br.d, J = 7.3 Hz), 6.17 (1H, d, J = 8.6 Hz), 6.35 (1H, dd, J = 8.9, 6.3 Hz), 7.20–7.40 (5H, m). MS (EI) m/z: 343 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{20}H_{25}NO_4$, 343.1784; found, 343. 1781. **33a-2**: a colorless gum. IR ν_{max} (neat) cm^{-1}: 3362, 1709, 1657, 1530, 703. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.40 (3H, d, J = 6.6 Hz), 1.50–1.80 (5H, m), 2.20 (1H, d, J = 15.0 Hz), 2.50 (1H, m), 2.58 (1H, br.d, J = 13.2 Hz), 2.73 (1H, d, J = 15.0 Hz), 3.28 (3H, s), 5.01 (1H, quint., J = 7.3 Hz), 5.95 (1H, br.d, J = 7.3 Hz), 6.11 (1H, d, J = 8.8 Hz), 6.27 (1H, dd, J = 8.8, 6.6 Hz), 7.18–7.38 (5H, m). MS (EI) m/z: 343 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{20}H_{25}NO_4$, 343.1784; found, 343.1792.

(1R, 2R, 4S, 1′R)-2-(1′-Phenylethyl)carbamoylmethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (33b-1) and (1S, 2S, 4R, 1′R)-2-(1′-phenylethyl)carbamoylmethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (33b-2). The title compounds **33b-1** and **33b-2** were synthesized from anhydride **15b** as described above. **33b-1**: a colorless gum; IR ν_{max} (neat) cm^{-1}: 3345, 2949, 1705, 1661, 1535, 1434, 708. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.44 (3H, d, J = 6.9 Hz), 1.60–1.80 (5H, m), 2.49 (1H, dt, J = 13.5, 4.0 Hz), 2.51 (1H, d, J = 14.4 Hz), 2.61 (1H, m), 2.73 (1H, d, J = 14.4 Hz), 3.45 (3H, s), 5.05 (1H, quint., J = 7.3 Hz), 6.06 (1H, br.d, J = 7.3 Hz), 6.27 (1H, d, J = 8.9 Hz), 6.38 (1H, dd, J = 8.9, 6.6 Hz), 7.20–7.40 (5H, m). MS (EI) m/z: 343 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{20}H_{25}NO_4$, 343.1784; found, 343.1807. **33b-2**: a colorless gum; IR ν_{max} (neat) cm^{-1}: 3357, 2953, 1709, 1668, 1540, 702. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.39 (3H, d, J = 6.9 Hz), 1.49 (1H, br.d, J = 13.7 Hz), 1.50–1.80 (4H, m), 2.39 (1H, dt, J = 13.7, 3.9 Hz), 2.51 (1H, d, J = 15.1 Hz), 2.52 (1H, m), 2.75 (1H, d, J = 15.1 Hz), 3.43 (3H, s), 5.00 (1H, quint., J = 7.3 Hz), 6.20 (1H, m), 6.21 (1H, d, J = 8.3 Hz), 6.30 (1H, dd, J = 8.6, 6.6 Hz), 7.18–7.38 (5H, m). MS (EI) m/z: 343 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{20}H_{25}NO_4$, 343.1784; found, 343.1786.

(1R, 2S, 4S)-2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (+)-(20a). To a solution of **33a-2** (21.8 mg, 0.064 mmol) in THF (0.15 ml) was added 4 M HCl (0.5 ml). After being stirred at ambient temperature for 2 days, the mixture was diluted with water and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to give a residue which was chromatographed on a silica gel column ($CHCl_3$-MeOH, 9:1) to give diacid (+)-**20a** (8.9 mg, 58%). $[\alpha]_D^{23}$ +7.7° (*c* 0.92, $CHCl_3$).

(1S, 2R, 4R)-2-Carboxymethyl-1-methoxy-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (−)-(20a). The

title compound (−)-**20a** was synthesized from amide **33a-1** as described above; $[\alpha]_D^{23} -6.8°$ (*c* 0.86, $CHCl_3$).

(1R,2R,4S)-2-Carboxymethyl-1-methoxy-bicyclo [2.2.2]oct-5-ene-2-carboxylic acid (+)-(20b). The title compound (+)-**20b** was synthesized from amide **33b-2** as described above; $[\alpha]_D^{23} +96°$ (*c* 0.16, $CHCl_3$).

(1S,2S,4R)-2-Carboxymethyl-1-methoxy-bicyclo [2.2.2]oct-5-ene-2-carboxylic acid (−)-(20b). The title compound (−)-**20b** was synthesized from amide **33b-1** as described above; $[\alpha]_D^{23} -97°$ (*c* 0.36, $CHCl_3$).

Results and Discussion

For the first screening of inhibitors, various types of bicyclic intermediate analogs in Fig. 1 were synthesized. Bicyclic compounds **4**, **6**,[14] **7**,[15] **10** and **11**[16] were prepared by literature procedures and others were synthesized by Diels-Alder reactions (Scheme 2). The relative configuration of **8** was tentatively assigned on the basis of selectivity found in similar substrate, and those of **9** and **12** were unassigned. Diastereomers **13a** and **13b** were converted to the corresponding α-hydroxy acids **14a** and **14b**, respectively. Since iodolactonization of **14b** under kinetically controlled conditions[17] yielded **17**, the relative configuration of **14b** was determined as shown in Fig. 1.

Next various analogs and derivatives of anhydrides **15a** and **15b** were prepared (Scheme 3) since these were found to be effective inhibitors as described below. Diels-Alder reaction of cyclopentadiene with itaconic anhydride gave a diastereomeric mixture of anhydrides **18a** and **18b** which were then converted to diacids **19a** and **19b**, respectively. The relative configuration of **18a** was determined on the basis of the NOE data (Scheme 3). Acidic hydrolysis of anhydrides **15a** and **15b** provided diacids **20a** and **20b**, respectively. As in the case of **14b**, the relative stereochemistry of diacid **20b** was elucidated by iodolactone formation and its NOE analysis (Scheme 3). In addition, hydrogenation of **20a** gave dihydro derivative **21**. Treatment of anhydrides **15a** and **15b** with hexylamine provided the corresponding amides **23a** and **23b**, respectively. Methanolysis of anhydrides **15a** in the presence of triethylamine gave the half ester **24**, which was further transformed into diester **25** by methylation with diazomethane. Diester **25** was selectively hydrolyzed with 2 M KOH to afford the alternative half ester **26**.

Introduction of additional functionality present in the bicyclic intermediate **3** by 1,3-dipolar addition was next conducted (Scheme 3). Treatment of diester **25** with phenylisocyanate in the presence of triethylamine provided **27** as a major product with a minor regioisomer (9:1). The structure of the adduct was determined by analysis of ^{1}H-NMR and NOE experi-

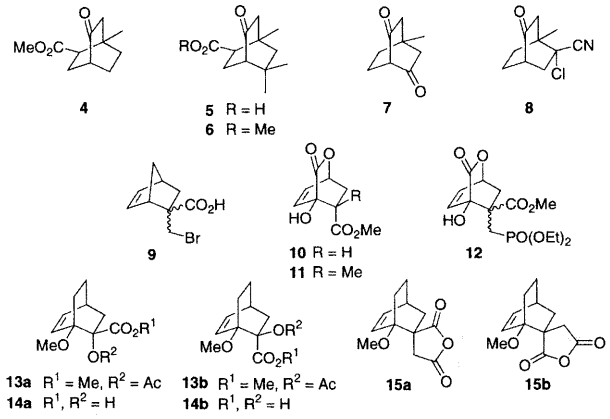
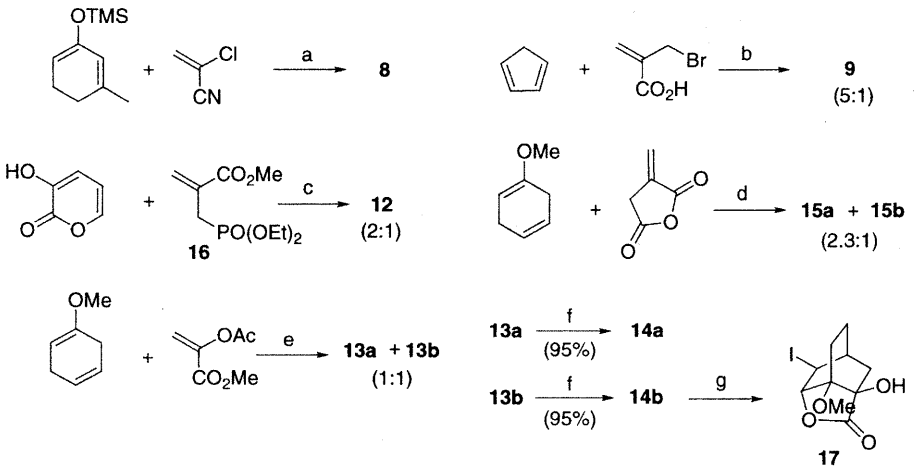
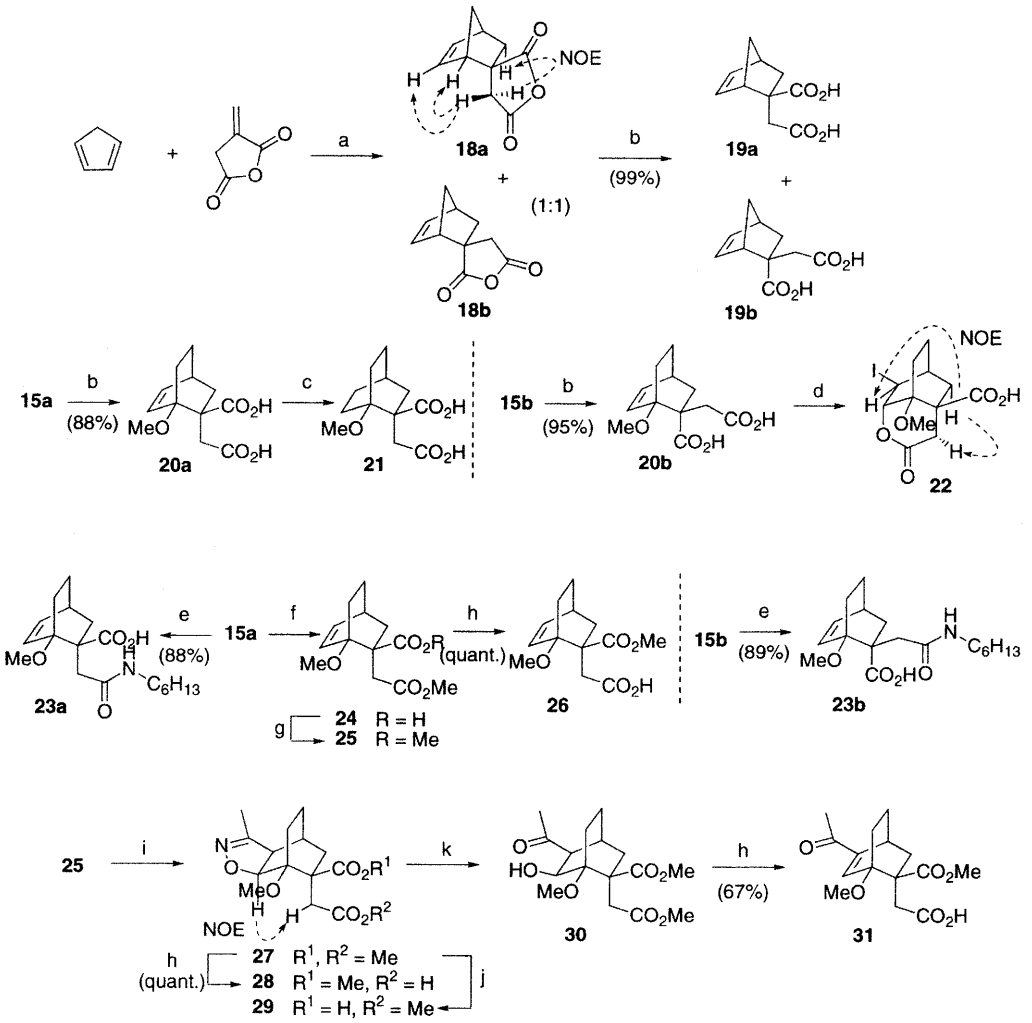


Fig. 1. Various Bicyclic Compounds Examined for Inhibitory Activity on Macrophomate Synthase.



Scheme 2. Synthesis of Bicyclic Inhibitors.

(a) toluene, 110°C (49%); (b) neat (50%); (c) Et_3N, $CHCl_3$ (51%); (d) neat, 60°C (63%); (e) neat, 150°C (29%); (f) 2 M NaOH, EtOH; (g) 1 M $NaHCO_3$, I_2, $CHCl_3$ (90%).



Scheme 3. Synthesis of Bicyclic Inhibitors.

(a) EtOH (27%); (b) TFA, THF-H_2O, heat; (c) H_2, Pd-C, MeOH (79%); (d) 1 M $NaHCO_3$, I_2, CH_2Cl_2 (90%); (e) $C_6H_{13}NH_2$, THF; (f) Et_3N, MeOH (99%); (g) CH_2N_2 (quant.); (h) 1 M NaOH, THF; (i) $EtNO_2$, PhNCO, Et_3N, C_6H_6, reflux (59%); (j) $AlBr_3$, THT (66%); (k) H_2, Rh-Al_2O_3, MeOH-H_2O-AcOH (62%).

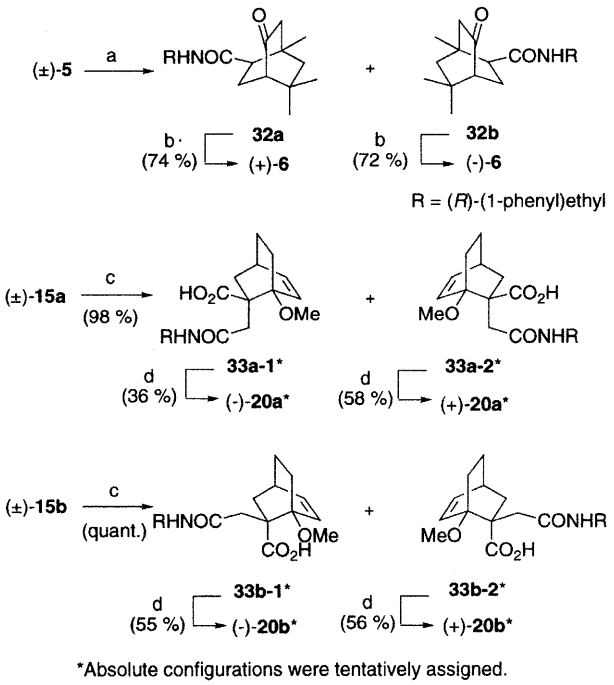
ments (Scheme 3). Alkaline hydrolysis and treatment with aluminum tribromide and tetrahydrothiophene of the adduct **27** gave half esters **28** and **29**, respectively. Reductive cleavage of the isoxazoline ring with Rh on alumina under a hydrogen atmosphere provided hydroxyketone **30**, which was further converted to half ester **31** by alkaline hydrolysis with concomitant dehydration.

In order to evaluate the inhibitory effect between enantiomers, optical resolutions of **6**, **20a**, and **20b** were employed. Condensation of acid **5** with (*R*)-(+)-phenethyl amine afforded diastereomeric mixture of amides **32a** and **32b** which were easily separated on a silica gel chromatography. The absolute configuration of amide **32a** was determined by X-ray crystallographic analysis* as shown in Scheme 4. In the cases of diacids **20a** and **20b**, similar amide formations of anhydrides **15a** and **15b** afforded **33a-1** and **33a-2**, **33b-1** and **33b-2**, respectively, which did not give suitable crystals for X-ray analysis. Attempts to apply Hoye's method[18] which was recently reported for the determination of absolute configuration of acid part of phenethylamide failed since the chemical shift differences of diastereomeric pairs *i.e.* **33a-1** and **33a-2** did not show clear positive and negative values, probably due to the presence of a carboxyl group adjacent to the amides. Structure was tentatively assigned to the one as shown in Scheme 4. Currently, suitable derivatization is being explored for determination of the absolute configuration with ^{1}H-NMR spectra.

In this paper, we described inhibition in terms of I_{50} values which were obtained from plots of 1/verocity *vs.* inhibitor concentration at oxalacetate concen-

* The X-ray crystallographic data (CCDC 144444) have been deposited to the Cambridge Crystallographic Data Center. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, CB2 1EZ, UK.

tration of K_m and $3K_m$ for oxalacetate analogs or at 2-pyrone concentration of those for bicyclic intermediate analogs. I_{50} values represent the concentration of inhibition for 50% inhibition under the condition investigated. At first, inhibitory activities of oxalacetate analogs were examined. Simple dicarboxylic acids such as malonate, succinate, maleate, fumarate, and itaconate showed no inhibition below 25 mM. In addition, keto acids such α- and β-ketoglutarates and acetoacetate did not inhibit macrophomate formation. The conformationally restricted diacid 2,2-dimethylmalonate was a weak inhibitor (I_{50} 8.1 mM) whereas relatively strong inhibitions ($I_{50}<2.6$ mM) were observed in the cases of 3-bromopyruvate and 3-hydroxypyruvate (Table 1). All of them revealed reversible inhibition since addition of a large excess of the substrates restored macrophomate synthase activity. Pyruvate, which can chelate to Mg(II) in the active site as oxalacetate, is shown to have weaker inhibition (K_i 104 mM) than those of the inhibitors shown above. Inhibitions of these analogs suggest that the C-3 heteroatom, which can form a hydrogen bond to a residue in the active site, significantly increases their affinity for the enzyme.



Scheme 4. Optical Resolutions of Bicyclic Inhibitors.
(a) (i) 0.1 M KOH/EtOH (93%); (ii) D-(+)-phenylethylamine, EDC, DMAP, Et_3N/CH_2Cl_2 (66%); (b) (i) 6 M HCl, reflux; (ii) CH_2N_2/ether; (c) D-(+)-phenylethylamine, THF; (d) 4 M HCl/THF. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

Next, the bicyclic intermediate analogs synthesized above were examined and I_{50} values obtained were summarized in Table 1. Compound **6** exhibited moderate inhibition (I_{50} 3.9 mM) but compounds **4**, **5**, **7**, and **8**, which possess essentially the same carbon framework showed no or negligible inhibitory activities. This indicates that appropriate location of functionality is important and *gem*-dimethyl groups in **6** are effective for inhibitory activity. Alternative carbon frameworks such as **9**–**12**, **14a**, and **14b** were also effective although the latter two compounds required α-hydroxy acid functionality. In the case of **14a** and **14b**, stereochemistry of the carbon attached to polar functionality did not show any difference (I_{50} 5.0, 4.5 mM). Among the compounds in Fig. 1, anhydrides **15a** and **15b** were found to be most effective (I_{50} 0.95, 1.1 mM). After this first screening, we decided to prepare several derivatives of relatively potent **15a** and

Table 1. Inhibition of Macrophomate Synthase Activity with Substrates or Intermediate Analogs

Compounds	I_{50} (mM)	I_{50}/K_m	Compounds	I_{50} (mM)	I_{50}/K_m
3-bromopyruvate	1.3	0.77	**19a**	4.0	2.4
3-hydroxypyruvate	2.6	1.5	**19b**	5.4	3.2
2,2-dimethylmalonate	8.1	4.8	(±)-**20a**	0.84	0.49
4	no inhibition		(+)-**20a**	13	7.8
5	no inhibition		(−)-**20a**	0.34	0.20
(±)-**6**	3.9	2.3	(±)-**20b**	0.80	0.47
(+)-**6**	4.3	2.5	(+)-**20b**	13	7.7
(−)-**6**	3.4	2.0	(−)-**20b**	0.41	0.24
7	17	10	**21**	9.5	5.6
8	no inhibition		**23a**	1.5	0.88
9	5.4	2.6	**23b**	2.1	1.2
10	4.3	2.5	**24**	5.7	3.4
11	5.7	3.3	**25**	3.6	2.1
12	3.0	1.8	**26**	1.0	0.61
13a	11	6.3	**27**	5.2	3.1
13b	14	8.3	**28**	3.3	1.9
14a	5.0	3.1	**29**	8.9	5.3
14b	4.5	2.6	**31**	55	33
15a	0.95	0.56			
15b	1.1	0.65			

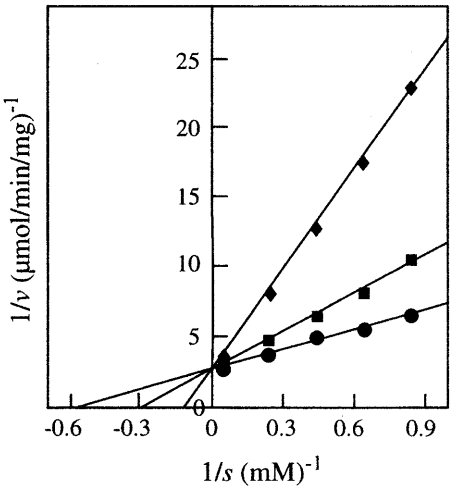


Fig. 2. Double Reciprocal Plots of the Inhibitors **6** and **20b**.
Shown are the data points observed from formation of macrophomate **1**. Inhibitor **6**: (◆); **20b** (■); without inhibitors (●). Concentrations of 2-pyrone **2** were 1.19, 1.56, 2.27, 4.17, 25.0 mM.

15b for systematic screening using the same molecular skeleton.

Structurally simple derivatives **19a** and **19b** showed moderate activity (I_{50} 4.0, 5.4 mM), suggesting that skeletons of **15a** and **15b** possess additional effects. Among simple derivatives, diacids **20a**, **20b**, mono esters **24**, **26**, and diester **25**, diacids are shown to be most potent inhibitors (I_{50} 0.84, 0.80 mM) and introduction of an ester group caused 1.2–7-times lower activity. Introduction of sterically demanding alkylamino groups in amides **23a** and **23b** did not significantly decrease the inhibition. This suggests that this type of modification could be used as an affinity ligand for macrophomate synthase. Difference between diastereomers **20a** and **20b** were again relatively small whereas dihydro derivative **21** unexpectedly reduced its activity, indicating the importance of the double bond for recognition.

The effects of additional functionalities which mimic the bicyclic intermediate **3** were examined. Modification of double bond in **20a** reduced their potency 4–11 times in **27–29**. Especially compound **31** showed 55 times lower activity. These indicate that the dicarboxylate moiety and double bond in **20a** are not compatible with the corresponding functionalities in the intermediate **3** and a different type of binding mode is involved. Enantiomers (−)-**6** and (+)-**6** did not showed any difference in their activity (I_{50} 4.3, 3.4 mM). On the other hand, significant differences between each enantiomer of **20a** and **20b** were observed (I_{50} 13, 0.34 mM; 13, 0.41 mM). These suggest that affinity of (−)-enantiomers of **20a** and **20b** are superior to (+)-ones and this resulted in strong inhibitions. Remarkable difference between enantiomers could provide information for structure of the active site if the X-ray crystal structure of macrophomate synthase is available.

For the representative inhibitors **6** and **20b**, a detailed kinetic study was conducted as shown in Fig. 2. K_i obtained in this study were 1.7 and 0.40 mM for **6** and **20b**, respectively. The K_i for moderately active inhibitor **6** is compatible with K_m of the substrate 2-pyrone **2** whereas the K_i for one of the most potent inhibitor **20b** is 4-times smaller than K_m of **2**. In fact, nearly complete inhibitions of the macrophomate synthase reaction were observed for compounds **20a** and **20b** at the same concentration (5 mM) of the substrate 2-pyrone **2**.

We have already established the structural requirement for one of the substrates, 2-pyrone.[10] In the study of substrate diversity, none of 2-pyrone having polar substituents was accepted as a substrate, suggesting that the diacid moiety in **20a** mimics oxalacetate and one or both carboxyl group(s) could bind Mg(II) in the active site. The importance of a double bond and strong inhibition of (−)-enantiomer suggests that the bicyclic framework and its spatial position are critical for the inhibition. Although anhydrides **15a** and **15b** possibly inhibit the activity irreversibly, they behave essentially the same as dicarboxylic acids since addition of a large excess of substrate restored macrophomate synthase activity. Based on kinetic studies on each and overall chemical steps,[6] we speculated that macrophomate synthase more tightly associates with the bicyclic intermediate **3** than the substrates and the product. This possibly explains why diacids **20a** and **20b** show stronger affinity than substrates.

In conclusion, we have synthesized various intermediate analogs and found an effective inhibitor **20a**, which shows 5-times higher affinity than the substrate 2-pyrone **2**. Discovery of the effective inhibitor would provide further insight into the reaction mechanism of this highly complex multistep transformation. Elucidation of the stereostructure of macrophomate synthase bound with the inhibitor would clarify the role of amino acid residues in the active site.

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