Phytochemistry, Vol. 20, No. 11, pp. 2565–2568, 1981.
Printed in Great Britain.

0031–9422/81/112565–04 $02.00/0

BIOLOGICALLY ACTIVE PHENOLICS FROM *LILIUM LONGIFLORUM**

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(*Revised received* 22 *December* 1980)

Key Word Index—*Lilium longiflorum*; Liliaceae; *trans-p*-coumaroyl; *cis-p*-coumaroyl; feruloyl; 2,3-dihydroxy-1,2-propanedicarboxylic acid esters; itaconic acid; biological activity.

Abstract—The new phenolics, the *trans-p*-coumaroyl and the feruloyl esters of 2,3-dihydroxy-1,2-propanedicarboxylic acid and itaconic acid, were isolated from *Lilium longiflorum*; the *cis-p*-coumaroyl ester was also detected. The biological activities of the *trans-p*-coumaroyl ester are described.

INTRODUCTION

In a previous paper [1], we reported the presence of a growth inhibitor in the bulbs of a variety of *Lilium longiflorum*. The growth inhibitor was detected by the rice seedling growth test and localized in the acidic fraction. This report describes the isolation and structure elucidation of three phenolics, as well as itaconic acid, derived from the growth inhibitory fraction and estimates the effect of the major phenolic on the rice and lettuce seedling growth test.

RESULTS AND DISCUSSION

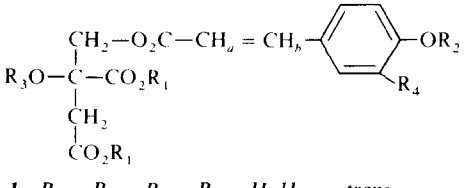
Fresh bulbs of *Lilium longiflorum* were homogenized with 80% acetone and the acetone extracts were partitioned between ether and H_2O. After adjusting to pH 3.0, the H_2O layer was extracted with ether, EtOAc and *n*-BuOH, successively. The growth inhibitory active fraction, EtOAc extracts, was repeatedly purified by column chromatography on Sephadex LH-20 and Avicel and monitored for growth inhibitory activity by the rice seedling growth test; the active fractions were recrystallized.

Compound **1**, $C_{14}H_{14}O_8$, gave an orange colour with diazotized benzidine. The UV spectrum showed the presence of a conjugated benzene ring (300 and 313 nm), and the IR spectrum demonstrated the presence of the following functions: *OH* (3400, 3000 cm^{-1}), *CO* (1730, 1680 cm^{-1}) and a benzene ring (1605, 1590 cm^{-1}). The ^1H NMR spectrum exhibited the presence of two pairs of phenyl protons as AB type (δ 6.81, 7.42, J = 8 Hz), *trans* vinyl protons (δ 6.32, 7.62, J = 16 Hz) and two methylene groups (δ 2.76, 3.00, each *d*, J = 16 Hz and δ 4.32, *s*). The FD–MS spectrum gave a $(M + 1)^+$ at *m/z* 311 and ions at *m/z* 621 $(2M + 1)^+$ and 643 $(2M + Na)^+$.

When **1** was treated with CH_2N_2 in MeOH, a diMe ester, **2**, was produced, which had two carbomethoxyl signals (δ 3.64, 3.75). The ^{13}C NMR spectrum showed a typical quaternary carbon (75.1 ppm), two types of methylene carbons (40.7, 68.4 ppm) and nine carbons of a *p*-coumaroyl moiety, which were determined by comparisons with Me-*p*-coumarate and tri-Me-citrate suggesting that **1** must be the *p*-coumaroyl ester of dihydroxydicarboxylic acid.

Mild acetylation of **2** resulted in a monoacetate, **3**, with an OH (3050 cm^{-1}) and an acetoxyl signal (δ 2.31) on phenolic OH. Further acetylation of **3** produced a diacetate, **4**, as a colourless syrup which created two kinds of acetoxyl signal, one alcoholic (δ 2.11) and the other phenolic (δ 2.31). An aliphatic OH is centred between two methylene groups as indicated by the lower field shift (0.26–0.47 ppm) of individual methylene after the acetylation of **3**.

Thus, the aliphatic dicarboxylic acid is 2,3-dihydroxy-1,2-propanedicarboxylic acid. For positive identification of this structure, **1** was hydrolysed with N NaOH at 90° under N_2 for 30 min and produced *p*-coumaric acid, which was identified by direct comparison (mmp, ^1H NMR) with an authentic sample. 2,3-Dihydroxy-1,2-propanedicarboxylic acid was determined by ^1H NMR followed by methylation to give a diMe ester which showed two pairs of methylene groups (δ 2.70, 3.04, each *d*, J = 17 Hz and δ 4.26, 4.48, each *d*, J = 10 Hz) and two carbomethoxyl groups (δ 3.83, 3.90) in the ^1H NMR spectrum and MS fragmentation, 193$(M + 1)^+$, 175$(M - OH)^+$, 161 $(M - OMe)^+$ and 133$(M - CO_2Me)^+$. Therefore, the original ester is 2-hydroxy-3-*O-trans-p*-coumaroyl-1,2-propanedicarboxylic acid (**1**).



1 $R_1 = R_2 = R_3 = R_4 = H$, $H_{a,b}$ = *trans*
2 R_1 = Me, $R_2 = R_3 = R_4 = H$, $H_{a,b}$ = *trans*
3 R_1 = Me, R_2 = Ac, $R_3 = R_4 = H$, $H_{a,b}$ = *trans*
4 R_1 = Me, $R_2 = R_3$ = Ac, $R_4 = H$, $H_{a,b}$ = *trans*
5 $R_1 = R_2 = R_3 = R_4 = H$, $H_{a,b}$ = *cis*
6 R_1 = Me, $R_2 = R_3 = H$, R_4 = OMe, $H_{a,b}$ = *trans*

*Part of this work was presented at the 100th Annual Meeting of the Pharmaceutical Society of Japan, 1980.

Since PC of the mother liquor of **1** indicates another phenolic compound (R_f 0.72; 2% HOAc), the fraction was further purified. Repeated use of column chromatography (Avicel) and a final preparative PC produced **5**, colourless syrup. **5** shows almost the same UV and IR spectrum as **1**. The ^{1}H NMR spectrum showed two methylene groups (δ 2.66, 2.82, J = 12 Hz and δ 4.24, 3.36, J = 8 Hz, respectively), two pairs of phenyl protons (δ 6.72, 7.64, J = 8 Hz) and *cis*-vinyl protons (δ 5.78, 7.66, J = 12 Hz). Thus, the structure of **5** is an isomer of **1** having a *cis*-*p*-coumaroyl group in the molecule. However, it is evident that **5** is contaminated with a small amount of **1** in spite of the rigorous purification procedures. *Cis*- to *trans*-isomerization occurs during purification.

The methylation of the mother liquor of **1** produces compound **2** as well as an additional minor component. The new compound was purified by preparative TLC using $CHCl_3$–MeOH (5:1) as a solvent to give compound **6**, a colourless syrup. **6** shows similar UV and IR spectra with **2**. The ^{1}H NMR spectrum of **6** showed a methoxyl signal (δ 3.71) and three aromatic protons (δ 6.72–7.02, *m*) suggesting that **6** must be the ferulate of 2,3-dihydroxy-1,2-propanedicarboxylic acid diMe ester. The mass spectrum of **6** gave M^+ at *m/z* 368 and ions 353, 338, 322, 177 and 147 in full agreement with its structure as ferulic acid ester. In order to confirm the structure, **6** was treated with N NaOH and found to migrate the same as ferulic acid on TLC. Thus, **6** is 2-hydroxy-3-*O*-*trans*-feruloyl-1,2-propanedicarboxylic acid diMe ester.

The mother liquor of **1** contains another compound which is not positive when tested with phenolic reagents, but is detectable by UV on fluorescent TLC plates. After purification by Sephadex LH-20 column chromatography and recrystallization, compound **7** was obtained as colourless plates, with a structure of $C_5H_6O_4$, and mp 154–158°, $[\alpha]_D$ 0° (in H_2O). The FD–MS showed ions 131($M+1$)$^+$, 130(M^+), 113 ($M-OH$)$^+$ and 86($M-CO_2$)$^+$. The IR spectrum exhibited CO (1705 cm^{-1}) and H_2C=C (1630, 920 cm^{-1}). The ^{1}H NMR spectrum showed simple signals which were a methylene proton (δ 3.43, *br s*) and two vinyl protons (δ 5.89, *br s* and δ 6.37, *br s*, respectively). The ^{13}C NMR spectrum of **7** indicated the existence of two carbonyl carbons (172.3, 178.1 ppm), two unsaturated carbons (133.0 ppm, *t* and 135.8 ppm, *s*) and methylene carbon (39.3 ppm). Thus, **7** was identified as itaconic acid by mmp and IR with an authentic sample. However, it is still unclear whether **7** is a genuine compound or an artefact originating from 2,3-dihydroxy-1,2-propanedicarboxylic acid.

Previously, dicaffeoyl-*d*-tartaric acid [2], mono-*p*-coumaroyl-*meso*-tartaric acid [3], 2-acetyl-3-(*p*-coumaroyl)-*meso*-tartaric acid [4], caffeoyl-*l*-malic acid [5, 6], 2-*O*-*p*-coumaryl-, 2-*O*-feruloyl- and 2-*O*-caffeoyl-hydroxycitric acid [7] have been isolated from *Chicorium intybus* [2], spinach leaf [3, 4], red clover [5], *Phaseolus vulgaris* [6] and corn plant [7], respectively. However, the ester having the dicarboxylic acid of a five-carbon unit in a molecule is the first example.

Bioassay

The effect of **1** on the rice seedling growth test is shown in Fig. 1.The second leaf sheaths of seedlings were inhibited by 17.8% at 250 ppm of compound **1**, 31.2% at 500 ppm and 45.5% at 1000 ppm; an inhibitory activity

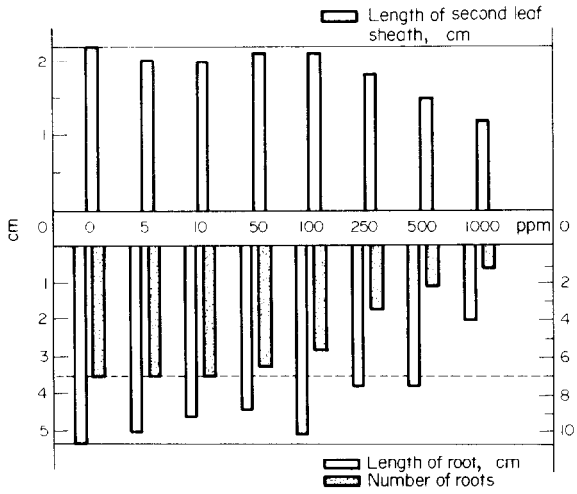


Fig. 1. Effect of 2-hydroxy-3-*O*-*trans*-*p*-coumaroyl-1,2-propanedicarboxylic acid on the growth of rice seedlings.

was also demonstrated to occur on the length of root. Compound **1** significantly inhibited the number of roots present by 5.8% at 50 ppm of **1**, 20% at 100 ppm, 52.5% at 250 ppm, 71.5% at 500 ppm and 84.3% at 1000 ppm, but did not inhibit this growth at concentrations of 50 ppm or less. This study showed that all seedlings treated with **1** at concentrations of 250–1000 ppm died after 3 days' incubation.

In the case of the lettuce seedling growth test, **1** inhibited the growth of the hypocotyl as well as the root as shown in Fig. 2. The effect of **1** on the length of the hypocotyl was dose-dependent. Like the rice seedling growth test, the lettuce seedling growth test showed a stronger root effect than hypocotyl effect; root inhibition was 15% at 50 ppm of **1**, 65% at 100–500 ppm and 80% at 1000 ppm. The lettuce seedlings did not die after prolonged exposure to high concentrations of **1** as did the rice seedlings described above.

EXPERIMENTAL

All mps are uncorr. ^{1}H NMR spectra were measured at 100 MHz and chemical shifts are given on (ppm) scale with TMS

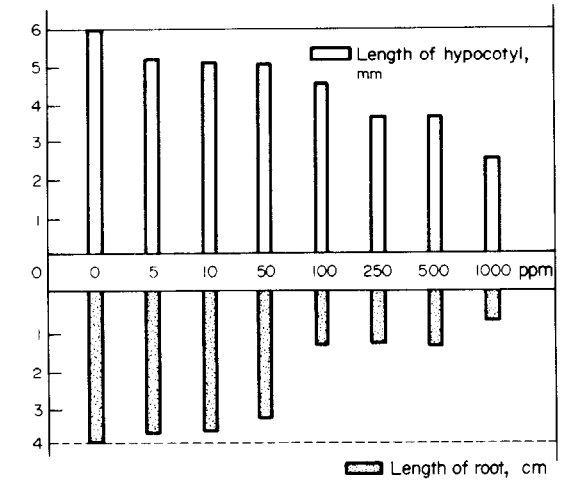


Fig. 2. Effect of 2-hydroxy-3-*O*-*trans*-*p*-coumaroyl-1,2-propanedicarboxylic acid on the growth of lettuce seedlings.

as internal standard. Diazotized benzidine reagent, I_2 vapour, 10% H_2SO_4 and UV were used for detection. Column chromatography was carried out with Sephadex LH-20(25–100 μm), Si gel 60 (60–200 μm, Merck) and Avicel.

Bioassay methods. Seeds of rice (*Oryza sativa* var. Tangin-bozu) were sterilized by usplon for 1 hr, then rinsed with H_2O for 4 hr. The sterilized seeds were transferred into a petri dish containing sterilized H_2O. The petri dish was kept under white fluorescent light at 25° for 3 days; H_2O was changed once a day. Germinating seeds having 1 mm of coleoptiles were employed for assay. Ten of these seeds were placed in a test-tube (4 × 2 cm) containing 1.0 ml of aq. test soln (adjusted to pH 4.5 with H_3PO_4) and different concns of the experimental compounds. The test-tubes were covered and kept at 25° under white fluorescent light for 4 days. The second leaf sheaths, root lengths and root numbers were measured. The control assay was done by using H_2O alone. The lettuce seedling growth test was done as previously described [8] with modification.

Isolation of **1**. Fresh bulbs of *Lilium longiflorum* (2 kg) were homogenized with 80% Me_2CO (10 l.) and stored overnight. After filtration, the filtrate was concd to near dryness. The extracts (60 g) were partitioned between Et_2O and H_2O. The H_2O soln was adjusted to pH 3.0 with dil. HCl and extracted with Et_2O, EtOAc and *n*-BuOH, successively. The EtOAc extracts (2.4 g), which inhibited the growth of rice seedlings at a concn of 1000 ppm, were chromatographed on Sephadex LH-20 using H_2O–MeOH and on Avicel using $CHCl_3$–MeOH–H_2O (5:3:1, bottom layer). The resultant pale yellow syrup (70 mg) was recrystallized with EtOAc–MeOH to produce colourless, crystalline **1**, mp 176–178.5°, $[\alpha]_D^{23}$ −6.6° (*c* 1.0, MeOH) (Found: C, 54.27; H, 5.05. $C_{14}H_{14}O_8$ requires: C, 54.19; H, 4.55%). UV λ_{max}^{MeOH} nm (log ε): 227 (3.98), 300 (sh.), 313 (4.25); IR ν_{max}^{KBr} cm^{-1}: 3400, 3000 (OH), 1730, 1680 (C=O), 1605, 1590 (C=C). ^1H NMR (CD_3OD): δ 2.76, 3.00 (1 H × 2, each *d*, J_{gem} = 16 Hz), 4.39 (2 H, *s*), 6.32 (1 H, *d*, *J* = 16 Hz, vinyl H), 6.81 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.42 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.62 (1 H, *d*, *J* = 16 Hz, vinyl H). FD-MS *m/z*: 311 $(M + 1)^+$, 621 $(2M + 1)^+$, 643 $(2M + Na)^+$.

Dimethyl ester of **1**. **1** was methylated with CH_2N_2 and recrystallized from $CHCl_3$ to give **2** as colourless needles, mp 165–167°, $[\alpha]_D^{20}$ −6.6° (*c* 0.55, MeOH). (Found: C, 56.50; H, 5.39. $C_{16}H_{18}O_8$ requires: C, 56.80; H, 5.36%). UV λ_{max}^{MeOH} nm (log ε): 227 (3.81), 300 (sh.) 313 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3450, 3380 (OH), 1740, 1690 (C=O), 1608, 1587 (C=C). ^1H NMR (Me_2CO-d_6): δ 2.73, 3.02 (1 H × 2, each *d*, J_{gem} = 15 Hz), 3.64, 3.76 (3 H × 2, each *s*, $-CO_2Me$), 4.33 (2 H, *s*), 6.32 (1 H, *d*, *J* = 15 Hz, vinyl H), 6.89 (2 H, *d*, *J* = 9 Hz, aromatic H), 7.54 (2 H, *d*, *J* = 9 Hz, aromatic H), 7.63 (1 H, *d*, *J* = 15 Hz, vinyl H), 8.93 (1 H, *s*, −OH). MS *m/z*: 338 (M^+), 279 $(M - CO_2Me)^+$, 147 (*p*-coumaroyl)$^+$. ^{13}C NMR (Me_2CO-d_6) ppm: 40.7, 51.8, 52.9, 68.4, 75.1, 114.5, 116.4, 126.5, 130.7, 145.8, 160.4, 170.2.

Acetylation of **2**. **2** (5.5 mg) was acetylated with Ac_2O (0.5 ml) at room temp. to give **3** as colourless needles (from hexane–$CHCl_3$), mp 77–79°, $[\alpha]_D^{23}$ −26.4° (*c* 0.39, $CDCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3050 (OH), 1750, 1640 (C=O), 1602, 1585 (C=C). ^1H NMR ($CDCl_3$): δ 1.62 (1 H, *br s*, OH), 2.31 (3 H, *s*, OAc), 2.78, 3.00 (1 H × 2, each *d*, J_{gem} = 16 Hz), 3.78, 3.83 (3 H × 2, each *s*, $-CO_2Me$), 4.33 (2 H, *s*), 6.38 (1 H, *d*, *J* = 16 Hz, vinyl H), 7.10 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.52 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.66 (1 H, *d*, *J* = 16 Hz, vinyl H). MS *m/z*: 380 (M^+), 338 (M − Ac)$^+$, 189 (*O*-Ac-*p*-coumaroyl)$^+$.

Acetylation of **3**. **3** (2.9 mg) was acetylated with Ac_2O–pyridine at 95° for 1 hr to give a colourless syrup, **4**: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750 (C=O), 1605, 1583 (C=C). ^1H NMR ($CDCl_3$): δ 2.11 (3 H, *s*, OAc) 2.31 (3 H, *s*, OAc), 3.04, 3.28 (1 H × 2, each *d*, J_{gem} = 12 Hz), 6.36 (1 H, *d*, *J* = 16 Hz, vinyl H), 7.08 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.52 (2 H, *d*, *J* = 8 Hz, aromatic H), 7.64 (1 H, *d*, *J* = 16 Hz, vinyl H). MS *m/z*: 422 (M^+), 380 (M − Ac)$^+$, 338 (M − 2Ac)$^+$, 280 (M − 2Ac − CO_2Me)$^+$.

Alkaline hydrolysis of **1**. **1** (20 mg) was dissolved in N NaOH. The mixture was heated at 90° under N_2 for 30 min. The reactant was passed through Amberlite IR-120. The eluate was extracted with Et_2O and after the Et_2O was evapd, the resultant *p*-coumaric acid (9 mg) was recrystallized with dil. MeOH and identified by direct comparison with an authentic sample (mmp, TLC and ^1H NMR). The aq. soln was evapd *in vacuo* to dryness. The residue was purified by prep. PC using Et_2O–HCO_2H–H_2O (5:2:1) as a solvent. The band of R_f 0.34 (detected by BCG) was cut off and extracted with H_2O. H_2O was evapd *in vacuo* to give 2,3-dihydroxyl-1,2-propanedicarboxylic acid (4 mg). ^1H NMR (pyridine-d_5): δ 3.12, 3.63 (1 H × 2, each *d*, J_{gem} = 16 Hz), 4.52, 4.84 (1 H × 2, each *d*, J_{gem} = 20 Hz). The acid was methylated by CH_2N_2 in MeOH to give a colourless syrup which was purified by prep. TLC using C_6H_6–EtOAc–EtOH (4:5:1) as a solvent. The band of R_f 0.47 (detected by I_2 vapour) was removed and extracted with EtOAc. The solvent was evapd to give diMe ester (1.2 mg). ^1H NMR ($CDCl_3$): δ 2.70, 3.04 (1 H × 2, each *d*, J_{gem} = 17 Hz), 3.84, 3.90 (3 H × 2, each *s*, $-CO_2Me$), 4.62, 4.84 (1 H × 2, each *d*, J_{gem} = 10 Hz). MS *m/z*: 193 $(M + 1)^+$, 175 (M − OH)$^+$, 161 (M − OMe)$^+$, 133 (M − CO_2Me)$^+$.

Purification of **5**. Half of the mother liquor of **1** was purified by Avicel and then on PC using 2% HOAc as solvent. The band of R_f 0.72 was cut off and extracted with MeOH. The MeOH was evapd *in vacuo* to give compound **5** (8 mg), colourless syrup. UV λ_{max}^{MeOH} nm: 227, 300 (sh), 313. ^1H NMR(CD_3OD): δ 2.66, 2.82 (1 H × 2, each *d*, J_{gem} = 12 Hz), 4.24, 4.36 (1 H × 2, each *d*, J_{gem} = 8 Hz), 5.78 (1 H, *d*, *J* = 12 Hz, *cis*-vinyl H), 6.72, 7.64 (2 H × 2, each *d*, *J* = 8 Hz, aromatic H), 7.66 (1 H, *d*, *J* = 12 Hz, *cis*-vinyl H).

Isolation of **6**. Half of the mother liquor of **1** was dissolved in MeOH and treated with CH_2N_2 at room temp. for 10 min. After evapn of the solvent, the product was purified by prep. TLC using $CHCl_3$–MeOH (5:1) as solvent. The band of R_f 0.71 was removed and extracted with $CHCl_3$–MeOH (1:1) to give compound **6** (5 mg), colourless syrup. UV λ_{max}^{MeOH} nm: 233, 298 (sh), 323. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3530 (OH), 1740 (C=O), 1604, 1583 (C=C). ^1H NMR($CDCl_3$): δ 2.76, 3.00 (1 H × 2, each *d*, J_{gem} = 12 Hz), 3.71 (3 H, *s*, OMe), 3.85, 3.94 (3 H × 2, each *s*, $-CO_2Me$), 6.28 (1 H, *d*, *J* = 16 Hz, vinyl H), 6.72–7.02 (3 H, *m*, aromatic H), 7.60 (1 H, *d*, *J* = 16 Hz, vinyl H). (Found: M^+ 368.1135. $C_{17}H_{20}O_8$ requires 368.1107). MS *m/z*: 368 (M^+), 353, 338, 322, 177, 147.

Alkaline hydrolysis of **6**. **6** (1 mg) was heated with N NaOH at 90° under N_2 for 30 min and then passed through Amberlite IR-120. The eluate was concd and the product identification as ferulic acid was determined by TLC. R_f 0.21 ($CHCl_3$–MeOH–3% HOAc (7:3:0.3)). R_f 0.83 (*n*-BuOH–HOAc–H_2O (4:1:5)).

Isolation of **7**. The first H_2O eluate which was obtained by purification of **1** on Sephadex LH-20 column chromatography was evapd. The residue was recrystallized from MeOH to give **7**, colourless plates, mp 154–158°: IR ν_{max}^{KBr} cm^{-1}: 1705 (C=O), 1630 (C=C), 920 (H_2C=C). ^1H NMR (D_2O): δ 3.43 (2 H, *br s*), 5.89 (1 H, *br s*), 6.37 (1 H, *s*). ^{13}C NMR (D_2O) ppm: 39.9, 133.0, 135.8, 172.3, 178.1. FD-MS *m/z*: 131 $(M + 1)^+$, 130 (M^+), 113 (M − OH)$^+$, 86 (M − CO_2)$^+$. **7** was identified by direct comparison with authentic itaconic acid (mmp, IR).

Acknowledgements—The authors thank Miss T. Komiya and Mr. K. Hatano for excellent technical assistance. Thanks are also due to Mr. Y. Tanaka, Miss M. Kawamura, Miss Y. Soeda and

Mr. I. Maetani and the members of the Central Analysis Room of this University for ^{1}H NMR, ^{13}C NMR, MS measurements and microanalyses.

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