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Antiinflammatory Flavonoids from *Artocarpus heterophyllus* and *Artocarpus communis*

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The antiinflammatory activities of the isolated flavonoids, including cycloartomunin (**1**), cyclomorusin (**2**), dihydrocycloartomunin (**3**), dihydroisocycloartomunin (**4**), cudraflavone A (**5**), cyclocommunin (**6**), and artomunoxanthone (**7**), and cycloheterohyllin (**8**), artonins A (**9**) and B (**10**), artocarpanone (**11**), artocarpanone A (**12**), and heteroflavanones A (**13**), B (**14**), and C (**15**) from *Artocarpus communis* and *A. heterophyllus*, were assessed *in vitro* by determining their inhibitory effects on the chemical mediators released from mast cells, neutrophils, and macrophages. Compound **4** significantly inhibited the release of β-glucuronidase and histamine from rat peritoneal mast cells stimulated with *P*-methoxy-*N*-methylphenethylamine (compound 48/80). Compound **11** significantly inhibited the release of lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP). Compounds **8**, **10**, and **11** significantly inhibited superoxide anion formation in fMLP-stimulated rat neutrophils while compounds **2**, **3**, **5**, and **6** evoked the stimulation of superoxide anion generation. Compound **11** exhibited significant inhibitory effect on NO production and iNOS protein expression in RAW 264.7 cells. The potent inhibitory effect of compound **11** on NO production in lipopolysaccharide (LPS)-activated macrophages, probably through the suppression of iNOS protein expression.

KEYWORDS: ***Artocarpus heterophyllus*; *Artocarpus communis*; moraceae; antiinflammatory; flavonoids**

INTRODUCTION

The plants of *Artocarpus* species distribute over the tropical and subtropical regions and have been used as traditional folk medicine in Indonesia against inflammation and malarial fever (*1*). In the previous papers the characterization of prenylflavonoids of *Artocarpus communis* (*A. communis*) and *A. heterophyllus* (*2*−*7*) and antiinflammatory, cytotoxic, antiplatelet, and scavenger and antioxidant properties of prenylflavonoids isolated from Formosan *Artocarpus* plants were reported (*8*−*12*). Mast cells play a central role in the pathogenesis of diseases such as allergic asthma, rhinoconjunctivitis, urticaria, anaphylaxis, and systemic mastocytosis and may well be important players in other chronic inflammatory disorders (*13*). The neutrophil is an important inflammatory cell. It can be triggered by a variety of inflammatory stimuli to produce highly reactive oxygen species which have potent microbicidal and inflammatory effects (*13*). Macrophages are important in nonspecific host resistance to microbial pathogens and serve as central regulators of the specific immune response (*14*). Upon activation, nitric oxide (NO), together with other chemical mediators, is released in response to bacterial endotoxin (LPS) (*15*). NO plays a central role in macrophage-induced cytotoxicity; however, excess NO may contribute to the pathophysiology of septic shock (*16*). The excess production of NO also can destroy functional normal tissues during acute and chronic inflammation (*17*). Hence, compounds with potent inhibition of chemical mediators released from mast cells, neutrophils, and macrophages would suggest a promising antiinflammatory agent. Continuing our screening for bioactive compounds as inhibitors of chemical mediators released from mast cells, neutrophils, and macrophages, the antiinflammatory effects of compounds **1**−**15** (Figure 1) (*2*−*7*) were examined.

MATERIALS AND METHODS

General Procedures. Optical rotations were obtained on a JASCO model DIP-370 digital polarimeter. UV spectra were obtained on a JASCO model UV−vis spectrophotometer. IR spectra were recorded on a Hitachi model 260-30 spectrophotometer. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Unity-400 spectrometer. MS were obtained on a JMS-HX100 mass spectrometer.

Chemicals. Compound 48/80, histamine, formyl-Met-Leu-Phe-(fMLP), mepacrine, trifluoperazine, heparin, bovine serum albumin,

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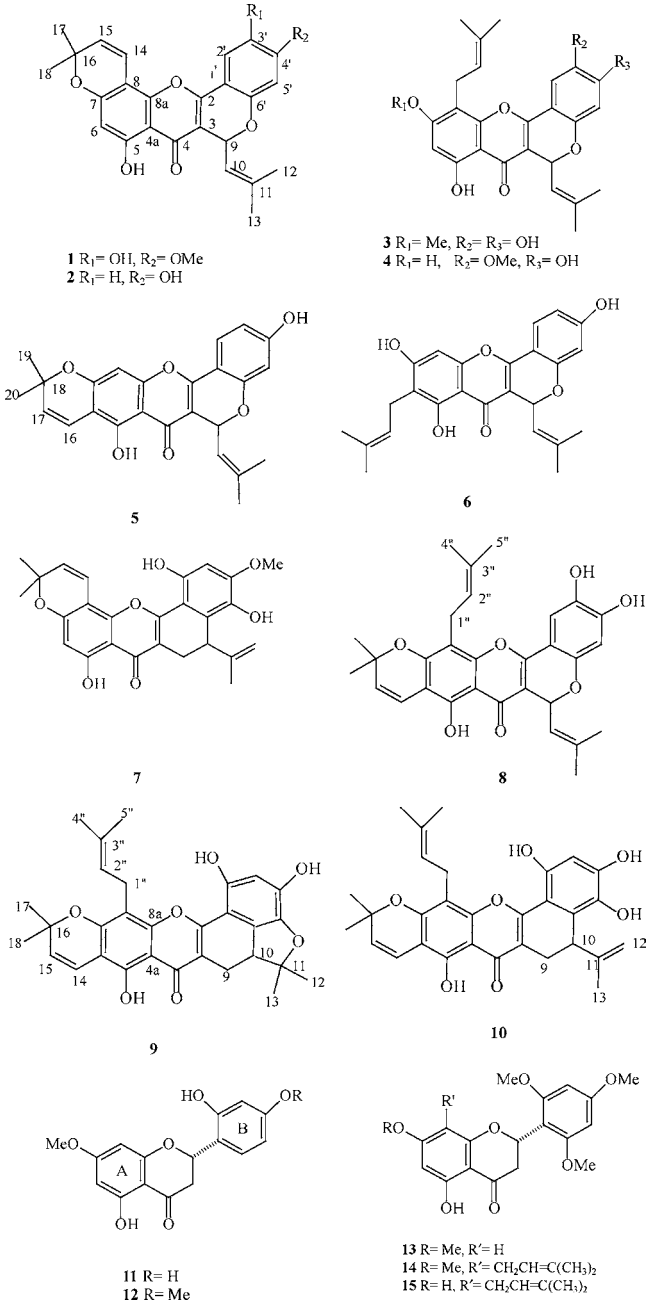

Figure 1. Structures of flavonoids isolated from *Artocarpus communis* and *Artocarpus Heterophgllus.*

phenolphthalein-β-D-GLUCURONIDASE, *O*-phthadialdehyde, cytochrome *c*, superoxide dismutase (type I, from bovine liver), bacterial LPS (*Escherichia coli*, serotype 0111: B4), and L-NAME were obtained from Sigma, St. Louis, MO. Hanks' balanced salt solution (HBSS) was obtained from Gibco Lab, Grand Island, NY. Dextran T 500 was purchased from pharmacia LKB, Taipei, Taiwan. Dimethyl sulfoxide (DMSO) was obtained from Merck, Taiwan. All culture reagents were obtained from Gibco BRL. Rabbit monoclonal anti-iNOS antibody was obtained from Santa Cruz Biotechechnology.

Plant Materials, **Extraction**, **and Isolation.** These items are described as those of previous reports (*2*−*7*).

Compound Identification of Cycloartomunin (1), Dihydrocycloartomunin (3), Dihydroisocycloartomunin (4), Cyclocommunin (6), Artomunoxanthone (7), Artocarpanone A (12), and Heteroflavanones A (13), B (14), and C (15). The physical and spectral data of these compounds have been described in previous reports (*2*−*7*).

Cyclomorusin (**2**) was obtained as yellow needles: mp 246−248 °C; $[\alpha]^{25}_D = +3°$ ($CHCl_3$, c, 0.016); UV (MeOH) λ_{max} 220, 255, 280, and 380 nm; IR ν^{KBr}_{max} (cm^{-1}) 3400, 1640, 1600; ^1H NMR (400 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); EIMS m/z 418 $[M]^+$, 403 $[M - 15]^+$, 385, 363, 347, 203, 194, 174.

Cudraflavone A (5) was obtained as yellow needles: mp 270−272 °C; $[\alpha]^{25}_D = +338°$ ($CHCl_3$, *c*, 0.06); UV (MeOH) λ_{max} 210, 260, 290, and 360 nm; IR ν^{KBr}_{max} (cm^{-1}) 3450, 1640, 1580; ^1H NMR (400 MHz, $CDCl_3$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $CDCl_3$), Table S1 (Supporting Information); EIMS *m*/*z* 418 $[M]^+$, 403, 363, 347, 203, 194, 174.

Cycloheterophyllin (8) was obtained as yellow needles: mp 220 °C; $[\alpha]^{23}_D = -2°$ (acetone, *c*, 0.1); UV (MeOH) λ_{max} 205, 231 (sh), 270, 300, 400 nm; IR ν^{KBr}_{max} (cm^{-1}) 3400, 1650, 1625, 1590; ^1H NMR (400 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); EIMS *m*/*z* 502 $[M]^+$, 487, 459, 448, 447, 431, 403, 236, 205, 189, 153, 69, 55, 43.

Artonin A (9) was obtained as yellow powders: $[\alpha]^{23}_D = -6°$ (acetone, *c*, 0.1); UV (MeOH) λ_{max} 210, 240 (sh), 295, 383 nm; IR ν^{KBr}_{max} (cm^{-1}) 3570, 1655, 1610; ^1H NMR (400 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); EIMS *m*/*z* 502 $[M]^+$, 487, 460, 459, 448, 447, 446, 431, 403, 388, 251, 215, 43.

Artonin B (10) was obtained as yellow needles: mp 202−204 °C; $[\alpha]^{22}_D = -4°$ (acetone, *c*, 0.1); UV (MeOH) λ_{max} 211, 237 (sh), 368, 394 nm; IR ν^{KBr}_{max} (cm^{-1}) 3500, 1655, 1610; ^1H NMR (400 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); EIMS *m*/*z* 502 $[M]^+$, 487, 459, 447, 446, 417, 403, 388, 215, 189, 165, 69, 43.

Artocarpanone (11) was obtained as colorless needles: mp 220−212 °C; $[\alpha]^{24}_D = -2°$ (acetone, *c*, 0.2); UV (MeOH) λ_{max} 206, 227, 285, 327 (sh) nm; IR ν^{KBr}_{max} (cm^{-1}) 3450, 1640, 1615; ^1H NMR (400 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information); ^{13}C NMR (100 MHz, $(CD_3)_2CO$), Table S1 (Supporting Information).

The above data of **2**, **5**, and **8**−**11** were consistent with those of reported data in the literature (*18*−*22*). The purity (>95%) of compounds, used for antiinflammatory tests, was determined by high-performance liquid chromatography (HPLC).

Biological Evaluation. Compound stock solution (30 mM in DMSO) was prepared and stored at −25 °C, and was diluted with DMSO to 1−20 mM range at room temperature before experiment. The final percentage of DMSO in the reaction mixture was less than 0.5% (v/v). Rat (Sprague Dawley) peritoneal mast cells (*23*) and peripheral blood neutrophils (*24*) were isolated and incubated with test compounds for 5 min at 37 °C before stimulation with 10 μg/mL of compound 48/80 for another 15 min or with 1 μM formyl-Met-Leu-Phe (fMLP) for another 45 min, respectively. The degranulation of mast cells and neutrophils was assessed by the determination of histamine and β-glucuronidase, and β-glucuronidase and lysozyme, respectively, in the supernatant (*23*, *25*). The total content of lysozyme and β-glucuronidase was measured from the Triton X-100 treated cells. In the superoxide anion generation experiments, neutrophils were stimulated with 0.3 μM fMLP/CB for 30 min in the presence of cytochrome *c*, and the superoxide anion generation was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction (*26*, *27*). Murine macrophage-like cell line RAW 264.7 cells were plated in a 96-well plate and incubated with test compounds for 1 h at 37 °C before stimulation with 1 μg/mL of lipopolysaccharide (LPS) for 24 h. NO in the cell medium was determined by the Griess reaction (*28*). In Western blot analysis, cells were washed with PBS twice and harvested in Laemmli SDS sample buffer. Cell lysates were separated by 10% SDS−PAGE, and electrophoretically transferred to poly(vinylidene difluoride) membranes. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk. Membranes were washed with TBST buffer and then incubated for 1 h with a monoclonal anti-iNOS antibody (1:1000 dilution). Following the wash with TBST buffer, horseradish peroxidase-labeled anti-mouse IgG (1:10000 dilution) was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents.

Table 1. Inhibitory Effects of **1**–**15** on the Release of β-Glucuronidase and Histamine from Rat Peritoneal Mast Cells Stimulated with Compound 48/80 (**A**), the Release of β-Glucuronidase and Lysozyme from Rat Neutrophils Stimulated with fMLP (**B**), and Superoxide Anion Formation from Rat Neutrophils Stimulated with fMLP (**C**)

	IC_{50}[a] (μM)				
	A		B		C
compnd	β-glucuronidase	histamine	β-glucuronidase	lysozyme	*n* mol/(10^6 cells)
1	>90 (4.6 ± 7.6)	>90 (11.2 ± 2.2)	>90 (–3.5 ± 5.0)	>90 (–43.9 ± 5.3)	ND
2	>3 (16.9 ± 12.5)	>3 (6.1 ± 2.4)	>3 (–2.7 ± 4.6)	>3 (–40.2 ± 10.2)	ND
3	>30 (5.3 ± 13.0)	>30 (11.2 ± 8.6)	>30 (13.2 ± 4.4)	>30 (–7.1 ± 8.3)	ND
4	72.6 ± 2.5	70.2 ± 3.2	>90 (0.9 ± 8.4)	>90 (6.6 ± 9.2)	>30 (–30.5 ± 5.8)
5	>3 (16.0 ± 1.4)	>3 (17.3 ± 1.0)	>3 (–10.7 ± 3.2)	>3 (–41.3 ± 11.2)	ND
6	>3 (16.0 ± 6.7)	>3 (14.1 ± 4.8)	>3 (–12.3 ± 3.2)	>3 (–35.2 ± 15.6)	ND
7	>90 (61.5 ± 9.9)	ND	>90 (21.6 ± 3.1)	>90 (36.8 ± 6.6)	>90 (15.3 ± 8.1)
8	>9 (48.8 ± 5.4)	>9 (35.0 ± 2.7)	>9 (5.3 ± 10.8)	>9 (–1.5 ± 13.5)	8.4 ± 1.2
9	>3 (–20.4 ± 15.4)	>3 (–24.7 ± 8.1)	>9 (–4.7 ± 9.8)	>9 (–19.1 ± 8.5)	>90 (–80.7 ± 6.4)
10	>9 (2.3 ± 4.1)	>9 (5.4 ± 15.9)	>30 (–57.7 ± 5.9)	>30 (–67.0 ± 1.0)	52.2 ± 17.4
11	>90 (–30.3 ± 7.6)	>90 (2.7 ± 15.8)	>30 (36.0 ± 11.7)	46.5 ± 5.1	19.8 ± 1.5
12	ND	ND	ND	ND	ND
13	>90 (–10.9 ± 10.8)	>90 (–3.9 ± 1.8)	>90 (9.2 ± 4.5)	>90 (4.6 ± 9.1)	>90 (14.9 ± 8.9)
14	>90 (–12.2 ± 10.1)	>90 (2.4 ± 4.2)	>90 (0.2 ± 0.9)	>90 (5.0 ± 1.1)	>90 (34.1 ± 7.7)
15	>30 (7.8 ± 3.2)	>30 (–8.4 ± 6.3)	>30 (5.9 ± 9.1)	>30 (4.7 ± 3.7)	>90 (46.9 ± 0.9)
positive control[b]	32.2 ± 3.6	48.5 ± 3.8	7.8 ± 0.6	9.0 ± 1.4	14.8 ± 1.7

[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data are presented as the mean ± sem (n = 3–5). ND: not determined. [b] Mepacrine and trifluoperazine were used as positive controls for A–C, respectively.

Statistical Analysis. Data are presented as the mean ± sem (standard error of the mean) from four to six separated experiments. Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the Student's *t*-test method for two-group comparison. $P = 0.05$ was considered significant. Analysis of linear regression (at least three data within 20−80% inhibition) was used to calculate IC_{50} values.

RESULTS AND DISCUSSION

The antiinflammatory activities of compounds **1**−**15** were studied in vitro on the basis of their effects on chemical mediators released from mast cells, neutrophils, and macrophages. Compound **4** significantly inhibited the release of β-glucuronidase and histamine from peritoneal mast cells stimulated with compound 48/80 in a concentration-dependent manner, while the other compounds did not reveal significant inhibitory effect on this response (Table 1). fMLP induced the release of β-glucuronidase and lysozyme from rat neutrophils. Compound **11** indicated significant and concentration-dependent inhibition of the release of lysozyme stimulated with fMLP, while the other compounds did not show significant inhibitory effect on this response (Table 1). fMLP also stimulated superoxide anion formation in rat neutrophils. Compounds **8**, **10**, and **11** showed significant and concentration-dependent inhibition of superoxide anion formation (Table 1). The suppression of protein kinase C (PKC) activity through the interaction with the regulatory region of PKC is involved in the inhibition of the superoxide anion generation by compound **8** in rat neutrophils (*12*). Compounds **10**, a pyranodihydroxanthone, and **11**, a flavanone, with different chemical structures from **8**, a prenylflavonoid, may have a different mechanism of action on the inhibition of superoxide anion generation in rat neutrophils.

As compared with the compounds shown above, compound **6** did not show significant inhibitory effect on superoxide anion formation in rat neutrophils stimulated with fMLP (Table 1); however, it induced a respiratory burst in rat neutrophils (*27*). The stimulation of respiratory burst by **6** is probably mediated by the synergism of PKC activation and $[Ca^{2+}]_i$ elevation in rat neutrophils (*27*). As shown in Table 2, addition of **2**, **3**, **5**, and **6**, like fMLP, into neutrophils suspensions evoked superoxide anion generation with the maximum effect at 90 μM for **2**, **3**, and **5** and 30 μM for **6**. The stimulation of superoxide anion generation by **6** reconciles with the previous report (*23*). The prenylflavonoids, **2**, **3**, **5**, and **6** may induce respiratory burst through the same mechanism of action as that of **6**. Further experiments are needed to elucidate their exact mechanism of action. The antiinflammatory activities of **11**−**15** were also studied in vitro on the basis of their inhibitory effects on chemical mediator released from macrophages. Treatment of RAW 264.7 macrophage-like cells with LPS (1 μg/mL) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media based on Griess reaction. As shown in Table 3 and Figure 2, LPS induced a significant increase of NO production, and this effect was concentration-dependently suppressed by **11**. The *O*-methylated at C-4′-OH (i.e. **12**) of **11** did not enhance the inhibitory effect on NO production. It indicates that the **11** increased in lipophilicity by *O*-methylation did not enhance the inhibitory effect on NO production. To determine whether the inhibition of NO production in RAW 264.7 cells is attributable to the decrease of iNOS protein expression, Western blot analysis was performed. Unstimulated cell expressed a very low level of iNOS protein, whereas LPS (1 μg/mL) induced a large

Table 2. Effects of Compounds on Superoxide Anion Generation in Rat Neutrophils[a]

compd (concn (μM))	superoxide formation (nmol)
fMLP (0.3)	1.78 ± 0.14
1 (30)	0.54 ± 0.32
(90)	0.02 ± 0.25
2 (30)	2.16 ± 0.02
(90)	4.18 ± 0.34
3 (30)	6.22 ± 1.68
(90)	6.70 ± 2.71
5 (30)	2.89 ± 0.86
(90)	3.87 ± 1.07
6 (30)	14.33 ± 0.31
(90)	12.98 ± 0.86

[a] Data are presented as means ± sem (n = 3–5). fMLP was used as a positive control.

Table 3. Inhibitory Effects of Compounds on the Accumulation of NO_2^- in the Culture Media of RAW 264.7 Cells in Response to LPS

compd	IC_{50}[a] (μM)
11	35.9 ± 2.89
12	>30 (18.5 ± 0.87)
13	>30 (–17.8 ± 2.04)
14	>30 (–17.4 ± 2.08)
15	ND
L-NAME[b]	6.1 ± 0.1

[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data are presented as mean ± sem (n = 3–5). ND: not determined. [b] *N*-(3-Aminomethyl)benzylacetamide was used as a positive control.

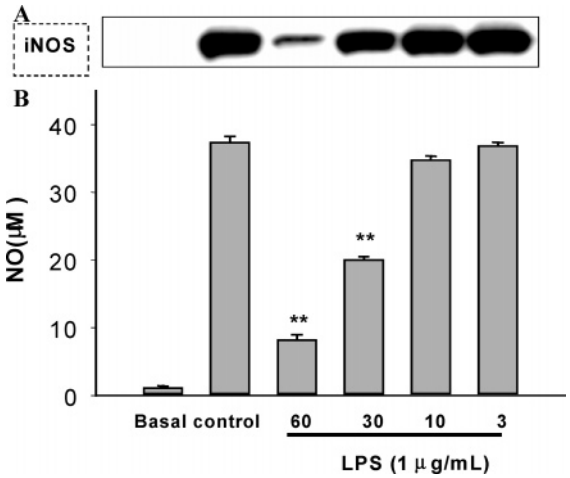

Figure 2. Effects of **11** on the expression of iNOS protein and NO production. (**A**) iNOS expression. RAW 264.7 cells were pretreated with **11** at 3, 10, 30, or 60 μM for 1 h, followed by stimulation with 1 μg/mL LPS. After 24 h, the expression of iNOS protein was analyzed by Western blotting. Similar results were obtained from three independent experiments. (**B**) NO production. RAW 264.7 cells were treated with **11** as described in **A**. NO production was measured and expressed as mean ± sem (n = 3).

amount of iNOS protein expression (Figure 2). Compound **11** inhibited the iNOS protein expression in a concentration-dependent manner. Thus, the blockade of iNOS expression has a critical role as evidenced from the parallelism of the inhibition of NO production and iNOS protein expression by **11**.

The present study verifies that **4**, **8**, **10**, and **11** exert inhibitory effects on the release of chemical mediators from inflammatory cells. NO plays a central role in macrophage-induced cytotoxicity and has been demonstrated to implicate in the pathology of central neurologic diseases and also in the peripheral tissue damage associated with acute and chronic inflammation (*29*, *30*) and septic shock (*16*). The present study also suggests that the inhibition of NO production by **11** in macrophages may have value in the therapeutic treatment or prevention of certain central as well as peripheral inflammatory diseases associated with the increase of NO production. Further experiments are needed to elucidate in vivo their antiinflammatory activities.

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Supporting Information Available: NMR data of **2**, **5**, and **8**−**11** shown in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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