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Azido-Coronatine: A Useful Platform for "Click Chemistry"-Mediated Probe Synthesis for Bioorganic Studies

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We report on the development of azide-coronatine as a useful platform for azide alkyne cycloaddition ("click chemistry")-mediated synthesis of molecular probes. (+)-Azido-coronatine was synthesized in 10 steps with 11% yield using improved synthesis of coronafacic acid, in which the highly *exo*-selective Diels-Alder reaction (*endo:exo* > 1:25) is the key step. Azido coronatine was as effective as the original coronatine in a stomatal opening assay, and was easily modified to a fluorescein isothiocyanate (FITC)-labeled probe with high yield.

Key words: coronatine; click chemistry; molecular probe; fluorescence; stomatal opening

Jasmonic acid (JA, **1**) is an important plant hormone involved in plant defensive behavior against biotic and abiotic stresses, including pathogen attack.[1] The mode of action of jasmonate has been verified through identification of the COI1-JAZ signaling module,[2,3] in which COI1 functions directly as JA receptor.[4] Coronatine (COR, **2**),[5] a phytotoxin isolated from *Pseudomonas syringae* pv. *atropurpurea*, is a conjugate of polyketide coronafacic acid and nonproteinogenic amino acid coronamic acid. COR (**2**) is considered a mimic of *N*-(−)-7-*iso*-jasmonoyl L-isoleucine (7-*iso*-JA-Ile, **3**), a genuine bioactive form of JA,[6] and has played an important part in the recent progress of JA research, as in the discovery of the *coi1* mutant[7,8] and the identification of the JA receptor.[2–4] However, it has remained unclear whether COI1 is the exclusive receptor of **1** in its various bioactivities. For example, COR (**2**) has stomatal opening activity[9] for *Ipomoea tricolor*, *Commelina communis*, and *Arabidopsis thaliana*, for which it is unclear whether COI1 acts as a receptor. In this research, the development of chemically modified forms of **2**, including fluorescence-labeled and photoaffinity probes, is indispensable. Observing the structure of **2**, it is easily recognized that the most feasible modification is one on the carboxylate functionality, such as esterification or amide formation. However, Toshima *et al.* reported that such modifications were very difficult because condensation by using carbodiimide reagent resulted in intramolecular cyclization to give anhydrocoronatine (**4**) with the oxazole ring (Fig. 1).[10] They concluded that modification of the carboxylate of **2** is not promising for the attachment of a linker. Hence, a practical method for the modification of **2** is required. Here, we report on the development of azide-COR (**5**) as a useful platform for azide alkyne cycloaddition (click chemistry[11,12])-mediated synthesis of useful derivatives, including molecular probes (Fig. 1).

Recently, copper-catalyzed azide-alkyne cycloaddition (CuAAC)[11,12] has been applied in the synthesis of various functional molecules, including molecular probes. The azide group is the most valuable bioorthogonal functionality.[12] It is used for labeling certain target proteins, which can be tagged subsequently by CuAAC or copper-free click chemistry (CFCC)[13–15] with fluorescence-dye or biotin. In a previous paper,[9] we reported the synthesis of four stereoisomers of COR using *exo*-selective Diels-Alder reaction at a key step. We examined structure-activity relationship of COR in stomatal opening activity, and reached the conclusion that the coronafacic acid moiety is more important for activity. Here the azide handle should be introduced into the ethyl group of coronamic acid because of the difficulty of chemical modification of carboxylate.

Materials and Methods

General procedure. The NMR spectra of the synthetic compounds were recorded on a Jeol JNM-Lambda400 (^{1}H (400 MHz) and ^{13}C (100 MHz), Jeol, Tokyo) using TMS as internal standard. The HRMS spectra of DART-TOF were recorded on a DART (Jeol). The IR spectra were recorded on a Jasco FT/IR-410 (Jasco, Tokyo). Specific rotations were measured on a Jasco DIP-360 polarimeter (Jasco). Silica gel column chromatography was performed on a Silica Gel 60N (Kanto Chemical, Japan). HPLC purification and chiral analysis were performed on a Jasco LC-2075 series HPLC system (Jasco). Reagents and solvents were purchased from Kanto Chemical and Wako Pure Chemical Industries.

*(+)-Azido-coronatine (**5**) and (−)-*ent*-*azido-coronatine (*ent-**5**).* To a solution of (−)-**14** (8.50 mg, 32.7 μmol), (±)-**13** (6.80 mg, 32.7 μmol), and Et_3N (6.61 mg, 65.3 μmol) in *N*-methylpyrrolidone (NMP, 1.0 ml) was added *O*-(7-azabenzotrazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HATU, 14.9 mg, 39.2 μmol) at 0 °C under an argon atmosphere. After stirring for 2 h, the reaction mixture was quenched with 5% aqueous $KHSO_4$. The mixture was extracted with EtOAc (3 × 5 ml), and the resulting organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. After evaporation, the residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 7/1–6/1) to give a mixture of condensation products. These compounds were dissolved in THF (0.16 ml) and CH_3OH (0.16 ml). To this solution was added 1 M aqueous LiOH (0.16 ml). After stirreing for 2 h, the reaction mixture was quenched

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Abbreviations: JA, jasmonic acid; COR, coronatine; 7-*iso*-JA-Ile, *N*-(−)-7-*iso*-jasmonoyl L-isoleucine; CuAAC, copper-catalyzed azide-alkyne cycloaddition; CFCC, copper-free click chemistry; FITC, fluorescein isothiocyanate; NMP, *N*-methylpyrrolidone; HATU, *O*-(7-azabenzotrazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate

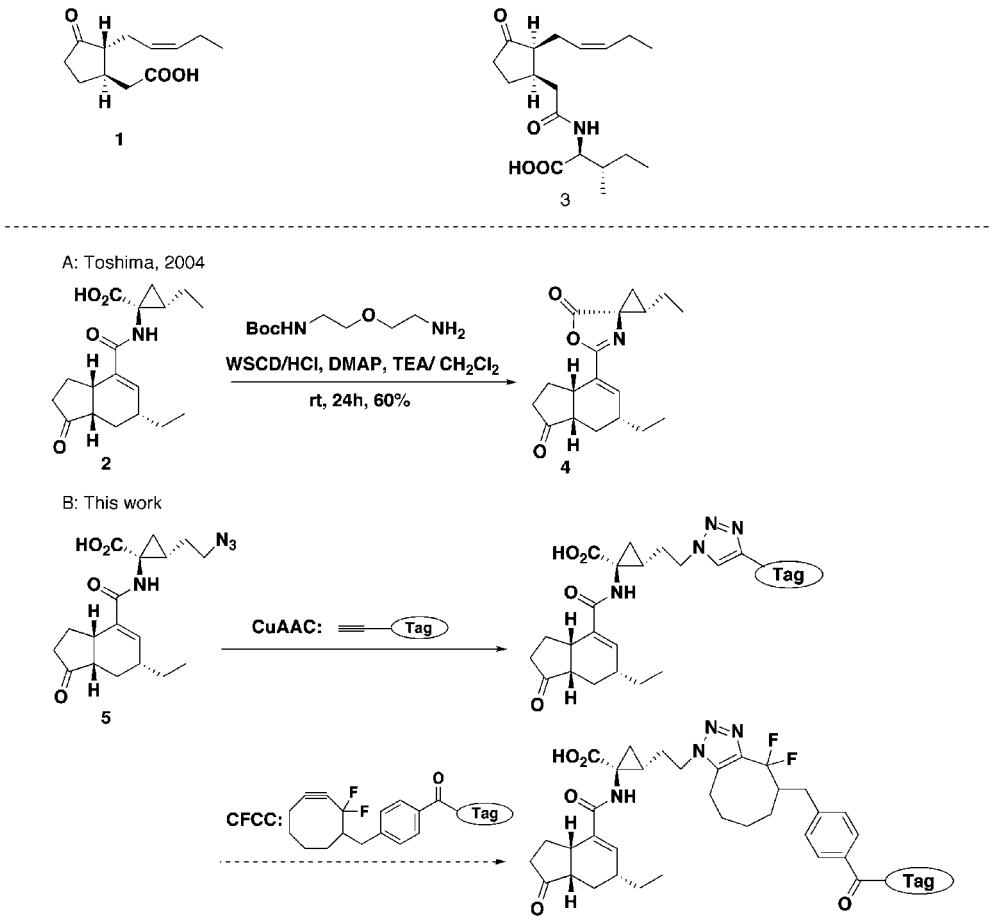


Fig. 1. Structures of JA (**1**), (+)-Coronatine (**2**), and (+)-7-*iso*-JA-Ile (**3**), and Coupling Reactions with Molecular Tags: (A) Toshima *et al.* in 2004 Using **2**, (B) This Study Using (+)-Azido-Coronatine (**5**).

with 5% aqueous $KHSO_4$. This mixture was extracted with EtOAc (3 × 5 ml), and the resulting organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. After evaporation, the residue was purified by silica gel column chromatography ($CHCl_3$/CH_3OH = 120/1–40/1) to give a mixture of (+)-**5** and its diastereomer (7.40 mg, 20.6 µmol, 63% in 2 steps) as a colorless oil. A mixture of (+)-**5** and its diastereomer (20.5 mg) was separated by HPLC on a triacontyl column (Develosil RPAQUEOUS, 20 × 250 mm, Nomura Chemical, Nagoya) with 56% aqueous CH_3OH containing 30 mM HCO_2NH_4 at 8.0 ml/min. Each fraction (2 mg 10 times, (+)-**5**; Rt = 24.0–24.5 min, diastereomer (+)-**15**; Rt = 24.5–25.0 min) was collected and concentrated *in vacuo* to give azide coronatine (+)-**5** (10.3 mg) as a colorless oil and diastereomer (+)-**15** (10.0 mg) as a colorless oil, respectively. The corresponding enantiomers, (−)-**5** and (−)-**15**, were obtained by the same procedure using (+)-**14** instead of (−)-**14**.

*(+)-**5***. NMR δ_H ($CDCl_3$): 0.99 (3H, t, J = 7.2 Hz), 1.07 (1H, dd, J = 12.0, 12.8 Hz), 1.30–1.43 (2H, m), 1.52 (1H, m), 1.55–1.65 (4H, m), 1.84–1.98 (2H, m), 2.18 (1H, m), 2.25–2.51 (4H, m), 3.14 (1H, m), 3.46 (2H, q, J = 6.0 Hz), 6.37 (1H, s), 6.46 (1H, s), δ_C: 11.3, 22.7, 25.9, 26.8, 27.8, 28.0, 29.4, 36.1, 37.3, 37.9, 38.1, 46.3, 50.9, 135.3, 137.7, 169.9, 175.2, 220.2. $[\alpha]^{23}_D$ +69° (*c* 0.5, $CHCl_3$). IR (film) cm^{-1}: 3300, 2926, 2097, 1735, 1655, 1625, 1458, 1269, 1185, 752. HRMS m/z ($M + H^+$) Calcd. for $C_{18}H_{25}N_4O_4$: 361.1870, Found: 361.1871.

*(−)-**5***. $[\alpha]^{23}_D$ −72° (*c* 0.5, $CHCl_3$).

*(+)-**15***. NMR δ_H ($CDCl_3$): 0.99 (3H, t, J = 7.2 Hz), 1.07 (1H, dd, J = 12.0, 12.8 Hz), 1.30–1.43 (2H, m), 1.52 (1H, m), 1.55–1.65 (4H, m), 1.84–1.98 (2H, m), 2.18 (1H, m), 2.25–2.51 (4H, m), 3.14 (1H, m), 3.46 (2H, q, J = 6.0 Hz), 6.37 (1H, s), 6.45 (1H, s), δ_C: 11.3, 22.4, 25.9, 26.8, 27.8, 28.0, 29.4, 36.1, 37.3, 37.9, 38.1, 46.3, 50.9, 135.3, 137.7, 169.9, 175.2, 220.2. $[\alpha]^{23}_D$ +8.8° (*c* 0.5, $CHCl_3$). IR (film) cm^{-1}: 3300, 2926, 2097, 1735, 1655, 1625, 1458, 1269, 1185, 752. HRMS m/z ($M + H^+$) Calcd. for $C_{18}H_{25}N_4O_4$: 361.1870, Found: 361.1864.

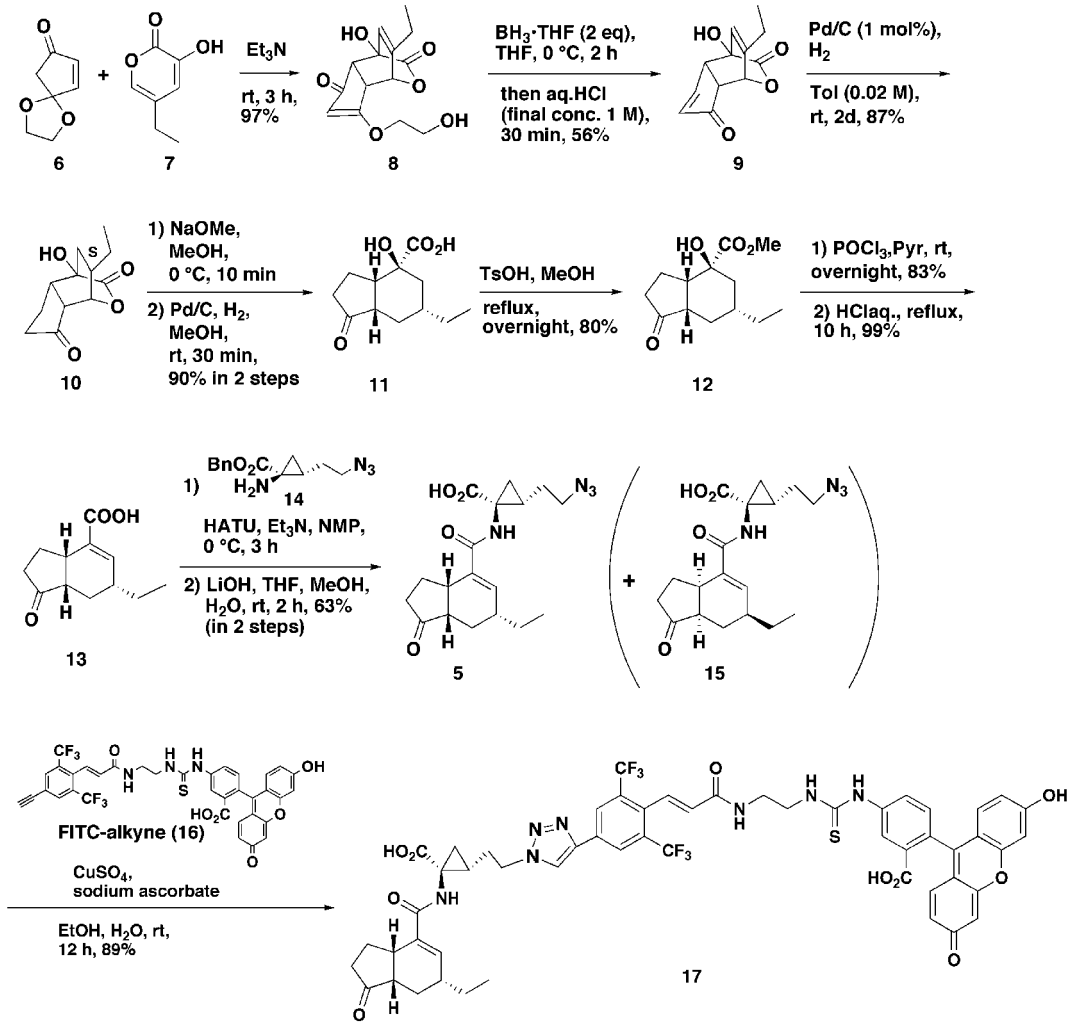
*(−)-**15***. $[\alpha]^{23}_D$ −9.0° (*c* 0.5, $CHCl_3$).

*(−)-**14***. NMR δ_H ($CDCl_3$): 1.19 (1H, dd, J = 4.6, 9.6 Hz), 1.25 (1H, m), 1.40 (1H, m), 1.71–1.86 (2H, m), 2.00 (2H, brs), 3.15–3.25 (2H, m), 5.17 (2H, s), 7.27–7.41 (5H, m), δ_C: 22.7, 26.8, 29.9, 40.1, 51.3, 67.1, 128.4, 128.6, 135.7, 174.3. $[\alpha]^{23}_D$ −14° (*c* 0.5, $CHCl_3$). IR (film) cm^{-1}: 3357, 2098, 1720, 1303, 1255, 1153. HRMS m/z ($M + H^+$) Calcd. for $C_{13}H_{17}N_4O_2$: 261.1346, Found: 261.1353.

*(+)-**14***. $[\alpha]^{23}_D$ +15° (*c* 0.5, $CHCl_3$).

8. NMR δ_H ($CDCl_3$): 1.11 (1H, t, J = 7.4 Hz), 2.22–2.36 (2H, m), 2.83 (1H, d, J = 7.4 Hz), 3.28 (1H, dd, J = 1.0, 7.4 Hz), 3.96–4.06 (2H, m), 4.16–4.20 (2H, m), 5.15 (1H, dd, J = 1.0, 2.0 Hz), 5.40 (1H, s), 6.05 (1H, dd, J = 1.0, 2.0 Hz), δ_C: 11.0, 24.6, 47.6, 50.6, 60.2, 74.0, 76.0, 76.9, 107.7, 127.8, 145.3, 172.5, 186.4, 201.9. IR (film) cm^{-1}: 3421, 2967, 2938, 2878, 1766, 1677, 1589, 1344, 1250, 1183, 1120, 1041. HRMS m/z ($M + H^+$) Calcd. for $C_{14}H_{17}O_6$: 281.1020, Found: 281.1033.

9. NMR δ_H ($CDCl_3$): 1.11 (1H, t, J = 7.4 Hz), 2.22–2.30 (2H, m), 2.73 (1H, dd, J = 2.2, 7.4 Hz), 3.43 (1H, dd, J = 2.0, 7.4 Hz), 3.68 (1H, brs), 5.21 (1H, dd, J = 2.0, 2.2 Hz), 6.06 (1H, dd, J = 2.0, 2.2 Hz), 6.37 (1H, dd, J = 2.0, 5.9 Hz), 7.75 (1H, dd, J = 2.2, 5.9 Hz), δ_C: 11.0, 24.6, 49.1, 49.9, 128.4, 138.8, 145.8, 162.8, 173.9, 204.2. IR (film) cm^{-1}: 3406, 2966, 2931, 1752, 1709, 1582, 1459, 1344, 1300, 1238, 1193, 1135, 999, 929, 889. HRMS m/z ($M + H^+$) Calcd. for $C_{14}H_{17}O_6$: 221.0804, Found: 221.0829.



Scheme 1. Syntheses of (+)-Azido-Coronatine (**5**) and FITC-Labeled Coronatine (**17**).

FITC-labeled coronatine (17). To a solution of (+)-**5** (1.10 mg, 3.05 μmol), FITC-alkyne **16** (2.40 mg, 3.25 μmol), and sodium ascorbate (643 μg, 3.25 μmol) in EtOH (330 μl) and 1 M aqueous $NaHCO_3$ (330 μl) was added $CuSO_4$ (104 μg, 0.652 μmol). After stirring for 12 h, the reaction mixture was quenched with 5% aqueous $KHSO_4$. This mixture was extracted with EtOAc (4 × 3 ml), and the resulting organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. After evaporation, the residue was purified by silica gel column chromatography ($CHCl_3/CH_3OH$ = 40/1–10/1) to give **16**[16] (3.30 mg, 3.00 μmol, 89%) as a yellowish solid. NMR δ_H (CD_3OD): 0.78–0.91 (4H, m), 1.08–1.43 (3H, m), 1.68 (1H, m), 1.92–2.28 (14H, m), 3.41–3.87 (7H, m), 6.15 (1H, d, J = 16.0 Hz), 6.35 (1H, s), 6.42–6.45 (2H, m), 6.56–6.60 (4H, m), 7.06 (1H, d, J = 8.2 Hz), 7.67 (1H, d, J = 9.6 Hz), 8.03 (1H, s), 8.37 (2H, s), 8.64 (1H, s). HRMS m/z ($M + H^+$) Calcd. for $C_{54}H_{48}F_6N_7O_{10}S$: 1100.3082, Found: 1100.3090.

Results and Discussion

The synthesis of azide-COR (**5**) is described in Scheme 1. We achieved on improvement in product yield in the synthesis of (±)-coronafacic acid by examination of the synthetic route. For example, *exo*-selectivity in the Diels-Alder reaction was improved to *endo:exo* > 1:25 by the use of **6** as substrate,[17] and the yields in the subsequent reductions were also improved by the use of **8** or α,β-unsaturated ketone (**9**) as intermediate. Fortunately, the reduction of **9** in toluene proceeded with high stereoselectivity to give (*S*)-**10** as a single product. Protected azido-coronamic acid ((−)-**14**) can be prepared with ease according to our previous procedures,[9] using (+)-(*R*)-4-azido-1,2-butanediol[18] as starting material instead of (+)-(*R*)-1,2-butanediol. After coupling between (±)-**13** and (−)-**14**, followed by HPLC purification, (+)-azido-COR (**5**) was obtained in 10 steps with 11% yield with its diastereomer (+)-**15** (10%).[19] The enantiomer of (+)-**5** ((−)-*ent*-**5**) and its diastereomer ((−)-*ent*-**15**) were obtained similarly.

The stereochemistry of (+)-**5** and (−)-*ent*-**5** was assigned by a comparison of the $[\alpha]_D$ value together with the results of bioassay. A comparison of $[\alpha]_D$ values among (+)-**5** ($[\alpha]^{23}_D$ +69°), (−)-*ent*-**5** ($[\alpha]^{23}_D$ −72°), (+)-**15** ($[\alpha]^{23}_D$ +8.8°), and (−)-*ent*-**15** ($[\alpha]^{23}_D$ −9.0°) strongly suggested that (+)-**5** corresponds to the naturally occurring stereoisomer of COR. Moreover, (+)-**5** was as effective as naturally occurring (+)-**2** in the stomatal opening assay using *I. tricolor*, whereas (−)-*ent*-**5** was not effective in the same assay (Fig. 2). Moreover, since (+)-azide-COR (**5**) was verified to maintain the bioactivity of original **1** (Fig. 2), it can be used as a platform for chemical probes.

Easy operation using click chemistry should provide molecular probes with various tags fitting any purposes, such as fluorescence-dye for detection of bindingsites, biotin for chemiluminescence detection, and epitope peptides for immuno precipitation. The resulting **5**

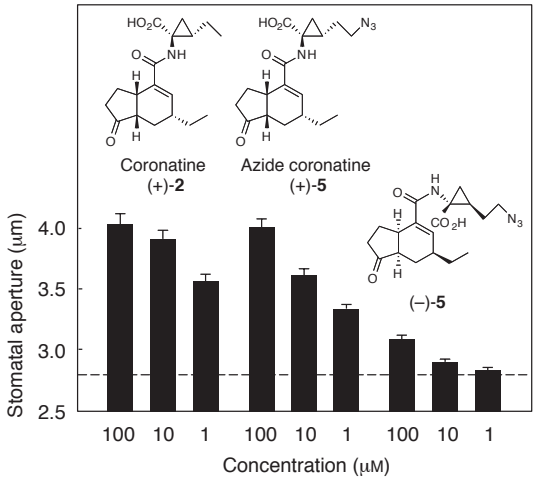


Fig. 2. Stomatal Opening Activities of (+)-Azido-Coronatine (**5**), (−)-Azido-*ent*-Coronatine (*ent*-**5**), and (+)-Coronatine (**2**) Using *Ipomoea tricolor*.

was used for CuAAC coupling with fluorescein isothiocyanate (FITC)-alkyne (**16**)[16] to give FITC-labeled COR (**17**) in almost quantitative yield (Scheme 1). Further bioorganic studies using **17** will be reported in elsewhere.

In conclusion, we have established a novel synthetic route for (+)-azido-COR (**5**) for practical use. (+)-Azido COR (**5**) was as effective as naturally occurring (+)-COR (**2**), and is a useful platform for the synthesis of various molecular probes as well as *in vivo* application using the CFCC technique.[15]

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19) Note: In ref. 9, COR (**1**) was obtained in 10 steps with *ca.* 5% yield.