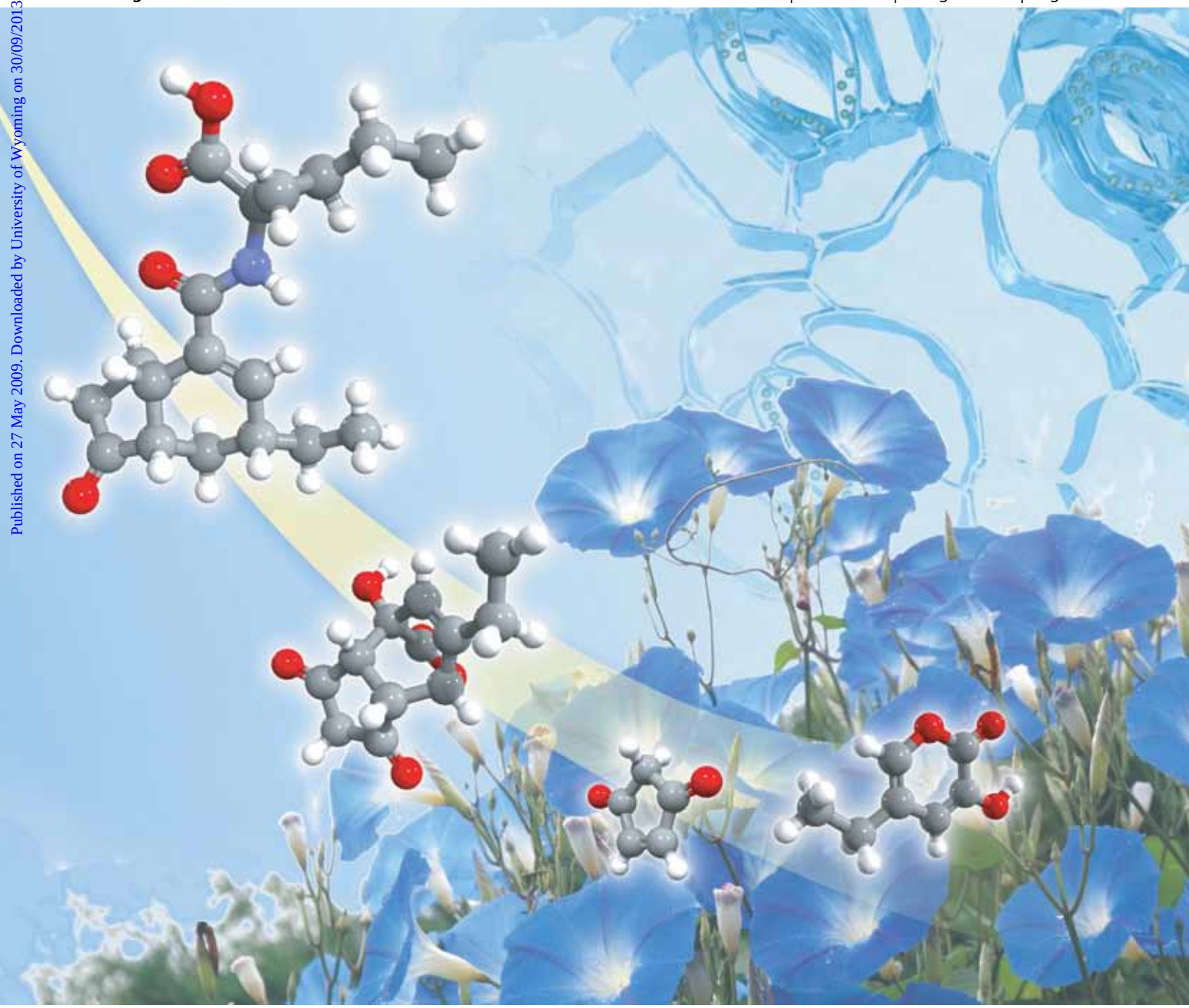
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 7 | Number 15 | 7 August 2009 | Pages 2993–3180



ISSN 1477-0520

RSC Publishing

Total syntheses of coronatines by *exo*-selective Diels–Alder reaction and their biological activities on stomatal opening†

Masahiro Okada,[a] Satoko Ito,[a] Akira Matsubara,[a] Izumi Iwakura,[b] Syusuke Egoshi[a] and Minoru Ueda*[a]

Received 13th March 2009, Accepted 17th April 2009
First published as an Advance Article on the web 27th May 2009
DOI: 10.1039/b905159g

The natural phytotoxin coronatine, which is composed of two individual parts, coronafacic acid and coronamic acid, exhibits various promising biological activities similar to jasmonic acid. Interestingly, coronatine induces stomatal opening involving the swelling of guard cells in which jasmonic acid is not involved as an endogenous regulator. We established syntheses of four stereoisomers of coronatine employing the *exo*-selective Diels–Alder reaction as a key step. Remarkable differences in stomatal opening activity were observed between enantiomers of coronatine. This result strongly suggests that the stereo structure of coronatine is very important for its stomatal opening activity. In addition, SAR studies suggested that coronatine operates as a molecular mimic of jasmonyl-L-isoleucine in plant guard cells.

Introduction

The natural phytotoxin coronatine (+)-**1** isolated from *Pseudomonas syringae* (*coronafacience*) var. *atropurpurea* induces chlorosis in the leaves of Italian rye.[1] Coronatine (+)-**1** is composed of two individual units, coronafacic acid (+)-**2** as an acid component and coronamic acid (+)-**3** as an amino acid component (Fig. 1).

Coronatine (+)-**1** has various promising biological activities including root tuberization and potato cell expansion.[2–5] Jasmonic acid (–)-**4** possesses similar biological activities but coronatine (+)-**1** usually shows greater bioactivities than (–)-**4** in various bioassays.[2] Interestingly, (+)-**1** induces swelling of the guard cells involved in stomatal opening,[6] whereas (–)-**4** is not effective in this bioassay. *cis*-12-Oxophytodienoic acid (+)-**5**, a biosynthetic precursor of (–)-**4**, also induces stomatal opening,[7] while methyl jasmonate (+)-**6** induces stomatal closing.[8] This situation is very confusing and make it desirable to reveal the mode of action of (+)-**1** in the regulation of stomatal movements.

Recently, we established a novel method for detecting the target protein of bioactive metabolites, called the enantiodifferential approach,[9] by which we can differentiate the genuine target protein which specifically recognizes the stereochemistry of the ligand from many nonspecific binding proteins in the living organisms. We planned to apply the enantiodifferential approach to the study of the chemical biology of (+)-**1**.

Firstly, it is necessary to examine the difference in bioactivity between the two enantiomers. However, there are no reports on the synthesis of enantiomeric (–)-**1**. We report herein on the syntheses of four stereoisomers of **1** including (–)-**1** and their biological activities in stomatal opening. Our synthetic route also enables further studies on the synthesis of molecular probes.

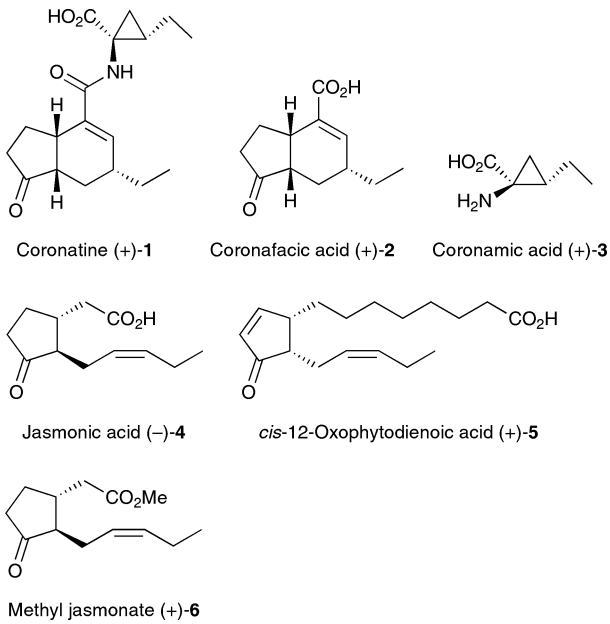


Fig. 1 Structures of coronatine (+)-**1**, jasmonic acid (–)-**4**, and their derivatives.

Result and discussion

Fifteen examples of the total synthesis of **2** have been reported,[10] but only a few examples of the total synthesis of **1** had previously been reported,[11] and there is no previously published report on the synthesis of (–)-**1**.

We planned to prepare both enantiomers of **1** from racemic coronafacic acid (±)-**2**, coronamic acid benzyl ester (+)-**7**, and its enantiomer (–)-**7**. Coupling each unit followed by HPLC separation will give four stereoisomers, (+)-**1** and its diastereomer (+)-**7**

[a]*Department of Chemistry, Tohoku University, 6-3 Aramaki-Aza Aoba, Aoba-ku, Sendai 980-8578, Japan. E-mail: ueda@mail.tains.tohoku.ac.jp; Fax: +81-22-795-6553; Tel: +81-22-795-6553*
[b]*Innovative Use of Light and Materials/Life, PREST, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

† Electronic supplementary information (ESI) available: ^{1}H NMR spectra and HPLC chromatograms. See DOI: 10.1039/b905159g

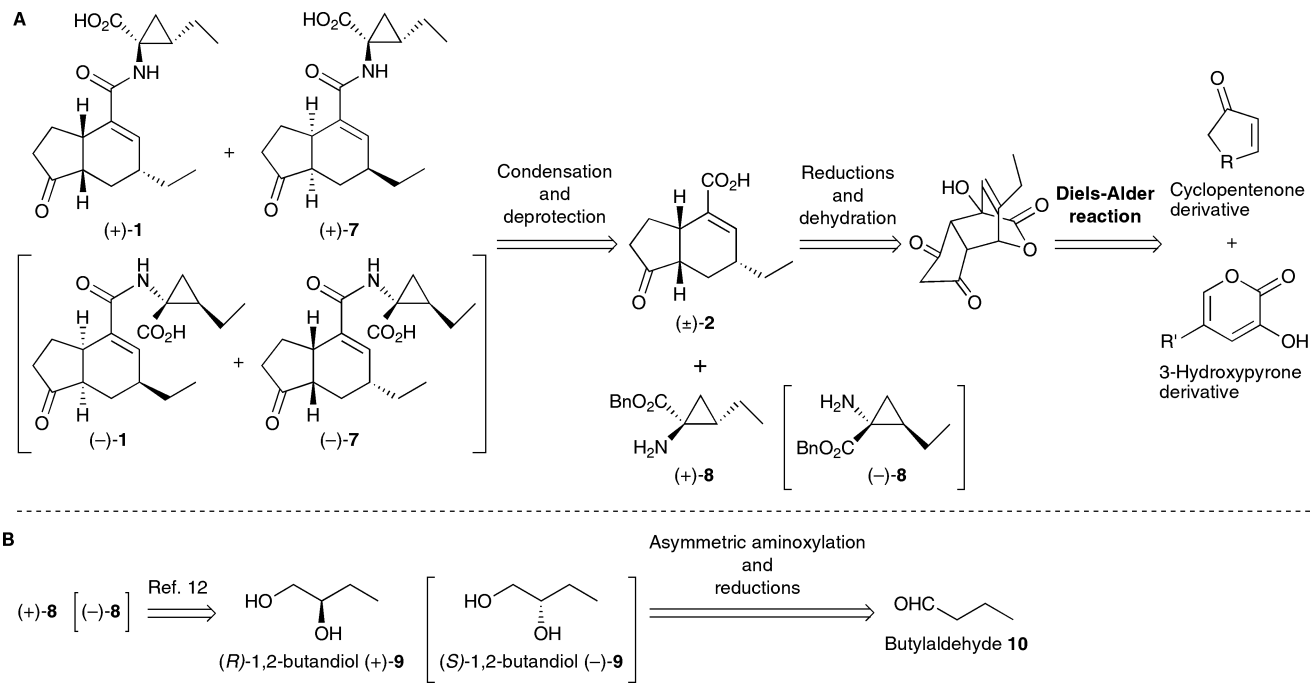


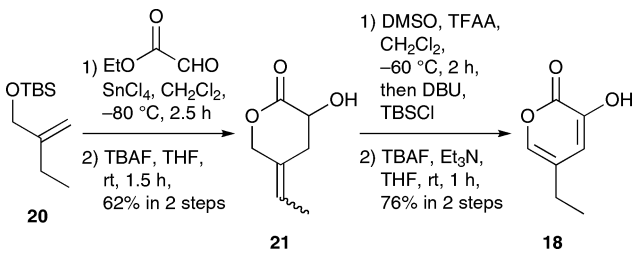
Fig. 2 Retrosynthesis of coronatine and their stereoisomers. **A**: Retrosynthesis of coronatine and their derivatives. **B**: Retrosynthesis of coronamic acid moieties.

from (±)-**2** and (+)-**8**, (−)-**1** and (−)-**7** from (±)-**2** and (−)-**8**, respectively (Fig. 2).

We planned to synthesize (±)-**2** by Diels–Alder reaction. It will be advantageous to go through a versatile intermediate for the preparation of various derivatives and the Diels–Alder reaction is very suitable for a synthesis of such intermediates. Diels–Alder reaction between a hydroxypyrone and a cyclopentenone with reduction and dehydration will afford (±)-**2** (Fig. 2A). Protected chiral coronamic acid, (+)-**8** and (−)-**8** can be synthesized respectively from (*R*)-1,2-butanediol (+)-**9** and (*S*)-1,2-butanediol (−)-**9**,[12] which can be prepared by using asymmetric α-aminoxylation of butylaldehyde (**10**) and successive reductions (Fig. 2B).[13]

We started with an investigation of the optimal conditions for the Diels–Alder reaction. As shown in Table 1, 3-hydroxypyrone (**11**) had not reacted with 2-cyclopentenone (**12**) at 100 °C after 1 day (entry 1) and the addition of triethylamine[14] resulted in the formation of the Michael-type adduct **13** (entry 2). When 4-hydroxy-2-cyclopentenone (**14**) was used as a dienophile, α,β-unsaturated ketone **15** was obtained as a Diels–Alder adduct in a low yield (entry 3). And then, when 4-cyclopentene-1,3-dione (**16**) was used (entry 4), the reaction accelerated dramatically to give Diels–Alder adduct **17** in a high yield with low stereoselectivity (*endo*:*exo* = 1:2). The stereochemistry of the resulting adduct was confirmed by the NOE relationship between H-2 and H-10. Under these conditions, we examined the Diels–Alder reaction using 5-ethyl-3-hydroxypyrone (**18**) as a dienophile, which gave the important intermediate of **2**. Diene **18** was prepared as shown in Scheme 1.

A stannic chloride mediated ene reaction between ethyl glyoxalate[15] and the TBS ether **20**,[16] deprotection of the TBS group, and intermolecular cyclization gave α-hydroxy lactone **21** as a 1:1 regioisomeric mixture. Swern oxidation and successive

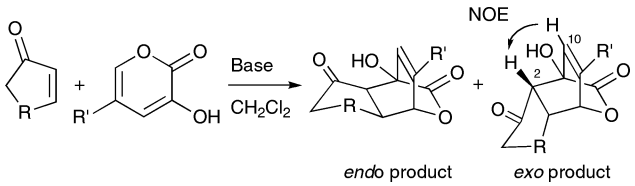


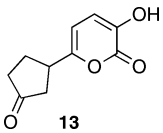
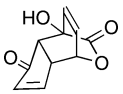
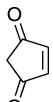
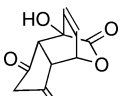
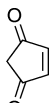
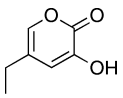
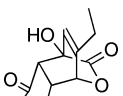
Scheme 1 Synthesis of 5-ethyl-3-hydroxypyrone **18**.

base-mediated isomerization gave **18**. The use of **18** improved the selectivity of the Diels–Alder reaction to give an *exo*-adduct selectively (*endo*:*exo* = 1:6) (Table 1, entry 5). This *exo*-selectivity was supported by DFT calculation (B3LYP/6-311+G**).[17] The ΔG* values of the *endo* transition sate (Fig 3A) and the *exo* transition state (Fig 3B) at 0 °C were calculated as 9.4 and 8.7 kcal/mol, respectively. Then the *endo* transition sate was estimated to be 0.7 kcal/mol less stable than the *exo* transition state. Thus, ratio of reaction kinetics was calculated to be 1:4. This *exo*-selectivity is most likely due to the steric hindrance between the ethyl group in **18** and the CH_2 group in **16** in transition state (Fig. 3).

Next, we tried to optimize the regioselective reduction of **19** (Scheme 2). Since direct reduction of **19** gave a complex mixture, the reduction was carried out on a vinylchloride intermediate. Triphosgene-mediated carbocyclization and successive *in situ* chlorination gave the relatively stable chlorovinylether **22** in a 31% yield with 28% recovered **19**. Hydrogenolysis of **22** resulted in stepwise reductions to give the desired monoketone **23** in a 74% yield with high stereoselectivity (11*S**:11*R** = 7:1). The stereochemistry of **23** was determined by the NOE between H-6 and H-11 (Scheme 2). Ring-opening of **23** by β-elimination was then carried out under basic conditions, followed by hydrogenolysis

Table 1 Base-mediated Diels–Alder reactions between 3-hydroxypyrone derivatives and 2-cyclopentenone derivatives



Entry	Dienophile	Diene	Base	Conditions	Product[a]	Yield[b] (*endo*:*exo*)
1	 **11**	 **12**	None	100 °C[c], 1 day	No reaction	
2	 **11**	 **12**	Et_3N (1 eq)	100 °C[c], 1 h	 **13**	60%
3	 **14**	 **12**	Et_3N (1 eq)	rt, 1 day	 **15**	21% (1:1)
4	 **16**	 **12**	Et_3N (1 eq)	rt, 10 min	 **17**	96%[d] (1:1.5)
5	 **16**	 **18**	Et_3N (1 eq)	0 °C, 10 min	 **19**	93%[d] (1:6)

[a] Diels–Alder adduct represented as *exo* product. [b] Isolation yield. The ratio of *endo*:*exo* was determined by NMR. [c] The reaction was carried out in a sealed tube. [d] The product was obtained as a Et_3N salt.

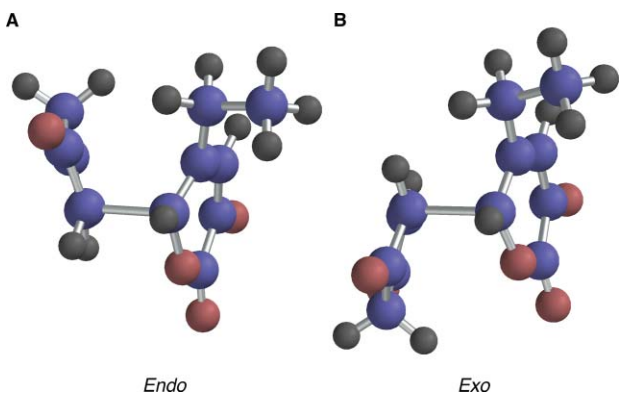
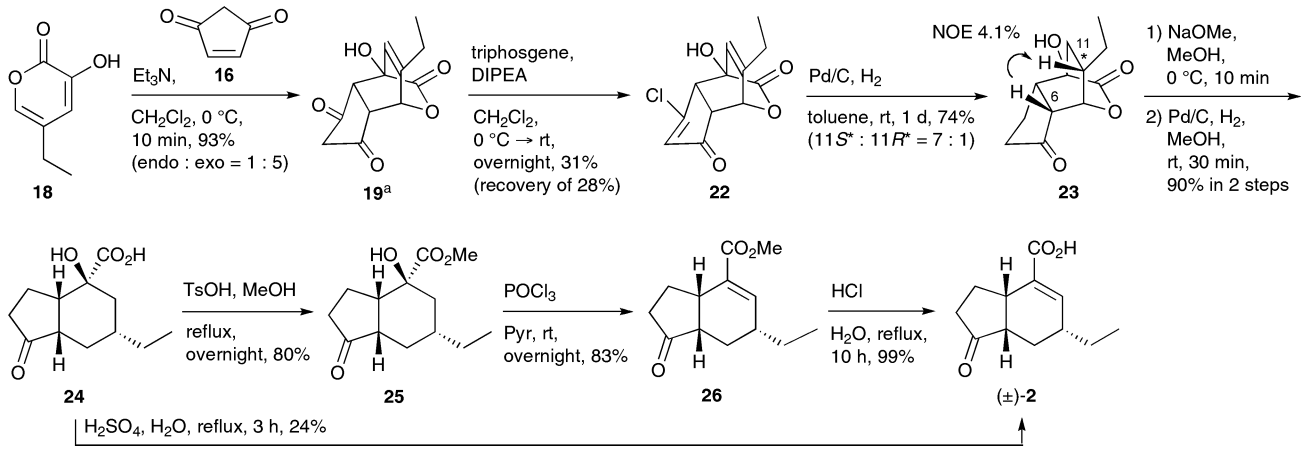


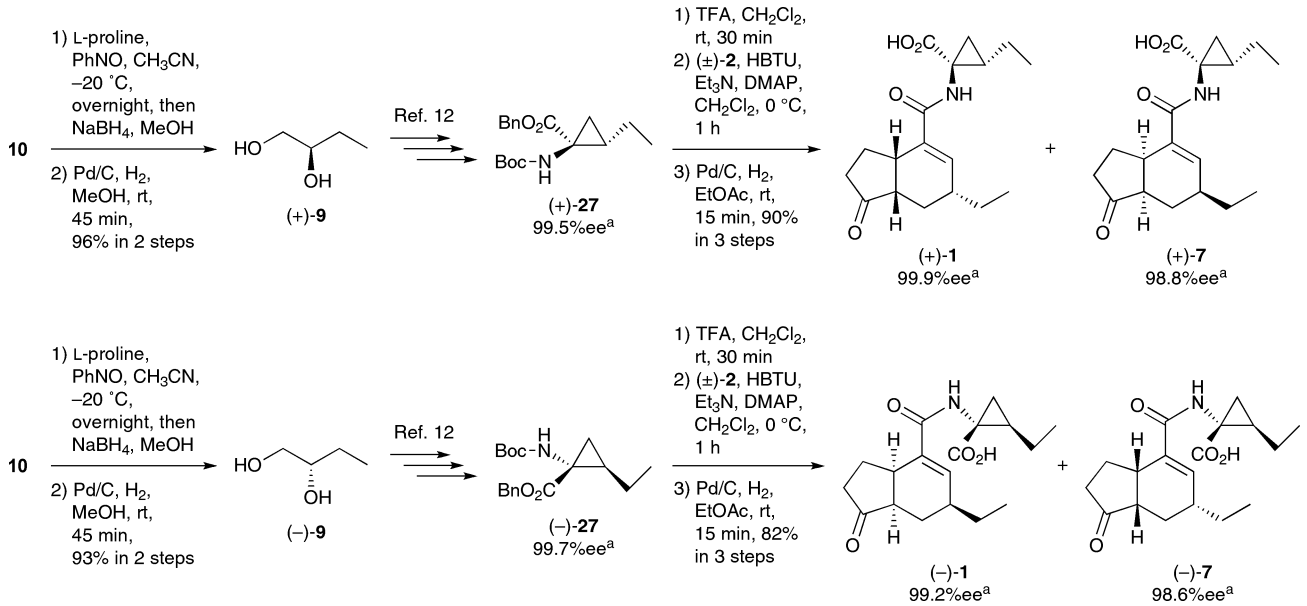
Fig. 3 The transition states for the Diels–Alder reaction between **16** and **18**. **A**: *Endo* transition state of **16** and **18**. **B**: *Exo* transition state of **16** and **18**.

to give **24**. Finally, methylesterification of **24**, dehydration of the resulting **25** with phosphorus oxychloride, and hydrolysis under acidic conditions gave (±)-**2**. Alternatively, sulfuric acid mediated dehydration of **24** also gave (±)-**2** in a single step, but the total yield was lower than that of the three-step procedure. Our synthetic route to (±)-**2** enables the preparation of various derivatives of **2**, including molecular probes. This is because the functional groups in the versatile intermediate **19**, such as the hydroxyl or carbonyl groups, can be used as a handle for further structural variations.

On the other hand, both enantiomers of the coronamic acid region, (+)-**27** and (−)-**27**, were synthesized from (+)-**9** and (−)-**9**, respectively (Scheme 3).[12] Enantioselective synthesis of (+)-**9** [or (−)-**9**] was carried out by asymmetric α-aminooxylation of butylaldehyde (**10**) using L-proline (or D-proline) as an organocatalyst and two successive reductions (Scheme 3).[13] Optical purities of both (+)-**27** and (−)-**27** were confirmed by chiral HPLC analyses to be 99.5%ee and 99.7%ee, respectively. This versatile method enabled the efficient preparation of optically pure coronamic acid (+)-**3** and its enantiomer (−)-**3** from common starting materials, and the preparation of the various derivatives. After deprotection of the Boc group, each enantiomer of **27** was condensed with (±)-**2** and successive deprotection of the benzyl group gave (+)-**1**



Scheme 2 Synthesis of coronafacic acid (±)-**2**. [a]The product was obtained as a Et_3N salt.



Scheme 3 Synthesis of naturally occurring coronatine (+)-**1** and stereoisomers (–)-**1**, (+)-**7**, and (–)-**7**. [a]Enantio excess was calculated by chiral HPLC analysis.

and (+)-**7**, or (–)-**1** and (–)-**7**, respectively (Scheme 3). The resulting mixture of two diastereomers was separated by HPLC. The stereochemistry of isolated (+)-**1** or (–)-**1** was confirmed by the comparison with spectral data of (+)-**1**, such as ^{1}H NMR, ^{13}C NMR, HRMS, and optical rotation, and another isolated diastereomer was identified as (+)-**7** or (–)-**7**. Successive chiral HPLC analyses under optimized conditions revealed the optical purities of four synthetic compounds; (+)-**1**, (–)-**1**, (+)-**7**, and (–)-**7** were 99.9%ee, 99.7%ee, 98.8%ee, and 98.6%ee, respectively.

We examined the bioactivity of these synthetic derivatives [(+)-**1** and (+)-**7**, or (–)-**1** and (–)-**7**] for stomatal opening of a morning glory, *Ipomoea tricolor* (Fig. 3).[7] Simultaneously, we examined the stomatal opening activities of racemic coronafacic acid (±)-**2**, naturally occurring coronamic acid (+)-**3**, racemic jasmonic acid (±)-**4**, and naturally occurring jasmonyl-L-isoleucine (–)-**28** which is considered to be an endogenous bioactive form of jasmonate in signal transduction.[18] Naturally occurring coronatine (+)-**1** induced significant stomatal opening at 1 μM in the dark, while (–)-**1** was not effective even at 100 μM. This result revealed that the stereochemistry of **1** is important for stomatal opening activity, and suggests the involvement of a specific target protein recognizing the stereochemistry of **1**. Additionally, (±)-**2** and (+)-**3** did not induce stomatal opening even at 100 μM, but (–)-**7** showed moderate biological activity at 10 μM. Thus, the coupling of the two components is indispensable for this bioactivity. Interestingly, (+)-**7** showed stomatal opening activity at 100 μM and was one-tenth as effective as (–)-**7**. These results suggest that the coronafacic acid region does not play a definitive role in stomatal-opening activity. It should be emphasized that (–)-**28** showed moderate bioactivity at 10 μM, but (±)-**4** did not induce stomatal opening even at 100 μM. The order of bioactivities is summarized as follows; (+)-**1** > (–)-**7** = (–)-**28** > (+)-**7** >> (–)-**1** = (±)-**2** = (+)-**3** = (±)-**4**. Earlier structure-activity-relationship studies on (+)-**1** and (±)-**4** also reported on tuber-inducing activity and expansion-inducing activity in potato tubers cells.[2] In these cases, (±)-**2** like (±)-**4** showed moderate tuber-inducing bioactivity

as opposed to stomatal opening activity. These results indicate that the pattern of specific interaction of coronatine in plant guard cells is different from that in typical jasmonic acid responses. These results suggested that (+)-**1** operates as a mimic for (−)-**28**, and not for naturally occurring (−)-**4**.

Conclusions

In summary, we established unique syntheses of coronatine (+)-**1** with its three stereoisomers, (−)-**1**, (+)-**7**, and (−)-**7**, using the Diels–Alder reaction. Stomatal opening activities of these synthetic derivatives indicate that the stereochemistry of **1** is important for stomatal opening activity and that our enantiodifferential approach is applicable to the study of the chemical biology of coronatine (+)-**1**. Structure-activity relationship studies of **1** suggest that (−)-**1** operates as a mimic of jasmonyl-L-isoleucine (−)-**32** in the plant guard cells. The synthesis of molecular probes based on a pair of enantiomers of **1** and the enantiodifferential approach for the identification of its target protein will be reported in due course.

Experimental section

General

NMR spectra of synthetic compounds were recorded on a Jeol JNM-Lambda400 [^{1}H (400 MHz) and ^{13}C (100 MHz), Jeol Inc., Japan] using TMS as internal standards. The ESI-MS spectra was recorded on a Bruker Esquire 4000 (Bruker Daltonics Inc., Germany). The HRMS spectra of ESI-TOF and DART-TOF were recorded on a Bruker APEX-III (Bruker Daltonics Inc., Germany) and on a DART (Jeol Inc., Japan), respectively. The IR spectra were recorded on a JASCO FT/IR-410 (JASCO Inc., Japan). The specific rotations were measured by a JASCO DIP-360 polarimeter (JASCO Inc., Japan). Elemental analyses were carried out on a CHN-corder MT-6 (Yanako Co., Ltd., Japan). Silica gel column chromatography was performed on Silica Gel 60 N (Kanto Chemical Co. Ltd., Japan). TLC was performed on Silica gel F254 (0.25 mm or 0.5 mm, MERCK, Germany) or RP-18F254S (0.25 mm, MERCK). HPLC purification and chiral analysis were performed on a Jasco LC-2075 series HPLC system (JASCO Inc., Japan). Reagents and solvents were purchased from Kanto Chemical Co. Ltd., Japan and Wako Pure Chemical Industries Co. Ltd., Japan. Racemic coronatin was purchased from Sigma-Aldrich Japan Co. Ltd., Japan.

Preparation of 3-hydroxypyrone derivatives

5-Ethylidene-3-hydroxytetrahydro-2*H*-pyran-2-one 21. The starting compound **20**[16] was easily prepared by reduction of 2-methylenebutanal, followed by protection of the resulting alcohol with TBSCl and imidazole. To a solution of **20** (1.90 g, 9.50 mmol) and ethylglyoxalate (1.02 g, 10.6 mmol) in CH_2Cl_2 (105 mL) was slowly added $SnCl_4$ (2.78 g, 10.6 mmol) at −80 °C under argon atmosphere. After being stirred at −80 °C for 2.5 h, the reaction mixture was quenched by aqueous $NaHCO_3$. The mixture was extracted with EtOAc (4 × 100 mL). The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give the ene product (2.85 g, 9.42 mmol, 99%) as a colorless oil. The product was carried on to the next step without further purification. To a solution of the product (1.23 g, 4.00 mmol) in THF (40 mL) at room temperature was added 1 M TBAF in THF (1.4 mL, 1.4 mmol). After being stirred for 1.5 h, the reaction mixture was quenched with H_2O. Then the mixture was extracted with EtOAc (4 × 50 mL). The 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 = 5/1) to give **21** (352 mg, 2.47 mmol, 62%) as a colorless oil, which was a 1:1 regioisomeric mixture; IR (film) 3410, 1749, 1444, 1377, 1338, 1301, 1244, 1213, 1176, 1084, 857, 796, 754, 721, 589, 462 cm^{-1}; *m*/*z* 143.0740 [M + H]$^+$, $C_7H_{10}O_3$ requires 143.0708. (*E*)-isomer; δ_H ($CDCl_3$) 5.63 (1H, m), 5.00 (1H, d, *J* = 14.2 Hz), 4.82 (1H, d, *J* = 14.2 Hz), 4.35 (1H, dd, *J* = 12.6, 7.1 Hz), 3.36 (1H, br s), 2.99 (1H, dd, *J* = 14.9, 7.1 Hz), 2.51 (1H, dd, *J* = 14.9, 12.6 Hz), 1.65 (3H, d, *J* = 7.6 Hz); δ_C ($CDCl_3$) 175.7, 127.2, 124.4, 72.0, 66.4, 30.9, 13.4. (*Z*)-isomer; δ_H ($CDCl_3$) 5.65 (1H, m), 4.82 (1H, d, *J* = 13.4 Hz), 4.59 (1H, d, *J* = 13.4 Hz), 4.51 (1H, dd, *J* = 12.0, 8.5 Hz), 3.41 (1H, br s), 3.16 (1H, dd, *J* = 16.4, 8.5 Hz), 2.32 (1H, dd, *J* = 16.4, 12.0 Hz), 1.65 (3H, d, *J* = 7.6 Hz); δ_C ($CDCl_3$) 175.5, 126.5, 123.4, 67.5, 65.5, 35.5, 12.9.

5-Ethyl-3-hydroxy-2*H*-pyran-2-one 18. To a solution of DMSO (352 mg, 4.51 mmol) in CH_2Cl_2 (9.0 mL) was added trifluoroacetic anhydride (574 mg, 2.73 mmol) at −60 °C under argon atmosphere. After the reaction mixture was stirred at −60 °C for 1 h, **21** (125 mg, 0.881 mmol) was slowly added. After the reaction mixture was stirred at −60 °C for 1 h, DBU (3.05 g, 30.1 mmol) was slowly added. The mixture was gradually warmed to −35 °C for 1 h with stirring, and then TBSCl (267 mg, 1.77 mol) was slowly added. After stirring overnight, the reaction mixture was quenched with saturated aqueous NH_4Cl. The mixture was extracted with EtOAc (4 × 10 mL). The 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/acetone = 50/1–10/1) to give oxidized compound. The compound was carried on to the next step immediately. To the residue at room temperature was added 1 M TBAF in THF (1.40 mL, 1.40 mmol). After stirring for 50 min, Et_3N (218 mg, 2.15 mmol) was added. After the reaction mixture was stirred for 10 min, Et_2O (10 mL) was added. The mixture was extracted with 1 M aqueous Na_2CO_3 (4 × 10 mL). The extracted water layer was acidified with $KHSO_4$, and then it was extracted with EtOAc (3 × 10 mL). The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4 saturated aqueous NaCl, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give **18** (93.5 mg, 0.667 mmol, 76%) as a yellow crystalline solid; δ_H ($CDCl_3$) 6.98 (1H, d, *J* = 2.0 Hz), 6.64 (1H, d, *J* = 2.0 Hz), 6.49 (1H, br s), 2.33 (2H, q, *J* = 7.6 Hz), 1.16 (3H, t, *J* = 7.6 Hz); δ_C ($CDCl_3$) 161.5, 141.7, 137.8, 122.3, 117.0, 23.0, 13.7; IR (film) 3325, 1696, 1642, 1562, 1461, 1431, 1370, 1330, 1295, 1252, 1142, 1115, 1051, 943, 773, 483 cm^{-1}; *m*/*z* 141.0588 [M + H]$^+$, $C_7H_9O_3$ requires 141.0552.

Typical procedure for Diels–Alder reaction

To a solution of 3-hydroxypyrone derivative (1.00 mmol) and cyclopentenone derivative (1.00 mmol) in CH_2Cl_2 (10 mL) was added Et_3N (1.00 mmol) at the appropriate temperature under

argon atmosphere (in a sealed tube if necessary). The reaction mixture was stirred until starting materials had disappeared on a TLC analysis, and then evaporated to dryness. The residue was purified by silica gel column chromatography with $CHCl_3$/MeOH solvent system containing 0.1% Et_3N to give the Diels–Alder adduct.

***rel*-(1*S*,2*S*,6*R*,7*R*)-11-Ethyl-1-hydroxy-8-oxa-tricyclo[5.2.2.0^{2,6}]-undeca-10-ene-3,5,9-trione 19 (from 16 and 18).** δ_H (CD_3OD) 5.97 (1H, q, J = 2.0 Hz), 5.22 (1H, s), 5.17 (1H, t, J = 2.0 Hz), 3.34 (1H, s), 3.16 (1H, dd, J = 7.3, 2.0 Hz), 3.02 (1H, d, J = 7.3 Hz), 2.30 (2H, qd, J = 7.3, 2.0 Hz), 1.10 (3H, t, J = 7.3 Hz); δ_C (CD_3OD) 197.8, 197.8, 174.9, 147.8, 130.0, 109.1, 78.8, 78.2, 51.4, 50.6, 23.4, 11.5; IR (film) 2969, 2523, 1767, 1752, 1653, 1560, 1387, 1297, 1140, 961 cm^{-1}; m/z 237.0774 [M + H]$^+$, $C_{12}H_{12}O_5$ requires 237.0763.

Synthesis of coronafacic acid

***rel*-(1*S*,2*S*,6*R*,7*R*)-3-Chloro-11-ethyl-1-hydroxy-8-oxa-tricyclo[5.2.2.0^{2,6}]undeca-3,10-diene-5,9-dione 22.** To a solution of **19** (104 mg, 0.308 mmol) in CH_2Cl_2 (3.0 mL) was added triphosgene (54.3 mg, 0.183 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred for 10 min, and then DIPEA (39.8 mg, 0.308 mmol) was slowly added. Then the reaction mixture was gradually warmed to room temperature and stirred for overnight. The reaction mixture was evaporated to dryness, and the residue was purified by silica gel column chromatography (*n*-hexane/acetone = 16/1–1/1) to give **22** (24.1 mg, 94.6 mmol, 31%) as a pale yellow oil and recovered **19** (29.6 mg, 87.7 mmol, 28%); δ_H ($CDCl_3$) 6.35 (1H, d, J = 1.0 Hz), 6.01 (1H, q, J = 1.9 Hz), 5.22 (1H, t, J = 1.9 Hz), 3.77 (1H, br s), 3.44 (1H, dd, J = 7.3, 1.0 Hz), 2.98 (1H, dd, J = 7.3, 1.9 Hz), 2.26 (1H, m), 1.06 (3H, t, J = 7.3 Hz); $^1\delta_C$ ($CDCl_3$) 198.9, 172.7, 169.9, 145.9, 135.4, 128.1, 78.0, 77.9, 53.5, 51.3, 24.4, 10.9; m/z 255.0414 [M + H]$^+$, $C_{12}H_{11}ClO_4$ requires 255.0424.

***rel*-(1*S*,2*S*,6*R*,7*S*,11*S*)-11-Ethyl-1-hydroxy-8-oxa-tricyclo-[5.2.2.0^{2,6}]undecane-5,9-dione 23.** To a solution of **22** (6.00 mg, 23.6 mmol) in toluene (1.0 mL) under argon atmosphere was added 10% Pd/C (7.70 mg, 7.24 mmol). The atmosphere was displaced with hydrogen, and the reaction mixture was stirred for 1 day at rt. After filtration with Celite, the filtrate was evaporated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 1/1) to give a 7:1 mixture of two stereoisomers of **23** (3.90 mg, 17.4 mmol, 74%, 11*S**:11*R** = 7:1) as a pale yellow oil; δ_H ($CDCl_3$) 4.77 (1H, s), 3.88 (1H, br s), 2.86 (1H, m), 2.79 (1H, dd, J = 7.8, 1.9 Hz), 2.71 (1H, d, J = 7.8 Hz), 2.27–2.09 (4H, m), 1.87–1.73 (2H, m), 1.47–1.25 (3H, m), 0.87 (3H, t, J = 7.3 Hz); δ_C ($CDCl_3$) 216.3, 176.7, 78.2, 72.6, 50.6, 41.6, 39.1, 37.3, 37.0, 26.3, 21.7, 11.0; IR (film) 3452, 1741, 1460, 1407, 1370, 1312, 1264, 1219, 1009, 960, 754 cm^{-1}; m/z 225.1098 [M + H]$^+$, $C_{12}H_{17}O_4$ requires 225.1121.

***rel*-(1*S*,2*S*,4*R*,6*S*)-4-Ethyl-2-hydroxy-7-oxo-bicyclo[4.3.0]-nonane-2-carboxylic acid 24.** To a solution of **23** (176 mg, 0.785 mmol) in MeOH (7.9 mL) was added NaOMe (42.4 mg, 7.52 mmol) at 0 °C under argon atmosphere. After the reaction mixture was stirred for 10 min, the reaction mixture was quenched with 5% aqueous $KHSO_4$. The mixture was extracted with EtOAc (3 × 50 mL). The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. After evaporation, to a solution of the residue in MeOH (7.9 mL) was added 10% Pd/C (40.2 mg, 0.378 mmol) under argon atmosphere. The atmosphere was displaced with hydrogen, and then the reaction mixture was stirred for 30 min at rt. After filtration with Celite, the filtrate was evaporated to give **24** (160 mg, 0.709 mmol, 90%). The resulting **24** was carried on to the next step without further purification because **24** was pure enough and highly polar; δ_H ($CDCl_3$) 2.61 (1H, dt, J = 12.5, 6.3 Hz), 2.46 (1H, dd, J = 19.3, 8.8 Hz), 2.32 (1H, dt, J = 12.7, 6.3 Hz), 2.19 (1H, dt, J = 19.3, 9.9 Hz), 1.98–1.91 (2H, m), 1.82–1.67 (5H, m), 1.35–1.25 (2H, m), 0.92 (3H, t, J = 7.5 Hz); δ_C ($CDCl_3$) 220.6, 178.5, 75.2, 46.7, 45.0, 37.0, 35.1, 32.0, 29.1, 28.0, 22.9, 10.9; IR (film) 3461, 2957, 1737, 1439, 1252, 1163, 1032 cm^{-1}; m/z 226.1205 [M + H]$^+$, $C_{12}H_{18}O_4$ requires 226.1205.

Methyl *rel*-(1*S*,2*S*,4*R*,6*S*)-4-ethyl-2-hydroxy-7-oxo-bicyclo-[4.3.0]nonane-2-carboxylate 25. To a solution of **24** (5.8 mg, 25.6 mmol) in MeOH (0.5 mL) was added $TsOH{\cdot}H_2O$ (3.4 mg, 17.9 mmol). After the reaction mixture was stirred and refluxed for one day, it was allowed to stand at room temperature. The reaction was stopped by the addition of aqueous $NaHCO_3$. Then the 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/acetone = 30/1–20/1) to give **25** (4.9 mg, 20.6 mmol, 80%) as a colorless oil; δ_H ($CDCl_3$) 3.81 (3H, s), 2.60 (1H, dt, J = 13.2, 6.2 Hz), 2.43 (1H, dd, J = 19.0, 8.4 Hz), 2.28 (1H, dt, J = 12.8, 6.2 Hz), 2.16 (1H, dt, J = 19.0, 10.0 Hz), 1.89 (1H, m), 1.81–1.61 (5H, m), 1.36–1.23 (2H, m), 0.91 (3H, t, J = 7.4 Hz); δ_C ($CDCl_3$) 219.0, 175.8, 75.2, 52.4, 46.6, 45.8, 36.9, 35.2, 32.0, 29.2, 28.1, 22.8, 10.9; IR (film) 3461, 2957, 1737, 1439, 1252, 1163, 1032 cm^{-1}; m/z 241.1452 [M + H]$^+$, $C_{13}H_{21}O_4$ requires 241.1440.

Methyl *rel*-(1*S*,4*R*,6*S*)-4-ethyl-7-oxo-bicyclo[4.3.0]non-2-ene-2-carboxylate 26. To a solution of **25** (98.9 mg, 0.412 mmol) in pyridine (4.0 mL) was added phosphorus oxychloride (1.32 g, 8.58 mmol) at 0 °C under argon atmosphere. The reaction mixture was gradually warmed to room temperature with overnight stirring. The reaction mixture was quenched with slow addition of cold H_2O, and then extracted with Et_2O (4 × 50 mL). 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/acetone = 60/1–16/1) to give **26** (76.2 mg, 0343 mmol, 83%) as a colorless oil. All spectral data of **26** were identical to those of reported one.[11]

Coronafacic acid (±)-2 (from 26). A suspension of **26** (69.6 mg, 0.313 mmol) in 3 M aqueous HCl (3.1 mL) was refluxed for 10 h. After the reaction mixture was quenched with H_2O, the mixture was extracted with EtOAc (4 × 10 mL). The resulting organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered. After evaporation, coronafacic acid (±)-**2** (64.6 mg, 0.310 mmol, 99%) was obtained as a colorless crystalline solid. All spectral data of (±)-**2** were identical to those reported.[11]

Coronafacic acid (±)-2 (from 24). To a suspension of **24** (2.3 mg, 10.2 mmol) in H_2O (0.5 mL) was added 98% H_2SO_4 (0.5 mL). After being refluxed for 3 h, the reaction mixture was quenched with H_2O. The mixture was extracted with EtOAc (4 × 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 preparative TLC ($CHCl_3$/MeOH = 10/1) to give (±)-**2** (0.50 mg, 2.40 mmol, 24%) as a colorless crystalline solid. All spectral data of (±)-**2** were identical to those reported.[11]

Synthesis of coronamic acid

(*R*)-1,2-Butanediol (+)-9. To a solution of butylaldehyde (**10**) (11.1 g, 153 mmol) and nitrosobenzene (5.48 g, 51.2 mmol) in CH_3CN (306 mL) was added L-proline (17.7 g, 154 mmol) at –20 °C. After the reaction mixture was stirred for 1 day, $NaBH_4$ (9.70 g, 256 mmol) in MeOH (102 mL) was added. Then the mixture was stirred for 40 min. The reaction mixture was quenched with 0.1 M potassium phosphate buffer (pH 7.0), and then extracted with EtOAc (4 × 200 mL). 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 15/1–1/1) to give α-aminoxylated product. The product was dissolved in MeOH (300 mL) and then was added 10% Pd/C (1.10 g, 0.945 mmol) to the solution under argon. The atmosphere was displaced with hydrogen, and the reaction mixture was stirred for 2 h. After filtration with Celite, the filtrate was evaporated to give (+)-**9** (4.43 g, 49.2 mmol, 96% in 2 steps) as a colorless oil. The resulting (+)-**9** was carried on to the next step without further purification because of its hygroscopicity. All spectral data of (+)-**9** were identical to those reported;[12] $[\alpha]_D^{19}$ +15.1 (*c* 1.4, EtOH).

(*S*)-1,2-Butanediol (–)-9. According to the above mentioned procedure, butylaldehyde (644 mg, 8.93 mmol), nitrosobenzene (314 mg, 2.93 mmol), and D-proline (100 mg, 0.880 mmol) gave (–)-**9** (246 mg, 2.73 mmol, 93% in 2 steps) as a colorless oil. All spectral data of (–)-**9** were identical to those reported;[12] $[\alpha]_D^{20}$ –15.4 (*c* 1.0, EtOH).

Boc-coronamic acid benzyl ester (+)-27 and (–)-27. According to the literature procedure,[12] (+)-**27** and (–)-**27** were synthesized from (+)-**9** and (–)-**9**, respectively. All spectral data of (+)-**27** and (–)-**27** were identical to those reported.[12] (+)-**27**; $[\alpha]^{25}_D$ +15.4 (*c* 0.55, $CHCl_3$), (–)-**27**; $[\alpha]^{26}_D$ –15.6 (*c* 0.50, $CHCl_3$). These optical purities were analyzed by chiral HPLC method using DAICEL Chiralcel OD (4.6 × 250 mm, DAICEL Co., Ltd., Japan) with 98% *n*-hexane-2% *i*-PrOH at 1.0 mL/min. Under these conditions, the analysis gave good separation of each enantiomer: (–)-**27** at R_t = 9.1 min and (+)-**27** at R_t = 10.5 min. Enantiomeric excess was calculated from the ratio of peak areas (mAu s) at 210 nm. Chiral HPLC analysis with 2 ng of the synthetic (+)-**27** gave a ratio of 6.10:2260 = (–)-**27**:(+)-**27**, which corresponded to 99.5%ee. According to the above mentioned procedure, chiral HPLC analysis of 4 ng of the synthetic (–)-**27** gave a ratio of 4340:6.28 = (–)-**27** : (+)-**27**, which corresponded to 99.7%ee.

Synthesis of coronatine and stereoisomers

Coronatine (+)-1 and its diastereomer (+)-7. To a solution of (+)-**27** (42.1 mg, 132 mmol) in CH_2Cl_2 (0.50 mL) was added TFA (0.5 mL) at 0 °C under argon atmosphere. After being stirred for 1 h, the reaction mixture was evaporated, and then the residue was dissolved in CH_2Cl_2 (0.88 mL) at 0 °C under argon atmosphere. To the solution were added TEA (26.7 mg, 264 mmol), DMAP (3.3 mg, 27.0 mmol), HBTU (66.7 mg, 176 mmol), and coronafcic acid (±)-**2** (18.3 mg, 87.9 mmol). After being stirred for 30 min, the reaction mixture was quenched with 5% aqueous $KHSO_4$. The mixture was extracted with EtOAc (3 × 10 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 = 16/1–1/1) to give a mixture of condensed compounds. The mixture was carried on to the next step immediately without further purification. The mixture was dissolved in EtOAc (1.6 mL) under argon and 10% Pd/C (16.4 mg, 15.4 mmol) was added. The atmosphere was displaced with hydrogen, and the reaction mixture was stirred for 1 h. After filtration with Celite, the filtrate was evaporated to dryness. A mixture of coronatine (+)-**1** and (+)-**7** (25.3 mg, 79.2 mmol, 90% in 3 steps) was obtained as a colorless crystalline solid.

Enantiomer of coronatine (–)-1 and its diastereomer (–)-7. According to the above mentioned procedure, (–)-**27** (23.8 mg, 75.4 mmol) and (±)-**2** (11.0 mg, 52.8 mmol) gave a mixture of (–)-**1** and (–)-**7** (13.9 mg, 43.5 mmol, 82% in 3 steps) as a crystalline solid.

HPLC separation

A mixture of coronatine (+)-**1** and its diastereomer (+)-**7** was separated by HPLC on a triacontyl column (Develosil RPAQUEOUS, 20 × 250 mm, Nomura Chemical Co., Ltd.) with 30% aqueous CH_3CN containing 0.1% TFA at 8.0 mL/min to give coronatine (+)-**1** (R_t = 80–82 min) and (+)-**7** (R_t = 83–85 min), respectively. Each solution was carefully concentrated *in vacuo* with attention to decomposition under acidic condition. A mixture of (–)-**1** and (–)-**7** was separated similarly by HPLC. All spectral data of coronatine (+)-**1** and its enantiomer (–)-**1** were identical those reported.[11]

Coronatine (+)-1. δ_H ($CDCl_3$) 6.47 (1H, br s), 6.35 (1H, s), 3.15 (1H, dt, *J* = 12.0, 6.1 Hz), 2.46 (1H, dt, *J* = 12.0, 6.1 Hz), 2.42–2.23 (3H, m), 2.21–2.11 (1H, m), 1.89 (1H, dt, *J* = 13.0, 4.8 Hz), 1.70–1.44 (6H, m), 1.39 (1H, dq, *J* = 14.6, 7.3 Hz), 1.31 (1H, dd, *J* = 8.8, 5.2 Hz), 1.06 (1H, td, *J* = 13.0, 10.7 Hz), 1.03 (3H, t, *J* = 7.3 Hz), 0.98 (3H, t, *J* = 7.5 Hz); δ_C ($CDCl_3$) 219.9, 174.2, 170.5, 137.8, 135.3, 46.4, 38.9, 38.1, 37.4, 36.2, 33.7, 28.1, 27.9, 26.0, 22.3, 20.8, 13.4, 11.3; IR (film) 3336, 2963, 2875, 1719, 1662, 1626, 1515, 1462, 1271, 1186, 1143, 755 cm^{-1}; $[\alpha]_D^{23}$ +73.1 (*c* 0.40, MeOH); *m/z* 320.1872 $[M + H]^+$, $C_{18}H_{26}NO_4$ requires 320.1862.

Enantiomer of coronatine (–)-1. $[\alpha]^{25}_D$ –74.2 (*c* 0.30, MeOH).

Diastereomer of coronatine (+)-7. δ_H ($CDCl_3$) 6.52 (1H, br s), 6.38 (1H, s), 3.16 (1H, dt, *J* = 12.9, 6.2 Hz), 2.45 (1H, dd, *J* = 12.9, 6.2 Hz), 2.41–2.25 (3H, m), 2.22–2.12 (1H, m), 1.89 (1H, dt, *J* = 13.0, 4.8 Hz), 1.70–1.45 (6H, m), 1.39 (1H, dq, *J* = 14.7, 7.3 Hz), 1.30 (1H, dd, *J* = 9.3, 5.5 Hz), 1.07 (1H, td, *J* = 13.0, 11.2 Hz), 1.04 (3H, t, *J* = 7.3 Hz), 0.98 (3H, t, *J* = 7.5 Hz); δ_C ($CDCl_3$) 219.9, 173.9, 170.8, 138.5, 135.1, 46.4, 39.1, 38.1, 37.4, 36.1, 33.7, 28.0, 27.7, 25.9, 22.0, 20.9, 13.4, 11.3; IR (film) 3323, 2926, 2878, 1732, 1660, 1624, 1519, 1462, 1272, 1187, 1144, 753 cm^{-1}; $[\alpha]_D^{22}$ +9.3 (*c* 0.50, MeOH); *m/z* 320.1875 $[M + H]^+$, $C_{18}H_{26}NO_4$ requires 320.1862.

Enantiomer of (–)-7. $[\alpha]^{26}_D$ –9.1 (*c* 0.20, MeOH).

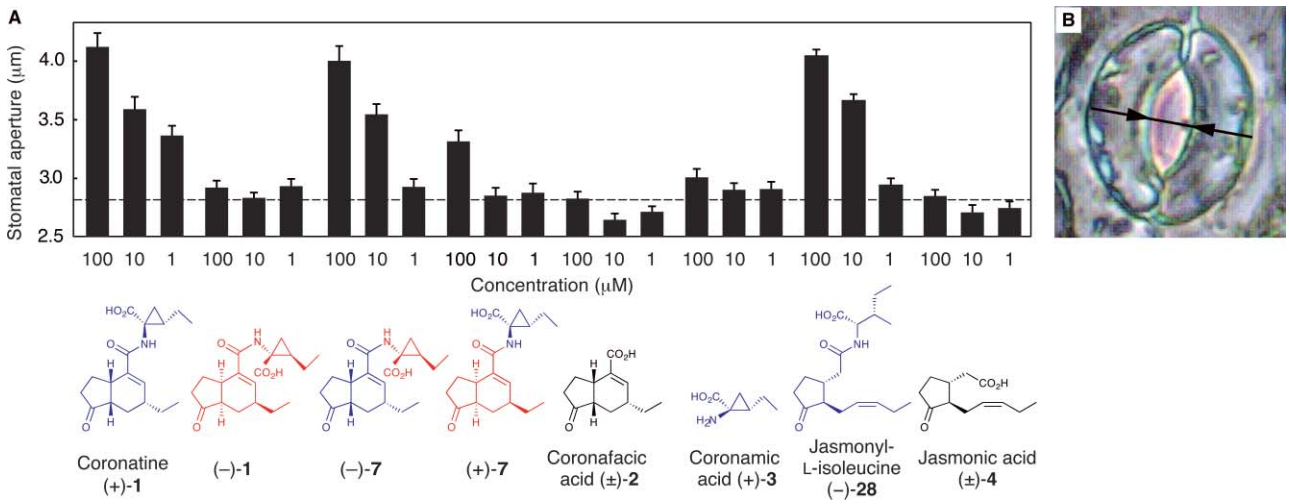


Fig. 4 Effects of synthetic coronatines and related compounds on stomatal aperture in epidermis of *I. tricolor* (**A**) and a stoma in epidermis of *I. tricolor* (**B**). **A**: The dashed line is a control which represents the mean of stomatal apertures in the buffer only lacking test samples. The bars represent the means of stomatal apertures and error bars indicate standard error of the means (n = 100). The chemical structures are color-coded according to the stereochemistry (blue; naturally occurring, red; enantiomeric, black; racemic). **B**: The arrow represents the width of stomatal aperture.

Chiral HPLC analysis

Coronatines (+)-1 and (−)-1. Optical purities were determined by chiral HPLC analysis on a Sumichiral OA-3100 (4.6 × 250 mm, Sumitomo Chemical Co., Ltd., Japan) with 90% *n*-hexane-9.6% EtOH containing 0.4% TFA at 1.5 mL/min. Under these conditions, the analysis reached to the good separation of each enantiomer: coronatine (+)-**1** at R_t = 16.6 min and (−)-**1** at R_t = 17.8 min. Enantiomeric excess was calculated from the ratio of peak areas (mAu s) at 230 nm. Chiral HPLC analysis of 6 ng of the synthetic (+)-**1** gave a ratio of 6200:3.98 = (+)-**1**:(−)-**1**, which corresponded to 99.9%ee.

According to the above mentioned procedure, Chiral HPLC analysis of 2 ng of the synthetic (−)-**1** gave a ratio of 6.38:1680 = (+)-**1**:(−)-**1**, which corresponded to 99.2%ee.

Diastereomers of coronatine (+)-7 and (−)-7. Optical purities were analyzed by chiral HPLC on a Sumichiral OA-3100 (4.6 × 250 mm, Sumitomo Chemical Co., Ltd., Japan) with 0.03 M NH_4OAc in MeOH at 1.0 mL/min. Under these conditions, the analysis reached to the good separation of each enantiomer: (+)-**7** at R_t = 16.2 min and (−)-**7** at R_t = 17.7 min. Enantiomeric excess was calculated from the ratio of peak areas (mAu s) at 220 nm. Chiral HPLC analysis with 2 ng of the synthetic (+)-**7** gave a ratio of 2150:13.3 = (+)-**7**:(−)-**7**, which corresponded to 98.8%ee. According to the above mentioned procedure, chiral HPLC analysis with 3 ng of the synthetic (−)-**7** gave a ratio of 21.9:3010 = (+)-**7** : (−)-**7**, which corresponded to 98.6%ee.

Bioassay of stomatal opening activity

The leaves from 4- to 6-week-old plants of *I. tricolor* were collected and floated on water, then kept in darkness for several hours to close the stomata. The abaxial epidermis of the leaves was peeled and submerged in 10 mM MES–KOH (pH 6.2) buffer containing 50 mM KCl and test sample. After the peels were incubated in darkness for 3 h, pictures of the stomata were taken using a microscope equipped with CCD camera. The stomatal apertures in photos were measured using Adobe Photoshop 7.0 software, and means of them with standard errors were calculated, as shown in Fig. 4.

Computational methods

The Gaussian 03 program was used for the calculations.[17] Geometric optimizations were performed using the B3LYP/6-311+G**//B3LYP/6-311+G** method and basis set. Calculations were performed without assuming symmetry. Frequency calculations were performed for all of the obtained structures at the same level. It was confirmed that all the frequencies were real for the ground states and one imaginary frequency existed for the transition state. Vectors of the imaginary frequencies directed the reaction mode and intrinsic reaction coordinate calculations were further performed to confirm that the obtained TSs were on the saddle points of the energy surface between the reactant and the product.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research No.20310128-00 from MEXT, Asahi Glass Foundation, and Toray Foundation. We also thank Dr. Mitsuru Shoji (Tohoku University) for helpful discussion. The authors are grateful to the Information Technology Center of the University of Electro-Communications (ITC of UEC) for their support with the DFT calculations.

Notes and references

1 A. Ichihara, K. Shiraishi, H. Sara, S. Sakamura, K. Nishiyama, R. Sakai, A. Furusaki and T. Matsumoto, *J. Am. Chem. Soc.*, 1977, **99**, 636–637.
2 Y. Koda, K. Takahashi, Y. Kikuta, F. Greulich, H. Toshima and A. Ichihara, *Phytochemistry*, 1996, **41**, 93–96.
3 S. Blechert, C. Bockelmann, M. Füßlein, T. v. Schrader, B. Stelmach, U. Niesel and E. W. Weiler, *Planta*, 1999, **207**, 470–479.
4 M. Melotto, W. Underwood and S. Y. He, *Annu. Rev. Phytopathol.*, 2008, **46**, 101–122.

5 R. Lauchli and W. Boland, *Chem. Rec.*, 2003, **3**, 12–21.
6 Y. Mino, Y. Matsushita and R. Sakai, *Ann. Phytopath. Soc. Jpn.*, 1987, **53**, 53–55.
7 T. Ohashi, Y. Ito, M. Okada and Y. Sakagami, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 263–265.
8 D. Suhita, A. S. Raghavendra, J. M. Kwak and A. Vavasseur, *Plant Physiol.*, 2004, **134**, 1536–1545.
9 Y. Nakamura, R. Miyatake and M. Ueda, *Angew. Chem., Int. Ed.*, 2008, **47**, 7289–7292.
10 The most recent report on the synthesis of coronafacic acid (±)-**2** is as follows; D. F. Taber, R. B. Sheth and W. Tian, *J. Org. Chem.*, 2009, **74**, 2433–2437. Other reports are cited therein.
11 S. Nara, H. Toshima and A. Ichihara, *Tetrahedron*, 1997, **53**, 9509–9524.
12 H. Toshima and A. Ichihara, *Biosci., Biotechnol., Biochem.*, 1995, **59**, 497–500.
13 Y. Hayashi, J. Yamaguchi, K. Hibino and M. Shoji, *Tetrahedron Lett.*, 2003, **44**, 8293–8296.
14 H. Okamura, T. Iwagawa and M. Nakatani, *Tetrahedron Lett.*, 1995, **36**, 5939–5942.
15 K. Mikami, M. Shimizu and T. Nakai, *J. Org. Chem.*, 1991, **56**, 2952–2953.
16 K. J. Hale and J. Cai, *Tetrahedron Lett.*, 1996, **37**, 4233–4236.
17 *Gaussian 03 (Revision D.02)*, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, Gaussian, Inc., Wallingford CT, 2004.
18 B. Thines, L. Katsir, M. Melotto, Y. Niu, A. Mandaokar, G. Liu, K. Nomura, S. Y. He, G. A. Howe and J. Browse, *Nature*, 2007, **448**, 661–666.