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A plant growth retardant related to chlamydocin and its proposed mechanism of action

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Abstract

A comparison of the plant growth retardant activity of the chlamydocin analogues, compound **1**, six derivatives from **1** and **2**, and two synthetic analogues revealed that there are two types of retardant in chlamydocin analogues. One, for example in compound **1**, requires an oxygen atom at C-8 of the 2-aminodecanoic acid moiety to show retardant activity. The other, for example in compound **8**, requires no oxygen atom at C-8 but requires a specific alkyl group chain length for activity. To determine the differences in mode of action of both types of retardant, rice seedlings were separately treated with compounds **1** and **8**, and after appearance of dwarfism, their endogenous ABA and GA_1 levels were determined and compared to those of the control. Treatment with **1** (10 nmol/plant) increased ABA levels 4 times higher than that of the control and decreased GA_1 levels to 20% of that of the control. Treatment with **8** (30 nmol/plant) did not affect the ABA level but decreased GA_1 content to 5% of that of the control.

Keywords: *Oryza sativa*; Gramineae; Plant growth retardant; Liquid phase synthesis; Cyclic tetrapeptide; Structure–activity relationship; ELISA; Abscisic acid; Gibberellins; Rice

1. Introduction

Plant growth retardants play important roles in agriculture. In the cultivation of grains, they can reduce lodging by shortening stem length and thus increase yield (Eastin, 1983). During a screening-based search for natural plant growth retardants among the metabolites of soil fungi, we found that cladospolide B and zygosporin D act as rice plant growth retardants (Fujii et al., 1995, 2000). We recently reported the isolation from *Peniophora nuda* of three chlamydocin analogues (**1**–**3**), which reduced the height of rice seedlings without blotching and wilting (Tani et al., 2001) (see Fig. 1 for structure). Compounds **1**, **2** and **3** have (*2S*, *9R*)-2-amino-9-hydroxy-8-oxodecanoic acid, (*2S*)-2-amino-8-oxodecanoic acid and (*2S*, *9R*)-9-acetoxy-2-amino-8-oxodecanoic acid moiety, in place of (*2S*, *9S*)-2-amino-9, 10-epoxy-8-oxodecanoic acid (aoe) moiety in chlamydocin. The data in our previous report indicated that the hydroxyl at the C-9 of the 2-aminodecanoic acid (ada) moiety is not essential for retardant activity, but that the ketone function at the C-8 of the ada moiety is important.

In this study we have tried to confirm the importance of the ketone function of the ada moiety in compound **1** for plant growth retardant activity by preparing some derivatives and determining their activities. In the course of this investigation we discovered a novel compound. Compound **8**, which was chemically derived from **1**, has no functionality in the alkyl side chain of the 2-aminooctanoic acid moiety, but still showed retardant activity. The shoots of rice seedlings treated with **8** were markedly curved, suggesting that the dwarfism induced by **8** was apparently different from that induced by **1**. We then tried to clarify the mode of action of these two compounds. We report here the plant growth retardant activity of compound **1**, six derivatives (**4**–**9**) from **1** and **2**, and two synthetic analogues (**10**, **11**), and the levels of endogenous abscisic acid (ABA) and GA_1 in the rice seedlings whose dwarfism was induced by **1** or **8**.

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2. Results and discussion

2.1. Preparation of test compounds

Compounds **4**–**8** were prepared from **1**, the major metabolite of the fungus, as shown in Fig. 1. The stereochemistry of the 8-hydroxyl of **4** and **5** was determined as follows. Compounds **4** and **5** were treated with 2,2-dimethoxypropane and *p*-toluenesulfonic acid to afford the corresponding acetonides, **4a** and **5a**. In the NOE experiments, irradiation of the H-8 of **4a** enhanced the H-9 methine signal, while irradiation of the H-8 of **5a** enhanced the H-10 methyl signal. These results revealed that the configuration of the 8-hydroxyl of **4** is *R* and that of **5** is *S*. Compound **9** was prepared from **2** by chemical conversion as shown in Fig. 1. Compounds **10** and **11** were synthesized according to the procedure reported by Pastuszak et al. (1982). These prepared compounds were checked by EIMS, and ^{1}H and ^{13}C NMR spectroscopy.

2.2. Plant growth retardant activities

Plant growth retardant activities for compounds **1** and **4**–**11** were determined with a microdrop bioassay using rice (cv. Koshihikari) seedlings (Table 1). The test compounds were applied to the rice seedlings at the dose of 30 nmol/plant. Compounds **1**, **4** and **5** reduced the length of the second leaf sheath to 58, 78 and 69% of the control, respectively. In the rice seedlings treated with **6** and **7**, the lengths of the second leaf sheaths were 51 and 67% of the control, respectively. These results indicated that the 8-oxo group in the ada moiety is important, but not essential for the retardant activity. Compound **5** was slightly more active than **4**, indicating that a small structural change, such as a change in the stereochemistry of only one carbon atom in the molecule, influences the retardant activity. Compound **9** completely lost its retardant activity. This result suggested that all of the compounds that have a chlamydocin framework need the oxygen atom at the C-8

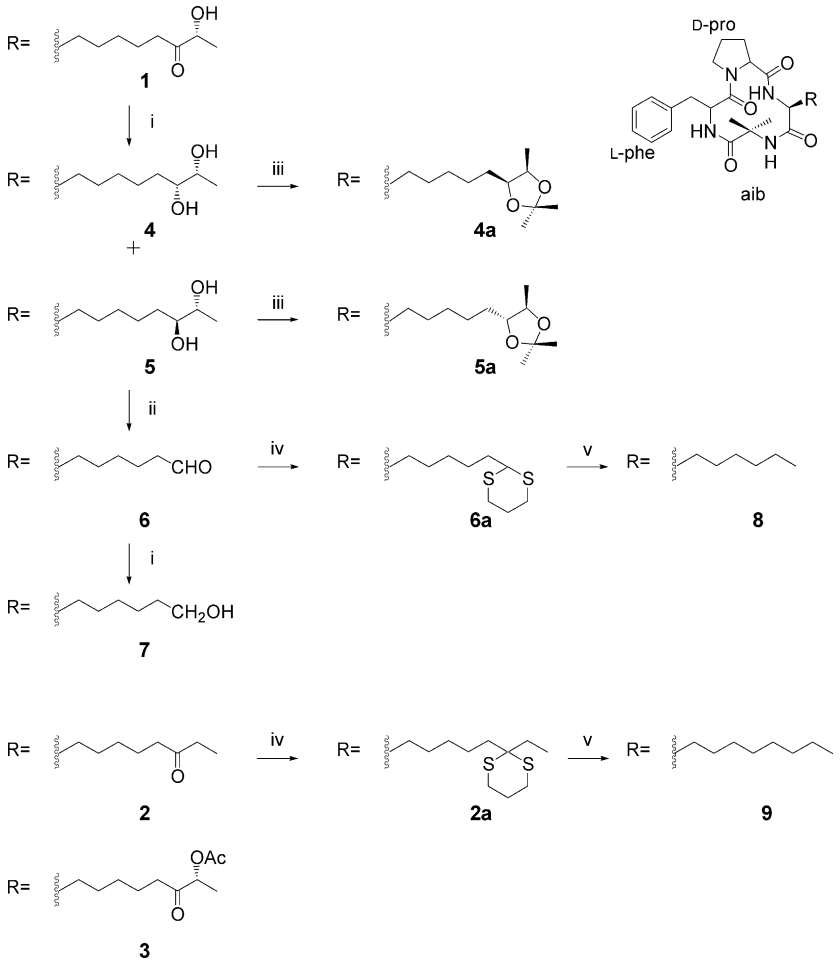

Fig. 1. Preparation of compounds **4**–**9**, reagents and conditions: i, $NaBH_4$, MeOH; ii, $NaIO_4$, aq. MeOH; iii, 2,2-dimethoxypropane, *p*-toluenesulfonic acid; iv, 1,3-propanedithiol, boron trifluoride-diethyl ether complex, CH_2Cl_2; v, Raney Ni (W-2), dioxane, reflux.

position of the ada moiety to exhibit their retardant activity. Surprisingly, compound **8** retained its retardant activity despite the absence of the oxygen atom at C-8. Actually, compound **8** reduced the length of the second leaf sheath to 70% of the control. Compounds **10** and **11** almost lost their retardant activity. The retention of retardant activity in compound **8** and the loss of activity in compounds **9**, **10** and **11** revealed that the length of the alkyl side chain in the compounds with no functionalities in the side chain is important for the retardant activity. Compound **6** was the most active among the compounds tested, presumably due to the additive effect of these two types of retardation. It is noteworthy that in the rice plant treated with **8** the shoots were remarkably curved compared with the control and with that treated with compound **1**. These phenomena suggested that the retardation mechanism of **8** is different from that of **1**.

2.3. Mechanism

Gibberellins are known to stimulate shoot elongation in higher plants. GA_1 is the active gibberellin in the rice plant, and promotes shoot elongation (Graebe, 1987). Abscisic acid (ABA) is involved in many physiological responses in plant development and sometimes shows inhibitory activity, although the role of this hormone is not clearly defined (Zeevaart and Creelman, 1988). To determine the mechanisms of the dwarfism induced by these two types of retardant, the changes of endogenous GA_1 and ABA levels caused by compounds **1** and **8** were investigated. Ten nmol/plant of **1** or 30 nmol/plant of **8** was applied to the seedlings as in the bioassay to equalize the retardation effect. The aerial parts of the treated rice seedlings were homogenized in 80% Me_2CO and filtrated. The filtrates were concentrated in vacuo and the d_6 ABA was added to the aqueous concentrate. This solution was subjected to a solvent fractionation to give the acidic ethyl acetate (AE) fraction, which was purified by HPLC after passing through a Sep-Pak C18 Cartridge. The ABA equivalent fractions from HPLC were combined and analyzed by LC–MS. On the other hand, the GA_1 equivalent fractions from HPLC were combined and analyzed by enzyme-linked immunosorbent assay (ELISA) after methylation with CH_2N_2. GC–SIM analysis after methylation and trimethylsilylation of the HPLC fractions confirmed the occurrence of GA_1 in these fractions. The hormonal levels determined in the treated plants are shown in Table 2. In the rice plants treated with **1**, the ABA level was four times higher than that of the control, and the GA_1 level was 20% of that of the control. In the rice plant treated with **8**, the ABA level was almost the same as that of the control, and the GA_1 level was 5% of that of the control. These results indicated that the increase in the ABA level and the decrease in the GA_1 level caused growth retardation in the case of **1**, and the dramatic reduction of the GA_1 level caused it in the case of **8**. We confirmed that the exogenously applied ABA reduced the height of the rice seedlings (data not shown), and thus it is reasonable that the accumulation of endogenous ABA causes such effects.

Chlamydocin (Closse and Huguenin, 1974) and HC-toxin (Walton et al., 1982) have been reported to cause inhibition of histone deacetylases (HDACs; Brosch et al., 1995). HDACs are important in the regulation of gene expression (Wolffe, 1996), and their inhibition causes cell differentiation and cell cycle arrest. If compound **1** has such inhibitory activity, the inhibition of the fundamental cellular process caused by **1** may enhance downregulation of GA_1 synthesis and also stimulation of ABA synthesis as an adaptation to stress. This presumption is very plausible from the structural point of view. There are important and interesting questions, such as whether both **1** and **8** inhibit HDACs, and which step in gibberellin biosynthesis they inhibit, that still remain to be solved. An investigation aiming to resolve such issues is in progress.

Table 1
Effects of compounds **1** and **4**–**11** (30 nmol/plant) on second leaf sheath length of rice (*Oryza sativa* L. cv. Koshihikari) seedlings

Compound	R[a]	Second leaf sheath length[b]
1	$(CH_2)_5COCH(R\text{-}OH)CH_3$	57.6±3.0
4	$(CH_2)_5CH(R\text{-}OH)CH(R\text{-}OH)CH_3$	78.1±2.0
5	$(CH_2)_5CH(S\text{-}OH)CH(R\text{-}OH)CH_3$	68.8±1.5
6	$(CH_2)_5CHO$	51.1±1.7
7	$(CH_2)_5CH_2OH$	67.5±1.9
8	$(CH_2)_5CH_3$	69.5±3.3
9	$(CH_2)_7CH_3$	97.3±1.9
10	CH_2CH_3	91.6±2.7
11	$(CH_2)_3CH_3$	90.6±3.8

[a] Refer to Fig. 1.
[b] Values are presented as percentage of the control. Data are mean±standard error ($n=5$).

3. Experimental

3.1. General experimental procedures

Optical rotation was measured with a Horiba SEPA-200 polarimeter, whereas NMR spectra were measured

Table 2
The endogenous levels of ABA and GA_1 in the aerial parts of the rice seedlings treated with **1** (10 nmol/plant) or **8** (30 nmol/plant)

	ABA (ng/g)	GA_1 (pg/g)
Control	2.2	315.9
1	9.1	60.3
8	2.3	15.9

with a JEOL JNM-ECP 500 NMR spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX505HA spectrometer (direct probe) and glycerol was the matrix used for FABMS. In EIMS the ionization voltage was 70 eV, and in CIMS the reaction gas was *iso*-butane. HPLC was carried out with a Cosmosil $5C_{18}$-AR column (Nacalai Tesque, 10×250 mm), a flow rate of 1.0 ml/min, and detection at 220 nm. Daisogel IR-60 was used for silica gel column chromatography and Merck Kieselgel 60 F_{254} for TLC.

3.2. Compounds **4** and **5**

A soln of **1** (25.6 mg, 0.048 mmol) and $NaBH_4$ (3.0 mg, 0.072 mmol) in dry MeOH (1.0 ml) was stirred for 15 min at room temp. The reaction mixture was diluted with 15 ml of brine (pH 2.0 with HCl), and the products were extracted with EtOAc (15 ml×3). The EtOAc extract was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by HPLC (70% MeOH) to give **4** (8.0 mg, 0.015 mmol, 31%, R_t 19.2 min) and **5** (11.5 mg, 0.022 mmol, 46%, R_t 20.5 min) as colorless oils. **4**: $[\alpha]_D^{25}$ −49° (*c* 0.10; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): L-α-aminodecanoic acid (ada) δ 1.15 (3H, *d*, *J* = 6.5 Hz, H-10), 1.25-1.84 (10H, *m*, H-3-7), 3.60 (1H, *m*, H-8), 3.78 (1H, *dq*, *J* = 3.5, 6.5, H-9), 4.19 (1H, *ddd*, *J* = 7.6, 7.8, 10.1 Hz, H-2), 7.11 (1H, *d*, *J* = 10.1 Hz, NH), α-aminoisobutyric acid (aib) δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.97 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.8, 10.1, 10.1 Hz, H-2), 7.19-7.29 (5H, *m*, H-5-9), 7.51 (1H, *d*, *J* = 10.1 Hz, NH), Pro *δ* 1.25–1.84 (2H, *m*, H-3 and 4), 2.14–2.34 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 8.1 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): *δ*16.7, 23.6, 24.7, 25.0, 25.3, 25.6, 26.5, 28.8, 29.1, 31.5, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 70.4, 74.8, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.6. EIMS *m*/*z* (rel. int.): 530 $[M]^+$ (11), 485 (17), 412 (14), 411 (13), 332 (12), 314 (13), 230 (11), 229 (26), 120 (32), 70 (100), 69 (11), 58 (66). **5**: $[\alpha]_D^{25}$ −48° (*c* 0.15; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 1.19 (3H, *d*, *J* = 6.2 Hz, H-10), 1.24–1.90 (10H, *m*, H-3-7), 3.32 (1H, *m*, H-8), 3.59 (1H, *dq*, *J* = 6.4, 6.2 Hz, H-9), 4.19 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.12 (1H, *d*, *J* = 10.1 Hz, NH), aib *δ* 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 6.02 (1H, *s*, NH), Phe *δ* 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *J* = *ddd*, 5.8, 10.1, 10.1 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.51 (1H, *d*, *J* = 10.1 Hz, NH), Pro *δ* 1.24–1.90 (2H, *m*, H-3 and 4), 2.12–2.35 (2H, *m*, H-3 and 4), 3.21 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.3, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 8.0 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 19.5, 23.6, 24.7, 25.0, 25.2, 25.3, 26.5, 28.8, 29.1, 33.1, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 70.9, 76.1, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.6. EIMS *m*/*z* (rel. int.): 530 $[M]^+$ (11), 486 (12), 485 (20), 430 (24), 412 (11), 411 (15), 332 (20), 314 (11), 229 (25), 120 (35), 70 (100), 69 (12), 58 (64), 57 (11), 55 (15).

3.3. Compound **4a**

A soln of **4** (3.2 mg, 0.0060 mmol) was dissolved in 2,2-dimethoxypropane (1.0 ml) and a catalytic amount of *p*-toluenesulfonic acid was added and stirred for 30 min at room temp., and the solvent was then evaporated in vacuo. The residue was purified by silica gel column chromatography to give **4a** (2.4 mg, 0.0042 mmol, 70%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada *δ* 1.13 (3H, *d*, *J* = 6.2 Hz, H-10), 1.23–1.85 (10H, *m*, H-3-7), 1.33 (3H, *s*, CH_3C), 1.44 (3H, *s*, CH_3C), 4.01 (1H, *m*, H-8), 4.22 (1H, *dq*, *J* = 7.3, 6.2 Hz, H-9), 4.18 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.08 (1H, *d*, *J* = 10.1 Hz, NH), aib *δ* 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.89 (1H, *s*, NH), Phe *δ* 2.95 (1H, *dd*, *J* = 5.7, 13.8 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.8 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.1 Hz, NH), Pro *δ* 1.23–1.85 (2H, *m*, H-3 and 4), 2.12–2.35 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.2, 8.3, 9.6 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.2, 7.6 Hz, H-2). EIMS *m*/*z* (rel. int.): 570 $[M]^+$ (36), 556 (23), 555 (49), 513 (20), 485 (26), 470 (26), 455 (23), 451 (21), 412 (35), 314 (21), 229 (26), 120 (34), 70 (100), 58 (70).

3.4. Compound **5a**

Compound **5** (8.0 mg, 0.015 mmol) was treated with 2,2-dimethoxypropane and *p*-toluenesulfonic acid under the same conditions as **4** to give **5a** (5.0 mg, 0.0088 mmol, 59%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada *δ* 1.24 (3H, *d*, *J* = 6.0 Hz, H-10), 1.26–1.86 (10H, *m*, H-3-7), 1.38 (3H, *s*, CH_3C), 1.39 (3H, *s*, CH_3C), 3.49 (1H, *m*, H-8), 3.69 (1H, *dq*, *J* = 8.0, 6.0 Hz, H-9), 4.18 (1H, *ddd*, *J* = 7.8, 7.8, 10.1 Hz, H-2), 7.08 (1H, *d*, *J* = 10.1 Hz, NH), aib *δ* 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.87 (1H, *s*, NH), Phe *δ* 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19–7.30 (5H, *m*, H-5-9), 7.50 (1H, *d*, *J* = 10.1 Hz, NH), Pro *δ* 1.26–1.90 (2H, *m*, H-3 and 4), 2.13–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.4, 7.5, 10.0 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.6, 8.5, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.3, 7.8 Hz, H-2). EIMS *m*/*z* (rel. int.): 570 $[M]^+$ (16), 556 (9), 555 (29), 513 (11), 485 (14), 470 (14), 455 (13), 451 (13), 412 (19), 314 (14), 229 (18), 120 (27), 70 (100), 58 (75).

3.5. Compound **6**

A mixture of **4** and **5** (39.6 mg, 0.075 mmol) in MeOH (2.0 ml) was added to a soln of $NaIO_4$ (19.3 mg) in H_2O

(2.0 ml) at 0 °C. After being stirred at room temp. for 1 h, the reaction mixture was diluted with 20 ml of H_2O. The products were extracted with EtOAc (18 ml×3). The EtOAc soln was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by HPLC (70% MeOH) to give **6** (33.9 mg, 0.070 mmol, 93%, R_t 21.5 min) as a colorless oil. $[\alpha]_D^{25}$ −51° (*c* 0.13; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): L-α-aminooctanoic acid (aoa) δ 1.21–1.85 (8H, *m*, H-3-6), 2.43 (2H, *dt*, 1.4, 7.4 Hz, H-7), 4.18 (1H, *ddd*, *J* = 7.6, 7.8, 10.3 Hz, H-2), 7.07 (1H, *d*, *J* = 10.3 Hz, NH), 9.76 (1H, *t*, *J* = 1.4 Hz, H-8), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.93 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.3, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.8, 10.3, 10.3 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.50 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ 1.21–1.85 (2H, *m*, H-3 and 4), 2.10–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.7, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.4, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.8, 23.5, 24.7, 25.0, 25.3, 26.5, 28.72, 28.73, 35.8, 43.7, 47.0, 53.4, 54.3, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.3, 175.6, 202.5. EIMS *m*/*z* (rel. int.): 484 $[M]^+$ (17), 428 (14), 427 (14), 399 (13), 384 (33), 365 (24), 356 (30), 286 (26), 229 (27), 128 (19), 120 (38), 70 (100), 58 (71).

3.6. Compound 7

A soln of **6** (11.2 mg, 0.023 mmol) and $NaBH_4$ (1.2 mg) in dry MeOH (1.0 ml) was stirred for 15 min at room temp. The reaction mixture was diluted with 10 ml of brine (pH 2.0 with HCl), after which the products were extracted with EtOAc (10 ml×3). The EtOAc extract was dried over Na_2SO_4 and evapd in vacuo. The residue was purified by HPLC (70% MeOH) to give **7** (9.3 mg, 0.019 mmol, 83%, R_t 22.8 min) as a colorless oil. $[\alpha]_D^{25}$ −54° (*c* 0.16; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 1.25–1.86 (10H, *m*, H-3-7), 3.63 (2H, *t*, *J* = 6.5 Hz, H-8), 4.19 (1H, *ddd*, *J* = 7.6, 7.8, 10.1 Hz, H-2), 7.10 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.98 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.8, 13.6 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.6 Hz, H-3), 5.16 (1H, *J* = *ddd*, 5.8, 10.1, 10.1 Hz, H-2), 7.17–7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.25–1.86 (2H, *m*, H-3 and 4), 2.12–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.6, 7.6, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.1, 8.7, 10.3 Hz, H-5), 4.66 (1H, *dd*, *J* = 1.6, 7.6 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.6, 24.7, 25.0, 25.4, 25.5, 26.5, 28.9, 29.0, 32.5, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 62.9, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.4, 175.6. EIMS *m*/*z* (rel. int.): 486 $[M]^+$ (12), 430 (16), 429 (18), 387 (19), 386 (69), 367 (30), 314 (14), 288 (29), 229 (51), 227 (18), 217 (13), 201 (13), 131 (10), 130 (24), 120 (48), 70 (100), 58 (96).

3.7. Compound 8

To a soln of **6** (33.9 mg, 0.070 mmol) in CH_2Cl_2 (2.5 ml) was added 1, 3-propanedithiol (0.0098 ml, 15.0 mg, 0.14 mmol) and boron trifluoride–diethyl ether complex (0.026 ml, 29.8 mg, 0.098 mmol) at 0 °C. After being stirred at room temp. for 30 min, the reaction mixture was diluted with EtOAc (10 ml). The EtOAc extract was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and evaporated in vacuo to give thioacetal **6a** (21.9 mg, 0.038 mmol, 54%) as a colorless oil. A soln of **6a** (21.9 mg, 0.038 mmol) in dioxane (2.0 ml) was refluxed for 20 min with Raney Ni (700 mg) freshly prepared from alloy. The soln was filtered, the metal washed thoroughly with methanol, and the filtrate and washings evaporated in vacuo. The residue was purified by prep. TLC [EtOAc–*n*-hexane, 4:6, R_f 0.51] and then HPLC (70% MeOH) to give **8** (10.5 mg, 0.022 mmol, 58%, R_t 26.8 min) as a colorless oil. $[\alpha]_D^{25}$ −63° (*c* 0.24; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): aoa δ 0.88 (3H, *t*, *J* = 7.1 Hz, H-8), 1.20–1.90 (10H, *m*, H-3-7), 4.18 (1H, *ddd*, *J* = 7.3, 7.6, 10.2 Hz, H-2), 7.07 (1H, *d*, *J* = 10.2 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.91 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.27 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.53 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.20–1.90 (2H, *m*, H-3 and 4), 2.14–2.36 (2H, *m*, H-3 and 4), 3.23 (1H, *ddd*, *J* = 7.3, 7.3, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 5.5, 8.6, 10.1 Hz, H-5), 4.66 (1H, *dd*, *J* = 2.1, 8.0 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 14.0, 22.5, 23.6, 24.7, 25.0, 25.5, 26.5, 28.9×2, 31.6, 35.8, 47.0, 53.4, 54.5, 57.8, 58.8, 126.7, 128.6, 129.0, 137.1, 171.8, 172.8, 174.5, 175.7. EIMS *m*/*z* (rel. int.): 470 $[M]^+$ (34), 370 (60), 351 (31), 229 (34), 120 (37), 70 (100), 58 (69).

3.8. Compound 9

Compound **2** (5.9 mg, 0.012 mmol) in CH_2Cl_2 (0.5 ml) was treated with 1,3-propanedithiol (0.017 ml, 2.6 mg, 0.024 mmol) and boron trifluoride–diethyl ether complex (0.046 ml, 5.2 mg, 0.017 mmol) under the same conditions used in the preparation of **6a** to give thioketal **2a** (5.1 mg, 0.0085 mmol, 71%) as a colorless oil. Compound **2a** (5.1 mg, 0.0085 mmol) was treated with Raney Ni (100 mg) under the same conditions used in the preparation of **8**. The product was purified by silica gel column chromatography followed by HPLC (90% MeOH) to give **9** (1.5 mg, 0.0030 mmol, 35%, R_t 26.8 min) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): ada δ 0.88 (3H, *t*, *J* = 6.9 Hz, H-10), 1.24–1.86 (14H, *m*, H-3-9), 4.18 (1H, *ddd*, *J* = 7.6, 7.6, 10.1 Hz, H-2), 7.07 (1H, *d*, *J* = 10.1 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.89 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19–7.29

(5H, *m*, H-5-9), 7.53 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.24–1.86 (2H, *m*, H-3 and 4), 2.14–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.3, 7.3, 10.1 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 10.1 Hz, H-5), 4.66 (1H,*dd*, *J* = 2.1, 7.6, H-2). EIMS *m*/*z* (rel. int.): 498 $[M]^+$ (34), 399 (22), 398 (63), 386 (18), 379 (30), 229 (34), 142 (15), 120 (36), 70 (100), 58 (66).

3.9. Compound **10**

3.9.1. N-(tert-Butyloxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyric acid methyl ester

L-α-Aminobutyric acid (103.0 mg, 1 mmol) in dioxane (1.0 ml) and H_2O (1.0 ml) was coupled with *N*-(*tert*-butyloxycarbonyl) azide (290 mg, 1.6 mmol) under alkali conditions to give *N*-(*tert*-butyloxycarbonyl)-L-α-aminobutyric acid (183.4 mg, 0.90 mmol, 90%). α-Aminoisobutyric acid methyl ester hydrochloride (10.5 mg, 0.070 mmol) that was prepared by the method reported by Pastuszak et al. (1982) in EtOAc (0.5 ml) was neutralized with triethylamine (0.0098 ml, 7.17 mg, 0.070 mmol) and coupled with the *N*-(*tert*-butyloxycarbonyl)-L-α-aminobutyric acid (15.2 mg, 0.075 mmol) by use of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) (18.9 mg, 0.075 mmol) in EtOAc (0.1 ml) to give the title compound (16.5 mg, 0.055 mmol, 79%).

3.9.2. N-(tert-Butyloxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester

N-(*tert*-Butyloxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyric acid methyl ester (16.5 mg, 0.055 mmol) in MeOH (0.11 ml) was saponified with 1 M NaOH (0.083 ml) to give the acid (14.2 mg, 0.049 mmol, 89%). L-Phenylalanyl-D-proline methyl ester trifluoroacetate (82.5 mg, 0.23 mmol), which was prepared by the method reported by Pastuszak et al. (1982), in $CHCl_3$ (2.0 ml) was neutralized with triethylamine (0.032 ml, 23.4 mg, 0.23 mmol) and coupled with the *N*-protected dipeptide acid (95 mg, 0.33 mmol) in $CHCl_3$ (0.8 ml) by use of 1-hydroxybenzotriazole monohydrate (HOBt) (40.2 mg, 0.26 mmol) in DMF (0.5 ml) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (56.5 mg, 0.26 mmol) in $CHCl_3$ (0.5 ml) to give the title compound (99.7 mg, 0.18 mmol, 78%).

3.9.3. Cyclo (L-α-aminobutyryl-α-aminoisobutyryl-L-phenylalanyl-D-prolyl) (**10**)

The *N*-protected tetrapeptide methyl ester (18.2 mg, 0.033 mmol) in MeOH (0.66 ml) was saponified with 1 M NaOH (0.50 ml) to give the acid (14.9 mg, 0.028 mmol, 85%). The *N*-protected tetrapeptide acid (20.5 mg, 0.038 mmol) in CH_2Cl_2 (0.5 ml) was coupled with *N*-hydroxysuccinimide (HOSu) (8.9 mg, 0.076 mmol) in DMF (0.2 ml) by use of DCC (9.1 mg, 0.042 mmol) in CH_2Cl_2 (0.2 ml). The succinimide ester was *N*-deprotected using TFA (0.38 ml). The TFA salt was dissolved in DMF (2.0 ml) and then cyclized by being slowly added to a large volume of pyridine (60 ml). The pyridine was evaporated in vacuo. The residue was purified by silica gel column chromatography followed by HPLC (85% MeOH) to give the cyclic peptide **10** (4.1 mg, 0.01 mmol, 26%, R_t 16.2 min) as a colorless powder. $[\alpha]_D^{25}$ −71° (*c* 0.19; EtOH). ^1H NMR (500 MHz, $CDCl_3$): aba δ 0.91 (3H, *t*, *J* = 7.3 Hz, H-4), 1.59–1.91 (2H, *m*, H-3), 4.11 (1H, *ddd*, *J* = 7.6, 7.6, 10.3 Hz, H-2), 7.09 (1H, *d*, *J* = 10.3 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.92 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.5 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.5 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.1 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.52 (1H, *d*, *J* = 10.1 Hz, NH), Pro δ 1.59–1.91 (2H, *m*, H-3 and 4), 2.13–2.36 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.4, 7.6, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.8, 8.7, 10.3 Hz, H-5), 4.66 (*dd*, *J* = 2.5, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 10.1, 22.3, 23.6, 24.7, 25.0, 26.5, 35.8, 47.0, 53.4, 55.9, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.9, 172.8, 174.4, 175.7. EIMS *m*/*z* (rel. int.): 414 $[M]^+$ (29), 371 (12), 314 (43), 302 (14), 295 (19), 229 (27), 120 (44), 70 (100), 58.

3.10. Compound **11**

3.10.1. N-(tert-Butyloxycarbonyl)-L-norleucyl-α-aminoisobutyric acid methyl ester

L-Norleucine (131.2 mg, 1 mmol) in dioxane (2.0 ml) and H_2O (1.0 ml) was coupled with *N*-(*tert*-butyloxycarbonyl) azide to give *N*-(*tert*-butyloxycarbonyl)-L-norleucine (197 mg, 0.85 mmol, 85%). α-Aminoisobutyric acid methyl ester hydrochloride (13.7 mg, 0.091 mmol) was neutralized with triethylamine and coupled with *N*-(*tert*-butyloxycarbonyl)-L-norleucine (23.2 mg, 0.10 mmol) by use of EEDQ to give the title compound (21.1 mg, 0.064 mmol, 70%).

3.10.2. N-(tert-Butyloxycarbonyl)-L-norleucyl-α-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester

The *N*-protected dipeptide methyl ester (48.5 mg, 0.15 mmol) was saponified with 1 M NaOH to give the acid (41.9 mg, 0.13 mmol, 87%). L-Phenylalanyl-D-proline methyl ester trifluoroacetate (189.8 mg, 0.53 mmol) was neutralized with triethylamine and coupled with the *N*-(*tert*-butyloxycarbonyl)-L-norleucine (253 mg, 0.80 mmol) by use of HOBt and DCC to give the title compound (195.2 mg, 0.34 mmol, 64%).

3.10.3. Cyclo (L-norleucyl-α-aminoisobutyryl-L-phenylalanyl-D-prolyl) (**11**)

The *N*-protected tetrapeptide methyl ester (46.0 mg, 0.084 mmol) was saponified with 1 M NaOH to give the acid (40.7 mg, 0.073 mmol, 87%). The *N*-protected tetrapeptide acid (7.0 mg, 0.013 mmol) was converted to

the corresponding *N*-hydroxysuccinimide ester. The succinimide ester was *N*-deprotected by use of TFA and then cyclized in pyridine. The crude product was purified by silica gel column chromatography followed by HPLC (85% MeOH) to give the cyclic peptide **11** (1.3 mg, 0.0030 mmol, 23%, R_t 20.3 min) as a colorless powder. $[\alpha]_D^{25}$ −44° (*c* 0.10; EtOH). ^{1}H NMR (500 MHz, $CDCl_3$): nle 0.90 (3H, *t*, *J* = 7.1 Hz, H-6), 1.14–1.85 (6H, *m*, H-3-5), 4.18 (1H, *ddd*, *J* = 7.6, 7.8, 10.3 Hz, H-2), 7.10 (1H, *d*, *J* = 10.3 Hz, NH), aib δ 1.34 (3H, *s*, H-4), 1.77 (3H, *s*, H-3), 5.97 (1H, *s*, NH), Phe δ 2.95 (1H, *dd*, *J* = 5.7, 13.6 Hz, H-3), 3.26 (1H, *dd*, *J* = 10.1, 13.6 Hz, H-3), 5.16 (1H, *ddd*, *J* = 5.7, 10.1, 10.3 Hz, H-2), 7.19–7.29 (5H, *m*, H-5-9), 7.54 (1H, *d*, *J* = 10.3 Hz, NH), Pro δ 1.14–1.81 (2H, *m*, H-3 and 4), 2.12–2.35 (2H, *m*, H-3 and 4), 3.22 (1H, *ddd*, *J* = 7.3, 7.4, 10.3 Hz, H-5), 3.86 (1H, *ddd*, *J* = 4.4, 8.3, 9.9 Hz, H-5), 4.66 (*dd*, *J* = 2.1, 7.8 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): 13.9, 22.4, 23.6, 24.7, 25.0, 26.5, 27.7, 28.6, 35.8, 47.0, 53.4, 54.4, 57.8, 58.8, 126.7, 128.6, 129.0, 137.0, 171.8, 172.8, 174.5, 175.7. EIMS *m*/*z* (rel. int.): 442 $[M]^+$ (34), 399 (10), 343 (12), 342 (51), 330 (15), 323 (25), 314 (12), 229 (35), 217 (10), 183 (12), 120 (44), 86 (19), 70 (100), 58 (80).

3.11. Spectral and analytical data for intermediates

Spectral and analytical data for **6a**, **2a**, L-α-aminobutyric acid, *N*-(*tert*-butyloxycarbonyl)-L-α-aminobutyril-α-aminoisobutyric acid methyl ester, *N*-(*tert*-butyloxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyric acid, *N*-(*tert*-butyloxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester, *N*-(*tert*-butyl-oxycarbonyl)-L-α-aminobutyryl-α-aminoisobutyryl-L-phenylalanyl-D-proline, *N*-(*tert*-butyloxycarbonyl)-L-norleucine, *N*-(*tert*-butyloxycarbonyl)-L-norleucyl-α-aminoisobutyric acid methyl ester, *N*-(*tert*-butyloxycarbonyl)-L-norleucyl-α-aminoisobutyric acid,*N*-(*tert*-butyloxycarbonyl)-L-norleucyl-α-aminoisobutyryl-L-phenylalanyl-D-proline methyl ester and *N*-(*tert*-butyloxycarbonyl)-L-norleucyl-α-aminoisobutyryl-L-phenylalanyl-D-proline are available on request by e-mail.

3.12. Biological assays

The retardant activity of compounds **1** and **4**–**11** was determined with the same procedures as reported previously (Tani et al., 2001).

3.13. Extraction and purification of plant hormones

Compound **1** (10 nmol/plant) or **8** (30 nmol/plant) in aq. MeOH was applied to the rice seedlings as in the bioassay described above. As a control only aq. MeOH was applied. After incubation for 72 h under continuous light, the aerial parts of the rice seedlings were collected. The weights of the plant materials collected were 8.2 (treated with **1**), 8.4 (treated with **8**) and 10.2 g (the control). Each plant material was homogenized in 60 ml of 80% aq. Me_2CO. The homogenate was filtered and the debris was further extracted twice with 80% aq. Me_2CO. The extracts were combined and concentrated in vacuo. The aq. residue equivalent to 7.2 g of plant materials was diluted up to 100 ml by adding H_2O and d_6-ABA was added to it. The amounts of d_6-ABA added were 144 (treated with **1**), 100 (treated with **8**) and 93 ng (the control). The aq. residue was adjusted to pH 2.5 with 6 M HCl, and extracted with EtOAc (80 ml×3). The EtOAc solution was extracted with saturated $NaHCO_3$ (50 ml×3). The $NaHCO_3$ solution was adjusted to pH 2.5 with 6M HCl, and extracted with EtOAc (180 ml×3). The EtOAc solution was dried over Na_2SO_4 and evaporated in vacuo. The residue was dissolved in a small amount of 80% aq. MeOH, and loaded onto a Sep-Pak C18 Cartridge (Millipore). The eluate eluted from the cartridge with 7.5 ml of 80% aq. MeOH was evaporated. The residue was subjected to HPLC with a column of Capcell Pak C18 (Shiseido, 6.0×150 mm) and eluted with 0.5% AcOH in 10% aq. MeCN (solvent A) and 0.5% AcOH in 80% aq. MeCN (solvent B) at 30 °C as follows: 0–10 min, elution with solvent A; 10–50 min, linear gradient of 0 to 100% solvent B. The flow rate of the solvent was 1.0 ml/min. Eluate was collected every 1 min as one fraction. The retention times of ABA and GA_1 were 31 and 19 min, respectively. The authentic GA_1 was a gift from Professor Takeshi Sassa (Yamagata University, Yamagata, Japan) and (+)-ABA was purchased from Sigma. The d_6-ABA was prepared according to the procedure of Milborrow et al. (1971), and its purity and labeling pattern were checked with ^{1}H NMR and EIMS.

3.14. LC–MS analysis for ABA

Negative electrospray mass spectra were obtained using a Quattro micro mass spectrometer (Micromass, electrospray voltage: 3.5 kV; desolvation temp.: 400 °C; sheath gas: N_2) coupled with a JASCO PU-980 Gulliver LC system equipped with a RP-18 GP column (Kanto Chemical, 250×2.0 mm, 5 μm). For LC, a gradient system was used that started from 0.25% AcOH in 10% aq. MeCN (solvent C) to 0.25% AcOH in 80% aq. MeCN (solvent D) at 40 °C as follows: 0–10 min, elution with solvent C; 10–50 min, linear gradient of 0 to 100% solvent D. The flow rate was 0.2 ml/min (injection volume: 10 μl). Multiple reaction monitoring (MRM) was obtained during LC analysis under the following conditions: cone voltage, 30 V; collision energy, 13 eV; collision pressure, 1.7 mT; collision gas, Ar. The MRM transitions from *m*/*z* 269 to 159 of the internal d_6-ABA and from *m*/*z* 263 to 153 of the natural ABA were monitored, and these product ions of 159 and 153 were used for quantitation.

3.15. GC–MS analysis for methyl trimethylsilyl GA_1

A portion of GA_1 equivalent fraction from ODS-HPLC was subjected to GC/SIM analysis after methylation with CH_2N_2 and trimethylsilylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. A Trace MS (Thermo) equipped with a Trace GC 2000 (Thermo) and a capillary column SPB™-1 (Supelco, 30 m×0.25 mm) was used. The ionization voltage was 70 eV. The GC conditions were as follows: injection temperature, 250 °C; initial temperature, 60 °C (1.5 min); linear gradient up to 200 °C at 60 °C/min, to 280 °C at 5 °C/min, and maintained at 280 °C (10 min). In the GC/SIM analysis, characteristic ions, m/z 506 (M^+) and 448 were monitored.

3.16. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed according to the procedure of Yamaguchi et al. (1990).

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