Pergamon

0031-9422(95)00809-8

Phytochemistry, Vol. 41, No. 6, pp. 1485–1489, 1996

0031-9422/96 $15.00 + 0.00

ISOLATION OF 5-(8′*Z*-HEPTADECENYL)-RESORCINOL FROM ETIOLATED RICE SEEDLINGS AS AN ANTIFUNGAL AGENT

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(*Received in revised form* 24 *August* 1995)

Key Word Index—*Oryza sativa*; Gramineae; rice; resorcinol; 5-alkylresorcinol; antifungal activity.

Abstract—A new antifungal substance against rice blast fungus (*Pyricularia oryzae*), 5-(8′*Z*-heptadecenyl)resorcinol, was isolated from etiolated rice seedlings together with a mixture of its homologues with C_{13}, C_{15}, and C_{17} saturated alkyl chains. Its structure was determined by ^{1}NMR, ^{13}C-NMR, and MS spectra. Its ED_{50} was *ca* 40 μg/ml. These 5-alkylresorcinols were newly produced after germination and the concentration reached 50 μg g^{-1} fresh weight on day 6.

INTRODUCTION

The seedling stage is assumed to be one of the most infectious stages in the life of the plant because young buds in soil are physically weak and surrounded by many microbes. On this assumption, we sought disease-defending substances specific to the seedling stage and different from those at the subsequent stages in plant growth. Cyclic hydroxamates [1] which are widely distributed as sugar conjugates in seedling stages of Gramineae have been assumed to play a role in defence against insects and microbes, especially in corn [2], wheat, and rye where they occur in high concentration. These metabolites also reach a maximum level within a week after germination, followed by a decrease with age in corn and wheat. We have identified disease-defending substances from seedling plants of cereals which contain little or no detectable amounts of these hydroxamates: i.e., rice and sorghum [3]. We report here the isolation of 5-(8′*Z*-heptadecenyl)resorcinol and a mixture of its homologues with saturated alkyl chains as antifungal substances from etiolated rice seedlings.

RESULTS AND DISCUSSION

Antifungal activity was examined in shoots subjected to mechanical injury and compared to untreated shoots. The activity was evaluated by a spore germination test using the rice blast fungus, *Pyricularia oryzae*. Aqueous MeOH extracts of the shoot tissues were partitioned between solvents and water. All except for *n*-butanol fractions possessed sufficiently high activity to completely inhibit the spore germination in the fresh weight concentration (Table 1). The activity for each fraction seemed to be due chiefly to preformed antifungal substances. Induced antifungal substances may exist in the acidic fraction of the injury-treated shoots, together with the preformed ones, since significant increase in activity was noted in the injury treatment.

The neutral fraction of the injury-treated shoots was prepared from 400 g fresh weight to isolate the antifungal substances.

Column chromatography of the TN fraction on Sephadex LH-20 gave a single active fraction, which was then purified by a prep. TLC on silica gel. The band of R_f around 0.5 with MeOH–$CHCl_3$ (7:93) gave a syrup of an active substance (64.0 mg). The ^{1}H NMR spectrum gave signals characteristic of resorcinol with an unsaturated alkyl substituent at the 5 position, 5-alkylresorcinol (AR) [4]. A doublet at δ6.25 ppm (2H, J = 2.3 Hz), triplet at δ6.17 ppm (1H, J = 2.3 Hz), and broad singlet at δ5.25 ppm (2H) identified as aromatic methines and phenols, a triplet at δ2.47 ppm (2H, J = 7.2 Hz), multiplet at δ1.55 ppm (2H), broad singlet at δ1.25 ppm (*ca.* 18H), and triplet at δ0.89 ppm (3H, J = 6.7 Hz) identified as benzylic methylene, homobenzylic methylene, methylenes, and methyl, and a multiplet at δ5.15 ppm (*ca.* 1H) and 2.00 ppm (*ca.* 2.7H) identified as olefinic methines and allylic methylenes were observed. However, the intensities of both the olefinic methines and allylic methyenes were about half the theoretical values. This suggested the active substance was a mixture of 5-alkylresorcinols (ARs) with both saturated and unsaturated alkyl substituents. GCEI-mass spectrometry analysis of the active substance as TMSi showed that there were five homologs; that is, in these mass spectra, the molecular ion peaks at *m/z* 436, 462, 464, 490, and 492 are comparable to the AR with the $C_{13:0}$, $C_{15:1}$, $C_{15:0}$, $C_{17:1}$, and $C_{17:0}$ alkyl substituents, respectively. Both negative and positive FAB-mass spectra (see Experimental) confirmed the results of the GC-mass spectrometry. In these mixtures the $AR_{17:1}$ was the most abundant component which amounted to *ca.* 50% of the total.

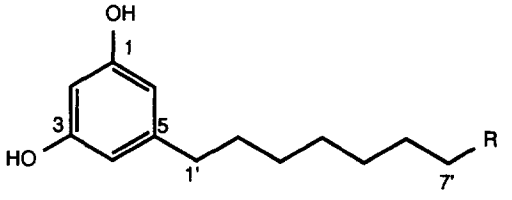
Table 1. Antifungal activity of fractions extracted from shoots with/without injury treatment on spore germination of *P. oryzae*

Conc.*	Inhibition %		
	Neutral fr.	Acidic fr.	Butanol fr.
1.0	89.7 (96.1)†	98.3 (100)	0 (7.5)
0.5	49.2 (68.4)	74.4 (100)	/
0.25	57.8 (32.0)	41.6 (100)	/
0.125	41.0 (4.5)	0 (49)	/

Shoots of 8-day-old etiolated seedling plants grown at 20–25° were used for extraction. Values represent the mean of four separate experiments.

* Conc. 1.0 represents a fresh weight concentration.

† Parentheses represent samples with injury treatment.



1 $AR_{13:0}$ R=$(CH_2)_5CH_3$
2 $AR_{15:1}$ R=$CH{=}CH(CH_2)_5CH_3$
3 $AR_{15:0}$ R=$(CH_2)_7CH_3$
4 $AR_{17:1}$ R=$CH{=}CH(CH_2)_7CH_3$
5 $AR_{17:0}$ R=$(CH_2)_9CH_3$

Separation of the mixture by silica gel TLC impregnated with $AgNO_3$ gave two bands: the $AR_{17:1}$ containing trace amounts of the $AR_{15:1}$ from the polar band and a mixture of $AR_{13:0}$, $AR_{15:0}$, and $AR_{17:0}$ from the lesser polar band. Positions of the double bond for the $AR_{15:1}$ and $AR_{17:1}$ were determined by charge-remote fragmentation FAB-mass spectra. Figure 1 shows these spectra for the pairs AR_{15} and AR_{17}. Similar mass fragmentation patterns as observed in several monounsaturated fatty acids [5, 6] were observed in those of the ARs; in the $AR_{17:1}$ and $AR_{15:1}$, two intense peaks (*m/z* 191 and 245) corresponding to cleavage of the allylic positions with three very low intense peaks (*m/z* 205, 219, and 231) were present. This indicates that the double bond is located at the 8-position in both cases. In addition, the FAB data for the $AR_{13:0}$, $AR_{15:0}$, and $AR_{17:0}$ showed also that these alkyl substituents were composed entirely of linear carbon chains.

The double bond was unequivocally determined to be *Z* stereochemistry from the ^{13}C NMR data of the known 3-(heptadec-8′*Z*-enyl)-1,2-dimethoxybenzene and its 8′-*E* isomer [7, 8]. The allylic (δ27.2 ppm) and olefinic (δ129.8 and 129.9 ppm) carbon signals of the native AR mixture and the $AR_{17:1}$ were coincident with those of 8′*Z* isomer (allylic carbons: δ27.18 ppm, olefinic carbons: δ129.82 and 129.87 ppm), but not those of 8′*E* isomer (allylic carbons: δ32.58 ppm, olefinic carbons: δ130.28 and 130.34 ppm). Thus, we determined the structures of the five 5-alkylresorcinol homologues as **1**–**5**. Among these homologues **4** is a new natural product and its shorter homologue, **2**, was identical with bilobol from *Gingko biloba* [9].

The dose–response curves of antifungal activity for the ARs against spore-germination of *Pyricularia oryzae* P_2 are shown in Fig. 2. At a concentration of 100 $\mu g\,ml^{-1}$ both types of AR caused almost complete inhibition. However, at concentrations lower than 100 $\mu g\,ml^{-1}$, **4** appeared to exhibit slightly higher activity than the mixture of ARs with the saturated alkyls. The ED_{50} of **4** was *ca* 40 $\mu g\,ml^{-1}$, 50 $\mu g\,ml^{-1}$ for the mixture of **1**, **3** and **5** and 45 $\mu g\,ml^{-1}$ for the mixture of **1**–**5** (data not shown).

The time-course of AR accumulation in etiolated rice seedlings is shown in Fig. 3. For extraction, the whole plant of the etiolated seedlings was used. Partially purified sample by ODS cartridge column was derivatized as TMSi and analysed by GCEI-MS. All components had a common base ion peak at *m/z* 268, which was formed by a fission at the benzylic position of the alkyl chains [10]. Therefore, the total amount of the AR was calculated using the mass chromatograms with the peak at *m/z* 268. The ARs were absent on day 2, first detected on day 4, increased linearly with time, and led to a concentration of 126 $\mu g\,g^{-1}$ fr. wt on day 10. The ARs were newly produced after germination and on day 6, the amount led to a concentration of 50 $\mu g\,g^{-1}$ fr. wt which is comparable to the ED_{50}. Significantly elevated concentration of the ARs in etiolated seedlings suggested that these ARs may play a role in defending an early seedling stage against microbes.

Several 5-alkylresorcinols have been found as deleterious substances in animals [10, 11] in rye, wheat, and barley (Gramineae), and some plant families (Anacardiaceae, Ginkgoaceae, and Proteaceae). In those ARs with unsaturated alkyls, 5-(3′*Z*-undecenyl)-resorcinol [13] from the vesicant wood of *Persoonia elliptica* (Proteaceae), 5-(8′*Z*-pentadecenyl)-resorcinol (bilobol) from *Ginkgo biloba* (Ginkgoaceae) [9], its 10′*Z* isomer [14] from the vesicant exudate of seed pods of *Grevillea pyramidalis* (Proteaceae), and 5-(12′*Z*-heptadecenyl)-resorcinol [15] from the peel of mango fruit (Anacardiaceae) have been reported; 5-(8′*Z*-heptadecenyl)-resorcinol however, does not appear to have been recorded. This is also the first finding of the ARs being present in the rice plant. Additionally, there are a few apparent differences between the ARs in rice and the three cereal plants. In the latter, ARs are present in the seeds themselves and consist of relatively longer alkyl chains of C_{13} to C_{29} which are accompanied by the corresponding mono and bisunsaturated alkyls as minor components [16], whereas in rice the ARs are not present in the seeds, but are newly produced after germination, and consist of relatively shorter carbon chains of C_{13} to C_{17}. As mentioned, in wheat and rye the ARs are present in the seeds, while the cyclic hydroxamates appear upon germination and accumulate. It is noteworthy that in rice the ARs, but not the

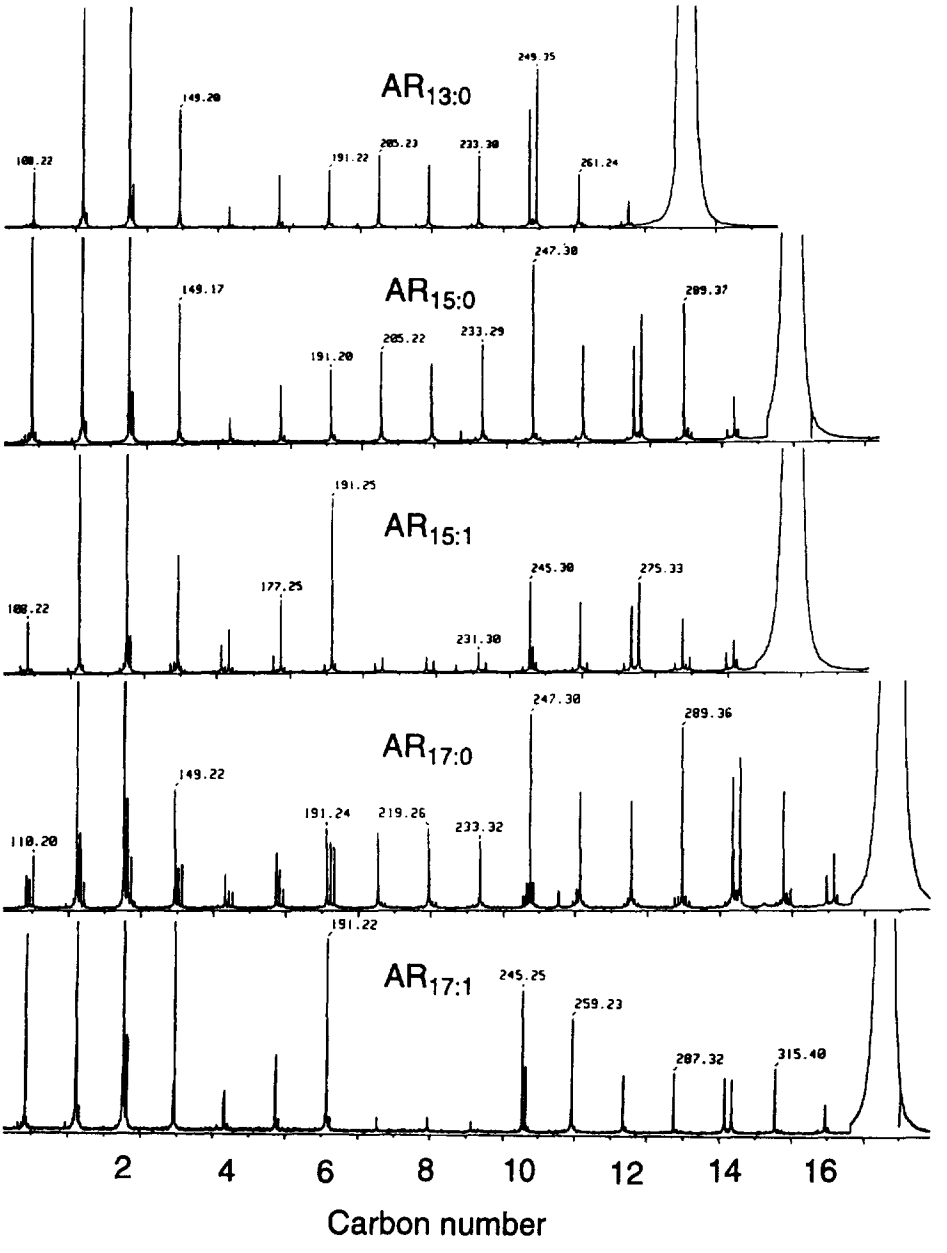


Fig. 1. Charge-remote fragmentation FAB-mass spectra of $[M - H]^-$ ions of ARs (carbon one is the benzylic position).

cyclic hydroxamates, accumulate during the etiolated seedling stage.

EXPERIMENTAL

General. NMR spectra were recorded with TMS as an internal standard. IR spectra were measured as films. FAB-MS and MS-MS data were measured using glycerol as a matrix. TLC was conducted with precoated plates (0.25 mm and 0.5 mm thickness) of silica gel F_{254}.

Preparation of rice seedlings. Rice seeds (*Oryza sativa* L. cv. RD-25: Indica type) were immersed at 30° for 2 days after sterilizing with 70% EtOH for 3 min. The immersed seeds were transferred on vermiculite and incubated at 25–27° in the dark.

Extraction of rice seedlings and solvent partitioning. The shoot tissues of 7-day-old etiolated rice seedlings (150 g fr.wt) were homogenized with: (a) 80% aq. MeOH (2L); or (b) water (400 ml) for one min. MeOH (1.6 l) was added to the latter homogenate after 1 hr. Each filtrate after 1 day was concd and partitioned between Et_2O and H_2O at pH 2. The Et_2O layer was extracted with 0.5 N NaOH aq., washed with H_2O, and evapd to dryness, giving an ether soluble neutral fr. The NaOH extract was extracted with EtOAc at pH 2, and the organic layer was

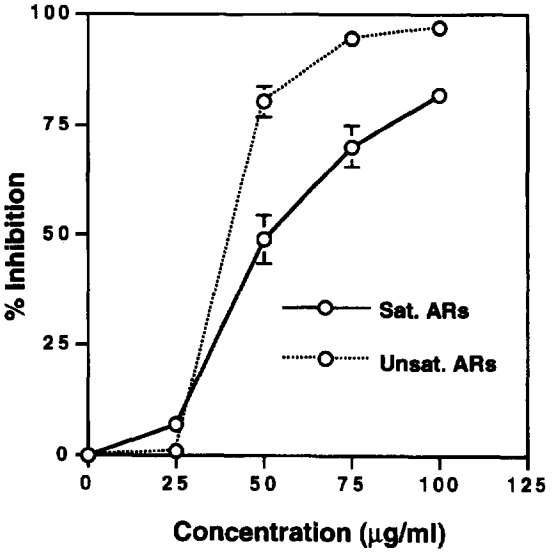


Fig. 2. Dose-response of ARs in spore germination on *P. oryzae* P_2. Sat. ARs: $Ar_{13:0,15:0,17:0}$, Unsat. ARs: $AR_{15:1,17:1}$. Inhibition % after 5 hr incubation at 25°. Data represent an avarage of two samples.

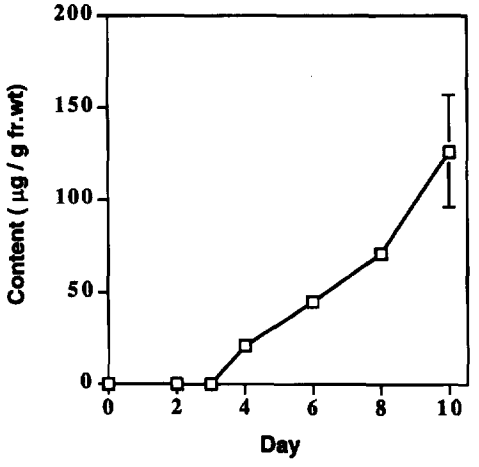


Fig. 3. Time-course of accumulation of ARs in etiolated rice seedlings. Whole etiolated rice seedlings at 25° were used for extraction. Data represent an avarage of three samples.

evapd to dryness after washing with H_2O, giving an EtOAc soluble acidic fr. The H_2O layer was extracted with *n*-BuOH, and the organic layer was washed with H_2O and evapd to dryness, giving a BuOH fr.

Isolation of antifungal substances. The shoot tissues of the 7-day-old etiolated rice seedlings (400 g fr.wt), after the injury treatment described above, were soaked in 80% MeOH for one day. The solvent partitioning of the concd filtrate gave TN (435 mg), TA (204 mg), and TB (298 mg) frs, respectively. The TN fr. was passed through a Sephadex LH-20 column with MeOH, giving 4 frs (TN-1: 72.3 mg, TN-2: 247.2 mg, TN-3: 96.0 mg, TN-4: 16.2 mg). The TN-3 fr. was then purified by prep. TLC on silica gel (0.5 mm thickness) with MeOH–$CHCl_3$ (7:93). The band of R_f around 0.5 which was visualized under UV light (258 nm) was scrapped and eluted with MeOH–$CHCl_3$ (10:90). The eluate was evapd to give a mixture of ARs (64.0 mg) as an active substance.

IR ν_{max} (film) cm^{-1}: 3300, 2900, 2850, 1620, 1595, 1460, 1300, 1140, 985, 820; UV λ_{max} (EtOH) nm: 276 and 281 ($E^{1\%} = 42$); ^1HNMR (270 MHz, $CDCl_3$): see below; GCEI–MS of its TMSi (see Time-course experiment) 20 eV, *m/z* (rel. int., %): 436 (M^+, 50), 421 (8), 310 (3), 272 (12), 268 (100) in $AR_{13:0}$, 462 (M^+, 62), 447 (10), 310 (6), 272 (12), 268 (100) in $AR_{15:1}$, 464 (M^+, 78), 449 (8), 310 (3), 272 (10), 268 (100) in $AR_{15:0}$, 490 (M^+, 56), 475 (8), 310 (6), 272 (12), 268 (100) in $AR_{17:1}$, 492 (M^+, 80), 477 (10), 310 (4), 272 (5), 268 (100) in $AR_{17:0}$; FAB-MS (negative) *m/z* $[M - H]^-$: 291 ($AR_{13:0}$), 317 ($AR_{15:1}$), 319 ($AR_{15:0}$), 345 ($AR_{17:1}$), 347 ($AR_{17:0}$); FAB-MS (positive) *m/z* $[M + H]^+$: 293 ($AR_{13:0}$), 319 ($AR_{15:1}$), 321 ($AR_{15:0}$), 347 ($AR_{17:1}$), 349 ($AR_{17:0}$); charge-remote fragmentation FAB-MS (see Fig. 1).

Separation of the ARs with saturated and unsaturated alkyls. The mixture of the ARs (57.2 mg) was purified on silica gel plates (20 cm × 20 cm × 0.25 mm, 6 plates) impregnated with 15% $AgNO_3$ in EtOH–H_2O (1:1). The plates were developed twice with C_6H_6–EtOAc (85:15) at 10 cm height followed by 15 cm height. The eluates of R_f 0.40–0.47 and R_f 0.28–0.36 gave the ARs with the satd alkyls (14.8 mg) as powders and ARs with the unsatd alkyls (11.3 mg) as a syrup in 45.6% total yield, respectively. The GCEI-MS analysis indicated that the latter was composed of the $AR_{17:1}$ containing 2% of $AR_{15:1}$ and the former, 31% of $AR_{13:0}$, 61% of $AR_{15:0}$, and 8% of $AR_{17:0}$.

The $AR_{17:1}$, EI-MS 20 eV, *m/z*: found, 346.2868 (M^+, $C_{23}H_{38}O_2$, calcd.346.2869); ^1H- NMR (270 MHZ, $CDCl_3$): δ6.25 (2H, *d*, J = 2.3 Hz, H-4 and H-6), 6.17 (1H, *t*, J = 2.3 Hz, H-2), 5.35 (2H, m, H-8′ and H-9′), 5.24 (2H, *br. s*, OH), 2.47 (2H, *t*, J = 7.2 Hz, H-1′), 2.00 (4H, *m*, H-7′ and H-10′), 1.55 (2H, *m*, H-2′), 1.25 (20H, *br. s*), 0.89 (3H, *t*, J = 6.7 Hz, H-17′); ^{13}CNMR (67.5 MHz, $CDCl_3$): δ156.4 (*s*, C-1 and C-3), 146.2 and 146.1 (*s*, C-5), 129.9 and 129.8 (*d*, C-8′ and C-9′), 108.0 (*d*, C-4 and C-6), 100.1 (*d*, C-2), 35.8 (*t*, C-1′), 31.8 (*t*, C-15′), 31.0 (*t*, C-2′), 29.7 *t*, 29.6 *t* × 2, 29.5 *t*, 29.4 *t*, 29.3 *t* × 2, 29.2 *t*, 27.2 (*t*, C-7′ and C-10′), 22.6 (*t*, C-16′), 14.0 (*q*, C-17′).

Time-course experiment. Whole seedling plants (*ca* 1 g fr. wt) were homogenized with MeOH (50 ml) and the filtrate was concd. The residue was placed on ODS cartridge column (Absorbex RP-18: 400 mg, Merck) using water (4 ml). The column was successively eluted with water (4 ml), 50% (4 ml), 75% (4 ml), and 100% MeOH (3 ml). To a concd MeOH eluate was added *N*-methyl-*N*-trimethylsilyltrifluoroacetimide (50 μl) and the mixture was allowed to stand for an hour at room temp. One μl of the sample was injected into GC equipped with a MS spectrometer, DB-1 column (0.53 mm i.d. × 15 m, 1.5 μm film thickness, column oven temp: 230°, He_2 at 10 ml min^{-1}; R_t (min.): $AR_{13:0}$ (4.8), $AR_{15:1}$ (8.2), $AR_{15:0}$ (8.7), $AR_{17:1}$ (14.4), and $AR_{17:0}$ (15.8). Total amounts of

ARs were calcd from the mass chromatograms with the ion at m/z 268.

Spore germination assay. Pyricularia oryzae P_2 was grown on oatmeal-agar medium at 25° in the dark for 7–10 days. Spores were collected by agitating with distilled water from cultures 2 days older under fluorescent light after removal of the spores from the culture plate. The spore suspension was filtered through tissue paper and adjusted to a density of about 10^5 spores ml^{-1} by addition of distilled water. Thirty μl of the spore suspension was added to a 30 μl of test sample (15% EtOH solution) and it was mixed well with a toothpick and incubated at 25° in the dark for 5 hr. The percentages of germinating and non-germinating spores were determined using a microscope.

Acknowledgement—This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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