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PHOTOSYNTHETIC INHIBITORS IN *EUCALYPTUS GRANDIS*

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Key Word Index—*Eucalyptus grandis*; Myrtaceae; inhibitors; photosynthetic electron transport; G-inhibitors; grandinol; homograndinol.

Abstract—Unlike the G-inhibitors isolated from the leaves of *Eucalyptus grandis*, synthetic G-inhibitors showed no inhibition of photosynthetic electron transport. These findings indicated that small amounts of impurities present in the natural samples were responsible for the inhibition of photosynthetic electron transport. In support of this, grandinol and homograndinol were isolated from the leaves of *E. grandis* and were shown to be the active photosynthetic electron transport inhibitory components in the *E. grandis* preparation of G-inhibitors.

INTRODUCTION

Grandinol [1] and the G-inhibitors [2, 3] are plant growth regulators (PGRs) that occur in leaves of *Eucalyptus grandis*. The content of G-inhibitors in adult leaves is higher (5–10 mg/g fr. wt) than that of grandinol (0.03 mg/g fr. wt). Both PGRs affect rooting of stem cuttings [4] and water loss by transpiration from excised shoots [5]. Grandinol is active at lower concentrations ($>10^{-6}$M) than G-inhibitors ($>10^{-4}$M) as an inhibitor of cress seed germination [6]. Furthermore, Sharkey *et al.* reported that G-inhibitors also inhibited photosynthetic electron transport at the site between photosystem II (PSII) and plastoquinone [7].

Since this initial study on photosynthetic electron transport inhibition, sufficient amounts of HPLC-pure G-inhibitors and their analogues have been synthesized [8], to allow the biological activities of the synthetic materials to be confirmed in all systems described above except the photosynthetic electron transport system. The inactivity of synthetic G-inhibitors in the photosynthetic electron transport system became evident during early trials associated with the present investigation and also from preliminary results obtained by Phillips [personal communication]. The difference in photosynthetic electron transport inhibitory activities between naturally occurring G-inhibitors and synthesized G-inhibitors suggested the possibility that impurities in the samples isolated from the leaves by Paton *et al.* [7] might be responsible for the inhibition of photosynthetic electron transport.

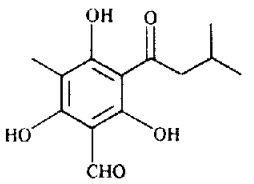
The samples of G-inhibitors used in the original study of photosynthetic electron transport inhibition [9] were obtained by recrystallization from the active fraction separated by column chromatography of adult-leaf extract and monitored by rooting bioassays. The photosynthetic electron transport inhibition was attributed to G-inhibitors in the absence of any evidence from UV and NMR studies [10] to show that the recrystallized fraction did not contain any compounds other than G-inhibitors. Nevertheless, the inconsistent results obtained with the original sample of natural G-inhibitors and pure synthetic G-inhibitors, clearly pointed towards the existence of some highly active trace contaminant in the sample of natural G-inhibitors. This situation was thus remarkably similar to that previously found for phaseic acid which had no photosynthetic electron transport activity when purified [11], although it was a major component in plant extracts that inhibited photosynthesis [12].

The present study identifies the active photosynthetic electron transport components in the fraction of G-inhibitors as grandinol and the closely related homograndinol. The isolation techniques required to separate these critical trace components from the G-fraction are described in detail. Most importantly, the photosynthetic electron transport results obtained with synthetic G-inhibitors, grandinol and homo-grandinol, are compared with those of natural substances isolated from adult leaves of *E. grandis*.

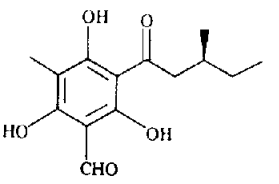
RESULTS AND DISCUSSION

Grandinol and homograndinol (for properties see Table 1) isolated from leaves of *E. grandis* showed obvious photosynthetic electron transport inhibition at 2×10^{-5} and 5×10^{-6}M respectively. These activities were confirmed by the use of the synthetic compounds. In contrast, synthetic G-inhibitors exhibited no photosynthetic electron transport inhibition even at 10^{-3}M (Table 2). Thus the natural occurring G-inhibitors used in the previous investigation [7] were very likely contaminated with small amounts of grandinol and homograndinol. Such contamination was demonstrated by HPLC analysis using multi-scan UV detection (Fig. 1). The quantities of the contaminating grandinol and homograndinol in the natural G-inhibitors were estimated as 0.12 and 0.10% respectively. In the previous paper [7], it was reported that 3 mM of natural G-inhibitors caused 60% photo-

Table 1. Physical and chemical properties of grandinol and homograndinol



Grandinol



Homograndinol

	Grandinol	Homograndinol
Mp	130—132°	153–155°
^{1}H NMR (ppm)	1.00 (6H, *d*), 2.05 (3H, *s*), 2.24 (1H, *m*), 3.02 (2H, *d*), 10.2 (1H, *s*)	0.92 (3H, *t*), 0.95 (3H, *d*), 1.28 (2H, *m*), 2.05 (3H, *s*), 2.34 (1H, *m*), 2.88 (1H, *dd*), 3.15 (1H, *dd*), 10.1 (1H, *s*)
IR (cm^{-1})	3340, 1650, 1630, 1600	3210, 1650, 1630, 1600
HRMS (*m/z*)	$M^+ = 252.0997$ ($C_{13}H_{16}O_5$)	$M^+ = 266.1169$ ($C_{14}H_{18}O_5$)
UV (nm)	276 (=28 000), 343 (=3800)	273 (=29 000), 340 (=3800)
$[\alpha]_D$	—	+14.1°

Table 2. Concentration for 50% photosynthetic electron transport inhibition by synthetic and natural product

	Synthetic	Natural product
Grandinol	8×10^{-5}M	8×10^{-5}M
Homograndinol	2×10^{-6}M	2×10^{-6}M
G-inhibitors	$>5 \times 10^{-2}$M*	$>5 \times 10^{-2}$M*†
		3×10^{-3}M‡

*Saturated aqueous solution of G.
†G-inhibitors' sample without contaminants.
‡G-inhibitor sample with 0.2% grandinol + homograndinol.

synthetic electron transport inhibition in spinach chloroplasts. However, the presence of about 0.2% grandinol plus homograndinol as contaminants with photosynthetic electron transport inhibition at about 0.003 mM, readily explains this apparent photosynthetic electron transport activity of natural G-inhibitors.

The photosynthetic electron transport inhibitors of *E. grandis* were indicated as PSII inhibitors by accurate measurement of stomatal conductance in comparison with DCMU. The fluorescence induction curve method [3] was adopted to determine the inhibition site of grandinol and homograndinol. The induction curves represent the pool size of the reducible primary and secondary electron acceptors under inhibited (A_i) and normal (A_n) conditions. The fluorescence induction of various PSII inhibitors by means of A_i/A_n values, with grandinol and homograndinol giving values of the same order as other PSII inhibitors is shown in Table 3. It is a reasonable expectation that inhibitors like grandinol and homograndinol will interfere with PSII electron transport, since other phenolic photosynthetic electron transport inhibitors are considered to have common functional groups [15].

While the initial physiological investigation [7] revealed photosynthetic electron transport inhibition in an extract of adult leaves of *E. grandis*, unequivocal identification of grandinol and homograndinol as the PGRs involved in this photosynthetic electron transport activity depended largely on the availability of synthetic preparations of these two PGRs as well as G-inhibitors. Another important factor was the need to develop special analytical methods before all these PGRs could be cleanly separated from extracts of adult leaves. Our results thus provide a good example of the complementary roles played by plant physiology and natural products chemistry in studies of this kind. We believe that the same approach has the potential to lead to identification of the molecules involved in photosynthetic electron transport inhibition reported for other plant extracts [16].

In the absence of good evidence to indicate the basis for photosynthetic electron transport inhibition by other plant extracts, the molecular basis of this phenomenon in green plants seems to be restricted to grandinol and homograndinol in *E. grandis*. Indeed, the only other naturally occurring compound that exhibit photosynthetic electron transport inhibition appears to be stigmatellin [17] which has been isolated from the non-photosynthetic myxo bacterium, *Stigmatella aurantiaca* [18].

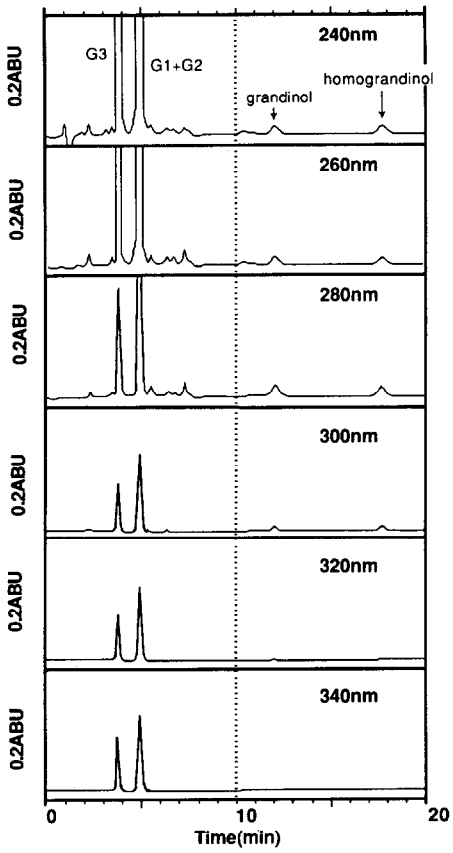


Fig. 1. Analysis of the quantities of the contaminating grandinol and homograndinol in G-inhibitors by HPLC using a multi-scan UV detector.

Table 3. The fluorescence induction rates of various photosynthetic electron transport inhibitors

Inhibitor	Concentration (μM)	A_i/A_n
DCMU	5	0.07
	10	0.06
Atrazine	5	0.08
	10	0.06
Ioxynil	5	0.07
	10	0.07
Grandinol	50	0.11
	100	0.07
Homograndinol	10	0.09
	20	0.07

The area values were estimated by fluorescence induction curves under inhibited (A_i) and normal (A_n) conditions [13].

EXPERIMENTAL

Bioassay for photosynthetic electron transport inhibition. Photosynthetic electron transport inhibition was measured by the Hill reaction, using chloroplasts isolated from the leaves of spinach (*Spinacia oleracea*), with a Clark-type O_2 electrode fitted into a water-jacketed cuvette [7]. All assays were carried out at 25°. The assay vol. was 2.0 ml, and Chl concn was usually 50 μg/ml. The site of inhibition of the compounds was confirmed by the fluorescence induction method [13] using isolated chloroplasts.

Isolation of photosynthetic electron transport inhibitors. Fresh leaves of *E. grandis* (11 kg) were extracted with 40 l MeOH. The extract was concd *in vacuo* to give *ca* 700 g of a gummy syrup, which was dissolved in aq. 95% MeOH (3 l) and applied on cellulose powder (2 kg) which was then percolated with 10 l of MeOH-saturated hexane. The eluant was concentrated and applied to a silica gel column (100 mm i.d. × 1000 mm), which was eluted with hexane–EtOAc mixtures (from 100% hexane to 100% EtOAc, 5% step gradient, each step 2 l). The eluate was fractionated (500 ml) to give 84 fractions. Although fractions between Nos 14 and 20 contained G-inhibitors, clear and substantial Hill inhibition was observed from fractions Nos 8 to 18. The photosynthetic electron transport inhibitory fractions were combined and concentrated to give 30 g of gum, which was rechromatographed on a silica gel column (50 mm i.d. × 500 mm) using acidic solvents (hexane–EtOAc mixtures containing 0.5% formic acid; 2% EtOAc gradient with 500 ml steps). The elution of active materials was detected between 8% and 12% EtOAc. The active fractions were further purified by prep. HPLC using a high-content ODS column (Senshu Pak 3151, column size: 20 mm i.d. × 300 mm, solvent system: MeCN–H_2O–HCO_2H, 140:59:1, flow rate: 10 ml/min, monitor: UV at 280 nm). Each peak was collected and assayed for photosynthetic electron transport inhibition. Two fractions (R_t 32 and 45 min) indicated strong activity. After evapn of the solvent *in vacuo* at 40–50°, the earlier fraction gave colourless crystals (grandinol, 40 mg) whereas the latter afforded an amorphous solid (homograndinol, 10 mg). Physico-chemical properties of grandinol and homograndinol are listed in Table 1. Mps: uncorr; ^{1}HNMR: 400 or 100 MHz.

Synthetic samples of G-inhibitors [7] and grandinol [13] were prepared by established procedures. For synthesis of the optically active homograndinol, *S*-(+)-2-methyl-1-butanol was used as the starting material of the side chain.

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