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EFFECTS OF BIOREGULATORS ON INDOLE ALKALOID BIOSYNTHESIS IN *CATHARANTHUS ROSEUS* CELL CULTURE

SIU-LEUNG LEE, KE-DI CHENG and A. IAN SCOTT*

Department of Chemistry, Texas A & M University, College Station, TX 77843, U.S.A.; * Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K.

Key Word Index—*Catharanthus roseus*; Apocynaceae; plant cell culture; indole alkaloids; biosynthesis; bioregulators; catharanthine; ajmalicine.

Abstract—Several carotenoid-inducers are effective in promoting indole alkaloid formation in *Catharanthus roseus* cell culture. Among the five compounds tested, viz. 1,1-dimethylpiperidine, 2-diethylaminoethyl-3,4-dichlorophenylether, 2-diethylaminoethyl-2,4-dichlorophenylether, 2-diethylaminoethyl-β-naphthylether and 2-diethylaminoethyl-3,4-dimethylphenylether, 1,1-dimethylpiperidine, 2-diethylaminoethyl-2,4-dichlorophenylether and 2-diethylaminoethyl-β-naphthylether at 5 ppm concentration increased total alkaloid production by up to *ca* 20% with concomitant increases in ajmalicine and catharanthine. Concentrations of 2-diethylaminoethyl-2,4-dichlorophenylether higher than 5 ppm caused growth inhibition and decrease in alkaloid synthesis.

INTRODUCTION

The development of a *Catharanthus roseus* cell culture and a cell-free system from it [1] have opened up new ways for theoretical studies and practical production of indole alkaloids from this plant. However, a search for new methods to induce the production of the 'late' alkaloids in large amounts is still necessary to render the system economically worthwhile.

2-(4-Chlorophenylthio)-triethylamine hydrochloride was first reported to induce accumulation of lycopene in citrus [2, 3] and *Blakeslea trispora* [4]. Several other amines [5, 6] were found to have similar effects, presumably by derepressing carotenogenesis. Although the site of action is unknown, the fact that rubber biosynthesis is also induced by 2-diethylaminoethyl-3,4-dichlorophenylether (**2**) implies that these synthetic regulators may be general inducers of terpenoid biosynthesis [7]. Furthermore, biosynthesis of indole alkaloids in *C. roseus* plants was observed to be promoted by some of these compounds (H. Yokoyama, personal communication). These results prompted us to test the compounds on a *C. roseus* cell line capable of producing *Heteroyohimbine*, *Strychnos*, *Iboga* and *Aspidosperma* alkaloids [8].

RESULTS AND DISCUSSION

Concentration effect of bioregulator 3

Compound **3** was added to the culture on day 0 and day 18 after inoculation, and the cells were grown for a total of 30 days. Growth was inhibited in the first case, but not in the latter showing that cell growth is unaffected once they reach the stationary phase (Fig. 1).

The pH of the medium was not affected at concentrations lower than 50 ppm while a higher concentration caused a shift from pH 5.8 to 6.2 which remained unchanged up to 500 ppm of **3**. The optimal concentration of **3** was found to be between 2 and 5 ppm (added on day 7) resulting in a 22% increase of total alkaloid per g dry wt. Increases in ajmalicine (**6**) (from 270 to 575%) and catharanthine (**7**) (from 82 to 146%) were observed (Fig. 2). Above 10 ppm, **3** inhibits alkaloid synthesis. At 100 ppm, only one CAS-lavender spot was detected on 2-dimensional TLC. Identification of this alkaloid is in progress.

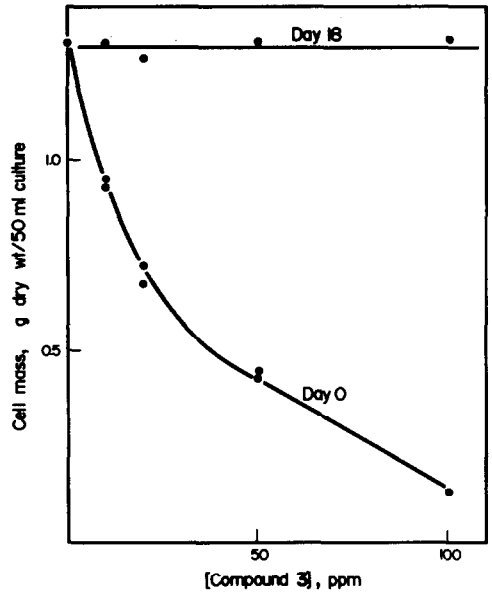


Fig. 1. Effect of different concentrations of 2-dithylaminoethyl-2,4-dichlorophenylether administered at different phases of *Catharanthus roseus* cell culture.

Effect of other synthetic bioregulators

Similar experiments were carried out with 1,1-dimethylpiperidine (**1**), 2-diethylaminoethyl-3,4-dichlorophenylether (**2**), 2-diethylaminoethyl-2,4-dichlorophenylether (**3**), 2-diethylaminoethyl-β-naphthylether (**4**) and 2-diethylaminoethyl-3,4-dimethylphenylether (**5**). The compounds were added to

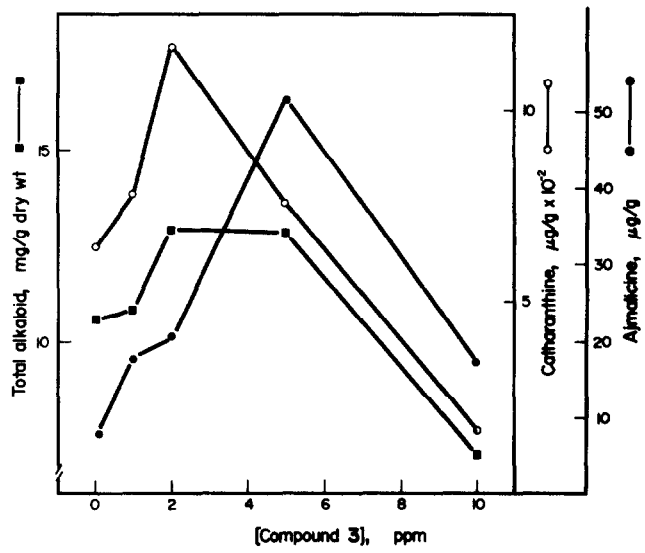
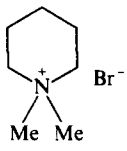


Fig. 2. Concentration effect of 2-diethylaminoethyl-2,4-dichlorophenylether on alkaloid production in *Catharanthus roseus* cell culture.



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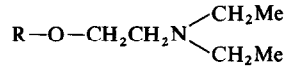

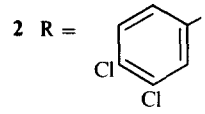

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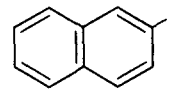

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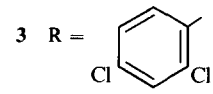

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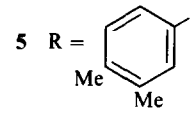

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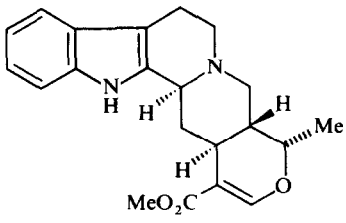

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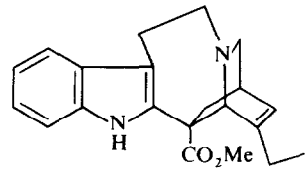

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C. roseus cell cultures at 5 ppm on day 7. Among the five compounds tested, **1**, **3** and **4** showed a *ca* 20% increase in total alkaloid with concomitant increase of ajmalicine and catharanthine (Fig. 3). The increase of monoterpene indole alkaloid may be explained as a result of induction of terpenoid precursors as in the reported cases. As tertiary amines, they might also act as oxygen scavengers, protecting oxidative degradation of the indole alkaloids. Enzymological study of the terpenoid pathway under the influence of these bioregulators will resolve this problem.

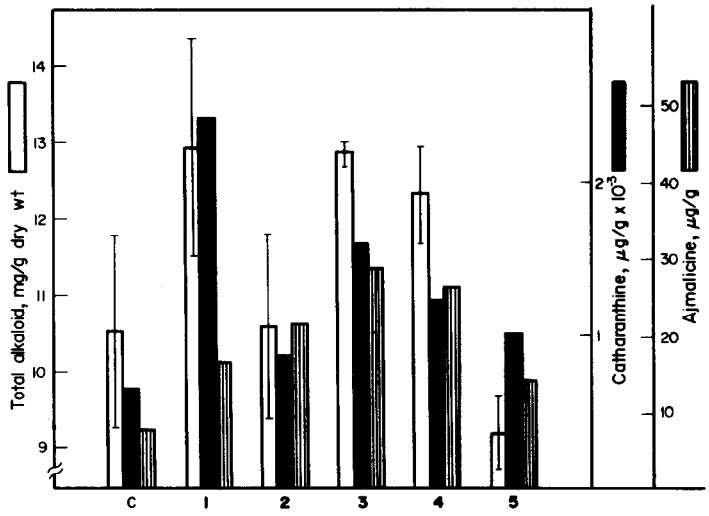

Fig. 3. Effect of different bioregulators on indole alkaloid production in *Catharanthus roseus* cell culture. C = control; **1** = 1,1-dimethylpiperidine; **2** = 2-diethylaminoethyl-3,4-dichlorophenylether; **3** = 2-diethylaminoethyl-2,4-dichlorophenylether; **4** = 2-diethylaminoethyl-β-naphthylether; **5** = 2-diethylaminoethyl-3,4-dimethylphenylether.

EXPERIMENTAL

C. roseus cell culture was maintained on SH medium [9] as previously described [8, 10]. The cell line used produces catharanthine as the major alkaloid (6–8% of total alkaloid) while ajmalicine constitutes 0.2–0.4% of the total alkaloid.

All bioregulators except **3** were gifts from Dr. Henry Yokoyama. Compound **3** was synthesized in our laboratory by the method of ref. [11]. They were added as free bases (except **1** as bromide) to the culture through a 0.45 μm membrane filter.

Total alkaloids were extracted by homogenization of freeze-dried cells in MeOH (Sorvall Omnimixer, full speed for 5 min). The MeOH extract was evapd under red. pres. and partitioned between H_2O (*ca* pH 3) and petrol. Then the aq. phase was basified to pH 8.5 with 1 M NaOH and extracted with CH_2Cl_2. Total alkaloids were determined by *A* at 280 nm compared to a standard curve constructed with weighed alkaloid mixtures (linear regression correlation coefficient >0.998).

Individual alkaloids were purified by HPLC (Waters μBondpak-C_{18} column, 3.9 mm × 30 cm; MeCN/10 mM Na-Pi buffer, pH 7.5; flow rate 1 ml/min) programmed with Perkin–Elmer 3B solvent delivery system as follows:

	T_{equil}	T_1	T_2	T_3	T_4	T_5
% MeCN	35	40	40	55	90	90
Time (min)	10	10	10	7	8	10
Curve		1	0	0	1	1

Alkaloids were monitored at 254 nm (Perkin–Elmer variable wavelength detector LC-75). R_ts for ajmalicine and catharanthine are 30.6 min and 36.6 min respectively. Purity of alkaloids were checked by Si gel TLC ($CHCl_3$–MeOH, 9:1), visualized by ceric ammonium sulfate (CAS) spray [12]. Quantitative determination of ajmalicine and catharanthine were made on the basis of peak areas on HPLC output, compared to standard curves (linear regression correlation coefficient >0.990).

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