Effect of 5-Azacytidine on the Formation of Secondary Metabolites in *Catharanthus roseus* Cell Suspension Cultures

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A cell suspension culture of *Catharanthus roseus* was treated during the growth phase with the nucleoside analogue 5-azacytidine, a powerful inducer of cell differentiation, as evidenced with animal and human cell lines. After induction the cell culture was further incubated in a production medium in the absence of the inducer for 10 days. The extraction of the freeze dried cells and analysis by high performance liquid chromatography showed an additional intense peak in the elution profile, not present in extracts of untreated cells. The structure of the newly synthesized metabolite, elucidated by mass spectrometry and ^{1}H and ^{13}C NMR, was a lignan type compound, lirioresinol B mono-β-D-glucoside. This compound has hitherto not been found in *Catharanthus roseus* plants or cell cultures.

Introduction

The main obstacle to the production of alkaloids in cell cultures of *Catharanthus roseus* is their low synthetic capacity compared to the whole plant [1]. This generally observed phenomenon may be due to the transition of the differentiated cell of the plant to the undifferentiated cell of the suspension culture. Some years ago it was observed that addition of dimethylsulfoxide to murine erythroleucemic cell cultures could stimulate the differentiation and thereby reactivate the capacity of the cells to synthesize hemoglobin which was lost after transformation by Friend viruses [2]. These findings resulted in the identification of a considerable variety of compounds which are active as differentiation inducers in this system. Amongst these are diamines [3], purine derivatives [4], and short chain fatty acids [5], for reviews see [6, 7]. Recently a nucleoside analogue, 5-azacytidine, was found to be a potent inducer of cell differentiation *via* inhibition of DNA methylation [8]. In plant cell cultures many attempts have been made to raise the production of secondary metabolites by varying the phytohormones, medium composition or by adding precursors [9, 10].

We wish to report on the influence of differentiation inducers, mainly 5-azacytidine, on the production of secondary compounds in *Catharanthus roseus* cell suspension cultures.

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Materials and Methods

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don were maintained in Murashige and Skoog medium [11] containing 3% sucrose and 2×10^{-6} M 2,4-dichlorophenoxyacetic acid. The suspension cultures were grown in 250 ml Erlenmeyer flasks containing 70 ml of culture medium at 26 °C in the dark on a gyratory shaker with 110 strokes/min. Subculturing was performed every two weeks using 2 g fresh weight cells as inoculum.

5-Azacytidine (Sigma Chemicals) was dissolved in water at a concentration of 7 mg/100 ml prior to application. The solution was added to the culture flask by pressing the respective amount through a sterile Millex filter unit (0.22 µm) to give a final concentration ranging from 70–140 µg per culture flask.

For the induction experiments the cultures were started by inoculation with 2 g cells in growth medium [11]. At days 3, 5 and 7 the cultures were treated with 1.0, 1.5 or 2.0 ml of the 5-azacytidine solution, respectively. The inducer has to be added repeatedly because of the short half life of azacytidine in solution [12]. The cultures were incubated until day 14, then the cells were separated by vacuum filtration through a porous glass filter and transferred to a production medium composed of MS-medium with 8% sucrose, one tenth of the original phosphate and nitrate, and devoid of phytohormones. The induced suspension cultures were incubated for a further two weeks. At the end of incubation the cells were harvested by vacuum

filtration, washed with distilled water and freeze dried. For HPLC and TLC analysis freeze dried cells were extracted with methanol. The crude extract was worked up to give an alkaline $CHCl_3$-extract, which was further purified by the enrichment technique with Extrelut cartridges (Merck, Darmstadt) as described [13]. HPLC analysis was performed with two Waters pumps M 6000 A, a M 660 solvent programmer and a Hewlett-Packard HP 1040 A photodiode array UV-detector with the pilot signal set to 280 nm. Spectra were recorded from 210 to 400 nm with a band width of 2 nm. Columns (25 cm × 4 mm) were packed with 10 µm Nucleosil RP-18 (Merck, Darmstadt). A linear solvent gradient of acetonitrile/0.005 molar aqueous triethylammonium formate buffer (pH 8.5) from 20:80 to 84:16 within 30 min was used. For TLC analytical and preparative silica gel 60 F_{254} coated plates (Merck, Darmstadt) were used. In both cases the plates were developed with CH_2Cl_2/MeOH 90:10. The spots were detected by spraying with a 1% solution of Ce (IV) ammonium sulfate in 60% phosphoric acid.

For the isolation of the new compound 40 g of lyophilized azacytidine treated cells were extracted with methanol in a Soxhlet apparatus for 6 h. The extract was diluted with an equal volume of acetic acid (10% v/v) and extracted twice with *n*-hexane. The aqueous phase was made alkaline with conc. ammonia and extracted with chloroform. After evaporation, the residue (250 mg) was redissolved in methanol and chromatographed on a Sephadex LH-20 column (80 × 3.5 cm) with methanol as the eluent. Fractions of 250 ml were collected. Fraction four contained the lignan and was evaporated to dryness (118 mg). Further purification was achieved by preparative TLC. The substance was extracted with methanol yielding 67 mg of pure compound.

$R_f = 0.26$ (CH_2Cl_2/MeOH 90 : 10). $[\alpha]_D^{20} = -13.8$ (c = 0.54 in methanol). UV: λ_{max} (log ε) = 217 (4.13), 226 (4.07, sh), 237 (4.04, sh), 271 (3.39), 280 (3.31, sh), 321 (2.88). MS (EI): *m/e* (%) 419 (42), 418 (82, M^+-glucose + 2H), 235 (20), 221 (15), 210 (32), 207 (18), 194 (22), 193 (40), 182 (32), 181 (100), 180 (30), 168 (45), 167 (95), 163 (40), 151 (41). High resolution MS: *m/e* 418.1649 (calc. 418.16277 for $C_{22}H_{26}O_8$). FAB-MS: *m/e* (%) 579 (M-H, 27), 417 (100). For ^1H and ^{13}C NMR data see Table I and II.

3 mg of the compound obtained after preparative TLC was acetylated at room temperature with acetic anhydride/pyridine (0.5 ml each) and a catalytic amount of dimethylaminopyridine. Excess reagents were removed *in vacuo* and the product was purified by preparative HPLC on Nucleosil RP-18 (25 cm × 4 mm, methanol/water 62:38, 2 ml/min). The main peak at 280 nm was collected to give 1.5 mg of pure compound.

^1H NMR (CD_3OD): $\delta = 6.58$ (2H, s), 6.55 (2H, s), 5.27 (3H, m), 5.04 (1H, d, 16 Hz), 4.78 (1H, d, 4Hz), 4.73 (1 H, d, 4 Hz), 4.30 (2 H, m), 4.22 (1 H, d, 5 Hz), 4.12 (1H, dd, 10, 2.5 Hz), 3.94 (2H, m), 3.69 (1H, m), 3.07 (2H, m), 2.32 (3H, s), 2.02 (12H).

EI mass spectra were recorded with an AEI MS 902 S mass spectrometer at a resolution of 1000. High resolution data were obtained by the peak matching method at a resolution of 30 000. Negative ion FAB mass spectra were recorded on a Kratos MS 50 spectrometer with a Kratos FAB source; glycerol was used as matrix. ^1H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker WM 400 NMR spectrometer at 400 and 100 MHz, respectively, and locked to the deuterium resonance of the solvent. Shifts are reported in ppm relative to TMS and couplings in Hz. Iterative spin simulations were carried out with a program based on LAOCOON III.

Results

Three days after inoculation in MS-medium [11] the suspension culture of *C. roseus* cells was treated with the 5-azaC solution to give a final concentration of 70, 105 or 140 µg per culture flask, respectively. Addition of the same amount of the drug was repeated twice at two day intervals. After a further three days of growth in the presence of the drug, the cells were withdrawn from the growth medium and transferred to a five-fold volume of production medium containing 8% sucrose, one tenth of the original phosphate and nitrate, and no phytohormones. This medium has recently been shown to enhance the alkaloid production of *C. roseus* cell cultures [14, 15]. After incubation in the production medium for ten days the cells were separated and lyophilized. The cells were extracted and the extract purified as described in Materials and Methods. The HPLC analysis of the purified extract of the azaC treated cells showed beside the normal alkaloid pattern, with catharanthine and ajmalicine as

the major constituents, an intense peak at 6.5 min, not present in untreated cells (Fig. 1). The UV-spectrum of this peak, obtained during the run with a photodiode array detector has an absorption maximum typical of an aromatic chromophore at 271 nm (Fig. 2). TLC analysis of the cell extract exhibited a new polar metabolite which developed an orange colour on spraying with Ce-IV-reagent.

For structure elucidation the new compound was isolated and purified by column chromatography on Sephadex LH-20 and preparative TLC. These procedures yielded a colourless amorphous substance whose EI mass spectrum showed a prominent peak at 418 mu. In addition a negative ion FAB mass spectrum exhibited a $[M-1]^-$ peak at 579 mu and a fragment ion at 417 mu corresponding to the loss of a hexose moiety $[M-C_6H_{11}O_5]^-$, indicating an unsubstituted hexose attached *via* an O-glycosidic bond. Hence the *m/e* 418 ion in the EI-ms is the aglycon resulting from thermal decomposition of the glycoside. The high resolution *ms* afforded an elementary composition of $C_{22}H_{26}O_8$ for the *m/e* 418 peak.

The ^1H and ^{13}C NMR data of the compound are shown in Table I. Extensive ^1H homonuclear decoupling experiments indicated the presence of two OCH_2CHCHO systems with almost identical shifts and the presence of a single sugar residue. Careful iterative spectral simulation allowed complete analysis of the latter and unambiguously established the presence of a β-glucopyranoside. The intensities of the proton and carbon signals of

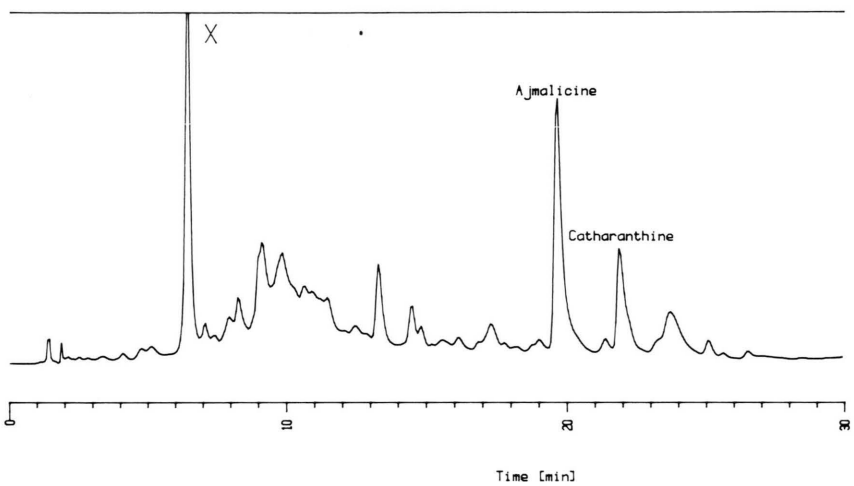


Fig. 1. HPLC diagramm at 280 nm of 5-azacytidine treated *Catharanthus roseus* cell culture with ajmalicine and catharanthine as major alkaloids and the new metabolite indicated with X. This diagramm could be reproduced with several induction experiments.

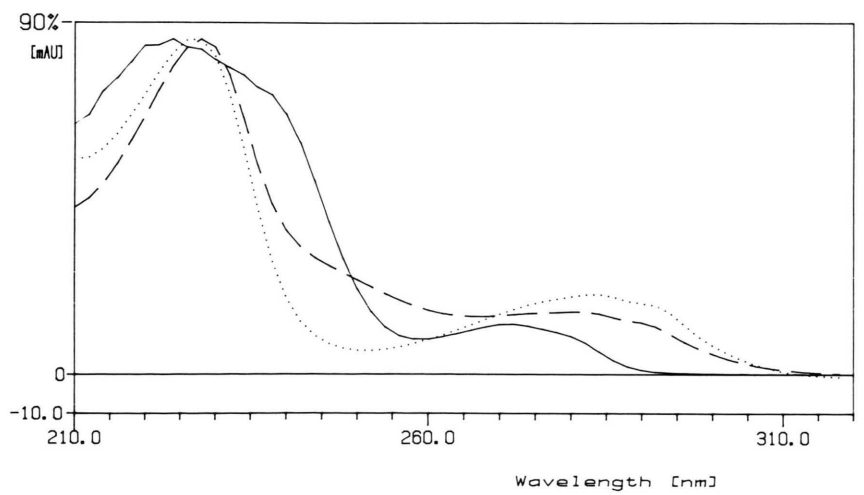
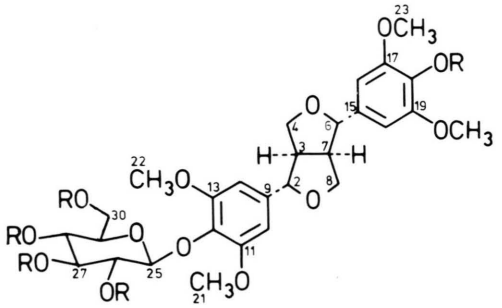


Fig. 2. UV-spectra of ajmalicine (...), catharanthine (- - - -) and the new metabolite (——).



1: R = H

2: R = Ac

Fig. 3. Structure of lirioresinol B mono-β-D-glucoside (1) and its acetylation product (2).

Table I. ^{1}H NMR data for the new metabolite in CD_3OD.

Proton	δ (Multiplicity)	Couplings [Hz]	
2; 6	4.79 (d); 4.74 (d) [a, b]		
3; 7	3.19 to 3.14 (m)		
4; 8 A	4.34 to 4.28 (m)		
B	3.96 to 3.92 (m)		
10; 14 } 16; 20 }	6.75 (s); 6.68 (s) [c]		
21; 22 } 23; 24 }	3.89 (s); 3.87 (s) [c]		
25	4.90 [d]	(25–26)	7.6
26	3.52	(26–27)	9.5
27	3.46	(27–28)	9.1
28	3.46	(28–29)	9.8
29	3.24	(29–30A)	2.4
30A	3.81	(29–30B)	5.1
30B	3.71	(30A–30B)	−12.0

[a] Protons H(2) to H(8) are part of a complex spin system.
[b] Interchangeable.
[c] Nuclear Overhauser enhancements (nOes) were observed for the signals at 6.75 and 6.68 upon irradiation of the methoxyl signals at 3.89 and 3.87 ppm. In addition irradiation of these latter signals caused nOes for the protons at 4.79 and 4.74 ppm respectively.
[d] Spin system analysed by the use of LAOCOON III.

Table II. ^{13}C NMR data for the new metabolite in CD_3OD.

Carbon	δ (Multiplicity) [a]	Carbon	δ (Multiplicity) [a]
2; 6	87.64 (d); 87.19 (d)	18	135.80 (s)
3; 7	55.71 (d); 55.45 (d)	21; 22	57.20 (q); 56.94 (q)
4; 8	72.96 (t); 72.83 (t)	23; 24	
9	139.63 (s) [b]	25	105.48 (d)
10; 14	104.88 (d) [c]	26	75.76 (d)
11; 13	154.43 (s)	27	77.86 (d)
12	137.37 (s)	28	71.45 (d)
15	132.45 (s) [b]	29	78.32 (d)
16; 20	105.07 (d) [c]	30	62.67 (t)
17; 19	149.65 (s)		

[a] Multiplicity in SFORD ^{13}C spectrum.
[b, c] Interchangeable.

the aglycon, together with the ^{1}H nuclear Overhauser enhancements (Table I) indicated a symmetry axis in the molecule and the substitution pattern within the aromatic residues. These data together with the formation of a pentaacetate under mild acetylating conditions are only compatible with the structure of the molecule shown in Fig. 3, which represents the monoglucoside of the known lignan, lirioresinol B [16]. Correlation of the ^{1}H and ^{13}C data by heteronuclear decoupling and comparison of the latter with literature data for similar systems [17, 18] confirmed the proposed structure as well as the configuration of the lignan. Thus the shifts for H(2) and H(6) are indicative of a diequatorial disposition of the aromatic moieties and this is confirmed by the ^{13}C shifts of the furan residues.

As judged by HPLC analysis (Fig. 1) the optimal response of the plant cells in suspension culture to the drug azaC was reached at a concentration of 105 µg per 70 ml of culture medium. Lowering the concentration of the inducer decreased the yield of the new metabolite, whereas a higher concentration did not result in a further stimulation of the cells. As azaC is known to be cytotoxic even at low concentrations [19] the viability of the treated cell cultures was confirmed by the fluoresceine diacetate staining method [20]. A retardation of growth in the presence of azaC compared to a control culture was not observed as indicated by the growth curves.

Other chemicals, also known to be potent inducers of differentiation in the Friend system such as hexamethylenebisacetamide [21] or sodium butyrate [5, 22] did not give rise to new secondary metabolites in the plant cell culture. Morphological changes of the plant cells responding to the drugs, as observed with some animal and human cell lines [23, 24], could not be detected under the light microscope.

Discussion

The results presented manifest for the first time an alteration of the secondary metabolism of cultured plant cells by a differentiation inducer known to be active in animal and human cell systems. Among many drugs tested in our *C. roseus* cell culture only 5-azacytidine exhibited a response of the plant cell by synthesizing a new metabolite, normally not found in that cell culture. The cytidine

analogue, 5-azacytidine, which can not be methylated, can be incorporated into replicating DNA thereby causing hypomethylated areas in the DNA [25]. This hypomethylation has been shown to correlate with gene activity and expression in a number of studies [26, 27]. Interestingly, sodium butyrate causing hyperacetylation of histones in mammalian cells, also correlated with gene activity [28] did not influence the synthesis of metabolites in *C. roseus* cells although this chemical caused hyperacetylation of histone H4 in plant cells [29]. Even though the hypomethylation of the DNA is inheritable we found that the plant cells lost their capability to synthesize the lignan type compound after a few passages. This may indicate that azaC not only interferes with DNA methylation but exerts its effect on gene expression by directly regulating the interaction of controlling proteins with the DNA [30]. The compound, lirioresinol B mono-β-D-glucoside, synthesized under the influence of azaC by the cell culture, has to our knowledge not been isolated from *C. roseus* plants or cell cultures. Only in callus cultures of *Vinca minor*, another plant of the Apocynaceae family, has the formation of lirioresinol B been reported [31]. Interestingly, from a large scale fermentation of untreated *C. roseus* cells 4-hydroxy-3,5-dimethoxycinnamic acid methylester, the biogenetic precursor of lirioresinol was isolated (W. Kohl, unpublished). This suggests that azaC treatment activated, *via* hypomethylation of DNA sequences or by direct interaction, the enzyme(s) for condensation of the two cinnamic acid moieties to form the lignan.

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[1] E. Teuscher, Pharmazie **28,** 6 (1973).
[2] C. Friend, W. Scher, J. G. Holland, and T. Sato, Proc. Natl. Acad. Sci. USA **68,** 378 (1971).
[3] R. C. Reuben, P. L. Khanna, Y. Gazitt, R. Breslow, R. A. Rifkind, and P. A. Marks, J. Biol. Chem. **253,** 4214 (1978).
[4] J. F. Gusella and D. Housman, Cell **8,** 263 (1976).
[5] A. Leder and P. Leder, Cell **5,** 319 (1975).
[6] P. A. Marks and R. A. Rifkind, Ann. Rev. Biochem. **47,** 419 (1978).
[7] R. C. Reuben, R. A. Rifkind, and P. A. Marks, Biochim. Biophys. Acta **605,** 325 (1980).
[8] P. A. Jones and S. M. Taylor, Cell **20,** 85 (1980).
[9] B. Deus, Production of Natural Compounds by Cell Culture Methods (A. W. Alfermann and E. Reinhard, eds.), p. 118, Gesellschaft für Strahlen- und Umweltforschung mbH, München 1978.
[10] G. Döller, ibid, p. 109.
[11] T. Murashige and F. Skoog, Physiol. Plant **15,** 473 (1962).
[12] J. A. Beisler, M. M. Abbasi, J. A. Kelley, and J. S. Driscoll, J. Medicin. Chem. **20,** 806 (1977).
[13] W. Kohl, B. Witte, and G. Höfle, Planta med. **47,** 177 (1983).
[14] K.-H. Knobloch and J. Berlin, Z. Naturforsch. **35c,** 551 (1980).
[15] M. H. Zenk, H. El-Shagi, H. Arens, J. Stöckigt, E. W. Weiler, and B. Deus, Plant Tissue Culture and its Biotechnological Application (W. Barz, E. Reinhard, and M. H. Zenk, eds.), p. 27, Springer Verlag, Berlin-Heidelberg-New York 1977.
[16] L. H. Briggs, R. C. Cambie, and R. A. F. Couch, J. Chem. Soc. **1968,** 3042.
[17] S. D. Jolad, J. J. Hoffmann, and J. R. Cole, J. Org. Chem. **45,** 1327 (1980).
[18] R. K. Chanduri and O. Sticher, Helv. Chim. Acta **64,** 3 (1981).
[19] F. Creusot, G. Acs, and J. K. Christman, J. Biol. Chem. **257,** 2041 (1982).
[20] J. M. Widholm, Stain Technol. **47,** 189 (1972).
[21] R. C. Reuben, Biochim. Biophys. Acta **588,** 310 (1979).
[22] W. Scher and C. Friend, Cancer Res. **38,** 641 (1978).
[23] S. M. Taylor and P. A. Jones, Cell **17,** 771 (1979).
[24] J. A. Wright, Exp. Cell Res. **78,** 456 (1973).
[25] P. A. Jones and S. M. Taylor, Nucl. Acid Res. **9,** 2933 (1981).
[26] J. L. Mandel and P. Chambon, Nucl. Acid Res. **7,** 2081 (1979).
[27] S. J. Compere and R. D. Palmiter, Cell **25,** 233 (1981).
[28] D. A. Nelson, M. Perry, L. Sealy, and R. Chalkley, Biochem. Biophys. Res. Commun. **82,** 1346 (1978).
[29] H.-A. Arfmann and E. Haase, Plant Sci. Lett. **21,** 317 (1981).
[30] R. L. P. Adams, J. Fulton, and D. Kirk, Biochim. Biophys. Acta **697,** 286 (1982).
[31] G. Kunech and M. Koch, Phytochemistry **14,** 1385 (1975).