Phytochemistry, Vol. 24, No. 10, pp. 2205–2209, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

HAEMCYTOCHROMES IN VALEPOTRIATE PRODUCING TISSUE CULTURES OF *CENTRANTHUS MACROSIPHON*

CHRISTIANE J. I. VIOLON and ANTOINE A. VERCRUYSSE

Dienst Farmacognosie, Fytochemie en Toxicologie, Vrije Universiteit Brussels, Laarbeeklaan 103, 1090 Brussels, Belgium

(*Revised received* 19 *December* 1985)

Key Word Index—*Centranthus macrosiphon*; Valerianaceae; biosynthesis; tissue culture; cytochrome b_5; cytochrome P_{450}; valepotriates.

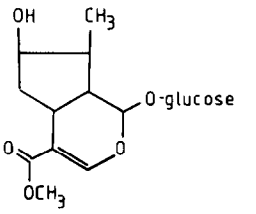
Abstract—Valepotriate and haemcytochrome (cyt b_5 and cyt P_{450}) contents were determined in tissue cultures of *Centranthus macrosiphon*. These cultures consisted of highly productive root organ cultures and poorly productive callus on solid media and highly productive root organ suspension cultures. High cyt b_5 contents were found in all the cultures (0.2–0.5 nmol cyt b_5 per mg protein). In the different tissue cultures the cyt P_{450} levels (which were very high in the root differentiated cultures: 0.4 nmol per mg protein) increased with increasing amounts of valepotriates. By analogy with the biosynthesis of loganin, a closely related secondary metabolite, an important function of cyt P_{450} dependent monooxygenase enzyme systems in valepotriate biosynthesis is suggested.

INTRODUCTION

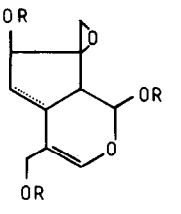
Valerian roots, known for their sedative action, contain three pharmacologically interesting groups of secondary plant products: valepotriates, volatile oils and alkaloids. Among these, the most significant contribution to the chemotaxonomic classification is made by the valepotriates. They occur in every genus and species of the Valerianeae tribe studied so far and were not found in any other plant family [1–4]. On the other hand volatile oils and alkaloids are only present in a few species of the Valerianaceae such as *Valeriana officinalis* [2]. Valepotriates were shown to be responsible for most pharmacological effects of valerian root preparations such as mild sedation and spasmolitic effects [5–7]. Recently valepotriates were shown to be cytotoxic *in vitro* to animal tissue cultures [8, 9]. They are polyesters of polyhydroxycyclopenta[c]pyran with isovaleric, acetic and β-acetoxy isovaleric acids, containing an epoxide. The three main valepotriates are the dienes (iso)valtrate and acevaltrate and the monoene didrovaltrate [10].

In previous work we have studied valerian secondary plant products by means of tissue cultures [11–14]. Valepotriates were shown to be formed and accumulated in tissue cultures of different Valerianaceae on which occasion no light is necessary for their biosynthesis [11, 14]. We localized valepotriates in intracellular lipid vesicles in *in vitro* cultures and roots of control plants. These droplets were shown to be closely related to the vacuolar membrane of the cell [12]. In relation to the biosynthesis of valepotriates we concluded that the level of valepotriate formation and consequently the amount of lipid droplets *in vitro* was clearly correlated with the differentiation level of the cultures. The differentiation level in its turn was influenced by the plant hormone content of the medium [14]. In this regard *Centranthus macrosiphon* cultures yielded the clearest response: high and stable production during several subcultivations in root organ cultures on media devoid of 2,4-D and steadily declining production, stabilized at a low level in callus cultures on media with 2,4-D.

The aim of this work was to get more insight into the origin of a high valepotriate biosynthesis in root organ cultures and a low biosynthesis in callus. The biosynthetic pathway of valepotriates remains, however, mostly unknown. Biosynthesis starting from mevalonic acid over geraniol, citral and iridodial was suggested by Hölzl [15] by analogy with other secondary iridoid plant products. Loganin, the key intermediate of many biosynthetic pathways (structure **1**), and valepotriates (structure **2**) possess the same heterogenic ring structure, identical position of (different) substituents, with a double bond at the same place as in monoene valepotriates. Loganin is a well known iridoid glycoside precursor [16] and has (together with secologanin) an important function in indole alkaloid biosynthesis: it yields the non-nitrogen containing moiety, which is synthesized with tryptamine by the strictosidine synthetase to cinchona, strychnos and rauwolfia alkaloids [17]. Loganin is biosynthesized starting from mevalonic acid over geraniol, which is hydro-



1



R = -acetic
-isovaleric
-acetoxyisovaleric

2

xylated to hydroxygeraniol. Ring closure yields deoxyloganin and loganin [18–23]. In investigations on the biosynthesis of *Catharanthus roseus* alkaloids, one step of loganin biosynthesis has been particularly well studied: the hydroxylation of geraniol to 10-hydroxygeraniol. The hydroxylase catalysing the reaction was demonstrated to be a cytochrome P_{450} dependent monooxygenase [20, 21]. Cyt P_{450} and cyt b_5 are haemproteins, having the function of electron-transport systems in monooxygenase catalysed reactions, often associated with the endoplasmatic reticulum [24].

Considering the possible biosynthetic relationship between loganin and valepotriates, the biosynthesis of the latter up to the polyhydroxycyclopenta[c]pyran might follow the same pathway as loganin biosynthesis and consequently pass through geraniol and hydroxygeraniol by mediation of cyt P_{450}. If this hypothesis holds true cyt P_{450} (and cyt b_5) should be present in valepotriate producing tissue cultures. We have therefore studied the presence of both haemcytochromes, cyt P_{450} and cyt b_5 in different tissue cultures of *C. macrosiphon*; in these cultures valepotriates were also determined. These parameters were analysed in 2½ year old and newly set up root organ and callus cultures, grown on solid media.

Culturing and harvesting for large scale purposes is often performed in suspension cultures. A suspension culture of high valepotriate-producing roots would also make out an excellent study tool for valepotriate biosynthesis studies. We have therefore set up such a culture, enabling us to study biomass increase, dry weight increase and valepotriate content simultaneously with the haemcytochromes at different time intervals of the growth cycle.

RESULTS

Cyt P_{450} estimations by the two methods of recording difference spectra (reduced CO versus oxidized CO and reduced CO versus reduced, see Experimental) gave identical results. Moreover it was possible, from the reduced CO versus oxidized CO spectra intended for cyt P_{450} estimations, to calculate simultaneously the cyt b_5 amounts from ΔA (424–409) using 185 mM^{-1} cm^{-1} as extinction coefficient. This is due to the fact that no or only very minute amouts of cyt P_{420} (in comparison with the cyt P_{450} and cyt b_5 amounts) were present in the analysed samples as estimated from the reduced CO versus reduced difference spectra. This last method is very suitable for determination of both cytochromes in one difference spectrum. Control of cyt P_{420} level is however necessary. We preferred to record the difference spectra according to the two methods in order to control eventual interference. Cytochrome determinations, performed in triplicate, yielded results within a variation of 5%.

Initial analyses on valepotriate rich root organ cultures of *C. macrosiphon*, which were 2½ years in culture, revealed the presence of cyt b_5 and P_{450} in (for plant material) considerable amounts. These analyses were performed on microsomal suspensions, obtained from resuspension of the 20 000–105 000 *g* pellet. The resuspended pellet (precipitated at 105 000 *g*) of the 3000–20 000 *g* supernatant also contained haemcytochromes. The amounts of microsomal haemcytochromes per mg microsomal protein and of 3000–20 000 *g* haemcytochromes per mg 3000–20 000 *g* protein were in the same order of magnitude. This signifies that haemcytochromes are not exclusively present in the endoplasmatic reticulum (microsomal fraction) but also in the 3000–20 000 *g* fraction, containing mitochondria and vacuolar particles. To enrich the final suspension in haemcytochromes, the 3000–105 000 *g* pellet was used for measurement of proteins and haemcytochromes in all further experiments.

In this way we determined haemcytochromes in 2½ year old root organ cultures (media I and IV) with high valepotriate content and in callus cultures (media II and III) with low valepotriate content of the same age; all cultures were grown on solid media (see Table 1). Both callus and root organ cultures contained cyt b_5, although the amount in callus was less than in root organ cultures. Cyt P_{450} on the other hand was only present in the root organ cultures and not in the callus. Cultures, grown on different media but with the same morphological type, gave similar valepotriate and cytochrome yields.

From previous work [14] we knew that valepotriate biosynthesis diminished steadily with ageing of callus cultures. It might therefore be interesting to analyse

Table 1. Valepotriate, cyt b_5 and cyt P_{450} contents in different valerian tissues

Plant material	Tissue cultures *C. macrosiphon*								Control plant *V. officinalis*
Morphological type	Root organ culture				Callus culture				Roots–rhizomes
Age of culture	2½ year		3 months		2½ year		3 months		Harvest in September
Solid medium*	I	IV	I	IV	II	III	II	III	—
Valepotriate content† (g/100 g dry wt)	6.2	6.2	7.2	6.9	0.1	0.1	1.7	1.5	0.2
Cyt b_5 (nmol/mg protein‡)	0.36	0.36	0.48	0.27	0.26	0.27	0.20	0.20	0.02
Cyt P_{450} (nmol/mg protein‡)	0.24	0.27	0.36	0.34	—	—	0.14	0.13	—

*Medium composition (see Experimental).

†Valepotriate content: Additive amounts of (iso)valtrate, acevaltrate and didrovaltrate.

‡Proteins and cytochromes were determined in the 3000–105 000 *g* resuspended pellet.

young callus tissue, with a relatively high biosynthetic capacity, having at that moment a harder and less spongy consistence. The different analysed parameters in new young tissue cultures are given in Table 1. Cyt b_5 was again present in both morphological types and again to a lesser extent in the callus. It was interesting that the young callus, producing valepotriates, contained cyt P_{450}. The amount of cyt P_{450} was, however, lower than in the young and old root organ cultures. Plotting cytochrome against valepotriate values of the different culture types shows that the cyt P_{450} levels of the tissues increase with increasing valepotriate content. Cyt b_5 on the other hand does not give such a clear view (see Fig. 1). As control plants we used roots and rhizomes of *V. officinalis* plants grown in the greenhouse. In this material we could only detect a very small amount of cyt b_5.

The analysis of the growth and production parameters during the first growth cycle of the batch culture of roots of *C. macrosiphon* revealed that the biomass increase and valepotriate production were promising. Roots (coming from 2½ year old root organ cultures on solid medium and cultured in liquid medium I) gained about ten-fold their inocula's fresh weight in 4 weeks and were highly productive (7 g/100 g dry wt). This situation persisted after subculture. The increase in biomass (fr wt), dry wt and valepotriate production during the third growth cycle is represented in Fig. 2. They are all expressed as absolute comparable amounts, starting from 2 g of inoculum. In relation to productivity the result is very favourable: starting from a 2 g inoculum, 87 mg of valepotriates are formed in *ca* 20 g of biomass, corresponding with 1.15 g of dry wt in 4 weeks. This valepotriate yield is about two-fold as high as that obtained previously on solid media [14]. The evolution of cyt b_5 and P_{450} content (nmol/mg protein) during the growth cycle is represented in Fig. 3. Cyt P_{450} shows a two-fold increase after 4 weeks. The amount of valepotriates expressed as g valepotriates per 100 g of dry wt, on the other hand remains almost constant during the whole growth cycle (mean 7.0, standard deviation 1.1 g/100 g dry wt).

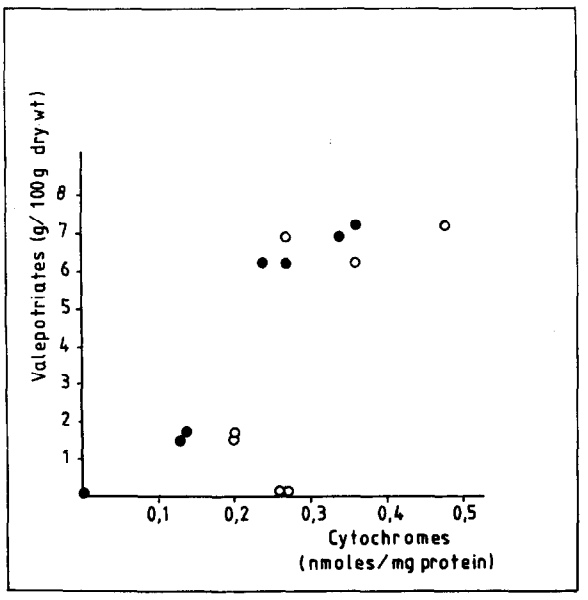


Fig. 1. Haemcytochrome content in relation to valepotriate content in different tissue cultures of *C. macrosiphon*. ○, Cyt b_5; ●, cyt P_{450}.

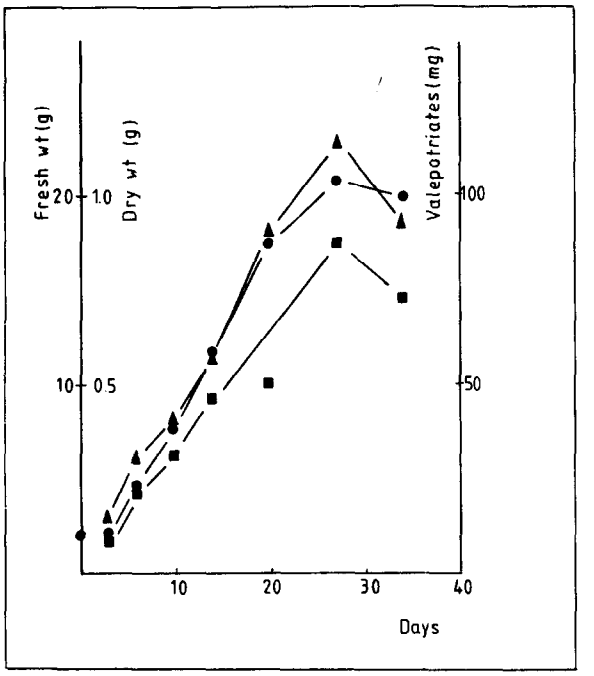


Fig. 2. Fresh wt, dry wt and valepotriate increase, during a growth cycle of *C. macrosiphon* root organ suspension cultures in liquid medium I. They are all expressed as absolute comparable amounts, starting from 2 g of inoculum. ●, Fresh wt; ▲, dry wt; ■, valepotriates.

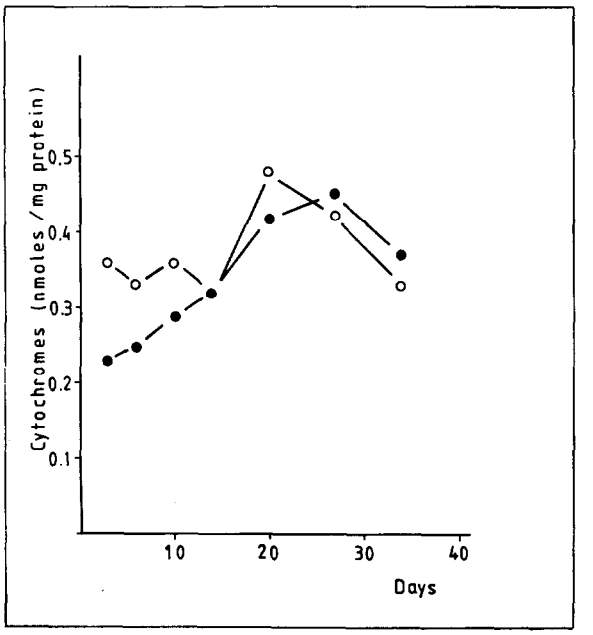


Fig. 3. Haemcytochromes in root organ suspension cultures of *C. macrosiphon*, during a growth cycle. ○, Cyt b_5; ●, cyt P_{450}.

DISCUSSION

The biosynthesis of valepotriates occurs to a much larger extent in differentiated root organ cultures than in callus cultures of valerian plants [14]. The high production in root organ cultures is stable during ageing whereas the production in callus is relatively high at the beginning of the culture (first passages) but thereafter decreasing steadily to a very low level [14]. 2,4-D probably exerts this influence on productivity. These phenomena most probably reflect differences in the biochemical systems necessary for biosynthesis, between the two morphological types.

We took as a model for the valepotriate biosynthetic pathway the (partly) analogous biosynthetic pathway of the chemically closely related loganin. This implies that valepotriate biosynthesis might also pass through 10-hydroxygeraniol. The hydroxylation of geraniol to its 10-hydroxyderivative is catalysed by a cyt P_{450} dependent monooxygenase system in loganin biosynthesis [18–23], which is one of the few cyt P_{450} dependent monooxygenase reactions, known in plant tissues.

The microsomal cyt P_{450} dependent monooxygenase enzyme reactions are well known for mammalian livers [25, 26]. In mammalian livers the microsomal cyt P_{450} dependent monooxygenases catalyse many oxidative reactions such as hydroxylation, oxidative *N*-demethylation and epoxidation. Another haemcytochrome, cyt b_5, is often associated with cyt P_{450}. Both have the function of electron transport systems [24]. In plants only four specific oxidative enzyme reactions have been shown to be cyt P_{450} dependent: kaurene oxidation [27] cinnamic acid [28], laurate [29] and relevant to this subject geraniol hydroxylation [21]. The reported amounts of cyt P_{450} in different fresh plant materials are mostly very low (0.001–0.01 nmol/mg microsomal protein) in comparison to non induced liver systems (0.6 nmol/mg microsomal protein) [30]. In *Catharanthus roseus* seedlings, possessing the cyt P_{450} dependent geraniol hydroxylase system, the cyt P_{450} amount was 0.1 nmol/mg protein [21]. In some cases, cyt P_{450}, not present in the fresh plant material, has been induced by wounding and ageing [31, 32].

Our results on haemcytochrome analysis in different tissues of *C. macrosiphon* were in this regard very significant. Undifferentiated as well as differentiated tissues always contained cyt b_5 in considerable amounts (0.2–0.5 nmol/mg protein). The amounts in callus were, however, always lower than those in root differentiated tissue cultures. The cyt P_{450} analyses on the other hand yielded evidence for a correlation between valepotriate and cyt P_{450} content. Indeed, both young (3 months) and old (2½ year) highly productive root organ cultures (6–7 g valepotriates/100 g dry wt) contained remarkably large amounts of cyt P_{450} (0.2–0.4 nmol/mg protein), whereas 2½ year old very poorly productive callus (0.1 g valepotriates/100 g dry wt) did not contain any detectable amounts of cyt P_{450}. Young and still productive callus tissue (1.5 g valepotriates/100 g dry wt) contained also cyt P_{450} (0.1 nmol/mg protein).

Both haemcytochromes and valepotriates were analysed at different time intervals during a growth cycle of well growing and highly productive root suspension cultures. Valepotriate production may be considered as constant during the whole growth cycle, parallel with biomass increase and not associated with a special phase of the growth curve. The amount of cyt b_5 was within 0.35–0.48 nmol/mg protein with a maximum 3 weeks after subcultivation. Cyt P_{450} raised from 0.25 to 0.45 nmol/mg protein during the growth cycle.

In root organ cultures cyt P_{450} was not only present in the microsomal fraction but to the same extent in the 3000–20 000 *g* fraction. This accords with the findings of Madyastha *et al.* [33], who found cyt P_{450} in corresponding fractions. They associated the cyt P_{450} dependent monoxygenase system with provacuolar fractions. It is worthwhile noting in this regard that we localized valepotriates previously in intracellular lipid droplets, associated with the vacuolar membrane of the cell [12].

In roots of *V. officinalis* control plants we could only detect small amounts of cyt b_5 and no cyt P_{450}; valepotriates were present in the roots (0.2 g/100 g dry wt). This finding is however not unexpected. Primary and secondary metabolism proceeds probably at a higher rate in the cell cultures than in control plants, in which growth and production is spread over different years. It is clear from this that due to their high primary and secondary metabolism rates, valerian tissue cultures are an excellent tool for biosynthesis studies.

It is likely that cyt P_{450} has a more limiting function in valepotriate biosynthesis than cyt b_5, which also takes part in the monooxygenase system, as cyt b_5 was present in relatively large amounts in all cultures, whether productive or not. The very high cyt P_{450} content in root differentiated tissue cultures and the absence of it in callus cultures of the same age most probably reflects only one of the underlying biochemical differences between the two morphological conditions, which are most often only perceived by difference in secondary production.

From the indication of a relationship between valepotriate content and cyt P_{450} level in valerian tissue cultures, we suggest that the cyt P_{450} dependent monooxygenase enzyme systems have an important function in valepotriate biosynthesis, as it does in loganin biosynthesis. Further work on monooxygenases, especially the geraniol hydroxylase activity, will probably give more decisive answers on the valepotriate biosynthetical pathway.

EXPERIMENTAL

Plant material. Two and a half year old well growing tissue cultures of *Centranthus macrosiphon* Boiss, cultured in permanent dark on solid media served for analysis. These cultures consisted of two morphological types: root organ cultures on media with NAA (medium I) and with NAA and kinetin (medium IV); callus cultures on media with NAA, kinetin and 2,4-D (medium II) and with NAA and 2,4-D (medium III). All plant hormones were present in a concn of 1 ppm in basal B_5 medium [34] with addition of sucrose (10 g/l.) and glycine (2 ppm). They were all subcultured every 5 weeks. Culture conditions and growth curves are described elsewhere [14]. Samples for analysis were taken 3 weeks after the previous subcultivation. Three months old *C. macrosiphon* cultures were obtained, cultured and sampled in the same way as the older cultures, described above. Root organ suspensions of *C. macrosiphon* were obtained by suspending *ca* 2 g of inoculum (from the 2½ year old root organ cultures on solid medium I) in 100 ml of liquid medium I per 250 ml conical flask. The flasks were incubated in permanent dark at 25° on a rotatory shaker (60 rpm). During the first growth cycle fresh and dry weight increase and valepotriate content were analysed. These revealed that the root suspensions grew very well and kept the high valepotriate biosynthesis capacity. During the third growth cycle fresh and dry weight increase, valepotriate and cytochrome content were measured at different time intervals in

these cultures. As controls we used the roots of *Valeriana officinalis* L. plants, grown in the greenhouse.

Valepotriate analysis. In all the above described plant material, valepotriates (sum of (iso)valtrate, acevaltrate and didrovaltrate) were quantified by spectrophotometry after TLC separation, according to our previously described method [14].

Fresh and dry weight determination during a growth cycle of C. macrosiphon *root organ suspension cultures.* To determine the increase in biomass and dry weight at different time intervals we proceeded as follows: *ca* 2 g of biomass (3 weeks after the previous subcultivation) were taken, weighed and inoculated aseptically in 100 ml of fresh medium I per 250 ml flask. The flasks were incubated (rotatory shaker, 60 rpm, dark, 25°). At different time intervals samplings were made: 30 flasks for the first three samplings, 20 flasks afterwards. The biomass of the samples was collected by filtering and weighed. Comparison with the inocula's fr. wt yielded the increase in biomass. This freshly collected material was blended and partly used for immediate cytochrome analysis. The remaining weighed fresh material was dried (< 40°), weighed again (dry wt) and used for valepotriate analysis.

Determination of cyt b_5, cyt P_{450} and proteins. Freshly harvested material (50 g) was ground in a mortar in the presence of sand (washed with buffer soln) during 5 min. 100 ml of buffer soln (0.1 M NaPi buffer pH 7) was then added and the mixture was ground again. Due to the toughness of roots of control plants an alternative homogenization method was necessary; the finely cut roots were mixed in buffer soln, followed by a Potter-homogenization. The homogenate, obtained in both ways, was then centrifugated at 3000 *g* for 20 min. The supernatant was ultracentrifuged at 105 000 *g* for 90 min. The resulting pellet was suspended in 15 ml buffer soln and homogenized in a Potter apparatus. In this suspension (2–3 mg proteins/ml) cytochromes and proteins were determined. Cyt b_5 and cyt P_{450} were determined according to the method of refs [25, 26]. Cyt b_5 was estimated from dithionite reduced versus oxidized difference spectra. Difference spectra of cyt b_5 containing spectra clearly showed α (558–560 nm), β (528–530 nm) and γ (424–426 nm) bands. Calculations were made from ΔA (424–409) with an extinction coefficient of 185 $mM^{-1}\,cm^{-1}$. Cyt P_{450} was estimated from dithionite reduced CO-saturated (CO saturation was obtained by bubbling CO during 90 sec through the reduced suspension) versus dithionite reduced difference spectra at ΔA (450–490) with an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. From the same suspensions we have recorded the dithionite reduced CO saturated versus oxidized CO saturated difference spectra, according to ref. [35], intended for determination of cyt P_{450} in mammalian liver microsomes, with the same extinction coefficient. All manipulations from homogenization until preparation of the sample and blank for spectral analysis were performed at 4°. Before recording the difference spectra, cuvettes were kept for 10 min at room temp, whereafter measurements remained stable for at least 1 hr.

Protein determinations in the suspensions were executed according to the method of ref. [36].

Acknowledgements—We are very grateful to Dr. Vera Rogiers and Dr. Guy Paeme for interesting discussions on the cytochrome determinations.

REFERENCES

1. Schild, W. and Seitz, W. (1971) *Ber. Dtsch. Bot. Ges.* **84**, 225.
2. Stahl, E. and Schild, W. (1971) *Phytochemistry* **10**, 147.
3. Stahl, E. and Schild, W. (1981) *Pharmazeutische Biologie, Drogenanalyse II, Inhaltsstoffe und Isolierungen*, p. 192. Gustav Fischer, Stuttgart.
4. Förster, W., Becker, H. and Rodriguez, E. (1984) *Planta Med.* **50**, 7.
5. von Eickstedt, K. W. and Rahman, S. (1969) *Arzneim. Forsch.* **19**, 316.
6. Wagner, H. and Jurcic, K. (1979) *Planta Med.* **37**, 84.
7. Wagner, H., Jurcic, K. and Schaette, R. (1980) *Planta Med.* **38**, 358.
8. Bounthanh, C., Bergmann, C., Beck, J. P., Haag-Berrurier, M. and Anton, R. (1981) *Planta Med.* **41**, 21.
9. Bounthanh, C., Richert, L., Beck, J. P., Haag-Berrurier, M. and Anton, R. (1983) *Planta Med.* **49**, 138.
10. Thies, P. W. (1968) *Tetrahedron* **24**, 313.
11. Violon, C., Van Cauwenbergh, N. and Vercruysse, A. (1983) *Pharm. Weekbl. Sci. Ed.* **5**, 205.
12. Violon, C., Dekegel, D. and Vercruysse, A. (1983) *Plant Cell Rep.* **2**, 300.
13. Violon, C., Sonck, W. and Vercruysse, A. (1984) *J. Chromatogr.* **288**, 474.
14. Violon, C., Dekegel, D. and Vercruysse, A. (1984) *J. Nat. Prod.* **47**, 934.
15. Hölzl, J. (1975) *Planta Med.* **28**, 302.
16. Inouyé, H. (1978) *Planta Med.* **33**, 193.
17. Waller, R. and Dermer, O. (1981) in *The Biochemistry of Plants. Vol 7, Secondary Plant Products*, (Stumpf, P. K. and Conn, E. E., eds) p. 317. Academic Press, New York.
18. Madyastha, K. M., Guarnaccia, R. and Coscia, C. J. (1971) *FEBS Letters* **14**, 175.
19. Madyastha, K. M., Guarnaccia, R., Baxter, C. and Coscia, C. J. (1973) *J. Biol. Chem.* **248**, 2497.
20. Meehan, T. D. and Coscia, C. J. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1043.
21. Madyastha, K. M., Meehan, T. D. and Coscia, C. J. (1976) *Biochemistry* **15**, 1097.
22. Scott, A. I., Lee, S. L. and Wan, W. (1977) *Biochem. Biophys. Res. Commun.* **75**, 1004.
23. Gröger, D. (1980) in *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V., eds) p. 128. Springer, Berlin.
24. West, C. A. (1980) in *The Biochemistry of Plants. Vol.* 2, *Metabolism and Respiration* (Davies, D. D., ed.) p. 317. Academic Press, New York.
25. Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370.
26. Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **239**, 2379.
27. Hasson, E. P. and West, C. A. (1976) *Plant Physiol.* **58**, 479.
28. Potts, J. R. M., Weklych, R. and Conn, E. E. (1974) *J. Biol. Chem.* **249**, 5019.
29. Salaün, J. P., Benveniste, I., Reichhart, D. and Durst, F. (1981) *Eur. J. Biochem.* **119**, 651.
30. Rich, P. R. and Bendall, D. S. (1975) *Eur. J. Biochem.* **55**, 333.
31. Benveniste, I., Salaün, J. P. and Durst, F. (1977) *Phytochemistry* **16**, 69.
32. Cotte-Martinon, M. G., Yahiel, V. and Ducet, G. (1974) *Phytochemistry* **13**, 2085.
33. Madyastha, K. M., Ridgway, J. E., Dweyer, J. G. and Coscia, C. J. (1977) *J. Cell Biol.* **72**, 302.
34. Gamborg, O. L. and L. R. Wetter (1975) *Plant Tissue Culture Methods*, p. 90. N.R.C., Ottawa.
35. Schoene, B., Fleischmann, R. A. and Remmer, H. (1972) *Eur. J. Clin. Pharmacol.* **4**, 65.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.