Phytochemistry, Vol. 31, No. 5, pp. 1609–1612, 1992
Printed in Great Britain.

0031-9422/92 $5.00+0.00
Pergamon Press plc

EFFECT OF NIFEDIPINE ON ALKALOID ACCUMULATION IN *CATHARANTHUS ROSEUS* CELL CULTURES

J. M. MÉRILLON, D. LIU, Y. LAURENT,* M. RIDEAU and C. VIEL*

Laboratoire de Biologie Cellulaire et Biochimie Végétale, Faculté de Pharmacie, F 37042 Tours Cédex, France; *Laboratoire de Pharmacochimie des Produits naturels et analogues structuraux, Faculté de Pharmacie, F 37042 Tours Cédex, France

(*Received in revised form* 30 *September* 1991)

Key Word Index—*Catharanthus roseus*; Apocynaceae; nifedipine; nitrophenylpyridine: nitrosophenylpyridine; aminophenylpyridine; channel blockers; biotransformation.

Abstract—The effect of nifedipine on both alkaloid accumulation and calcium uptake in *Catharanthus roseus* cell cultures was investigated. It was shown that nifedipine inhibited ajmalicine accumulation through a mechanism other than blocking of the calcium channel uptake. Moreover, nifedipine was biotransformed into its 4-(2′-aminophenyl)-pyridine derivative.

INTRODUCTION

1,4-Dihydropyridines (DHPs) are calcium channel blockers that bind specifically to a site (referred to as the DHP receptor) in the $\alpha 1$ sub-unit of the L-type voltage-sensitive calcium channels of animal cells [1, 2]. They have been used in plant cell studies to demonstrate the involvement of calcium ions as a second messenger in the response to different signals. However, their inhibitory effect on Ca^{2+} influx into plant cells has often been postulated rather than clearly demonstrated [for example: 3–5]. Binding of labelled 1,4-DHPs to plant membranes has been reported [6,7], but specific binding sites for the tritiated 1,4-DHP (+) PN 200-110 could not be identified on carrot protoplasts, and no blocking of Ca^{2+} influx was observed with several drugs in this series [8].

While studying the regulation of indole alkaloid biosynthesis, we previously found that several cytokinins (CKs) could enhance alkaloid accumulation in *Catharanthus roseus* cell suspensions [9]. On the basis of the inhibitory effect of the calcium chelatant EGTA and of several calcium entry blockers (CEBs) on the CK-enhanced alkaloid accumulation, we expected that CKs act, at least partially, through an increase in the level of intracellular Ca^{2+} [10]. However, the effect of the DHPs was different from that of other CEBs since in spite of their inhibitory properties against CK-enhanced alkaloid accumulation, they did not inhibit Ca influx in *C. roseus* protoplasts. Moreover, we could not detect specific binding of (+) PN 200-110 to *C. roseus* microsomes [10].

The experiments reported in this paper were undertaken to investigate in greater detail the effect of DHPs on CK-enhanced alkaloid accumulation in *C. roseus* cells. We also show that the cells are able to biotransform nifedipine into its 4-(2′-aminophenyl)-pyridine derivative.

RESULTS AND DISCUSSION

Effect of nifedipine and its corresponding 4-(2′-nitrophenyl)-and 4-(2′-nitrosophenyl)-pyridine on the CK-enhanced alkaloid accumulation

Typical results showing the effect of some DHPs on alkaloid accumulation in *C. roseus* cell line C20 are shown in Table 1. Cells grown in 2,4-D-supplemented medium did not accumulate alkaloids. Suppressing 2,4-D from the culture medium for one transfer induced an accumulation of alkaloids. Adding a CK to the 2,4-D-free culture medium at day 3 increased the alkaloid content. Co-adding CK and nifedipine (**1**), nimodipine (**2a**) or nitrendipine (**2b**) inhibited the alkaloid accumulation without affecting dry weight.

In order to assess the specificity of the inhibitory properties of nifedipine, we prepared two nifedipine derivatives, **3a** and **b**, lacking the dihydropyridine ring which is known as an essential structural feature in the nifedipine molecule for typical calcium-modulating properties [2], and we tested their effect on alkaloid accumulation in *C. roseus* cells. Compounds **3a** and **b** were prepared through photochemical irradiation [11] and aromatization of **1**, respectively. A dimer **4** was also obtained during photoirradiation but its effect was not investigated here; similar reactions are known on photoirradiating nitrophenyldihydropyridines [12, 13].

At the concentrations used, neither **1**, **3a** nor **3b** affected the growth (expressed as dry wt) (Fig. 1); **1** and **3a** inhibited alkaloid accumulation at concentrations higher than 10 μM; **3b** enhanced alkaloid accumulation at 5–20 μM and was inhibitory at 40–60 μM. These results show that the DHP moiety is not essential for inhibiting alkaloid content. Therefore, as the question arises

Table 1. Effect of DHPs on growth of *C. roseus* cells and on their alkaloid accumulation*

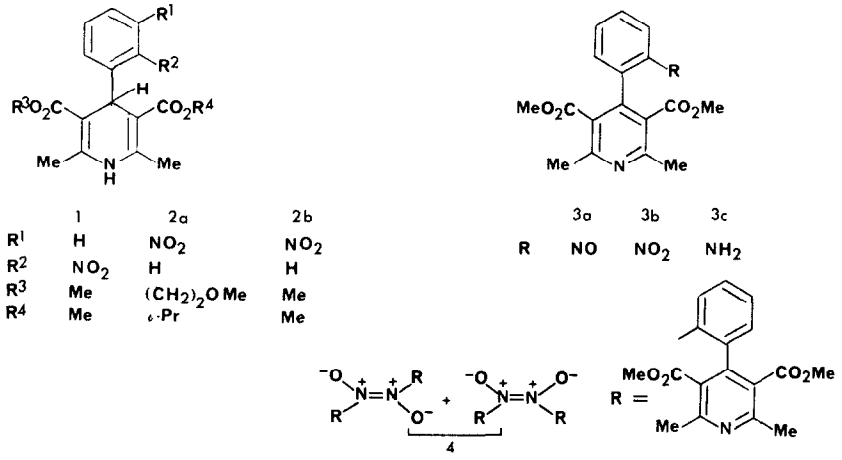
Culture conditions*	Growth (mg dry wt)	Ajmalicine ($\mu g\,g^{-1}$ dry wt)	($mg\,l^{-1}$)
Maintenance medium	550 (15)†	traces	traces
−2,4-D	521 (3)	699 (32)	7.28
−2,4-D + BAP‡	458 (19)	2380 (384)	21.80
−2,4-D + BAP + 1§	454 (26)	727 (81)	6.60
−2,4-D + BAP + 2a	471 (13)	890 (203)	8.38
−2,4-D + BAP + 2b	457 (27)	401 (75)	3.66

*Except for maintenance medium, 2,4-D was deleted from the media. Basal medium = B5 medium [22].

†Results given at day 9 ($n = 3$, s.e. in parentheses).

‡Benzylaminopurine (BAP) at 5 μM was added at day 3.

§DHP and DHP derivatives (20 μM) were added at day 3.



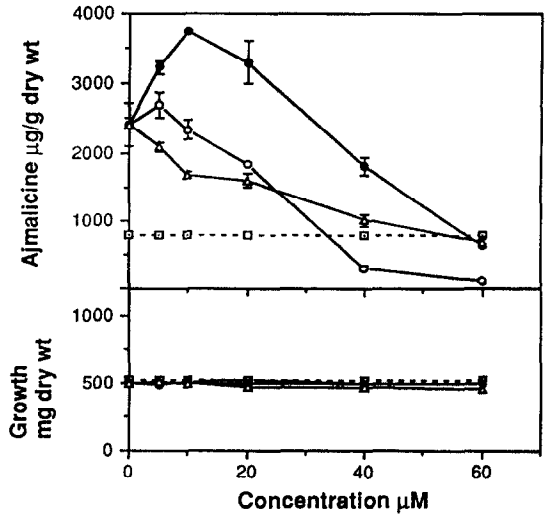


Fig. 1. Effect of **1** (○), **3a** (△) and **3b** (●) on growth and alkaloid contents in *C. roseus* cells. The drugs were added at day 3 in a 2,4-D-free culture medium supplemented with zeatin (5 μM). Cells were harvested at day 9. The broken line shows the ajmalicine content in cells grown in a 2,4-D-free culture medium.

whether the DHPs act on alkaloid accumulation through calcium channel modulation, we further studied the effect of **1** on calcium uptake in *C. roseus* cells.

Effect of nifedipine on $^{45}Ca^{2+}$ *uptake*

We previously tested the effect of several drugs belonging to different classes of CEBs on $^{45}Ca^{2+}$ uptake in *C. roseus* protoplasts: all the drugs, except DHPs, were inhibitory. Nifedipine slightly enhanced Ca^{2+} influx at 10–50 μM [10]. Yet, this DHP was found to block Ca^{2+} uptake in soybean [14] and *Chara* [15] cells. In spite of addition of protease inhibitors in the enzyme mixture used for preparing the protoplasts, it remains a possibility that DHP receptors could have been damaged during treatment of the cells. Thus, we investigated the effect of nifedipine on $^{45}Ca^{2+}$ uptake by using *C. roseus* cells in place of protoplasts. We used two different wash treatments to allow removal of Ca^{2+} from cell walls [16, 17]. Nifedipine never inhibited Ca^{2+} uptake in *C. roseus* cells, while the other CEBs were inhibitory at concentrations which were active on alkaloid accumulation (Table 2). This result, disagreeing with those reported on calcium uptake in refs [14, 15], might be due to various types of Ca^{2+} channels depending on the cells [17], but it is clear

that nifedipine inhibits alkaloid accumulation in *C. roseus* cells through a mechanism not blocking the calcium channels.

Biotransformation of nifedipine by C. roseus *cell cultures*

While investigating the effect of nifedipine on the CK-enhanced alkaloid accumulation, we observed the appearance in seven-day-old cells of a Dragendorff's reagent positive metabolite giving a blue fluorescent spot on irradiation at 365 nm. Preparative isolation of the compound was achieved through column and partition chromatography on silica gel. From the spectroscopic data, the structure of **3c** was assigned to be 2,6-dimethyl 3,5-di(carbomethoxy) 4-(2′-aminophenyl)-pyridine: in the ^{1}H NMR, the two characteristic singlets for DHP structure at δ5.5 and 6 in **1a** were missing in **3c**. Two singlets (6H each) at δ2.68 and 3.5 could be assigned to methyl groups at C-2 and C-6 and to carbomethoxyl groups at C-3 and C-5 respectively. Three proton groups centred at δ7.2, 7.58 and 8.1 were consistent with the phenyl ring protons. The signal at δ4.13 (*ca* 2H, *s*) was attributable to the amino group at C-2′ (cf. signals at δ3.73, 4.75 and 3.6 for *o*-anisidine, 2-aminopyridine and 2-aminobiphenyl, respectively [18, 19]). The first peaks at *m/z* 282 in the EI and at *m/z* 283 in the CI mass spectra were in accord with the presence of two nitrogens in the molecule. These values were not in agreement with the molecular formula of **3c**, but can be explained through the loss of one molecule of methanol with formation of the intermediary lactam. Also in agreement with **3c** was the UV spectrum showing two λ_{max} at 240 and 267 nm (compare to λ_{max} at 230 and 280 nm for aniline [20]).

In conclusion, (i) at least for some cells, nifedipine might lead to misleading effects concerning the mediation of signals by calcium. Obviously, this substance has to be used with caution and it is necessary to verify its effect on calcium uptake; (ii) nifedipine is biotransformed through aromatization of the dihydropyridine ring and reduction of the nitro group to an amino group. This biotransformation differed from the one observed in animal and human cells, in which **1** is metabolized into its pyridine derivative, the monocarboxylic acid and the corresponding monohydroxymethyl compound [21]. The reduction of the nitro group is known with yeast and bacteria [22], but has not been reported in plant cell cultures [23]. However, the position of the nitro group on the phenyl moiety seems of importance as feeding experiments with **2a** or **b** did not lead to biotransformation of these compounds. Moreover, the inhibitory effect of **1** on alkaloid accumulation is not attributable to its biotransformation product **3c** as **2a** and **b** also have the same inhibitory properties.

Table 2. A comparative effect of nifedipine and other CEBs on calcium uptake in *C. roseus* cells*

Wash treatment†	CEBs (μM)	Ca^{2+} uptake (% of control)‡
1	nifedipine 50	95§
	verapamil 500	59
	Co^{2+} 100	70
2	nifedipine 50	118
	verapamil 500	67

*Cells were treated with CEBs for 30 min before adding $^{45}Ca^{2+}$ and were harvested at $t=0$ and $t=10$ min.

†Wash treatment 1: 87 mM sucrose + 5 mM KCl + 2 mM $LaCl_3$ in 25 mM Tris–MES, pH 6.7; wash treatment 2: 116 mM sucrose + 1 mM EGTA.

‡Controls are taken as 100%: treatment 1: 0.9 nmol min^{-1} 10^6 cells^{-1}; treatment 2: 1.8 nmol min^{-1} 10^6 cells^{-1}.

§Non-significant difference at the $P=0.05$ level, when treatment was compared with control.

EXPERIMENTAL

Mps: uncorr. ^{1}H NMR spectra were run at 220 MHz in $CDCl_3$ with TMS as the int. standard; the coupling constants were in agreement with literature values [24]. EIMS and CIMS were performed at 70 eV (CIMS with NH_3). Reported fragments are those $\geq$10% in intensities, except for molecular or pseudomolecular peaks. TLC: precoated silica gel 60F 254 (Merck). Prep. TLC: laboratory-made 1 mm-coated silica gel H (Merck); in both cases, toluene–EtOAc (1:1) was used as solvent; spots were located under UV (254 and 365 nm) or with Dragendorff's reagent, and compounds were recovered from the adsorbent with MeOH–$CHCl_3$ (1:1). CC: 70–230 mesh silica gel 60 (Merck). UV spectra were determined in EtOH. Photoirradiation was carried out with an Hg lamp HPL 125 W (Philips). CKs, DHPs and CEBs were from Sigma. $^{45}CaCl_2$ was purchased from Dupont de Nemours. Opti Phase "Hi Safe II" liquid scintillation cocktail was from LKB.

Plant material and growth conditions. Cell suspensions of *C. roseus* (cell line C20) were propagated in Gamborg's B5 nutrient medium [25] supplemented with 58 mM sucrose and 4.5 μM 2,4-D [10]. Experiments were performed through subculturing the cells for one transfer in 2,4-D-free culture medium. The drugs to be studied were added at day 3 in dim light. The cells were grown in the dark for 9 days. DHPs and DHP derivatives were dissolved in DMSO. The final DMSO concn did not exceed 1%; controls contained DMSO as appropriate.

Calcium uptake into cells. Three-day-old cells (600 mg fr. wt) were incubated in 12 ml of a medium containing 25 mM Tris–MeS (pH 6.7), 88 mM sucrose, 5 mM KCl and (except for controls) the drug to be studied. Cells were pre-incubated for 1 hr at 25° under shaking; Ca uptake was initiated through addition of 100 μM $CaCl_2$ and $^{45}CaCl_2$ (3.7 kBq ml^{-1}). At $t=0$ and $t=10$ min, aliquots (1 ml) were rapidly filtered through glass fibre filters (Whatman GF/A). The cells were washed (x 3, 2 ml) with either an ice-cold soln of 116 mM sucrose plus 1 mM EGTA [16] or an ice-cold soln of 88 mM sucrose, 25 mM Tris–MES (pH 6.7), 5 mM KCl and 2 mM $LaCl_3$ [17]. Cells and filters were suspended in 10 ml premixed liquid scintillation cocktail and the radioactivity was measured 12 hr later.

Alkaloid contents. Ajmalicine was dosed through spectrofluorometry as described previously [10].

Photoirradiation of **1**. A soln of 346 mg (1 mmol) of **1** in 1 l MeOH was photoirradiated 20 cm from the source. The reaction was monitored by TLC every 5 min (R_f **1** 0.45). After 45 min, solvent was evapd under red. pres. The residue (310 mg) was dissolved in $CHCl_3$–MeOH (1:1) and purified through prep. TLC to give 295 mg (85%) of **3a** and 24 mg (4%) of **4**, dimer of **3a**.

2,6-*Dimethyl* 3,5-*di*(*carbomethoxy*) 4-(2′-*nitrosophenyl*)-*pyridine* (**3a**). Green crystals; mp 105° R_f 0.62. UV λ_{max}^{EtOH} nm: 204, 222, 283, 310 (*sh*). ^{1}H NMR ($CDCl_3$) δ: 2.68 (6H, *s*, Me-2, Me-6), 3.38 (6H, *s*, CO_2Me-3, CO_2Me-5), 6.54 (1H, *dd*, H-4′ or H-6′), 7.46 (2H, *td*+*dd*, H-5′ and H-4′ or H-6′), 7.72 (1H, *td*, H-3′); EIMS *m/z* (rel. int.): 328 $[M]^+$ (17), 314 (24), 298 $[M-NO]^+$ and/or $[M-2Me]^+$ (31), 284 (22), 283 (16), 282 $[M-(Me+OMe)]^+$ (70), 281 $[M-(Me+OMe+H)]^+$ (13), 270 (20), 269

$[M-CO_2Me]^+$ (100), 268 (10), 267 $[M-(NO+OMe)]^+$ and/or $[M-(2Me+OMe)]^+$ (42), 254 (17), 253 (41), 252 $[M-(NO+OMe+Me)]^+$ (40), 251 $[M-(2OMe+Me)]^+$, (56), 237 $[M-(CO_2Me+OMe+H)]^+$ (13), 236 $[(M-(NO+2OMe)]^+$ and/or $[M-(2Me+2OMe)]^+$ (12), 224 (15), 223 (15), 209 (21), 196 (10), 195 (10), 194 (11), 193 (32), 192 (10), 189 (12), 181 (16), 180 (19), 169 (11), 168 (14), 167 (12), 166 (14), 155 (10), 153 (27), 152 (49), 151 (11), 141 (17), 140 (32), 139 (27), 138 (10), 128 (14), 127 (22), 126 (20), 91 (11), 89 (12), 87 (12), 77 (27), 76 (14), 75 (12); CIMS *m/z* (rel. int.): 329 $[MH]^+$ (100), 328 $[M]^+$ (4), 269 $[M-CO_2Me]^+$ (8).

Dimer of compound **3a** (**4**). Beige crystals; R_f 0.55. UV λ_{max}^{EtOH} nm: 204, 222, 278, 310 (*sh*); EIMS *m/z* (rel. int.): 656 $[M]^+$ (7), 641 (25), 640 (45), 582 (17), 581 (37), 565 (11), 521 (21), 315 (17), 314 (77), 313 (10), 299 (19), 298 (100), 284 (19), 283 (12), 282 (39), 281 (10), 269 (14), 268 (13), 267 (13), 266 (19), 254 (16), 253 (16), 252 (24), 251 (30), 224 (17), 223 (19), 209 (11), 197 (12), 196 (14), 195 (13), 194 (10), 193 (13), 180 (11), 168 (11), 167 (12), 153 (15), 152 (24), 149 (24), 141 (11), 140 (14), 139 (12), 129 (12), 127 (18), 85 (16), 84 (10), 83 (15), 81 (16), 77 (11), 73 (24), 71 (25), 69 (30), 67 (11), 61 (10), 60 (78), 59 (19), 57 (23), 55 (30); CIMS *m/z* (rel. int.): 657 $[MH]^+$ (8), 656 $[M]^+$ (6), 643 (12), 642 (46), 641 (100), 640 (53), 345 (16), 315 (11), 314 (28), 313 (10), 299 (17), 298 (49), 284 (16), 283 (39), 282 (30), 251 (12).

Aromatization of compound **1**. Nifedipine was treated in two different ways. (A): Mixt. of 173 mg (0.5 mmol) of **1**, 346 mg of 10% Pd on charcoal and 17 ml of phenetole were heated under refluxing. After 20 hr, the pH was brought to 1 (0.1 M HCl) and phenetole was discarded. After alkalinization of the aq. layer with solid Na_2CO_3 and extraction with toluene, the solvent was dried on Na_2SO_4 and evapd under red. pres. The residue was taken up in $CHCl_3$–MeOH (1:1), and purified by prep. TLC, giving 145 mg (82%) of **3b**. (B): Mixt. of 346 mg (1 mmol) of **1** and 492 mg (2 mmol) of chloranile in toluene were heated under reflux for 8 hr. Further purifications were carried out as in procedure A and gave 245 mg (71%) of **3b**.

2,6-*dimethyl* 3,5-*di*(*carbomethoxy*)4-(2′-*nitrophenyl*)-*pyridine* (**3b**). Colourless crystals: mp 119°; R_f 0.57. UV λ_{max}^{EtOH} nm: 206, 270 (*sh*). ^{1}H NMR ($CDCl_3$) δ: 2.61 (6H, *s*, Me-2, Me-6), 3.48 (6H, *s*, CO_2Me-3, CO_2Me-5), 7.2 (1H, *dd*, H-4′ or H-6′), 7.6 (2H, 2 *td*, H-5′ and H-4′ or H-6′), 8.18 (1H, *dd*, H-3′); EIMS *m/z* (rel. int.): 313 $[M-OMe]^+$ (7), 299 (20), 298 $[M-NO_2]^+$ and/or $[M-(OMe+Me)]^+$ (100), 127 (10); CIMS *m/z* (rel. int.): 346 (45), 345 $[MH]^+$ (100), 298 $[M-NO_2]^+$ and/or $[M-(OMe+Me)]^+$ (12).

Biotransformation of compound **1**. A soln of **1** (34.6 mg, 1 mmol) in DMSO was added at day 3 to C20 cells grown in a 2,4-D-free B5 medium (final concn 100 μM). Cells were harvested at day 7 and rapidly washed with ice-cold H_2O. Freeze-dried cells were extracted with toluene (Soxhlet, 10 hr). The solvent was concd under red. pres. and acidified with 5% aq. H_2SO_4. The acid phase was treated with 28% NH_4OH until basic (pH 10) and extracted with toluene or CH_2Cl_2. After drying on Na_2SO_4 and solvent evapn, the residue was dissolved in EtOAc–toluene (1:1) and mixed with silica gel. After evapn under red. pres., the mixture was put on top of a column containing silica gel (60 × 1.2 cm i.d.) and subjected to toluene, EtOAc and MeOH as successive eluents. Fractions containing **3c** were purified by prep. TLC giving 3 mg of **3c**.

2,6-*Dimethyl* 3,5-*di*(*carbomethoxy*)4-(2′-*aminophenyl*)-*pyridine* (**3c**). Whitish crystals; R_f 0.31. UV λ_{max}^{EtOH} nm: 202, 234 (*sh*), 238, 267, 340. ^{1}H NMR ($CDCl_3$) δ: 2.68 (6H, *s*, Me-2, Me-6), 3.50 (6H, *s*, CO_2Me-3 and CO_2Me-5), 4.13 (2H, *s*, NH_2-2′), 7.20 (1H, *dd*, H-4′ or H-6′), 7.58 (2H, 2 *td*, H-5′ and H-4′ or H-6′), 8.10 (1H, *dd*, H-3′); EIMS *m/z* (rel. int.): 283 $[M-MeO]^+$ (17), 282 $[M-MeOH]^+$ (100), 281 (12), 252 (16), 251 $[M-(MeOH+Me)]^+$ (99), 224 (10), 223 $[M-(MeOH+CO_2Me)]^+$ (38), 208 $[M-(MeOH+CO_2Me+Me)]^+$ (16), 196 (11), 180 (17), 153 (13), 152 (10), 149 (15), 128 (12), 127 (28), 126 (23), 125 (14), 111 (11), 105 (10), 102 (12), 101 (10), 81 (13), 71 (12), 69 (11); CIMS *m/z* (rel. int.): 284 $[MH-MeO]^+$ (19), 283 $[MH-MeOH]^+$ (100).

REFERENCES

1. Rampe, D. and Triggle, D. J. (1989) *TIPS* **10**, 388.
2. Godfraind, T., Miller, R. and Wibo, M. (1986) *Pharmac. Rev.* **38**, 321.
3. Wacker, I. and Schnepf, E. (1990) *Planta* **180**, 492.
4. Reiss, H.-D. and Herth, W. (1985) *J. Cell. Sci.* **76**, 247.
5. Roberts, A. W. and Haigler, C. H. (1990) *Planta* **180**, 502.
6. Hetherington, A. M. and Trewavas, A. J. (1984) *Plant Sci. Letters* **35**, 109.
7. Dolle, R. (1988) *Physiol. Plant.* **73**, 7.
8. Graziana, A., Fosset, M. Ranjeva, R., Hetherington, A.-M. and Lazdunski, M. (1988) *Biochemistry* **27**, 764.
9. Kodja, H., Liu, D., Mérillon, J.-M., Andreu, F., Rideau, M. and Chénieux, J.-C. (1989) *C.R. Acad. Sc.* **309** (III), 453.
10. Mérillon, J.-M., Liu, D., Huguet, F., Chénieux, J.-C. and Rideau, M. (1991) *Plant Physiol. Biochem.* **29**, 289.
11. Berson, J.-A. and Brown, E. (1955) *J. Am. Chem. Soc.* **77**, 447.
12. Schönberg, A., Schenck, O. and Neumüller, O. (1968) *Preparative Organic Photochemistry*, pp. 266–273. Springer, Berlin.
13. Boyer, J. (1969) in *The Chemistry of the Nitro and Nitroso groups*, Part 1 (Feuer, H., ed.), pp. 252–255. Interscience, New York.
14. Waldmann, T., Teblick, W. and Kauss, H. (1988) *Planta* **173**, 88.
15. MacRobbie, E.-A.-C. and Banfield, J. (1988) *Planta* **176**, 98.
16. Klein, J.-D. and Ferguson, I.-B. (1987) *Plant Physiol.* **84**, 153
17. Tester, M. and MacRobbie, E.-A.-C. (1990) *Planta* **180**, 569.
18. Bhacca, N. and Johnson, L. (1962–1963) *NMR Spectra.* Varian Associates, Palo Alto.
19. Pouchert, C. and Campbell, J. (1974) *The Aldrich Library of NMR Spectra.* Aldrich, Milwaukee.
20. Gillam, A., Stern, E. and Jones, E. (1954) *An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry.* Edward Arnold, London.
21. Rämsch, K.-D., Graefe, K.-H., Scherling, D., Sommer, J. and Ziegler, R. (1986) *Am. J. Nephrol.* **6**, suppl. 1, 73.
22. Simon, P. and Meunier, R. (1970) *Microbiologie Industrielle et Génie Biochimique*, pp. 155–156. Masson, Paris.
23. Suga, T. and Hirata, T. (1990) *Phytochemistry* **29**, 2393.
24. Pretsch, E., Clerc, T., Seill, J. and Simon, W. (1983) *Tables of Spectral Data for Structure Determination of Organic Compounds. Chemical Laboratory Practice*, H255–H260. Springer, Berlin.
25. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) *Exp. Cell Res.* **50**, 151.