ELSEVIER

Biophysical Chemistry 52 (1994) 259–265

Biophysical Chemistry

Cercospora beticola toxins. Part VI: preliminary studies of protonation and complexation equilibria

V. Mikes [a], S. Lavernet [b], M.-L. Milat [a], E. Collange [b], M. Pâris [b], J.-P. Blein [a,*]

[a] *Unité Associée Phytopharmacie Phytobiologie Cellulaire, INRA-Université de Bourgogne, BV 1540, 21034 Dijon Cedex, France*

[b] *Laboratoire de Chimie de Coordination, Université de Bourgogne, BP 138, 21034 Dijon Cedex, France*

Received 18 February 1994; accepted in revised form 14 June 1994

Abstract

The biological activity of *Cercospora beticola* toxins might be enhanced by the complex formation with magnesium. Therefore, protonation and complexation equilibria of beticolins were studied. Beticolins carry three dissociable functions (H_3B) two of which dissociate at a physiological pH. In the presence of magnesium, the neutralisation and protonation curves provide evidence for the formation of complexes. At physiological pH, the uncharged complex, $Mg_2H_2B_2$, is the predominant form. The non-ionised forms of free beticolin-1 and -2 fluoresce in a 50% dioxan–water solution and their emission maxima shift to higher wavelengths in water. The dianion HB^{2-} is non-fluorescent both in water and in less polar media. The formation of the $Mg_2H_2B_2$ complex which strongly fluoresces in nonpolar media is confirmed by a marked increase in fluorescence at 520 nm and by a shift of the excitation maximum.

Keywords: Protonation equilibrium; Magnesium complex; Beticolin; Fluorescence; Potentiometry

1. Introduction

Cercospora beticola is the causal agent of cercosporiose, the most important leaf disease of sugar beet [1]. Two kinds of toxins are produced by several *C. beticola* strains. They include a red pigment called cercosporin [2,3] which can initiate the peroxidation of membrane lipids [4] and yellow compounds named beticolins. One of them was previously known as CBT. Scarce data concerning the chemical structure of CBT have been published in the last twenty years [1,5]. Milat et al. determined the structure of beticolin-2 on the basis of monocrystals by X-ray diffraction [6]. The structure of beticolin-1 was deduced by comparing NMR and mass spectrometry data [7]. Recently, two groups have isolated a compound called cebetin B [8,9], which is a dimeric chelate of cebetin A (cebetin A and beticolin-1 have the same structure).

The biological activities of CBT have been extensively described [10–15]. Macri et al. [13] have reported an inhibitory effect of CBT on ATP dependent proton translocating activity and suggested a primary effect on ATPase(s) associated with endo membranes. Blein et al. [14] have shown that the inhibition of plasmalemma ATPase by CBT is stronger at high pH and is competitive to ATP.

In order to understand the mode of action of beticolins and their interactions with biological membranes and ATPases, the protonation and complexation equi-

Abbreviations: Mes, 2-(N-morpholino)ethanesulfonic acid; CBT, *Cercospora beticola* toxin.

* Corresponding author. UA 692, INRA, BV 1540, F-21034 Dijon Cedex, France.

SSDI 0301-4622(94)00096-3

libria of beticolins in solution had to be studied first. The formation of a CBT–magnesium complex might be responsible for the inhibitory action on ATPase.

In water or in a hydro-organic solution of metal ions M, protons H and a ligand L, a complex equilibrium is established which can be described by the following equation (charges were omitted for clarity):

$$pM + qH + rL \Leftrightarrow M_pH_qL_r. \quad (1)$$

The equilibrium constant β, which is a general stability constant of the species $M_pH_qL_r$, is defined as follows [16]:

$$\beta_{pqr} = \frac{[M_pH_qL_r]}{[M]^p[H]^q[L]^r},$$

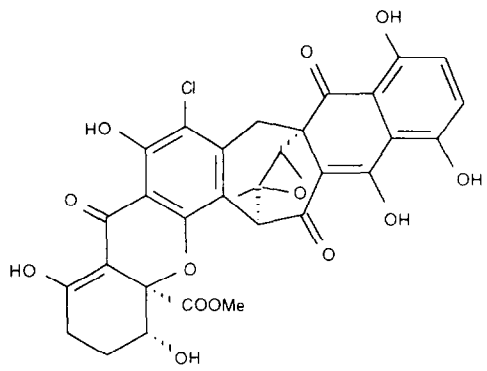
where [H], [M], [L] and $[M_pH_qL_r]$ are the concentrations of the respective species. In order to maintain the activity coefficients at the constant level the equilibrium constant is determined at a fixed ionic strength. This ionic strength is kept stable through the addition of a supporting electrolyte at a concentration far in excess (about 100-fold) of that of the reacting ionic species.

In the absence of the metal ion ($p = 0$), the equation describes the simple protonation of the ligand L. In this case, the stability constants are identical with the protonation constants of the ligand. If $p \neq 0$ the formation of mononuclear ($p = 1$) or polynuclear ($p > 1$) complexes takes place. The computation of stability constants of the various complexes is based on the fact that the concentration of each complex $M_pH_qL_r$ can be expressed as follows:

$$[M_pH_qL_r] = \beta_{pqr}[M]^p[H]^q[L]^r.$$

The three mass constraints corresponding to the total ligand, metal and initial hydrogen concentrations (L_t, M_t, H_t), written in terms of the concentration of the individual components M, H, L and overall stability constant β_{pqr}, give rise to a set of simultaneous equations which can be solved for each component. Several programs are described for their treatment [16]. We used the program PROTAF (PROtonation AFfinment, [17]) allowing not only the computation of the stability constants with the standard deviations but also enabling the adjustment of various parameters (concentrations, ΔpH, etc.) and the determination of the species distribution from the given stability constants. The program allows a direct treatment of accurately determined experimental curves on which a calculated titration curve is adjusted.

Beticolin -1



Beticolin- 2

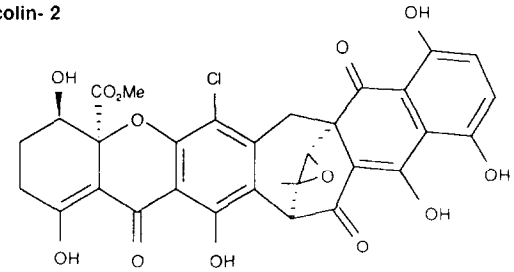


Fig. 1.

In parallel, these equilibria were investigated by fluorescence spectroscopy which is a powerful tool to study metal–ligand interactions thanks to its higher sensitivity.

On the basis of these combined results, we were able to determine the predominant species of beticolin-1 and -2 (Fig. 1) which might be the inhibitory form prevaling at the physiological conditions.

2. Experimental

2.1. Potentiometric measurements

Since the solubility of beticolins in water is low the titration was performed in a 50% (v/v) water–dioxan solution (dioxan was freshly distilled under a flux of nitrogen). The solutions of 4×10^{-4} M beticolins with the supporting electrolyte 0.5 M $NaNO_3$ were titrated with 0.1 M NaOH in the absence or presence of the metal ion at 25 ± 0.1°C. 1 mM HNO_3 was added to initiate the titration in the acidic region and to cover a sufficiently large pH range to reveal all possible spe-

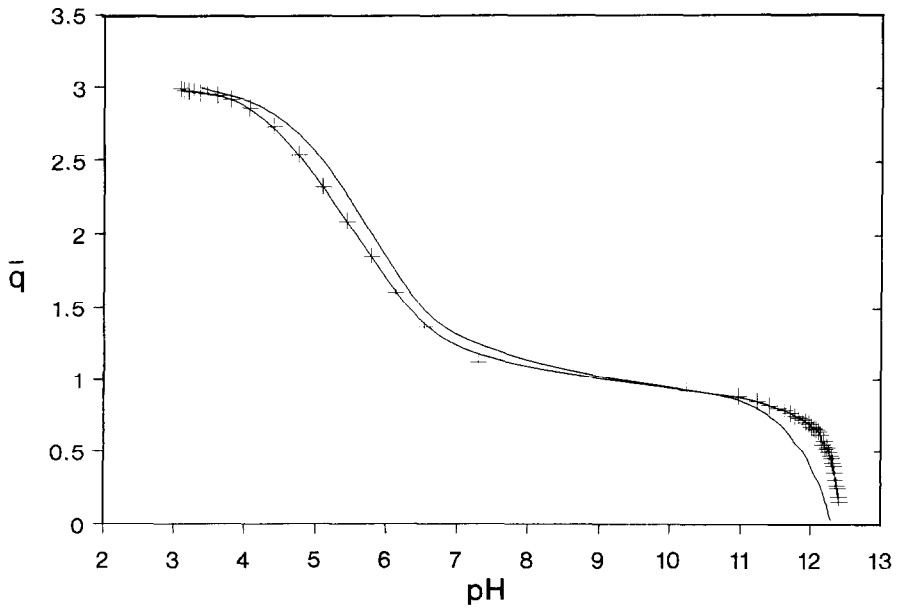


Fig. 2. Normalized protonation curve of beticolin -1 (+) 0.404 mM and of beticolin-2 (—) 0.392 mM in 50% (v/v) water–dioxan and 0.5 M $NaNO_3$ at 25°C.

cies. The pH was measured with a precision of 0.01 unit. The titration was carried out with the autotitrator Metrohm (702 SM Titrino) equipped with a combined glass electrode calibrated daily before each series of measurements with Bates standard buffers (pH 4.008 and 9.196). The parameter of van Huitert [18] correcting pH of the medium of 50% (v/v) water–dioxan and 0.5 M $NaNO_3$ defined as $\Delta pH = pH(exact) - pH$ (measured) was determined by the titration of a strong acid by a strong base in the defined medium and was −0.16 unit.

In order to determine the number of exchanged proton the titration curves were normalized by means of the following equation:

$$q = \frac{H_t + [H] + [OH]}{L_t}, \qquad (2)$$

where q is the mean proton number, H_t and L_t the total acid and ligand concentrations, respectively.

In the absence of metal, this normalized protonation curve directly indicates the number of protons fixed on the ligand L. The log K^H values correspond to the pH values for $q = 0.5$, 1.5, 2.5, etc. The comparison between the protonation curve of the free ligand and that of the metal–ligand mixture at a defined ratio L_t/M_t reveals the complexation of the metal cation.

2.2. *Spectroscopic measurements*

The fluorescence was measured in a 50% (v/v) dioxan–Mes/Tris (50 mM) solution if not stated otherwise. The spectra were obtained with a Shimadzu RF 5001 PC spectrofluorimeter. The determination of the absorption spectra was carried out with a DU 7400 spectrophotometer (Beckman). Owing to the fact that only the non-dissociated form of beticolins fluoresces (see below), the overall protonation constants of beticolins were calculated from the excitation fluorescence spectra using the adjusted Henderson–Hasselbach equation:

$$F = F_1 - (1/K^H) \cdot (F/[H]), \qquad (3)$$

where [H] is the concentration of protons, F the fluorescence intensity, K^H the protonation constant of the acid and F_1 the fluorescence of the non-dissociated

Table 1
Protonation constants of beticolins in 50% (v/v) dioxan–water. The values in parentheses are the standard deviations

Compound	log K	log K_2^H	log K_3^H
beticolin-1	12.06 (±0.05)	6.32 (±0.02)	4.92 (±0.02)
beticolin-2	12.21 (±0.03)	6.45 (±0.01)	5.18 (±0.01)

Table 2
Stability constants of the complexes of beticolins with magnesium ion in 50% (v/v) dioxan–water. The values in parentheses are the standard deviations

Compound	log β_{232}	log β_{222}	log β_{212}	log β_{202}
beticolin-1	43.88 (±0.04)	38.34 (±0.05)	28.01 (±0.07)	16.86 (±0.06)
beticolin-2	42.08 (±0.06)	36.95 (±0.07)	26.73 (±0.11)	15.68 (±0.09)

form (BH_3). The protonation constant was calculated from a plot of F versus $F/[H]$ where $1/K^H$ was the slope and F_1 the intersection with the y axis.

2.3. Purification of beticolins

Beticolins-1 and -2 were purified from a mycelial extract of a *C. beticola* strain (CM) according to Milat et al. [6].

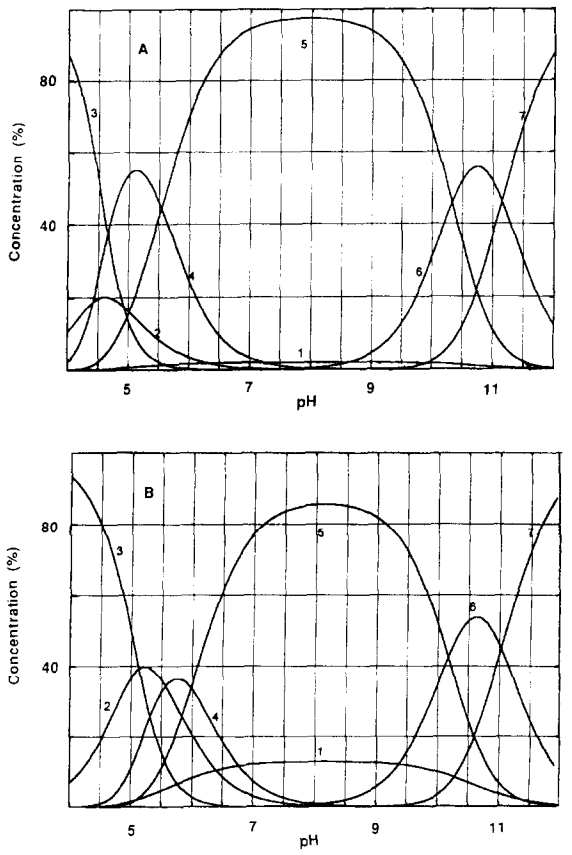


Fig. 3. Distribution of the individual forms of beticolins in a mixture with 1 mM magnesium ions in 50% (v/v) dioxan–water. A 7.8 μM beticolin-1, B 7.8 μM beticolin-2 1 = HB^{2-}, 2 = H_2B^-, 3 = H_3B, 4 = $Mg_2H_3B^+$, 5 = $Mg_2H_2B_2$, 6 = Mg_2HB^- 7 = $Mg_2B_2^{2-}$.

3. Results and discussion

3.1. Protonation constants of beticolins

The neutralisation curves of beticolin-1 and -2 showed two significant jumps. The first one at pH 5 and the second one at pH 9. In the protonation curves (Fig. 2) the loss of two protons between pH 3 and 7 could be clearly distinguished from the loss of the third proton observed in more alkaline conditions. Obviously, only three of six dissociable functions in the beticolin structure actually dissociated in the selected medium. The protonation constants, expressed as log K^H, are shown in Table 1. Beticolin-1 was more acidic than beticolin-2 and the dissociation of two hydroxyle functions was observed in the physiological pH range. Above pH 12, the response of the glass electrode is less accurate so that the first protonation constant is less precise as shown by standard deviation values and by the shape of the protonation curve.

3.2. Complexation of beticolins with magnesium

The mixtures of beticolin : magnesium 1/1 or 2/1 (mol/mol) were titrated with 0.1 M NaOH. A shift of the neutralisation and protonation curves was observed in comparison with the curves of free beticolins (data not shown). The shift was the most striking at pH 5–6 and 10–12 and more obvious for the ratio $L_t/M_t = 1$ than 2. Stability constants of beticolin-1 and -2 did not differ much (Table 2). The distribution of the individual forms of 7.8 μM beticolin-1 and -2 with 1 mM magnesium ions in 50% dioxan is shown in Fig. 3. The uncharged complex $Mg_2H_2B_2$, the predominant form in the pH range form 6 to 8, carries two functions which can dissociate at a higher pH.

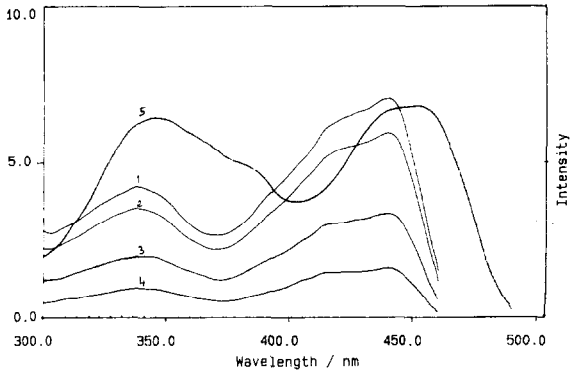


Fig. 4. Fluorescence excitation spectra of beticolin-1 in 50% (v/v) dioxan–water. The emission wavelength was adjusted to 470 nm (beticolin) and to 520 nm (beticolin–magnesium). 5.2 μM beticolin – 1, 1 = pH 3.0, 2 = pH 5.0, 3 = pH 5.8, 4 = pH 6.2, 5 = pH 5.7 with 1 mM $MgCl_2$.

3.3. *Fluorescent properties of beticolins*

In 50 mM Mes/HCl pH 3, the non-dissociated form of beticolin-1 showed three absorption maxima: 440, 421 and 336 nm. In a solvent of low polarity, the first two bands shifted to higher energy (434 and 418 nm) whereas the third maximum moved to lower energy (339 nm). This indicates that the first two bands correspond to the transition with higher dipole moment whereas the third band (339 nm) to the lower dipole moment in the excited state [19]. The opposite behaviour was observed in the case of the dianion of beticolin-1 which has two maxima (432 and 343 nm) in water. In a less polar solvent, the first maximum shifted to lower energy (436 nm) and the second one to higher energy (341 nm). This result confirms the lower dipole moment and the higher dipole moment in the excited state corresponding to the first and second band, respectively. The absorption maxima of beticolin-2 were very similar to those of beticolin-1 and only the molar absorption coefficients of these molecules were found to be somewhat different.

The dissociation of the first hydroxyl group of beticolin-1 did not result in any apparent shift of the absorption maxima with respect to the non-ionised form. Only a 15% increase in the molar absorption coefficient at 339 nm was observed at a 50% dioxan solution at pH 5.2.

The molecule of beticolin-1 and -2 are composed of two separated conjugated moieties. The first one has a hydroxyquinone structure and the second one consists of a partially hydrogenated xanthone moiety. The first excited singlet state of the non-dissociated dihydroxyanthraquinones was characterised by a lower dipole moment as a result of a charge-transfer excitation from the hydroxyl group to the carbonyl moiety [20]. The dissociation of two protons of dihydroxyanthraquinones resulted in a bathochromic shift of the absorption maximum. The excited singlet state of the dianion was characterised by the higher dipole moment [20,21]. Thus, the maximum at 339 nm of beticolins should be attributed to the transition of the hydroquinone moiety.

Figs. 4 and 5 show the fluorescence excitation and emission spectra of beticolin-1. The non-ionised form BH_3 showed excitation maxima at 439, 416 and 338 nm and had a broad emission maximum at 470 nm. This fluorescence emission maximum of the non-ionised form BH_3 of both beticolins was red-shifted to 500 nm in water. In 1 M sulfuric acid, the emission maximum remained unchanged at 500 nm which confirms that the form is non-ionised. The red-shift of the emission maximum in more polar solvents is generally observed for substances showing a dipole moment in the excited state higher than that in the ground state [19]. So, this emission should correspond to the band excited at 416 and 439 nm which is the emission band of the xanthone moiety.

As the pH increased the fluorescence intensity of beticolins decreased without any shift of the excitation maxima. The plots of the emission at 470 nm versus pH revealed that the dissociation giving rise to the form BH caused only small changes of fluorescence intensity (not shown). At pH 7.4, the fluorescence of both beti-

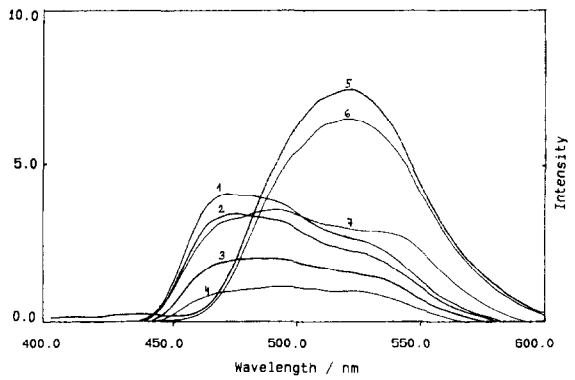


Fig. 5. Fluorescence emission spectra of beticolin – 1 in 50% (v/v) dioxan–water. The excitation wavelength was 340 nm. 5.2 μM beticolin-1, without magnesium: 1 = pH 3.0, 2 = pH 5.0, 3 = pH 5.8, 4 = pH 6.2, with 1 mM $MgCl_2$: 5 = pH 6.7, 6 = pH 5.7, 7 = pH 4.2.

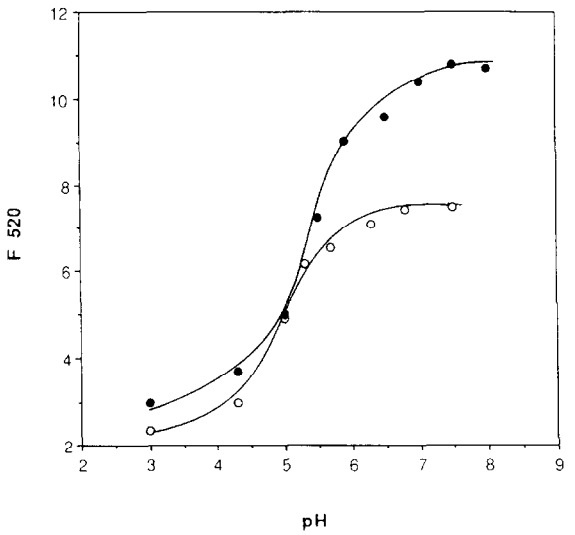


Fig. 6. The effect of pH on the fluorescence of the mixture of beticolins with magnesium chloride in 50% (v/v) dioxan–water. Excitation was measured at 340 nm, emission at 520 nm, (○) 5.2 μM beticolin-1, (●) 5.2 μM beticolin-2.

colins at 470 nm was negligible. So, the form BH^{2-} does not fluoresce. However, the emission spectrum revealed a very weak fluorescence at 520 nm even at higher pH. It corresponds probably to the emission of the anion or the dianion.

The overall protonation constants were calculated from the fluorescence titration curves of beticolins using Eq. (3) and the plots of F versus $F/[H]$. The measurements were made at two excitation maxima at 338 and 440 nm and the emission was measured at 470 nm. At this wavelength, the fluorescence of the dissociated form was neglected. We obtained straight lines for both beticolins showing correlation coefficients between 0.97 and 0.99. The respective values of log K^H for beticolin-1 and -2 were found to be 5.77 and 6.02. It confirms the potentiometric data showing that beticolin-1 is more acid than beticolin-2. The fluorometrically determined values of log K^H are lower than the second protonation constants determined by means of the the pH metric method (Table 1). This is probably due to the high ionic strength used in the latter measurements. Moreover, if this dissociation was followed in 50 mM Mes/Tris instead of 50% dioxan–Mes/Tris (50 mM) we obtained the log K^H values of 4.51 and 5.11 for beticolin-1 and -2, respectively. This indicate that the less polar dioxan solution altered the protonation constants of beticolins. This is confirmed by our potentiometric measurements of protonation constants of some flavonoids (unpublished results). We found the values of log K^H higher by 0.6 unit in dioxan solution in comparison with previously published values in water [22,23].

The addition of magnesium chloride to beticolin-1 caused: a) a red shift of the excitation maxima to 448 and 344 nm, b) a marked change of the emission spectra and c) an increase in the fluorescence intensity at 519 nm as a result of the formation of the complex (Figs. 4 and 5). An identical effect of magnesium was observed in the case of beticolin-2. The fluorescence intensity of the complex beticolin-1 with magnesium in water was much lower than in 50% dioxan but higher than the negligible fluorescence of free beticolin in water.

The effect of pH on the formation of the complex is shown in Fig. 6. At pH 3, the emission spectra of the beticolin–$MgCl_2$ mixtures were identical to the spectra of free beticolins and no complex was formed. With rising pH the fluorescence intensity at 520 nm increased for both beticolins studied. The complex of beticolin-2 with magnesium was more fluorescent than that of beticolin-1. The comparison of these fluorescence curves with the calculated distribution curves of the individual complexes forms (Fig. 3) confirms that the form fluorescent at 520 nm is the uncharged complex $Mg_2H_2B_2$.

As in the case of the protonation constants, the fluorometric curves of the complex slightly shifted to the acidic region when compared with the distribution curves determined by potentiometry (Fig. 3).

The results we obtained confirm that the uncharged complex of beticolin and magnesium is the predominant form in the experimental conditions used to study the inhibitory effects of beticolins on ATPases and that it could be the true inhibitory species. The interaction of these compounds with membranes is being presently investigated.

Acknowledgements

We wish to thank C. Descoins for his encouragement, the Conseil Regional de Bourgogne and MRT for financial support.

References

[1] E. Schlösser, Phytopathol. mediterr. 10 (1971) 154.
[2] S. Kuyama and T.J. Tamura, J. Am. Chem. Soc. 79 (1957) 5725.
[3] R.J.J.C. Lousberg, U. Weiss, C.A. Salemink, A. Arnone, L. Merlini and G. Nasini, J. Chem. Soc. D Chem. Commun. (1971) 1463.
[4] M.E. Daub and M. Ehrenshaft, Physiol. Plant. 89 (1993) 227.
[5] G. Assante, R. Locci, L. Camarda, L. Merlini and G. Nasini, Phytochemistry 16 (1977) 243.
[6] M.-L. Milat, T. Prangé, P.-H. Ducrot, J.-C. Tabet, J. Einhorn, J.-P. Blein and J.-Y. Lallemand, J. Am. Chem. Soc. 114 (1992) 1478.
[7] P.-H. Ducrot, M.-L. Milat, J.-P. Blein and J.-Y. Lallemand, J. Chem. Soc. Chem. Commun., in press.
[8] M.A.F. Jalal, M.B. Hossain, D.J. Robeson and D. van der Helm, J. Am. Chem. Soc. 114 (1992) 5967.
[9] A. Arnone, G. Nasini, L. Merlini, E. Ragg and G. Assante, J. Chem. Soc. Perkin Trans. 1 (1993) 145.
[10] F. Macri and A. Vianello, Physiol. Plant Pathol. 15 (1979) 161.
[11] F. Macri, A. Vianello, R. Cerana and F. Rasi-Caldogno, Plant Sci. Letters 18 (1980) 207.
[12] F. Macri and A. Vianello, Plant Sci. Letters 22 (1981) 29.
[13] F. Macri, P. Dell' Antone and A. Vianello, Plant. Cell Environ. 6 (1983) 555.
[14] J.-P. Blein, I. Bourdil, M. Rossignol and R. Scalla, Plant Physiol. 88 (1988) 429.
[15] F. Macri, A. Vianello and P. Dell' Antone, Phytopathol. mediterr. 22 (1983) 109.
[16] A.E. Martell and R.J. Motekaitis in: Determination and use of stability constants, 2nd Ed. (VCH Publishers, New York, 1992).
[17] R. Fournaise and C. Petitfaux, Analusis 18 (1990) 242.
[18] L.G. van Huitert and C.G. Haas, J. Am. Chem. Soc. 75 (1953) 451.
[19] L. Brand and B. Withold in: Methods in enzymology 11, ed. C.H.W. Hirs (Academic Press, New York, 1967) p. 776.
[20] T.S. Koch and R.P. Rava, Biophys. Chem. 36 (1990), 187.
[21] L.W. Johnson, M.A. Savory, C. Pope, M. Foresti and J.R. Lombardi, J. Chem. Phys. 86 (1987) 3048.
[22] V.P. Nikitin, I.E. Makashaeva and M.T. Golovkina, Zh. Obshsch. Khim. 48 (1978) 395.
[23] V.P. Nikitin and M.T. Golovkina, Zh. Obshsch. Khim. 53 (1982) 1463.