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CHARACTERIZATION OF MAGNESIUM AND CALCIUM TENUAZONATE FROM *PHOMA SORGHINA*

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Key Word Index—*Phoma sorghina*; fungi; tetramic acids; magnesium and calcium tenuazonate; onyalai; mycotoxicosis.

Abstract—*Phoma sorghina* Sacc., the fungus implicated in the aetiology of onyalai, a haematologic disorder, produces magnesium and calcium tenuazonate as toxic constituents.

INTRODUCTION

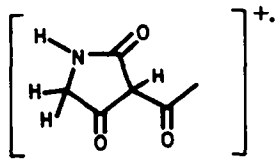
Onyalai is a haematologic disorder which occurs widely amongst the Black African population south of the Sahara [1]. It is an acute purpuric disease with the distinctive feature of haemorrhagic bullae in the mouth [2] but the aetiology of the disease remains obscure. Rabie *et al.* [3] studied the microflora from the households of patients suffering from onyalai; a mycological investigation of millet (*Pennisetum typhoides*) and grain sorghum (*Sorghum vulgare*) led to the isolation of several toxigenic fungi [3]. *Phoma sorghina* Sacc., an ubiquitous fungus in tropical and subtropical regions was the predominant type; furthermore, eight out of eleven isolates of this fungus from Ovamboland in South West Africa proved to be toxigenic [3]. From animal experiments, pathological and histopathological studies (damage to the vascular system), Rabie *et al.* [3] implicated a mycotoxin from *P. sorghina* in the aetiology of onyalai.

This paper reports the characterization of toxic constituents obtained from a toxigenic strain of *P. sorghina*, designated JF_1, isolated from grain sorghum obtained in Sekhukhuneland, Transvaal.

RESULTS AND DISCUSSION

The fungus was grown on maize as previously described [3] and the dried mouldy material extracted and subjected to solvent partition as described in the Experimental. Assay in day-old chickens indicated a concentration of toxicity in the 90% MeOH fraction. This material was tested for the cytotoxic cytochalasins [4] and the commonly known mycotoxins [5] but none was present. The material was subsequently fractionated by column chromatography on Si gel under pressure in $CHCl_3$–MeOH and the fractions investigated by UV spectroscopy. The fractions were essentially transparent except for a group which showed strong absorption at 236 and 277.5 nm. The latter fractions yielded a colourless, amorphous powder which was homogenous on several Si gel TLC systems. The powder was insoluble in water, slightly soluble in $CHCl_3$ and very soluble in MeOH. It can be detected on TLC plates by spraying with ethanolic $FeCl_3$ (red–brown); 2,4-DNP (yellow) and 1% $Ce(SO_4)_2$ in 6 N H_2SO_4 (grey–brown).

The IR spectrum of the sample recorded in nujol showed very strong absorption at 3300 (condensed N-H from lactam) 1615 and 1470 cm^{-1}. Absorption at 1615 cm^{-1} is generally characteristic of enolised β-diketones [6]. The presence of this unit explains the $FeCl_3$ colour reaction and the green colour obtained upon treatment with aqueous $Cu(OAc)_2$ and its immobility by TLC on Al_2O_3. Amongst fungal metabolites, these data indicated the presence of either a tetramic or an α-acyltetronic acid moiety. In the high resolution MS a M^+ was not recorded even at 240°, however, only a weak fragment was observed at *m/e* 141.0420, corresponding to $C_6H_7NO_3$ (fragment a); this fragment is the base peak in the MS of tenuazonic acid.

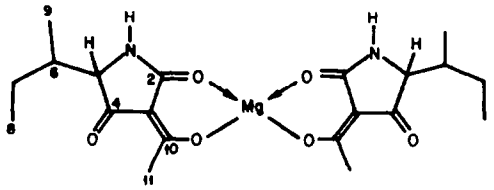


FRAGMENT a

This clearly suggests a tetramic acid as furthermore evidenced by UV: (log ϵ)* λ_{max}^{MeOH} 236 nm (4.19) and 277.5 nm (4.39) and CD: ($\Delta\epsilon$* in MeOH)$^{20°}$ 235 nm (−3.7) and 275 nm (−6.9). The UV spectrum remained unchanged upon addition of methanolic KOH; upon acidification (2 N HCl) the peak at 236 nm decreased in intensity and shifted to 215 nm, the high wave-length peak shifted only to 275 nm.

The PMR spectrum (100 M Hz, CD_3OD) showed: δ 3.58–3.80 (1 H, *m*, C-5); δ 2.34 (3 H, *s*, C-11); δ 1.8–2.02 (1 H, broad *m*, C-6); δ 1.2–1.5 (2 H, unstructured *m*, C-7),

* Calculations based on a calculated MW of 395 (see later).



Magnesium Tenuazonate Complex

Fig. 1.

δ 0.98 (3 H, *d*, J = 7 Hz, C-9); δ 0.88 (3 H, *t*, J = 7 Hz, C-8). The above data are clearly compatible with an α-acetyl-γ-(α'-methylpropyl) tetramic acid, eg tenuazonic acid [7]. However, the sample failed to dissolve in aqueous $NaHCO_3$ or to react with Et_2O/CH_2N_2 or Ac_2O/Py. The enolic functionality in the sample was therefore, protected. A solution of the sample was analysed by atomic absorption spectrometry for Ca^{2+}, Mg^{2+}, Zn^{2+}, Na^+ and K^+ using the line 422.7, 285.2, 213.8, 589 and 766.5 nm, respectively. Mg^{2+}, Ca^{2+} and Na^+ were present in a ratio (ppm) of 10.5:2:1.5. Only trace amounts of Zn^{2+} and K^+ were detected. The sample therefore consists mainly of the covalent magnesium tenuazonate complex $(C_{10}H_{13}NO_3)_2Mg$ together with the analogous calcium complex. Sodium tenuazonate is a water soluble salt and is probably absorbed on the other complexes. Acidification of the sample and extraction into $CHCl_3$ gave tenuazonic acid, identical in detail to authentic tenuazonic acid by direct comparison and conversion into copper tenuazonate.

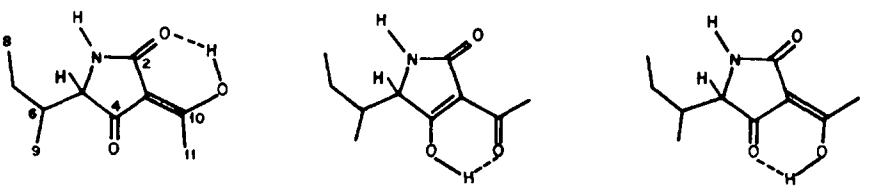
In tenuazonic acid there are three O atoms to which the metal ion could conceivably be coordinated. A recent X-ray crystallographic investigation [8] of copper-*bis*-tenuazonate *mono*-hydrate $[Cu(TA)_2.H_2O]$ established that the chelate is formed between the enolic C_{10}–O and the amide C_2–O. By analogy, the Group IIA cations (Mg^{2+} and Ca^{2+}) will probably occupy the same position in these complexes as shown in Fig. 1; a supposition substantiated by virtually identical IR spectra. The stability of the Group IIA complexes are apparently due to the strength of these cations as Lewis acids [9], the chelate effect and the favourable size of the chelate ring. Mg^{2+} is known to complex with electron donating ligands, the rate constant is *ca* $10^5\ sec^{-1}$ and virtually independent of the ligand [10]. The rate-determining step is attributable to the loss of coordinate water from the Mg^{2+}. Magnesidin is related to these complexes in being a novel magnesium-containing antibiotic which is produced by cultures of *Pseudomonas magnesiorubra* [11]. The magnesium is linked to complex tetramic acids in magnesidin. The isolation of tenuazonic acid from liquid cultures of *Alternaria tenuis* required acidification [7]. These covalent complexes should therefore be regarded as the "natural" form of tenuazonic acid. Tenuazonic acid may play an important role in the control mechanism of fungi (eg *A. tenuis* or *P. sorghina*) by complexation with bivalent cations.

The $Cu(TA)_2.H_2O$ showed broad CD Cotton effects at 230–240 nm ($\Delta\epsilon$-5.22) and 280–288 nm ($\Delta\epsilon$-3.23) in MeOH and are similar to those described for the magnesium–calcium tenuazonates, see before. The complexes, therefore, contain the same absolute configuration as reported for tenuazonic acid which is formally derived from L-isoleucine [12]. Holzapfel *et al.* [13] reported that tenuazonic acid showed three Cotton effects at 240 nm ($\Delta\epsilon$-2.34) 280 nm ($\Delta\epsilon$-2.8) and 320 nm ($\Delta\epsilon$ *ca* -0.4). The long wave-length Cotton effect was also observed by McGrath *et al.* [14] in α-acetyl-γ-(β-indolyl)methyl tetramic acid at 320 nm ($\Delta\epsilon$-1.0) and was attributed by Holzapfel *et al.* [13] to an $n \rightarrow \pi^*$ transition within the enolized β-diketone. It is of importance to note that this transition was not observed in metal stabilised planar chelates of tenuazonic acid. In solution tenuazonic acid can formally exist in an equilibrium of the three indicated tautomers (see Fig. 2); each differently stabilised by their hydrogen bonding capabilities. The structural assignment and ratio of the tautomers of tetramic acids are outside the scope of this paper and require detailed PMR and ^{13}C NMR investigations.

C. W. Holzapfel (personal communication, 1965) studied the toxigenicity of several South African isolates of *Alternaria tenuis* cultivated on maize. Tenuazonic acid was characterized as the mycotoxin of *A. tenuis*. Meronuck *et al.* [15] isolated it as the major toxin from *Alternaria alternata* (*A. tenuis*, *A. longipes* and *A. alternata* are morphologically indistinguishable [16]). Tenuazonic acid was isolated from the broth of *Pyricularia oryzae* Cavara, the causal fungus of the rice blast disease [17]; it does exhibit a conspicuous stunting effect on seedling growth of rice [18]. Tenuazonic acid was investigated for its antineoplastic [19,20] and anti-viral activities [21] and extensively for the blocking of the step in peptide bond formation in protein synthesis by human tonsil and pig liver ribosomes [22]. The highly biologically active tenuazonic acid, as the magnesium and calcium complexes, therefore apparently represents the toxicity of *P. sorghina*, its potential role in haematologic disorders, eg onyalai will require further investigation. The toxicity of the tetramic acids e.g. cyclopiazonic acid [23] and tenuazonic acid might be related in part to their ability to complex selectively *in vivo* with trace metals.

EXPERIMENTAL

PMR spectra were recorded at 100 MHz (CD_3OD or $CDCl_3$; TMS standard). 20 MHz ^{13}C NMR spectra were obtained from soln in CD_3OD using 10 mm sample tubes spectral width 4500 Hz, pulse delay 0–3 sec; data points 8192;



Enolic Tautomers of Tenuazonic Acid

Fig. 2.

number of transients *ca* 20 K. Column chromatography was performed on Si gel H.

Isolation of the tenuazonates. Dried, milled mouldy maize (650 g) was extracted with $CHCl_3$–MeOH. Evaporation of the solvent gave a crude extract (48 g) which was then partitioned between hexane–90% MeOH. The 90% MeOH fraction (4 g) was separated by chromatography on Si gel H (500 g) under pressure (0.66 kg/cm^2). The column was developed with $CHCl_3$–MeOH (8.8:1.2); 160 fractions (each 20 ml) were collected. The fractions were analysed by Si gel TLC ($CHCl_3$–MeOH; 8.5:1.5); a dark absorbent spot appeared at R_f 0.3 (red–brown coloration with ethanolic $FeCl_3$) in fractions 90–115. Combination of these fractions gave the tenuazonates (250 mg) as an amorphous powder, homogenous by chromatography in several solvents. The UV, CD, IR, PMR and MS characteristics of this powder were described in the text. The powder analysed for: C, 56.35; H, 7.65, N, 5.20%.

The powder (14 mg) dissolved in MeOH–H_2O (1:1) (25 ml) was analysed by atomic absorption spectrometry: Mg^{2+}, Ca^{2+}, and Na^+ were present in a concn of 21, 4 and 3 ppm, respectively. From these figures a theoretical MW of 395 was calculated for the powder and used in the calculation of log ϵ and $\Delta\epsilon$.

Acid treatment of tenuazonate powder. The powder (120 mg) in MeOH–H_2O (1:1) was treated with a slight excess of 0.1 N HCl. The mixture was extracted into $CHCl_3$, the $CHCl_3$ layer washed with H_2O, dried and filtered to yield tenuazonic acid (83 mg) as an homogenous oil. This material was identical to tenuazonic acid (obtained from its Cu complex by acidification [7]) by UV, IR, ^{13}C NMR and MS. The tenuazonic acid obtained from *P. sorghina* was converted into the Cu-*bis*-tenuazonate-*mono*-hydrate, mp 175°.

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