Full Papers

Mushroom toxins of the genus *Cortinarius*

I. R. Tebbett and B. Caddy

Forensic Science Unit, University of Strathclyde, 204 George Street, Glasgow (Scotland), 6 December 1983

Summary: 3 major components of the toxic fungus *Cortinarius speciosissimus* have been isolated and their structures determined as cyclic polypeptides. 2 of these compounds have been shown in laboratory animals to cause nephrotoxicity characteristic of Cortinarius mushroom poisoning.

The largest genus of mushrooms in Europe is the *Cortinarius* group about 250 examples of which are found in the United Kingdom. Of these several are known to be toxic and many others are suspected of being so. Grzymala first described the characteristic features of poisoning by the fungus *Cortinarius orellanus* following a study of 135 patients in Poland[9]. Toxic symptoms only became apparent after an unusual latent period of 3–20 days after ingestion; these included an intense thirst together with gastric disturbances, vomiting and persistent headaches. Some subjects also experienced a constant sensation of coldness and in severe cases renal function was impaired and oliguria and sometimes anuria observed. Autopsies following 19 deaths showed the presence of renal lesions characteristic of toxic interstital nephritis. Similar toxic reactions have been reported following the ingestion of *Cortinarius speciosissimus* and *C. gentilis* in Scotland[17] and Finland[12] respectively and recently *C. splendens* in France[7]. A toxic substance was isolated from *C. orellanus* by Grzymala and called by him orellanine[10]. Antkowiak and Gessner considered orellanine and its decomposition product orelline to have a bipyridyl structure[2]. However other workers have suggested that these 'compounds' are mixtures. Testa reported the separation of upto 10 toxic fractions from a methanolic extract of *C. orellanus* all of which showed polypeptide characters[23]. Gamper[6] and Kurnsteiner and Moser[13] although unable to verify Testa's results, were able to distinguish both a slow and a fast acting toxin from similar extracts. Although there is uncertainty as to the nature of the toxins, all workers were of the opinion that toxicity was associated with those compounds which show blue fluorescence when irradiated with ultra violet light. This correlation would appear to be emphasized by the observation of blue fluorescent material in the kidney tubules of laboratory animals poisoned with *Cortinarius orellanus*[3]. Preliminary structural elucidation of Cortinarius toxins was therefore concentrated on such a fluorescent compound (Cortinarin A) isolated from *Cortinarius speciosissimus*[4]. This paper reports the probable structures of this compound together with 2 other major components of *Cortinarius speciosissimus*, Cortinarins B and C.

Experimental

Specimens of *Cortinarius speciosissimus*, collected in August, 1982 from the North of Scotland were air dried at 40 °C for several hours and stored over anhydrous silica gel until required. The dried material (10 g) was powdered and soxhlet extracted with petroleum ether (40–60) for 1 h prior to a similar 6 h methanolic extraction. The methanol extraction was evaporated to dryness under reduced pressure and the residue redissolved in a minimum volume of methanol. Pure extracts of Cortinarins A and C were obtained by preparative thin layer chromatography (TLC) using silica gel G plates (Merck) (20 × 20 cm × 0.5 mm) developed with cyclohexane:ethyl acetate (3:1).

Cortinarin A was located as a fluorescent streak under ultra violet light at 254 nm (Rf 0.50) and Cortinarin C which is non-fluorescent, by spraying 1 edge of the plate with acidified p-dimethylaminobenzaldehyde reagent (pDAB) (Rf 0.30). Cortinarin B was similarly obtained by TLC with silica plates but using butanol:acetic acid:water (6:2:2) as the mobile phase. Cortinarin B also shows blue fluorescence under UV light. Appropriate areas of the chromatoplates containing the 3 compounds were removed and immediately eluted with methanol. The isolated Cortinarins were hydrolysed by refluxing with 6 M hydrochloric acid at 110 °C for 8 h. After dilution with water, the hydrolysate was neutralized with silver carbonate, filtered and the filtrate analyzed on an automated amino acid analyzer.

Isolation of tryptophan derivative (fig. 3 IV)

Cortinarin A (20 mg) was subjected to mild hydrogenation by heating with Raney nickel (50 mg) in boiling methanol (20 ml) for 2 h. The mixture was filtered and the filtrate evaporated to dryness under reduced pressure. The residue was then hydrolysed with 6 M HCl (1 ml) at 110 °C for 6 h. The acid solution containing the free amino acids was evaporated to dryness under reduced pressure and the residue redissolved in a minimum volume of distilled water (5 ml). This solution was applied to a 50 cm × 2.5 cm column of Sephadex LH20[5]. The column was eluted with 1% aqueous acetic acid and 10-ml fractions collected. These fractions were monitored by both UV absorbance at 270 nm and TLC on silica gel G plates [0.2 mm (Merck)] with butanol:acetic acid:water (4:1:1) as eluent. One fraction which showed maximum UV absorbance and the presence of a single spot on TLC which reacted with both acidified pDAB and ninhydrin reagents was evaporated to dryness to yield approximately 5 mg of a pure tryptophan derivative. A substituted indole was liberated from this compound by incubation with 20 units of

tryptophanase[11] (Sigma) in a reaction mixture containing 0.2 ml M/l phosphate buffer (pH 8.3) and 20 µl barium pyridoxal phosphate (Sigma) (100 µg/ml). This mixture was incubated at 37 °C for 10 min prior to the addition of the isolated tryptophan moiety (5 mg) in 2 ml of distilled water. The solution was then further incubated overnight at 37 °C. The reaction was terminated by the addition of 0.2 ml trichloroacetic acid and the liberated indole (3 mg) extracted by shaking with toluene (2 × 2 ml aliquots). After removal of the solvent the indole was redissolved in methanol for thin layer chromatographic and UV spectral analyses. The sample in deutero methanol was also used for recording the 250 MHz H^1NMR spectrum. These characters were compared with those of 4-methoxy indole which was prepared by the methylation of psilocin (4-hydroxy N,N, dimethyl tryptamine) with diazomethane followed by liberation of the indole with tryptophanase as described above.

Amino acid sequencing

Cortinarin A (10 mg) was prepared for amino acid sequencing by an initial treatment with Raney nickel (50 mg) in boiling methanol (20 ml) for 2 h. After this time the solution was filtered and the filtrate evaporated to dryness under reduced pressure. The residue was then partially hydrolysed by the addition of trifluoroacetic acid (Sigma) (10 ml) the reaction being allowed to proceed for several hours at room temperature. The sample was dried under a stream of nitrogen and the amino acid sequence of the resulting linear peptide (fig. 3 V) determined by Edman degradation employing dansylation of the terminal amino acid[8]. Dansyl amino acids were identified by comparison with standards (Sigma) using 2 dimensional TLC on polyamide plates (15 × 15 cm) (BDH). The chromatographic plates were developed with water: 90% formic acid (70:3) in an initial vector followed by benzene: glacial acetic acid (9:1) in a 90° vector[25]. Dansylated amino acids were identified as fluorescent spots under UV light.

Configurations of the amino acids

The configurations of the amino acids present in the Cortinarins were determined by incubation of a total acid hydrolysate of the peptides with both D and L amino acid oxidase, followed by determination of the undegraded amino acids[18]. Cortinarin A (2 mg) was hydrolysed with 6 M HCl for 6 h. The dried residue was dissolved in 0.2 M tris buffer (50 µl) at pH 7.9 and 80 µl of a 2% solution of L amino acid oxidase (Sigma) added. The mixture was incubated overnight at 37 °C and the non-degraded amino acids dansylated[8].
Identification of the dansyl amino acids was by 2-dimensional tlc on polyamide plates as described above. A further 2 mg of peptide was similarly hydrolysed and incubated with 1 ml of D amino acid oxidase (5 mg/ml) (Sigma) overnight in the presence of 50 units of catalase (Sigma). The dried residue was dansylated and the non-degraded amino acids identified by TLC.

Confirmation of amino acid sequence by mass spectrometry

The linear form of Cortinarin A (5 mg) prepared by treatment with Raney nickel and TFA as described above (fig. 3 V) was acetylated and permethylated prior to low resolution mass spectrometry[14]. The terminal amino groups of the peptide were acetylated by reaction with excess acetic anhydride/methanol (1:4) at room temperature for 3 h.
The reaction was terminated by evaporation of the reactants under a stream of nitrogen. This process results in the acetylation of basic free amino groups in the peptide; hydroxyl groups are not affected. The N-acetylated peptide was then permethylated. A base was prepared by heating sodium hydride in dimethylsulphoxide (50 mg/ml) at 90 °C for 15 min. A slight excess of this base was added, when cool, to the acetylated peptide dissolved in 0.1 ml of dimethyl-sulphoxide, followed by an excess of methyl iodide. The reaction was allowed to proceed at room temperature for 1 h in a stoppered tube after which the premethylated product was isolated by dilution with water and extraction into chloroform (1 ml). This reaction leads to methylation of amide nitrogen [$-CONH_2 \rightarrow CON(CH_3)_2$; $-CONH \rightarrow CON(CH_3)$], hydroxyl groups and carboxylic functions. Low resolution mass spectrometry was then performed on this derivatized peptide.

Preparation of a sulphoxide of Cortinarin B

A mixture of 30 vol. hydrogen peroxide (0.5 ml) and glacial acetic acid (2 ml) was added to 5 mg of Cortinarin B and the reaction allowed to proceed for 24 h at room temperature. The resulting sulphoxide of Cortinarin B was dried under vacuum and redissolved in methanol. The thin layer chromatographic and ultra violet spectral characteristics were compared with those of a metabolite removed from the kidneys of mice poisoned with Cortinarins A or B.

Results and discussion

The 3 isolated Cortinarins were found to be soluble in methanol and ethanol, more soluble in water and very soluble in dilute aqueous alkali. They are insoluble or only poorly soluble in apolar solvents such as ether, chloroform and ethyl acetate. Elemental analysis of Cortinarin A showed the presence of carbon, hydrogen, sulphur, nitrogen and by implication oxygen. The reactions of the Cortinarins with various chromogenic reagents are summarized in the table.
Cortinarins A, B and C only showed a positive reaction with ninhydrin after prior treatment with concentrated hydrochloric acid for 2 min at 100 °C. This is consistent with each Cortinarin being a polypeptide and is in

Reactions of Cortinarins A, B, C with various chromogenic reagents

	Cortinarin A	Cortinarin B	Cortinarin C
2% Ferric chloride solution	No reaction	Blue-grey	No reaction
Pauly's reagent	Pale yellow	Red	Pale yellow
Acidified pDAB	No reaction	No reaction	Purple
Neutral ninhydrin + heat	No reaction	No reaction	No reaction
Acid ninhydrin + heat	Purple	Purple	Purple

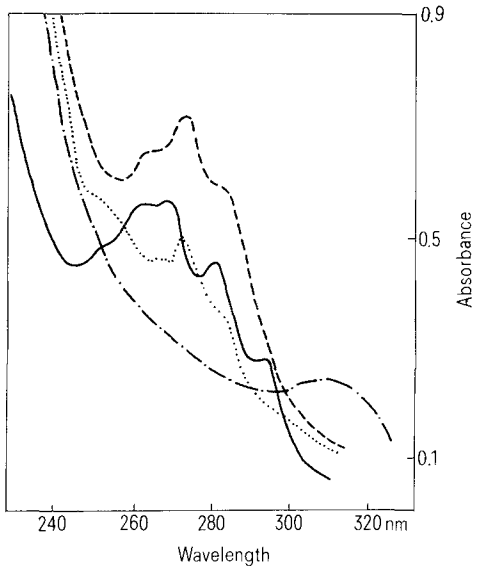

Figure 1. UV spectra in methanol of: Cortinarin A, – – – –; Cortinarin B, ...; Cortinarin B and 2 drops of sodium hydroxide, – · – · –; Cortinarin C ——.

agreement with Testa's observations for *Cortinarius orellanus*. The reactions of Cortinarin B with both ferric chloride and Pauly's reagent suggest that this compound contains a phenolic moiety. Only Cortinarin C produces a dark blue color with acidified p-dimethylaminobenzaldehyde a reaction characteristic of indoles. The ultra violet spectra of Cortinarins A, B and C are represented in figure 1. The patterns of Cortinarins A and B differ only by the presence of an inflexion at about 250 nm in the latter. The spectrum of Cortinarin B also shows a bathochromic shift on addition of 2 drops of alkali. This property is again characteristic of the presence of a phenolic group. The spectra of both Cortinarins A and B showed a strong similarity to that of tryptophan and more closely to that of phalloidin, a toxic principle of the fungus *Amanita phalloides*, but at a lower wavelength. The UV spectrum of Cortinarin C was found to be similar to that of psilocin (4-hydroxy, NN, -dimethyltryptamine) a hallucinogenic component of the fungus *Psilocybe semilanceata*, except that there was no bathochromic shift on addition of alkali. It was however identical to the UV spectrum of 4-methoxy-NN-dimethyltryptamine prepared by the methylation of psilocin with diazomethene. The fluorescence spectrum of Cortinarin A in methanol showed a maximum emission at 378 nm and a maximum excitation at 327 nm. This wavelength difference of 51 nm may be indicative of a substituted indole or N heterocyclic ring. This inference together with the UV spectra would suggest that Cortinarins A and B as well as C contain an indole moiety. However in the case of Cortinarins A and B the indole must be substituted in both the 2 and 3 positions in order to explain the lack of reaction of these compounds with acidified pDAB. I. R. spectroscopy of Cortinarin A, in the form of a KCl disc, showed poorly resolved bands at 1560 and 1640 cm^{-1} consistent with carboxylate ion and the presence of an aromatic nucleus. A strong band at 3250 cm^{-1} associated with N–H stretching frequency was also present while strong bands at 2900–2800 cm^{-1} may be associated with C–H stretching. The infra red spectra of Cortinarin B and C showed only minor differences to that of Cortinarin A. Extra bands were shown by Cortinarin B at 3400 and 1040–1050 cm^{-1} and could be assigned to OH stretch and C–O stretch respectively. Amino acid analysis showed the presence of threonine, ornithine, phenylalanine, lysine, glycine, alanine, valine, leucine, isoleucine and 1 other unidentified compound which gave a positive reaction with ninhydrin. Cortinarin B produced similar results and Cortinarin C differed only in the presence of a tryptophan derivative instead of the unidentified compound. This latter compound was identified by 2 dimentional thin layer chromatography and by its reaction with both acidified pDAB and ninydrin reagents. All of the data at this stage seemed to indicate a remarkable similarity between these compounds and the cyclic polypeptide liver toxins of the Amanita species of mushrooms previously described by Wieland[24] (fig. 2). This similarity was further substantiated by hydrogenation of Cortinarins A and B with Raney nickel. A single non-fluorescent product was obtained in each case which reacted strongly with acidified pDAB giving a purple color. The ultra violet spectra of Cortinarins A and B after hydrogenation showed a shift to shorter wavelength of about 6 nm and were similar to the pattern of Cortinarin C. Furthermore subsequent partial hydrolysis of the hydrogenated Cortinarin with trifluoroacetic acid resulted in a single compound which reacted with both acid pDAB and ninydrin reagents. These reactions are consistent with Cortinarin A and B being cyclic polypeptides of similar structure to the Amanita toxins. Further analysis of total acid hydrolysates of Cortinarins A and B by 2-dimensional tlc of the dansylatd amino acids[25] showed that both a tryptophan derivative and cysteine were present. In addition the UV spectrum of the acid hydrolysate of these compounds showed a single band at 300 nm which was considered to be due to the presence of an oxindole (fig. 3 III). However amino acid analysis of a hydrolysate of Cortinarin A or B which had been previously treated with Raney nickel showed the absence of cysteine and the ultra violet spectrum was again similar to that of Cortinarin C (fig. 3 IV).

These observations indicated that the chromophores of Cortinarins A and B consisted of a condensation product of cysteine with a tryptophan derivative (fig. 3 I). The substituted tryptophan unit was isolated by column chromatography on Sephadex LH20[5] from a total acid hydrolysate of Cortinarin A which had been previously

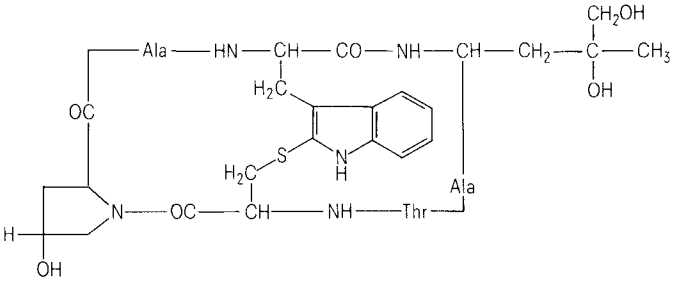

Figure 2. Structure of the hepatotoxin phalloidin isolated from the fungus *Amanita phalloides*.

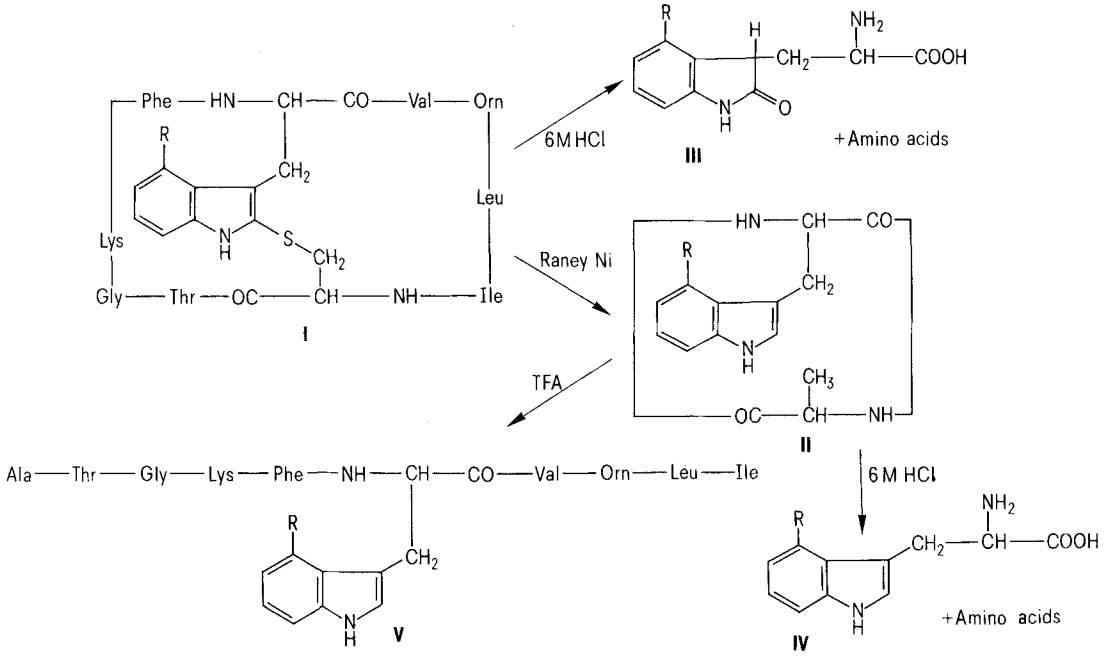

Figure 3. I R = OCH_3 Cortinarin A; R = OH Cortinarin B; II R = OCH_3 Cortinarin C.

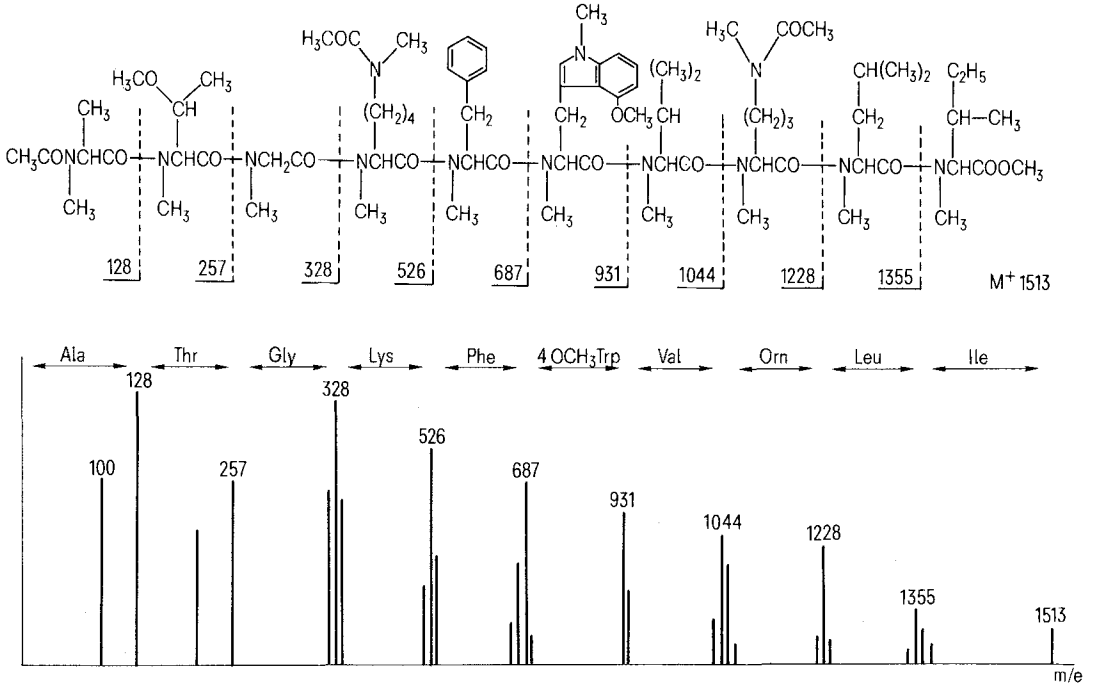

Figure 4. Low resolution mass spectrum of the linear form of Cortinarin A after acetylation and permethylation.

hydrogenated with Raney nickel. The free indole was then liberated by incubation with tryptophanase[11]. This indole was found to have identical ultra violet, tlc and 250 MHz H^1NMR characteristics to 4 methoxy indole prepared in a similar manner from methylated psilocin. The amino acid sequence of Cortinarin A was found to be: alanine (from cysteine), threonine, glycine, lycine, phenylalanine, 4 methoxy tryptophan, valine, ornithine, leucine and isoleucine. This sequence was subsequently confirmed by the low resolution mass spectrometry of an acetylated and permethylated derivative of the linear form of Cortinarin A[14] (fig. 4)[1]. All of the amino acids were found to have the L configuration with the exception of D threonine. Again, this is also the case with Amanita toxins. The probable structures of Cortinarins A, B and C can thus be represented as in figure 3I. The 3 compounds are chemically interconvertable, methylation of Cortinarin B with diazomethane produces Cortinarin A and hydrogenation of Cortinarin A with Raney nickel results in the formation of Cortinarin C.

Distribution of the Cortinarins

Some 60 different species of *Cortinarius* have now been screened by thin layer chromatography to determine the presence of the Cortinarins[22]. Cortinarin A was found to be present to some extent in all of the species examined with the exception of *Cortinarius violaceus* which is one of the few species within the genus *Cortinarius* considered to be edible. Only 3 species have so far been found to contain Cortinarin B. These are *Cortinarius orellanus, C. orellanoides* and *C. speciosissimus* which also contain relatively high concentrations of Cortinarin A and are reported to be the 3 most toxic species within the genus *Cortinarius*. Cortinarin C was observed in all 60 species examined. Both adsorption phase[20] and reversed phase[21] high performance liquid chromatographic (HPLC) systems have been developed which allow the quantification of Cortinarins A, B and C in mushroom extracts and give a method of determining the relative toxicities of individual species. The concentrations of Cortinarin A ranged from 0.47% dry weight for *Cortinarius speciosissimus* to 0.004% w/w for *C. croceofolius*. Concentrations of Cortinarin B were determined for *C. speciossisimus* (0.60% w/w), *C. orellanus* (0.52% w/w) and *C. orellanoides* (0.47% w/w). It was apparent from these results that toxicity is proportional to the sum of the concentrations of Cortinarins A and B. No such correlation could be made between Cortinarin C content and toxicity and this compound was considered to be non-toxic.

Toxicology

Initial toxicity studies have been carried out on Cortinarins A and B by i.p. administration to male BKA mice. Of the mice poisoned with Cortinarin A (5 mg) 1 died after 4 days and the others when killed 2 weeks after dosing, showed kidney damage which ranged from mild to severe. Mice given Cortinarin B (5 mg) also showed similar renal damage with 2 mice dying after 4 days. In both cases the kidneys of the poisoned mice were enlarged and also showed a blue fluorescence when examined under UV light. This compound was removed from the kidneys by methanolic extraction and analysed by thin layer chromatography. An ultra violet spectrum of the compound was also obtained. Results were consistent with the isolated compound being a sulphoxide of Cortinarin B. This suggests that Cortinarin A might undergo O-demethylation and S-oxidation prior to exerting its effect on renal tissue. These observations are in agreement with the work of Nieminen[15] who has produced evidence that the active toxin of *Cortinarius speciosissimus* is a product of liver metabolism.

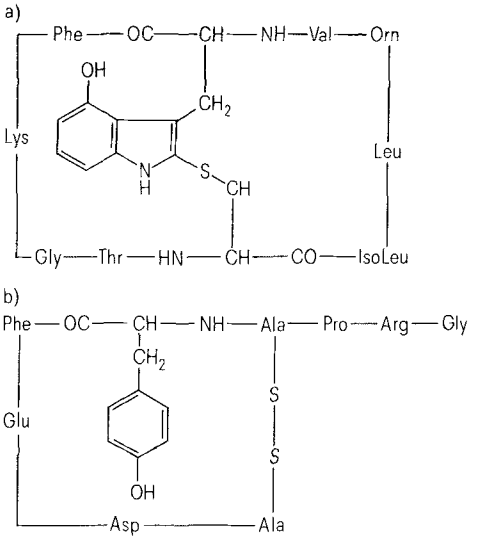

Figure 5. Comparison of the structures of (a) vasopressin and (b) Cortinarin B.

Although the mechanism of action of the Cortinarins is at present unknown, there are a number of similarities between these toxins and the posterior pituitary hormone vasopressin[19]. Both compounds act on the distal tubules and collecting ducts of the nephron resulting in water retention.

Vasoconstriction, causing hypertension and a sensation of coldness, increased gut motility and stimulation of the uterus are also pharmacological effects produced by both Cortinarius toxins and vasopressin. There are also similarities in the structure activity relationships of these compounds. The following characters are responsible for vasopressin activity[16]. The presence of a cyclic structure, a phenolic group at position 2, phenylalanine at position 3, and the presence of a basic amino acid (fig. 5). Cortinarin B and the metabolite of Cortinarin A isolated from kidney tissue both possess cyclic structures, a phenolic group in the form of a hydroxy indole which is next to phenylalanine and a basic amino acid, lysine. It seems likely therefore that the toxins act at similar sites to vasopressin in the kidney but unlike the hormone have an extremely long half life.

1 The work was carried out with support from the Scottish Home and Health Department grant No. K/MRS/50/C350 and the Science and Engineering Research Council.

2 Antkowiak, W.Z., and Gessner, W.P., The Structures of Orellanine and orelline. Tetrahedron Lett. *21* (1979) 1931.
3 Bouska, I., Detection of U.V. fluorescence in renal tissue following poisoning with *C. orellanus*. Cseka. Mycol. *34* (1980) 188.
4 Caddy, B., Kidd, C.B.M., Robertson, J., Tebbett, I.R., Tilstone, W.J., and Watling, R., *Cortinarius speciosissimus* toxins – a preliminary report. Experientia *38* (1982) 1439.
5 Faulstich, H., Buku, A., Bodenmuller, H., and Wieland, T., Virotoxins: Actin-Binding Cyclic Peptides of *Amanita virosa* mushrooms. Biochemistry *19* (1980) 3334.
6 Gamper, R., Untersuchungen über die Giftstoffe der Pilze *Cortinarius orellanus* Fr. und *C. speciossisimus* Kühn. Thesis, Innsbruck 1977.
7 Gerault, A., Intoxication collective de type orrellanien provoquée par *Cortinarius splendens* R.Hy. Bull. Trimest. Soc. Mycol. *97* (1981) 67.
8 Gray, W.R., Sequence analysis with dansyl chloride, in: Methods in Enzymology vol.25, p.333. Eds C.H.W. Hirs and N.T. Serge. Academic Press, New York 1972.
9 Grzymala, S., Massenvergiftung durch den Orangefuchsigen Hautkopf. Z. Pilzkunde *23* (1957) 139.
10 Grzymala, S., L'isolement de l'orellanine, poison du *C. orellanus* Fries et L'étude de ses effets anatomopathologiques. Bull. Trimest. Soc. Mycol. Fr. *78* (1962) 394.
11 Gunsalus, I.C., Courtland, C., Galeener, C.C., and Stamer, J.R., Tryptophan Cleavage, in: Methods in Enzymology, vol.2, p.238. Eds S.P. Colowick and N.O. Kaplan. Academic Press, New York 1965.

12 Hulmi, S.P., Forstrom, J., Sipponen, P., and Vilska, J., Mushroom poisoning by *C. speciosissimus*. A report of four cases. Duodecim, Helsinki *90* (1974) 1044.
13 Kurnsteiner, H., and Moser, M., Isolation of a leathal toxin from *Cortinarius orellanus*. Mycopathologia *74* (1981) 65.
14 Morris, H.R., Williams, D.H., and Ambler, R.P., Determination of the sequences of protein-derived peptides and peptide mixtures by mass spectrometry. Biochem. J. *125* (1971) 180.
15 Nieminen, L., Mottonen, M., and Heikkila, H., Damage caused by two Finnish mushrooms, *Cortinarius speciossisimus* and *Cortinarius gentilis* on the rat kidney. Z. Naturforsch. *30C* (1975) 668.
16 Sawyer, H., Neurohyophysial hormones. Pharmac. Rev. *13* (1961) 225.
17 Short, A.I.K., Watling, R., MacDonald, M.K., and Robson, J.S., Poisoning by *Cortinarius speciossisimus*. Lancet *2* (1980) 942.
18 Sieber, P., Riniker, B., Brugger, M., Kamber, B., and Rittel, W., Menschliches Calcitonin. VI. Die Synthese von Calcitonin M. Helv. chim. Acta *53* (1970) 2135.
19 Tebbett, I.R., Mechanism of action of Cortinarius toxins – a hypothesis, in preparation.
20 Tebbett, I.R., and Caddy, B., Analysis of Cortinarius mushrooms by HPLC. J. Chromat. *268* (1983) 535.
21 Tebbett, I.R., and Caddy, B., Analysis of Cortinarius toxins by reversed phase HPLC. J. Chromat., in press.
22 Tebbett, I.R., Kidd, C.B.M., Caddy, B., Robertson, J., and Tilstone, W.J., Trans. Br. Mycol. Soc., in press.
23 Testa, E., *Cortinarius orellanus* Fr. non Quelet. Indagini sulla tossicita dei funghi del genre Cortinarius. Rass. micol. tic. *2* (1970) 89.
24 Wieland, T., and Wieland, O., The toxic peptides of Amanita species, in: Microbial Toxins, vol. 8, p.249. Eds S. Kadis, A. Ciegler and S.J. Ajl. Academic Press, New York 1972.
25 Woods, K.R., and Wang, K.T., Separation of dansyl-amino acids by polyamide layer chromatography. Biochim. biophys. Acta *133* (1967) 369.

0014-4754/84/050441-06$1.50 + 0.20/0

Polarographic activity of the antitumor drug cis-dichlorodiammineplatinum (II). The effect of hydrolysis and trans-isomerization of the drug[1]

O. Vrána, V. Kleinwächter and V. Brabec[2]

Institute of Biophysics, Czechoslovak Academy of Sciences, 61265 Brno (Czechoslovakia), 2 May 1983

Summary. Electrochemical activities of cis-dichlorodiammineplatinum(II) (cis-DDP) and its trans isomer were studied by classical and differential pulse polarography (d.p.p.). It was shown that both isomers yielded a polarographic step or peak at about – 1.6 V (vs. Ag/AgCl), which corresponded to electroreduction of the complex and to catalytic hydrogen evolution. This signal was easily measurable with the aid of d.p.p. and was suitable for investigation of the extent of hydrolysis and trans-isomerization of cis-DDP leading to the formation of toxic products. The detection limit for determination of cis-DDP and its trans isomer by d.p.p. was 1×10^{-6} mol/l.

Introduction

Cis-dichlorodiammineplatinum(II) (cis-DDP) is a cytostatically active compound which is now widely used in combination therapy of different types of tumors[6,12,15]. However, the treatment is often accompanied by toxic effects, some of which could be ascribed to the action of platinum derivatives occurring in the drug sample as a consequence of improper handling or storage. It has been shown, for instance, that hydrolyzed cis-DDP is extremely toxic in vivo[17], even though it can be rapidly converted into the parent compound at the relatively high chloride ion concentration of the extracellular medium. Another process which could negatively affect the antitumor activity of the cis isomer is ultraviolet radiation-induced transformation into its trans-form (trans-DDP)[13], which is more toxic and cytostatically inactive[16].

Hydrolysis of cis-DDP as a function of chloride ion concentration, pH and temperature was studied by several authors[7,9,10,11,14], and the resulting products were identified. In a connection with investigation of the use of polarographic methods for the determination of platinum in biological materials[4,21], we tested the polarographic behavior of cis-DDP and found suitable conditions for simple, rapid and precise determination of the fractions of intact and hydrolyzed cis-DDP in solution and for the detection of trans-DDP in samples of cis-DDP.

Material and methods

Cis-DDP was obtained from Research Institute of Pure Chemicals, Lachema (Brno, Czechoslovakia). Trans-DDP was a generous gift of Professor B. Rosenberg. All cis-DDP and trans-DDP solutions analyzed in this work were prepared at low illumination intensity and stored in darkness at 28 ± 1 °C. They were used only after 6-day equilibration unless otherwise stated. The diaquodiammineplatinum(II) (cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$), was prepared by the addition of 1 equivalent of silver nitrate to cis-DDP. After standing overnight in the dark at 28 °C, the silver chloride formed was removed by filtration. Human albumin was obtained from Imuna (Šarišské Michalany, Czechoslovakia).

Polarographic measurements were performed with an EG&G PARC Polarographic Analyzer, Model 174A. A 3-electrode system was used, comprising an EG&G PARC Static Mercury Drop Electrode (SMDE) Model 303 (medium drop size), a spectrosopic graphite counter electrode and a silver/silver chloride (saturated KCl) reference electrode. All polarograms were recorded at 25 °C at a voltage scan rate of 0.002 V s^{-1}; drop time control of the Model 174A Polarographic Analyzer was set at 1.0 sec. Differential pulse polarographic (d.p.p.) curves were obtained with a pulse amplitude of −25 mV. The test solutions were always deoxygenated by purging with argon for about 6 min; an argon atmosphere was maintained in the cell throughout data