Cortinarius speciosissimus toxins – a preliminary report

B. Caddy, C. B. M. Kidd, J. Robertson, I. R. Tebbett, W. J. Tilstone and R. Watling[1]

Forensic Science Unit, University of Strathclyde, George Street, Glasgow GA 1XW (Scotland), and Royal Botanic Gardens, Edinburgh (Scotland), 1 July 1982

Summary. Methanolic extracts of *Cortinarius speciosissimus* yielded a fluorescent compound which was crystallized and shown to be a cyclic polypeptide. The compound, or an analogue, has been found in most members of the genus *Cortinarius.*

Most mushroom poisonings in the world have been associated with the genus *Amanita*[2] and the nature of the toxins and their mode of action have been well characterized[3]. Probably the next major group of toxic mushrooms belong to the genus *Cortinarius.* Problems of toxicity associated with this genus are highlighted by reports of deaths following the ingestion in Poland of *Cortinarius orellanus* Fries[4] and of renal failure caused by consumption of *Cortinarius speciosissimus* Kühn and Romagn in Scotland[5] and Finland[6]. The search for an effective treatment for this type of poisoning is undoubtedly hampered by a lack of knowledge as to the exact nature of toxins involved and therefore of their mode of action. In particular there appears to be considerable confusion as to the major toxin present in *Cortinarius orellanus.* Antkowiak and Gessner[7] claim to have isolated a toxic bipyridyl compound, orellanine, while Testa[8], Gamper[9] and Kürnsteiner and Moser[10] have suggested that orellanine might be a mixture of several substances. All workers, however, seem to be of the opinion that toxicity resides with those compounds which show fluorescence when irradiated with UV light. What follows therefore is a summary of preliminary work carried out on the isolation and part characterisation of a fluorescent material isolated from *Cortinarius speciosissimus* and its apparent occurrence in other *Cortinarius* species.

Experimental. Specimens of *Cortinarius speciosissimus* collected in October 1980 and 1981 were air dried at 40 °C for several hours and stored over silica gel until used. These and all other species reported here were identified microscopically[11–14]. The dried material was powdered and soxhlet extracted with petroleum ether (40–60) for 1 h prior to a similar 6-h methanolic extraction. The methanolic extract was evaporated to dryness under reduced pressure and the residue redissolved in a minimum volume of methanol. An alternative 2-/3-h cold extraction showed no qualitative difference to the previous method but was quantitatively inferior. TLC analysis was carried out using silica gel G plates (Merck) developed in solvent mixtures of butanol-acetic acid-water (6:2:2), butanol-acetic acid-water-chloroform-ethanol (55:15:15:5:10), cyclohexane-ethyl acetate (3:1) and methanol-ammonia (100:1). When used in a preparative mode a methanolic extract of 9 g of powdered mushroom was applied to the silica gel plate as a single streak, the plate developed in cyclohexane-ethyl acetate solvent and the appropriate blue fluorescent streak eluted from the plate with methanol. Chromatography was also carried out on the methanolic extract redissolved in water and applied to a 30 × 2.5 cm column of Sephadex LH20 as described by Wieland[15]. Evaporation of the aqueous eluate under reduced pressure gave a white crystalline material. The isolated material was hydrolyzed by refluxing with 6 M HCl at 110 °C for 8 h. After dilution with water, the hydrolysate was neutralized with silver carbonate, filtered and the filtrate analyzed on an automatic amino acid analyser.

Results and discussion. The methanolic extracts of *Cortinarius speciosissimus* when examined by TLC always showed, when irradiated with UV light at 254 nm, a single strong blue fluorescent spot at R_f-values of 0.90 for the 2 butanol-acetic acid systems, 0.49 for the cyclohexane-ethyl acetate system and 0.08 for the methanol-ammonia system. This progressive decrease in R_f-value with decrease in polarity of the solvent is consistent with the fluorescent compound being very polar in nature. A compound which

Chromatographic screening of the genus *Cortinatius* for the presence of the fluorescent material isolated from *Cortinarius speciosissimus*

*Myxacium**	*Phlegmacium**	*Serieocybe*	*Cortinarius**	*Leprocybe*	*Dermocybe**	*Hydrocybe/ Telamonia**
C. pseudosalor (++)	*C. rapaceus* (−)	*C. subargentatus* (+)	*C. violaceus*** (−)	*C. gentilis* (++)	*C. cinnamomeus* (+)	*C. decipiens* (++)
				C. betuletorum (++)		
C. mucosus (++)	*C. crocolitus* (−)	*C. anomalus* (+)		*C. callisteus* (++)	*C. semisanguineus* (++)	*C. glandicolor* (+)
C. elatior (+)	*C. subpurpurascens* (−)	*C. lepidopus* (+)		*C. pholideus* (++)		*C. hinnuleus* (+)
		C. tabularis (+)		*C. orellanus* (+++)		*C. torvus* (+)
				C. orellanoides (+++)		
				C. speciosissimus (+++)		
				C. bolaris (++)		
				C. rubicundulus (++)		

*These are the 7 subgenera of the *Cortinarius* genus. **This species yields a blue fluorescent spot at a similar but different (higher) R_f than the one under investigation. The fluorescent intensity is given in brackets on an arbitrary scale expressed by weak (+), fairly strong (++), very strong (+++) and absent (−). Authorities for the species in the table follow the 'New Checklist of British Agarics and Boleti'[16]. Voucher material has been deposited in the Royal Botanic Garden, Edinburgh (E).

showed identical chromatographic characteristics to the blue fluorescent material identified by analytical TLC was isolated by both preparative TLC and column chromatography and shown to melt at approximately 270 °C with decomposition yielding a yellow colored product. The initial product was found to be soluble in methanol and ethanol but more soluble in water and very soluble in dilute aqueous alkali. It was insoluble or only poorly soluble in dilute aqueous alkali. It was insoluble or only poorly soluble in apolar organic solvents, such as ether, chloroform and ethyl acetate. The solubility is in accord with that of the compound isolated by Kürnsteiner and Moser[10] from *C. orellanus* but is at variance with the data reported by Antkowiak and Gessner[7]. Elemental analysis showed the presence of carbon, hydrogen, sulphur, nitrogen and chlorine (and by implication, oxygen). When treated with a neutral solution of ferric chloride there was no reaction but in hydrochloric acid solution an orange color resulted. Furthermore, neutral ninhydrin solution when sprayed on to a TLC plate on to which a solution of the unknown compound had previously been applied produced no reaction. However, prior treatment with concentrated hydrochloric acid for 2 min at 100 °C resulted in a purple color from the ninhydrin spray. The results of both these reactions are consistent with the unknown compound being a polypeptide, a view expressed by Testa[8] as being consistent with his observations for *C. orellanus*.

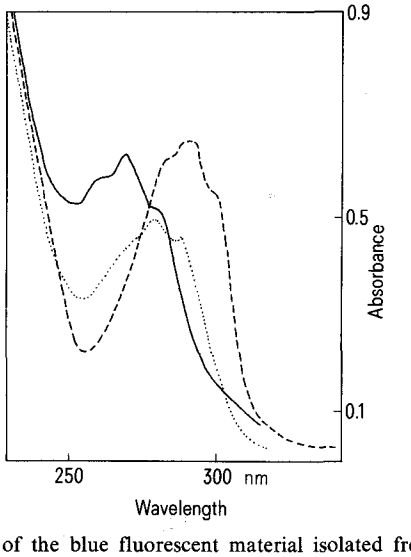
Acid hydrolysis of the isolated compound showed, when submitted to automated amino acid analysis, the presence of threonine, ornithine, phenylalanine, lysine, glycine, alanine, valine, isoleucine, leucine, tyrosine and one other unidentified compound, thus confirming the nature of the fluorescent material as a polypeptide.

Spectroscopic analysis of the isolated compound showed poorly resolved bands at 1560 and 1640 cm^{-1} in the IR consistent with carboxylate ion and the presence of an aromatic nucleus. A strong band at 3250 cm^{-1} associated with an N-H stretching frequency was also present, while strong bands at 2900–2800 and at 1020 cm^{-1} may be associated with C-H stretching and either C=S or S=O stretching respectively.

The fluorescence spectrum showed a maximum excitation at 327 nm and a maximum emission at 378 nm. This wavelength difference (51 nm) may be indicative of a substituted indole or N-heterocyclic ring. The former was further substantiated by the UV-spectra in methanol which showed a pattern very similar to that of tryptophan only at a lower wavelength (fig.).

MS and NMR data have so far remained inconclusive. However, the spectral data are consistent with the compound being a polypeptide and may also indicate that the unknown hydrolysis product is a tryptophan analogue. All data so far seem to indicate a remarkable similarity between this unknown compound and the cyclic polypeptide toxins previously isolated from species of *Amanita* and identified by Wieland[2,15] and his group of workers. While the material isolated from *Cortinarius speciosissimus* does not respond to ninhydrin, a reagent which requires a free peptide end group, reduction with Raney Nickel and mild hydrolysis with trifluoro acetic acid, as described by Wieland[2,15], yields a single product which then reacts with the reagent. This last series of reactions represents further evidence that the isolated peptide is cyclic in nature.

A preliminary screen by TLC for the presence of the unknown fluorescent compound in other members of the 7 subgenera of the genus *Cortinarius* has also been undertaken and the results are shown in the table. Of the species examined all but 4 show a fluorescent spot at the same R_f-value as that shown by the compound isolated from *C. speciosissimus* although in some of the species it is present at a much lower concentration. No fluorescent material was found in any of the 3 samples from the subgenus *Phlegmacium* which were tested. Crystalline material has been isolated from both *Cortinarius betuletorum* Moser and *Cortinarius pseudosolar* J. Lange and shown to have identical spectral and chromatographic properties to that of material isolated from *C. speciosissimus*. Toxicity testing of the compound isolated from *C. speciosissimus* has been carried out and will be reported at a later date as will a detailed study of the chromatographic examination of the members of the genus *Cortinarius*.



UV-spectra of the blue fluorescent material isolated from *Cortinarius speciosissimus* (–), tryptophan (· · · · · ·) and phalloidin as described by Wieland (– – –).

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2 Bornet, A., PhD Thesis, University of Zurich, Zurich 1980.
3 Wieland, T., Science *159* (1968) 946.
4 Grzymala, S., Z. Pilzk. *35* (1957) 249.
5 Short, A.I.K., Watling, R., MacDonald, M.K., and Robson, J.S., Lancet (1980) 942.
6 Hulmi, S.P., Siddonen, P., Forsstrom J., and Volska, J., Duodecim, Helsinki, *90* (1974) 1044.
7 Antkowiak, W.Z., and Gessner, W.P., Tetrahedron Lett. *21* (1979) 1931.
8 Testa, E., Rassegna Micologica ticinese *2* (1970) 89.
9 Gamper, R., PhD Thesis, University of Innsbruck, 1977.
10 Kürnsteiner, H., and Moser, M., Mycopathologia *74* (1981) 65.
11 Kühner, R., and Romagnesi, H., Flore analytique des champignons superieures de France. Paris 1953.
12 Moser, M., and Kleine, B., Kryptogamenflora, vol. 2B/2, 4th edn. Stuttgart 1978.
13 Orton, P.D., Cortinarius I: Myxacium and Phlegmacium. Naturalist, Suppl. 1955.
14 Orton, P.D., Cortinarius II: Inoloma and Dermocybe. Naturalist, Suppl. 1958.
15 Wieland, T., and Wieland, O., in: Microbial toxins, vol. 8, p. 249. Eds S. Kadis, A. Ciegler, and S.J. Ajl. Academic Press, New York and London 1972.
16 Dennis, R.W.G., Orton, P.D., and Hora, F.B., Trans. Br. mycol. Soc., suppl. *43* (1960) 1.