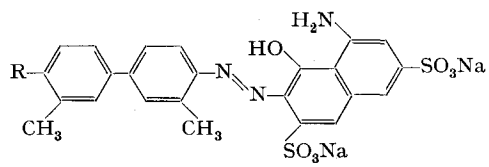
The So-Called Red Impurity of Trypan Blue

Characterization of impurities of trypan blue is of interest because they may be responsible for, or contribute to, the teratogenic and carcinogenic effects of commercial products. The so-called red impurity has been separated from trypan blue by extraction with ethanol[1], acetone[2] or methyl ethyl ketone[3] or by passing through a cellulose column[2,4]. However, resolution of a red fraction from trypan blue into 2 components was obtained by paper chromatography in aqueous ethanol, while another reddish impurity, called purple, was also separated[5].

Recently, the structure I was assigned to the red impurity of trypan blue which was extracted with methyl ethyl ketone[6]. Furthermore, it was established that the impurity differed from II. The results in this laboratory show that the major component of our red fraction is I and that another component is II.



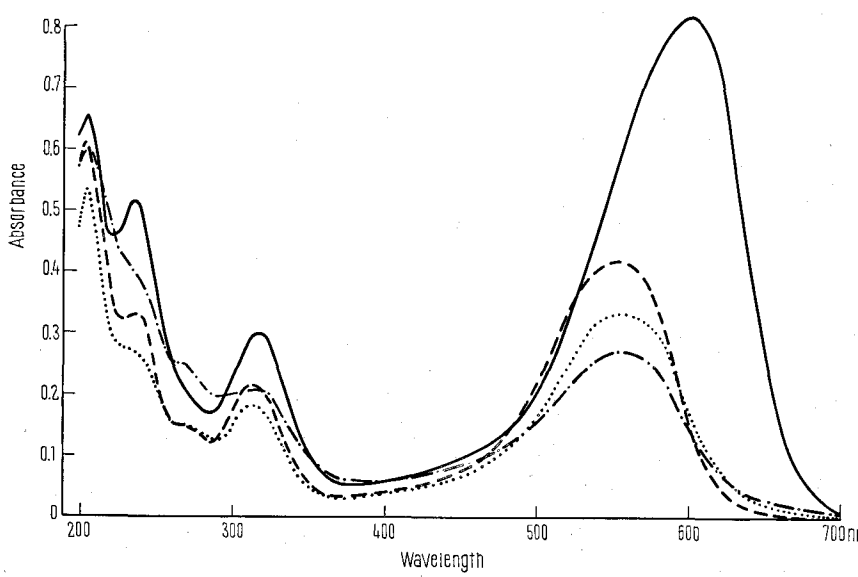
I R = H
II R = OH
III R = NH_2

The red fraction was obtained by chromatography of a trypan blue (Merck) solution in a methyl ethyl ketone – water – ethanol mixture on alumina[5]. The 2 components were separated by descending chromatography on paper in 80% aqueous ethanol, eluted from the paper with water and dried in vacuo. The yield of the fastest moving red-violet component (Rf 0.40) was 3 times that of the slower blue-violet component (Rf 0.30). Their absorption spectra are compared in the Figure with those of the blue main component and a purple impurity, both purified on alumina[5] followed by paper chromatography in water. The spectra of the 3 impurities are strikingly similar and are distinct from the spectrum of the blue component because their absorbance in the visible region is less and because they have a shoulder between 260 and 270 nm.

The components of the red fraction were reduced with sodium dithionite and subjected to paper electrophoresis at pH 4[7]. The electropherograms were examined for coloured compounds, ultraviolet absorbing spots and compounds which became visible after spraying with Ehrlich's reagent. The purified blue component of trypan blue was treated in the same way and served as a standard. Reduction of the blue dye yielded *o*-tolidine which moved to the cathode and 2-amino-H acid (2,8-diamino-1-naphthol-3,6-disulphonic acid) which gave a fast yellow and a slower blue band in the direction of the anode. When the electropherograms from the reduction products of the red-violet and blue-violet components of the red fraction were compared with those of the blue standard, it was clear that on reduction both components gave 2-amino-H acid. In the direction of the cathode both gave amines with half the mobility of *o*-tolidine, suggesting that their amines had only one NH_2 group. The amine of the red-violet component was yellow when sprayed with Ehrlich's reagent and that of the blue-violet component orange. The amines obtained after elution from the electropherograms with chloroform were submitted to mass spectrometry. Their molecular weights were 197 and 213, respectively, suggesting that they differed from *o*-tolidine by replacement of one NH_2 by H or OH. Thus structure I must be assigned to the red-violet major component and structure II to the blue-violet minor component. Their formation during synthesis of trypan blue must be attributed to decomposition of 1 diazonium group of tetrazotized *o*-tolidine to give H or OH, respectively.

These components are distinct from the blue and purple components because they are preferentially eluted by aqueous ethanol or other organic solvents. They can there-

[1] J. L. HARTWELL and L. F. FIESER, Org. Synthesis *16*, 12 (1936).
[2] J. W. KELLY, Stain Tech. *33*, 79 (1958).
[3] F. BECK and J. B. LLOYD, J. Embryol. exp. Morph. *11*, 175 (1963).
[4] F. BECK, B. SPENCER and J. S. BAXTER, Nature, Lond. *187*, 605 (1960).
[5] J. DIJKSTRA and J. GILMAN, Nature, Lond. *191*, 803 (1961).
[6] J. B. LLOYD and F. E. FIELD, Experientia *26*, 868 (1970).
[7] J. B. LLOYD and F. BECK, Stain Tech. *39*, 7 (1964).



Absorption spectra of components of trypan blue: blue (———), redviolet (----), blue-violet (....) and purple (-.-.-.-). All 0.001% in water.

fore be related to fraction 1-V1 of KELLY[2], who found that their removal did not leave the blue fraction entirely pure. This may be explained by the presence of the purple component which is not eluted from an alumina column with aqueous organic solvents[5]. The absorption spectrum of the purple dye resembles those of the red-violet and blue-violet components and consequently this dye will also be recognized as a red impurity. In fact, the capillary test of HARTWELL and FIESER[1] detects preferentially this component which on paper chromatography with water as solvent moves in front of the other components with an Rf of 0.90. This dye may have been separated by KELLY[2] as fraction 1-V2 by passing an aqueous solution of trypan blue through a cellulose column. The purple dye has probably structure III because electrophoresis after reduction with sodium dithionite revealed *o*-tolidine in the direction of the cathode. This dye is active in producing reticulosis in the liver of rats and is presumably responsible for the carcinogenic activity of commercial trypan blue[5].

It may be concluded that the so-called red impurity of trypan blue consists of one or more components depending on the method which is used for detection or extraction. Aqueous organic solvents usually extract a mixture of I and II while water preferentially elutes a third impurity (presumably III), the absorption spectrum of which resembles the spectra of the other reddish components.

Zusammenfassung. Die sogenannte rote Verunreinigung von Trypanblau wurde als Gemisch von 3 Azofarbstoffen erkannt, voneinander getrennt und in ihrer Struktur aufgeklärt. Eine dieser Substanzen scheint für die kanzerogene Aktivität des kommerziellen Trypanblaus verantwortlich zu sein.

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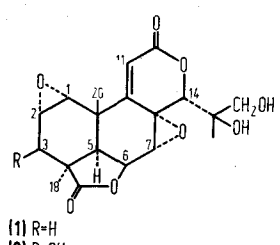
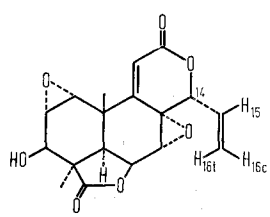
Plant Growth Inhibitory Lactones from *Podocarpus neriifolius:* Structure of Podolactone E

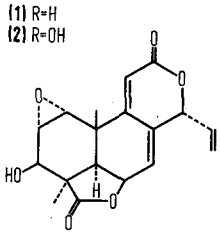
Earlier[1] we reported the bark of a *Podocarpus* species (c.f. *P. neriifolius* D. Don ex Lamb.) from Northern Queensland to contain two plant growth inhibitory compounds, podolactones A (**1**) and B (**2**). Further fractionation of the extract has afforded 4 other norditerpene lactones of the same type, podolactones C, D, E and the known lactone inumakilactone B (**3**)[2], identified by direct comparison with an authentic sample[3]. Podolactone E and inumakilactone B exhibit high activity as inhibitors of cell expansion in an assay system employing pea stem segments[4], inhibiting growth of hook and apical segments of pea stems at concentrations considerably lower than those required for the other podolactones and related compounds. The activities, expressed as the concentration necessary to limit growth of hook segments in 24 h to half that of the control, of podolactones A, B, E, inumakilactone B and abscisic (a convenient standard inhibitor) were respectively 60, 200, 6, 10 and 500×10^{-7} *M*. Podolactone E, which is shown to have the structure (**4**), is the most active inhibitory compound so far examined with this system.



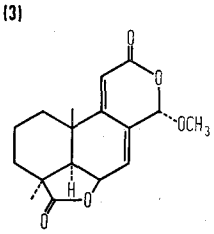
(1) R=H
(2) R=OH



(3)



(4)



(5)

Chemical shifts (δ) and coupling constants (Hz) of proton resonances[a]

a) From first-order analysis

	Podolactone E	Inumakilactone B[2]	Antibiotic LL-Z1271α[6]
1-H	3.69d	3.63d	
	(4.5)	(4.0)	
2-H	3.56dd	3.51dd	
	(4.5, 6.0)	(4.0, 5.6)	
3-H	4.69d	4.67d	
	(6.0)	(5.6)	
5-H	2.11d	2.16d	
	(5.5)	(5.2)	(5.3)
6-H	5.06m	5.10dd	
	(5.5, 4.0, 1.7)	(5.2, 1.2)	(5.3, 4.7, 1.0)
7-H	6.24m	3.95d	
	(4.0, 1.8, 2.0)	(1.2)	(4.7, 1.8, 2.0)
11-H	6.51d	6.78s	
	(1.8)		(1.8)
18-H_3	1.47s	1.41s	
20-H_3	1.52s	1.51s	

b) From computer analysis

	Podolactone E	Inumakilactone B
14-H	5.56	5.41
15-H	6.09	5.98
16c-H	5.48	5.42
16t-H	5.53	5.56
$J_{14,15}$	7.6	7.81
$J_{14,16^c}$	−0.6	−0.57
$J_{14,16^t}$	−0.7	−0.69
$J_{15,16^c}$	10.6	10.27
$J_{15,16^t}$	17.3	17.35
$J_{16^c,16^t}$	1.3	1.40

[a] In [$^2H_5$]pyridine.

[1] M. N. GALBRAITH, D. H. S. HORN, J. M. SASSE and D. ADAMSON, Chem. Commun. *1970*, 170.
[2] S. ITÔ, M. SUNAGAWA, M. KODAMA, H. HONMA and T. TAKAHASHI, Chem. Commun. *1971*, 91.
[3] We are grateful to Professor S. ITÔ, Tohoku University, for this sample.
[4] D. ADAMSON, V. H. K. LOW and H. ADAMSON, in *Biochemistry and Physiology of Plant Growth Substances* (Eds. F. WIGHTMAN and G. SETTERFIELD; Runge Press, Ottawa 1968), p. 505.