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## CHARACTERISATION AND ASSESSMENT OF ANTIFUNGAL ACTIVITY OF CHEMICALLY MODIFIED BERBERINE.

D.T.U. ABEYTUNGA, B.N. PATHIRATNE and W.M.S.R. RATNAYAKA

*Department of Chemistry, University of Colombo, Colombo 3*

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**Abstract:** Berberinechloride was treated with Sodiumborohydride, Sodiumcyanide, Sodiumazide and Sodiumamide to obtain tetrahydroberberine, 8-cyano-8(1H)-berberine, berberine azide and 8-amino-8(1H)-berberine respectively. 8-cyano-8(1H)-berberine and berberine azide completely inhibit the growth of *Rhizoctonia*, *Alternaria*, *Colletotrichum* and *Fusarium* at or below 0.025% concentration.

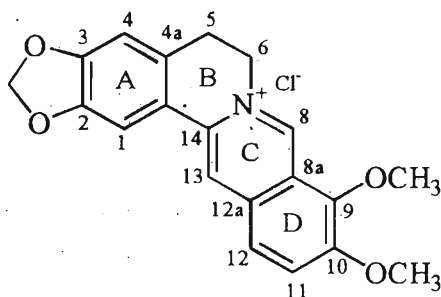
**Key words:** Alkaloid, *Alternaria*, antifungal activity, berberine, *Colletotrichum*, *Fusarium*, *Rhizoctonia*.

### INTRODUCTION

There are a number of reports in the literature<sup>1</sup> on the isolation of berberine alkaloids from plants of the Annonaceae, Berberidaceae, Convolvulaceae, Lauraceae, Menispermaceae, Papaveraceae, Ranunculaceae and Rutaceae. Pharmacological investigations<sup>2</sup> of berberine alkaloids have shown them to have antimicrobial, antileukemic, antiulcerous, gastric antisecretory activities and uterine stimulating effects.

*Coscinium fenestratum* (venivel) is a woody climber which belongs to the Family Menispermaceae. The water extract of the stem of this plant is widely used in Ayurvedic medicine in Sri Lanka. The major constituents of the plant have been identified as alkaloids<sup>3</sup> of which berberine is the principal component.

A closer look at the berberine skeleton indicates the possibility of its easy conversion into a quinolizidine nucleus. Quinolizidine alkaloids<sup>4</sup> are known to be poisons in small concentrations. The structural analogues of quinolizidine; indolizidine and pyrolizidine alkaloids have interesting biological properties.<sup>5</sup> The present study is part of an investigation into the structure-activity relationship of tetrahydroberberine derivatives which could be produced using the readily available berberine. Antifungal activity was the chosen bioassay due to the literature precedence of antifungal activity shown by the isoquinoline<sup>6</sup> alkaloids. Our previous work has shown that 8-amino-8(1H)-berberine is active against *Cladosporium*.<sup>7</sup> The antimicrobial activity of 8-alkyl and 8-phenyl berberine have also been reported in recent literature.<sup>8</sup>



### Berberinechloride

#### METHODS AND MATERIALS

##### *Synthesis of derivatives of berberine:*

Berberine.HCl (Sigma-Aldrich) in methanol or ethanol was treated with  $\text{NaBH}_4$ ,  $\text{NaCN}$ ,  $\text{NaN}_3$  and  $\text{NaNH}_2$  at  $29^\circ\text{C}$  for up to 3 hours to obtain tetrahydroberberine (2), 8-cyano-8(1H)-berberine (3), berberine azide (4) and 8-amino-8(1H)-berberine (5) respectively. Compounds 2, 3 and 5 were recrystallised from methanol, while 4 which was shown to be very polar, was recrystallised from dichloromethane. Compounds were characterised using NMR spectroscopic methods. FAB and electrospray methods were used in mass spectral analysis.

An attempt to convert compound 3 into the tetrahydroderivative by hydrogenation ( $\text{H}_2/\text{Pd}$ ) and reduction ( $\text{NaBH}_4$ ) failed. The hydrogenation resulted in compound 6 while reduction gave 2. The conversion of the nitrile group in compound 3 into a carboxylic acid moiety using  $\text{NaOH}$  and  $\text{H}_2\text{SO}_4$  also failed. Treatment of compound 4 with  $\text{NaBH}_4$  and  $\text{H}_2/\text{Pd}$  gave tetrahydroberberine.

##### *Measurement of antifungal activity:*

The fungus was first grown on potato dextrose agar (200g potato, 20g glucose, 30g agar and 1000ml distilled water) for 7 days at  $29^\circ\text{C}$ . The fungus forms sclerotia readily on the agar medium.

Stock solutions of 10,000 ppm were made from the test compounds. The required concentrations of the compounds (g/ 100 ml) were obtained by mixing the amount of stock solution shown with 15 ml of molten agar. The agar was then poured into petri dishes and allowed to set.

Amount of stock solutions used to obtain required concentrations:

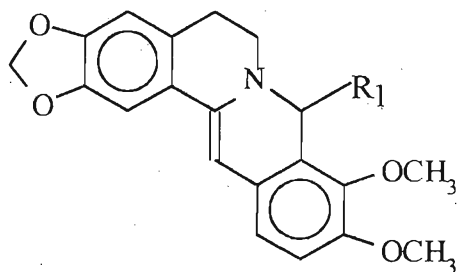
% concentration (g/100ml)	0.005	0.01	0.015	0.02	0.025
Amount of stock solution ( $\mu$ l)	75	150	225	300	375

A single sclerotium of the fungus was placed at the centre of the agar medium and the experiment was triplicated for each concentration. The diameter of the fungal mat was measured after 4 days at 29°C.

## RESULTS

The reaction of berberine.HCl with  $\text{NaBH}_4$ , KCN,  $\text{NaN}_3$  and  $\text{NaNH}_2$  gave tetrahydro berberine (2), 8-cyano-8(1H)-berberine (3), berberine azide (4) and 8-amino-8(1H)-berberine (5) in 45%, 53%, 65% and 70% respectively. 8-cyano-8(1H)-berberine and 8-amino-8(1H)-berberine are novel compounds.

Compound 2 was characterised using NMR spectroscopy. The assignments of protons and carbons are tabulated in Table 1 and Table 2 respectively.



3 $R_1 = \text{CN}$	8-cyano-8(1H)-berberine	4 $R_1 = \text{N}_3$ berberine azide
5 $R_1 = -\text{NH}_2$	8-amino-8(1H)-berberine	
6 $R_1 = =\text{O}$	8-oxyberberine	

Table 1:  $^1\text{H}$  NMR spectral data of 2

Proton	Chemical shift $\delta$ (J in Hz)
1	6.72 s
4	6.58 s
5 ax	3.08 m
5 eq	2.64m
6 ax	2.62 m
6 eq	3.17 m
8 ax	3.52 d (15.9)
8eq	4.23 d (15.8)
11	6.77 d (8.4)
12	6.86 d (8.4)
13 ax	2.79 dd (15.8,11.4)
13 eq	3.22 dd (15.7,3.6)
14	3.52m
$\text{OCH}_3$	3.83,3.84
s	
-	5.90s
$\text{OCH}_2$	

Table 2:  $^{13}\text{C}$  NMR Spectral data of 2

Carbon	Chemical shift $\delta$ (J in Hz)
1	105.4
2	145.8*
3	145.0*
4	108.3
4a	127.7#
5	29.5
6	51.3
8	53.8
8a	127.6#
9	150.2
10	146.1*
11	110.9
12	123.8
12a	128.5#
13	36.3
14	59.5
14a	130.7
9- $\text{OCH}_3$	60.1
10- $\text{OCH}_3$	55.8
$\text{O-CH}_2\text{-O}$	100.7

\*, # Assignments may be interchanged.

The decomposition temperatures of Compounds 3, 4, and 5 were noted as 169-171°C, 160-162°C and 105-107°C respectively. Compounds 3, 4 and 5 were studied using NMR methods and the protons and carbons were assigned as in Table 3 and 4. However there is an ambiguity in the assignment of quaternary carbons.  $^1\text{H}$  NMR data of Compound 6 was comparable to that reported for oxyberberine.<sup>9</sup>

Table 3:  $^1\text{H}$  NMR spectral data of compounds 3,4,5,6

Hydrogen	Compound : chemical shift $\delta$ (J in Hz)			
	3	4	5	6
1	6.13s	7.48s	6.08s	6.72s
4	6.58s	6.88s	6.60s	6.70s
5	2.89m	3.28t(6.2)	2.72 ddd (15.5, 5.7,3.8) 3.33 ddd (15.5,9.1,4.7)	2.89t (6.15)
6	3.33m	4.97 t (6.2)	3.70 ddd (11.6,9.1,3.8), 3.85 m	4.29 t (6.15)
8	5.75 s	9.81 s	5.62 s	
11	6.87 d (8.4)	7.98 d (9.3)	6.92 d (8.4)	7.27 d (8.7)
12	6.81 d (8.4)	7.93 d (9.3)	6.82 d (8.4)	7.32 d (8.7)
13	7.15 s	8.46 s	7.12 s	7.22 s
-OCH <sub>2</sub> O-	5.95 s	6.10 s	5.92 s	6.00 s
OCH <sub>3</sub> 's	3.85,3.96	4.10,4.24	3.85,3.90	3.95,4.01

The bioassay results are shown in Fig 1 and Fig 2.

The results obtained from the antifungal assays against *Rhizoctonia solani* are represented in bar diagrams in Fig 1. The diameter of the fungal mat in cm (Y axis) against % amounts (0.005, 0.01, 0.015, 0.02, 0.025) of the compound are plotted. The standard deviation among 3 readings are indicated on top of the bars.

The antifungal activity of the compounds 3 and 4 which showed strong activity against *R. solani* was then tested against three other fungal species and are presented in Fig.2. The diameter of the fungal mat in each fungal species in cm (Y-axis) against % amounts ( 0.005, 0.01, 0.015, 0.02, 0.025) of the compound is shown here.

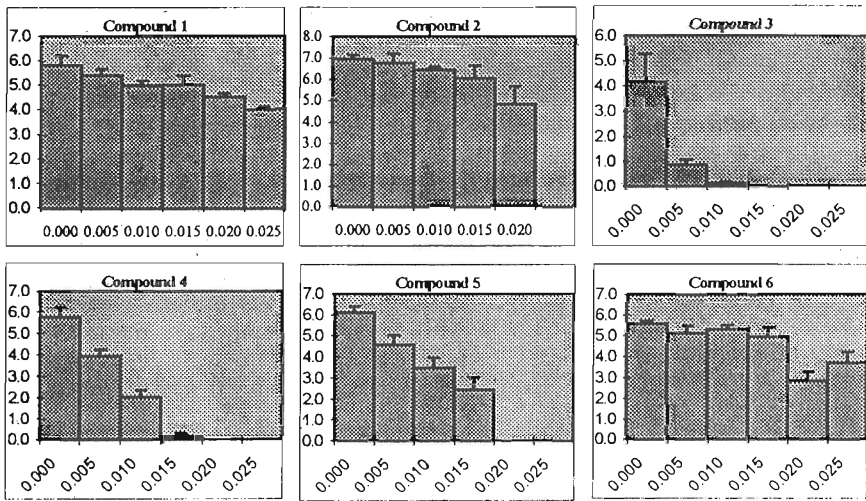


Figure 1: Changes in diameter of fungal mat of *Rhizoctonia solani* in cm (Y axis) with concentration (g/100ml)

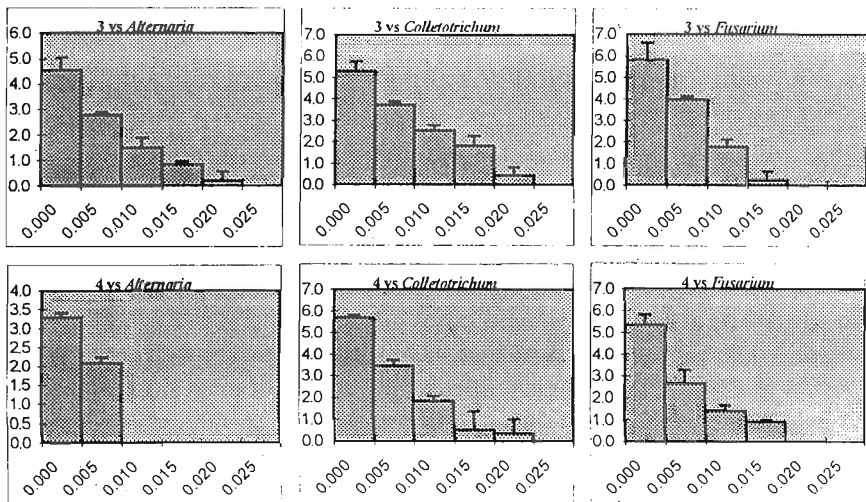


Figure 2: Changes in diameter of fungal mat of three fungi in cm (Y axis) with concentration (g/100ml) of compounds 3 and 4.

Table 4:  $^{13}\text{C}$  NMR spectral data of compounds 3,4,5,6

Carbon	Compound: chemical shift $\delta$			
	3	4	5	6
1	98.1	105.0	97.4	104.6
2	146.9	148.3	146.7	148.4
3	144.4	144.2	146.4	147.3
4	107.8	108.2	108.1	107.9
4a	124.1	121.7	125.0	129.9
5	29.5	27.0	30.5	28.6
6	47.9	55.7	51.6	39.3
8	49.9	144.6	73.4	160.1
8a	138.3	137.8	137.6	123.7
9	150.6	150.6	149.7	151.4
10	147.7	150.3	147.4	149.4
11	113.8	126.6	114.6	118.9
12	119.7	122.9	118.6	122.3
12a	128.4	133.3	129.4	132.3
13	104.0	119.9	104.1	101.3
14	116.5	119.8	114.8	119.3
14a	126.9	129.8	128.7	135.6
-OCH <sub>2</sub> O-	101.1	102.0	101.0	101.4
9-OCH <sub>3</sub>	60.9	61.8	60.9	61.6
10-OCH <sub>3</sub>	56.0	56.7	56.3	56.8
CN	116.9	-	-	-

## DISCUSSION

Comparison of the chemical shift of H-14 in compound 2 with the closely related tetrahydropalmatine in the literature<sup>10</sup>, indicates the conformation of 2 with respect to B/C ring fusion to be *trans*. This is in conformity with the chemical shift

difference of the angular proton of *cis* and *trans*-quinolizidines<sup>11</sup> where  $\Delta\delta_{\text{cis-trans}} = 0.27$  to  $0.33$  ppm.

The chemical shift assignments of protons and carbon were made using the 2DXHCORR and COSY pulse sequences. The downfield chemical shift of proton attached to C-8 in compound 4 and the observation of two triplets for protons attached to C-5 and C-6 confirms that the azide is not attached to C-8 in compound 4.

Mass spectral analysis of the compounds 3,4, 5 under FAB gave a base peak at 336, which corresponds to the stable berberine cation showing that the berberine structure is very stable and the products prefer to acquire the flat berberine skeleton even under mild ionisation technique. The base peak under electrospray for all three compounds was at 336, with the molecular ion being noted for compound 5 alone at 352.2.

This mass spectral results indirectly confirms the results obtained from the treatment of 8-cyano-(8H)-berberine with  $\text{NaBH}_4$ . It is clear that nucleophilic reactions on carbon 8 make the berberine skeleton to be slightly deviated from the otherwise flat structure. Hence it always prefers to revert back to the starting berberine cation. It is very clear that the reduction of 13,14 double bond in 8-cyano-(8H)-berberine is not a favourable way to obtain the quinolizidine nucleus.

Berberine.HCl (1), tetrahydroberberine (2) and 8-oxyberberine (6) did not have promising activity against *Rhizoctonia solani*. Compounds 3 and 4 showed very good activity against the same fungus. Both compounds indicated almost 100% inhibition at or above 0.15% concentration. They completely inhibited the growth of *Alternaria*, *Colletotrichum* and *Fusarium* at or below 0.025% concentration.

It is not clear whether the activity of compound 3 is due to the compound itself or due to the release of cyanide into the medium resulting in a stable berberine cation.

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