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The Yellow Toxins Produced by *Cercospora Beticola*. Part II[1] : Isolation and Structure of Beticolins 3 and 4.

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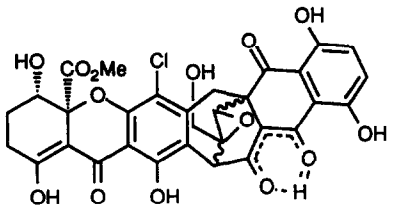
***Abstract** : Among the secondary metabolites produced by Cercospora beticola, two new compounds have been isolated and their structures elucidated by NMR and MS analysis : they have the same basic skeleton as beticolins 1 and 2 and were named beticolins 3 and 4.*

The pathogenic fungus, *Cercospora beticola,* which is responsible for leaf spot disease on sugar beet produces toxins of complex structures, mainly cercosporin, a red compound and CBT (*Cercospora beticola toxin*), a yellow one. They have been extensively studied but CBT structure has remained unknown for many years. We have recently identified structures of two yellow toxins, beticolins **1** and **2**[1], which correspond to a new class of compounds : the first one comigrates with CBT[2] and both exhibit the same biological activities as those reported for CBT[3]. Recently, another structure has been reported for a toxin also isolated from *C. beticola*[4]. It presents many similarities with beticolin 1.

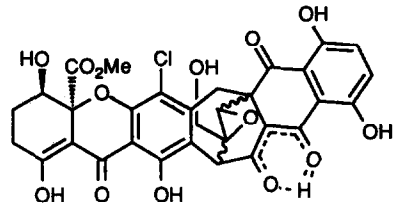
In the present paper, we report the structures of two new compounds belonging to the beticolin family. They were named beticolins **3** and **4.**

The beticolins were extracted from the mycelium of *C. beticola* and purified by silica gel column chromatography and recrystallisation[5]. They are bright yellow compounds : beticolin **3** (mp=268°C, $[\alpha]_D$=+664 (c=0.089, CH_2Cl_2)) and beticolin **4** (mp=220°C, $[\alpha]_D$=+545 (c=0.304, CH_2Cl_2)). Analysis by ammonia DCI-MS indicated (e.g. occurence of $(M+NH_4)^+$, MH^+ and $(M+NH_4-H_2O)^+$ ions) a molecular weight of 654 for both compounds. The molecular formula was determined to be $C_{31}H_{23}ClO_{14}$ for **3** and **4** according to HREIMS[6] and measurements of isotopic ion ratios[7].

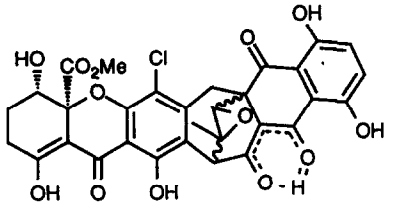
The ^1H NMR data (400 MHz, CD_3COCD_3) of beticolins **3** and **4** show the same basic features as those found for beticolins **1** and **2** respectively. The chemical shifts and the coupling constants are summarized and compared in the following tables. The only difference is the disappearance of the signal corresponding to the protons of methyl 16' and the presence of a new spin system of three coupled protons. Addition of D_2O induces the transformation of this spin system into an AB system (J=12 Hz for both compounds) and the collapse of the signal at 4.0 ppm (4.35 ppm). This led us to conclude to the presence of a CH_2OH subunit at C-16'. This result is in agreement with the ^{13}C data which exhibit a resonnance at 69.5 ppm (64.9 ppm) corresponding to a methylene carbon ; only one methyl carbon is thus observed which corresponds to the methyl ester (δ=54.8 ppm (54.9 ppm)) for beticolin **3** (and **4**). No significant NOE effects are observed in any beticolins, therefore the relative stereochemistry between the C-11'-C-12'-C-13'-C-14' and C-15 are not clearly established for **1**, **3** and **4**.



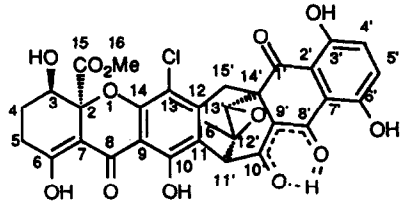
Beticolin 3



Beticolin 4

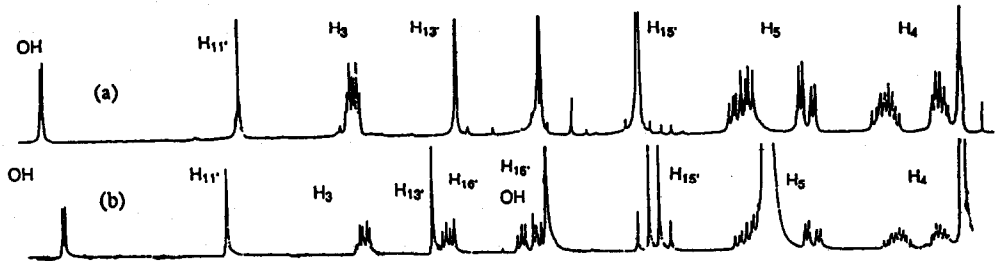


Beticolin 1



Beticolin 2

Beticolins **3** and **4** were also examined by low collision energy MS-MS experiments. CAD spectra of the MH^+ ions obtained[8] under ammonia DCI source conditions showed the same significant daughter ions for both compounds: i) m/z 637, 619, 595, 587, 583, 577, 559, 487, 467, 441, all these ions[9] (except m/z 487: +18 amu) being shifted by +16 amu relatively to daughter ions formed from **1** and **2** , ii) m/z 353, 219, 197 and subsequent ions[10] 179, 169, 141, 137 and 109 in common with the two former compounds.



^1H spectra (400 MHz) of beticolin **1** (a) beticolin **3** (b) in CD_3COCD_3 (expanded region : 2.2 to 5.9 ppm)

	1	**3**	Δδ	**2**	**4**	Δδ
3	4.57 dt (11.4, 4.5, 4.5)	4.68 dt (11, 5, 5)	0.09	4.15 t (4, 4)	4.65 m	0.5
4	2.33 qd (11.4, 5.2)	2.46 qd (11, 5.5, 5)	0.13	2.1 m	2.95 m	0.85
	2.11 tdd (11.4, 4.5, 0.8)	2.3 tdd (11, 5, 1)	0.19	2.1 m	2.95 m	0.85
5	2.91 ddd (18, 11.4, 5.2)	3.1 ddd (18, 12, 5.5)	0.19	2.92 ddd (13, 10, 5)	2.95 m	0.03
	2.64 ddd (18, 5.7, 0.8)	2.81 ddd (19, 6)	0.17	2.51 dd (13, 4)	2.61 dd (15, 5)	0.1
16	3.74 s	3.82 s	0.08	3.67 s	3.82 s	0.15
4'	7.31 d (13.4)	7.43 d (10)	0.12	7.45 d (9.8)	7.6 d (9.5)	0.15
5'	7.39 d (13.4)	7.5 d (10)	0.16	7.4 dd (9.8)	7.55 d (9.5)	0.15
11'	4.95 d (1.3)	5.23 d	0.28	4.68 bs	5.05 d (1.5)	0.37
13'	4.04 d (1.3)	4.4 bs	0.36	4.05 bs	4.35 d (1.5)	0.3
15'	3.31 bs	3.43 d (16)	0.12	3.45 d (15)	3.42 d (16)	-0.03
		3.52 d (16)	0.19	3.55 d (15)	3.51 d (16)	-0.05
16'	1.7 s	3.94 dd (12, 6)		1.55 s	4.05 dd (12, 6)	
		4.15 dd (12, 6)			3.68 dd (12, 6)	
OH		4.0 t (6,6)			4.35 t	

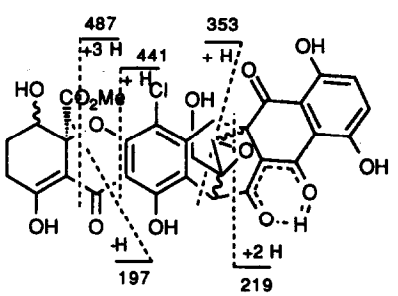
Table 1 : ^{1}H chemical shifts (ppm) (coupling constants (Hz)) (400 MHz, CD_3COCD_3)

	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**
2	86.9	84.5	85.1	84.5	1'	183.4	182.2	181.5	183.6
3	71.1	65.0	69.6	64.9	2'	114.2	114.4	112.4	112.2
4	25.4	23.4	24.1	22.5	3'	155.0	151.3	153.8	152.1
5	28.1	23.2	26.1	23.4	4'	130.9	129.2	128.6	128.6
6	181.0	180.7	179.5	180.7	5'	127.7	125.7	126.2	126.1
7	101.0	99.2	104.7	99.5	6'	158.1	156.7	156.5	156.1
8	187.1	185.6	185.4	185.4	7'	112.4	111.5	110.9	110.9
9	106.3	104.3	104.9	104.2	8'	186.6	182.3	184.6	182.0
10	157.5	155.7	153.9	156.1	9'	103.2	101.5	101.9	101.9
11	116.4	114.6	114.8	114.5	10'	202.0	200.1	200.4	200.3
12	114.7	113.0	112.5	112.9	11'	44.2	42.6	38.7	38.2
13	144.5	143.2	142.9	142.4	12'	58.8	53.0	58.6	52.9
14	155.6	153.2	155.6	153.7	13'	60.0	59.8	60.9	58.7
15	169.9	169.2	169.0	169.3	14'	49.1	47.7	47.5	47.5
16	53.1	52.5	54.8	54.9	15'	40.0	38.1	38.3	38.4
					16'	19.4	16.2	69.5	64.9

Table 2 : ^{13}C chemical shifts (ppm) (100.3 MHz, CD_3COCD_3)

	197	179	169	137
1	100	46	88	26
2	78	36	100	15
3	100	79	81	28
4	88	81	100	33

Table 3: Relative intensities of the low mass daughter ions in the MH^+ CAD spectra.



This similarity between **3** and **4** in the decomposition pathways is not dependent on the collision energy domain studied (20-70 eV). Strong similarity between beticolin **1** and **2** CAD spectra of the corresponding MH^+ ions was also observed which is consistent with a common skeleton for this new species of molecules. Furthermore, examination of the relative intensity ratios of the low mass region ions shows (Table 3) the same tendancies when comparing **3** and **4** (or **1** and **2**) which is due to the same structural difference for each pair (e.g. stereochemistry at C_3).

The effects of beticolins **3** and **4** on plasma membrane H^+-ATPase were studied. They display a similar inhibitor activity to beticolin **1** and **2**. Detailed studies on their biological activities are in progress and will be published elsewhere.

These results lead us to conclude that CBT, which was previously considered as a unique compound, consists in a family of at least 5 identified members.

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References and Notes

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2. Assante, G. ; Locci, R. ; Camarda, L. ; Merlini, L. ; Nasini, G. *Phytochemistry* **1977**, *16*, 243. Beticolin 1 comigrates with a standard given by Assante G. and Nasini G. in the conditions described 1)[5]
3. Blein, J.-P. ; Bourdil, I. ; Rossignol, M. ; Scalla, R. *Plant. Physiol.* **1988**, *88*, 429.
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5. After extraction with ethyl acetate, the beticolins were separated by flash chromatography using silica gel pretreated with $Ca(H_2PO_4)_2,H_2O$ and H_3PO_4[1] and eluted with $CHCl_3$. They were analysed on pretreated TLC, using two systems : 1) $CHCl_3$/MeOH/CH_3COOH : 100/2/1 and 2) Hexane/ethyl acetate 1/1. They crystallized from ethyl acetate/hexane.

Rf	1	2	3	4
syst. 1	0.42	0.70	0.25	0.40
syst. 2	0.45	0.54	0.10	0.30

6. Beticolin 3: found m/z 654.0797, calcd for $C_{31}H_{23}O_{14}Cl^{35}$ m/z 654.07763; found m/z 595.0631 corresponding to $(M\text{-}CO_2CH_3)^+$, calcd for $C_{29}H_{20}O_{12}Cl^{35}$ m/z 595.06433. Beticolin 4: found m/z 654.0777 and 595.0616 (see beticolin 3 for calcd).
7. Relative intensities found for ions m/z 654 to 658: 100, 30.1, 36.2, 10.1, 2.3 (beticolin 3) and 100, 33.4, 41.3, 11.9, 3.6 (beticolin 4); calcd 100. 35.7, 41.O, 13.1, 3.1 for $C_{31}H_{23}O_{14}Cl$.
8. The MS-MS spectra were obtained with a Nermag R30-10 triple quadrupole instrument using the following source conditions: T 120°C, filament current 100 mA, electron energy 95 eV, NH_3 pressure 10^{-4} Torr in the source housing. Laboratory energy was 50 eV and argon was used as collision gas in the second quadrupole at a 3 x 10^{-2} Torr pressure.
9. Ions from m/z 637 to m/z 559 correspond to the loss (or combination of losses) of H_2O, HCO_2CH_3, CH_3OH from the MH^+ ion m/z 655.
10. Ions m/z 179, 169, 141, 137 and 109 formally result from ion m/z 197 by losses of H_2O, CO, 2CO, (CO+CH_3OH) and (2CO+CH_3OH) respectively.

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