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ENANTIOMERIC PURITY OF SCYTALONE FROM DIFFERENT FUNGAL SOURCES

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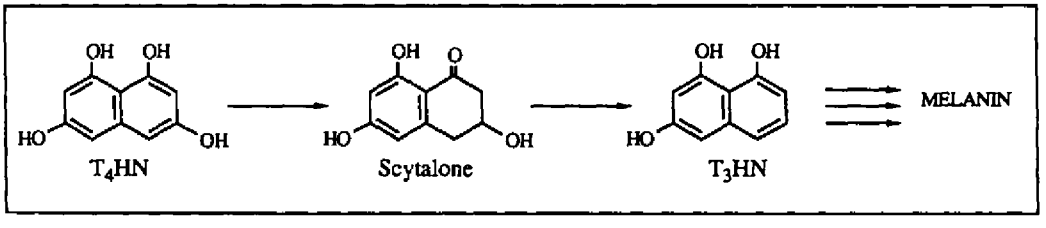
Abstract : The enantiomeric purity of scytalone biosynthesized by different fungi has been examined. In contrast with some previous statements the scytalone samples proved to be optically pure, although they exhibit a very low $[\alpha]_D^{20}$ and have the same absolute configuration.

INTRODUCTION

Scytalone 1 is an intermediate in the biosynthesis of melanin, a black pigment produced by pathogenic fungi (1). It is biosynthesized from tetrahydroxynaphthalene (T_4HN) (scheme 1) by a NADPH dependent dehydrogenase belonging to class B (2). Surprisingly there were descripancies about the enantiomeric purity of scytalones from



Scheme 1 : Biosynthesis of fungal melanin

different sources. Although their samples had a very small $[\alpha]_D$, Aldridge et al. concluded that they were optically active (comparison of the melting points and IR spectra of natural and racemic samples) and had the same absolute configuration (weak negative Cotton effect at 220 nm) (3) (table 1). Bell et al. purified a scytalone sample from Verticillum dahliae with $[\alpha]_D = +32°$ and a strong negative Cotton effect (4). Wheeler and Stipanovic compared this sample with a low $[\alpha]_D$ sample previously described (3) using optically active lanthanide shift reagent (5) and concluded the V. dahliae sample was optically pure whereas the scytalones form other sources were racemic (5).

Table 1 : Characteristics of scytalone from different sources

ORIGIN	$[\alpha]_D^{25}$	CD (nm)	mp °C	Ref.
Phialophora lagerbergii	0(C=1.68,MeOH)	Weak (-) (220)	167-168	3
Scytalidium sp.	0	Weak (-) (220)		3
			160-168	7
Verticillium dahliae	+32(C=0.25,EtOH)	θ_{307}=+4625 θ_{218}=-33.325	164-168.5	4
Colletotrichum lagenarium			165	8
Wangiella dermatitidis	-1.37(C=0.146, EtOH)			5
Racemic			185-190	

We have reexamined the question of the enantiomeric excess of scytalone from different sources.

EXPERIMENTAL SECTION

General Procedures. ^1H NMR spectra were recorded on a Brucker AC200 Spectrometer and chemical shifts are expressed in ppm relative to CD_3CN. Optical rotations were measured on a Perkin Elmer 141 Polarimeter. Circular dichroism spectra were run on a Jobin Yvon Autodichrograph V. Melting points were measured on a Kofler hot stage apparatus and are uncorrected. T.L.C. were carried out on silica gel precoated plates from Merck. Protein concentrations were determined with the Bio-Rad protein assay kit (9) with bovine serum albumin as standard.

Strains. Brm-1 mutant from V. dahliae kleb. strain T_9 was obtained from the American Type Culture Collection (ATCC 44571). P. oryzae car. strain G_{11} was a gift of Dr. LEBRUN (Laboratoire de Cryptogamie, Orsay).

Growth conditions. Cultures of V. dahliae for metabolite isolation were grown on liquid sucrose-nitrate medium (10), modified by addition of $CuSO_4$, $5H_2O$ (0.1 µg/mL), while shaking at 24°C in the dark during 3 weeks. P. oryzae strain was maintained on Tanaka medium (11)-yeast extract agar medium and cultures for enzymes extraction were grown on liquid Tanaka-yeast extract medium at 26°C in the dark for 5-6 days.

Enzymes. Alcohol dehydrogenase from Thermoanaerobium brockii (EC 1.1.1.2.) was from Sigma Chemical Co. Mycelia from P. oryzae were frozen in liquid nitrogen and ground manually in a mortar. The powder was suspended in KH_2PO_4 buffer (50 mM, pH 7) centrifuged and the proteins were precipitated with ammonium sulfate. Following dialysis, the enzyme preparation was dialyzed and kept frozen at - 80° C before use. This solution hence refered to as crude enzymatic preparation contained both naphthalene reductase and scytalone dehydratase. Further purification of naphthalene reductase was achieved by ion exchange chromatography.

Enzymatic assay. Enzymatic reactions were carried out under argon. Enzyme (about 1 mg protein) in KH_2PO_4 buffer (2 mL, 0.1 M, pH 7), NADPH (2.6 mole) and T_4HN (2.6 mole, dissolved in Ethanol 95°) were incubated at 25°C for 2 hours. After adjusting the pH to 4 with H_3PO_4 1N, the reaction mixtures were extracted twice with ethyl acetate ; the organic layers were dried over Na_2SO_4, concentrated under reduced pressure, and analyzed by thin layer chromatography using synthetics scytalone, T_3HN, vermelone and DHN (2) as references ($CHCl_3$/MeOH : 9/1, v/v as elution solvent).

Chemicals. CD_3CN (99 %) was from CEA (Saclay). Tris (3-((heptafluoropropyl)-hydroxymethylene)-d camphorato) Europium (III) ($Eu(hfc)_3$) was purchased from Aldrich. $NADP^+$, NADPH, dithiothreitol and EDTA were from Sigma Chemical Co. Tricyclazole was a gift of Rhône-Poulenc Agrochimie.

Isolation of (+) scytalone from Brm-1 V. dahliae. After centrifugation (5 000 g, 15') of 3 weeks old liquid cultures of Brm-1 (2 L), the supernatant was acidified to pH 4 with H_3PO_4 1N, extracted 3 times with ethyl acetate. The organic layer was dried over Na_2SO_4, concentrated and treated with charcoal in acetone. After filtration and removal of the solvent, the residue was chromatographed on silica gel ($CHCl_3$/MeOH : 8/2, v/v as eluent solvent), followed by TLC plate ($CHCl_3$/MeOH : 9/1, v/v) to afford pure scytalone (110 mg) as white crystals. mp = 166-167°C. $[\alpha]_D^{20}$ = + 1.4 - + 2.4° (C = 0.5, 95 % EtOH). ^1H NMR and UV spectra were identical with synthetic scytalone corresponding spectra.

Production of (+) scytalone from P. oryzae was achieved by enzymatic reduction of T_4HN (2) with naphthalene reductase partially purified and NADPH regenerating system under argon. $NADP^+$ (26 µmole), isopropanol (40 mmole) and alcohol dehydrogenase from T. brockii (10 U) were incubated at 30°C for 20 min. in KH_2PO_4 buffer (257 mL, 50 mM, pH 6, EDTA 1 mM, DTT 1 mM). Following addition of naphthalene reductase from P. oryzae (3 mL, 12.5 mg protein) and T_4HN (50 mg, 260 mole dissolved in ethanol 95° (5 mL)), the incubation was continued for 20 hours. Extraction, purification and analysis of scytalone were achieved as above. mp = 164-166°C. $[\alpha]_D^{20}$ = + 0.9 - +1.9° (C = 0.5, 95 % EtOH).

(-) scytalone from P. oryzae. Synthetic scytalone (20 mg, 103 mole dissolved in ethanol 95° (5 mL), tricyclazole (2.5 mole in ethanol (2 mL)) and crude enzymatic preparation (10 mL, 300 mg protein) were incubated in KH_2PO_4 buffer (83 mL, 0.1 M, pH 7.5) during 15 hours. Extraction of the remaining scytalone was performed as above. Purification was achieved by 2 consecutives TLC : first : $CHCl_3$/MeOH 9/1 as eluent and second : ethyl acetate/cyclohexane 75/25.

Circular dichroism spectra of various scytalones were measured in ethanol 95° from 220 to 400 nm. Synthetic racemic scytalone (6.2 10^{-5} M, θ_{220} = 0) ; (+) scytalone from V. dahliae (6.6 10^{-5} M, θ_{220} = - 4 130) ; (+) scytalone from P. oryzae (5.8 10^{-5}, θ_{220} = - 4 110) ; (+) scytalone from S. macrospora (5.8 10^{-5} M, θ_{220} = - 4 095) ; (-) scytalone from P. oryzae (5.6 10^{-5}, θ_{220} = + 4 050).

^1H NMR spectra of scytalones in presence of chiral shift reagent $Eu(hfc)_3$. To a solution of synthetic scytalone in CD_3CN (0.4 mL, 50 mM) in a NMR tube, were added increasing amounts of 0.2 M shift reagent solution (prepared using a freshly open bulb of CD_3CN) from 0 to 1.2 equivalents. For the determination of the enantiomeric purity of the

natural scytalones, the sample (5 mg, 25.77 μ mole) was dissolved in a CD_3CN solution of the reagent (0.5 mL, 52 mM) and the spectra were recorded the same day.

RESULTS AND DISCUSSION

Scytalone from different sources was extracted and extensively purified by column chromatography on silica gel. The purity was checked by thin layer chromatography and ^1H NMR spectra. The S. macrospora sample was obtained from Dr. BOUILLANT (12) and repurified according to our methods before rotation determinations. The scytalone from V. dahliae was produced as already described by growing mutant Brm-1 which accumulates scytalone (4). (+) scytalone from P. oryzae was obtained by reducing 1,3,6,8 tetrahydroxynaphthalene with a partially purified cell free extract coupled with a NADPH regenerating system. The optical rotations and the melting points of the V. dahliae, P. oryzae and S. macrospora scytalone samples purified in this work are quoted in table 2. The three samples melted in the 164-167°C range previously described for the scytalones from natural sources (see table 1), i.e. about 20°C lower than the racemic sample. This difference which was in agreement with Aldridge's observation (3) suggested that all the samples examined so far were optically active, with the exception of the scytalone from W. dermatitidis for which no melting point was available (5). Scytalone being only sparingly soluble in ethanol*, in all cases the values were only slightly positive ranging 1.4 to 1.9° which corresponded to actual reading of 0.007-0.010°** (table 2). It is thus understandable that most authors concluded to an $[\alpha]_D = 0$.

Table 2 : Physical constants of scytalones obtained in this study

ORIGIN	$[\alpha]_D^{20}$ (C = 0.5, 95 % EtOH)	mp °C	CD θ_{220}
V. dahliae	+ 1.9 ± 0.5	166-167	- 4 130
S. macrospora	+ 1.55 ± 0.54	165-167	- 4 095
P. oryzae (+)	+ 1.4 ± 0.5	164-166	- 4 110
(-)			+ 4 050
Racemic		185-190	

* : The $[\alpha]_D$ determinations were run with a 5 mg/mL solution in ethanol (C = 0.5).

** : 10 cm path length cell thermostated at 20°C.

Although they descrided a melting point in the normal range, the $[\alpha]_D = +32°$ reported by Bell et all. (4) for the scytalone from V. dahliae significantly deviates from values described so far (table 1) and especially from the $[\alpha]_D$ for scytalone from the same source that we obtained (table 2). It is probable that their sample was contaminated by an impurity with a very high $[\alpha]_D$*.

The circular dichroism spectra were recorded in ethanol between 400 and 220 nm (figure 1). In all cases, the CD was slightly negative at 220 nm with a θ_{220} in the - 4 100 range (table 2). It is thus clear that (+) scytalones from P. oryzae, V. dahliae and S. macrospora had the same absolute configuration and that this absolute configuration holds for scytalones from Scytalidium and P. lagerbergii which exhibit also a slightly negative CD at 220 nm (3). Once again the CD of V. dahliae sample of Bell et al. deviates (4), with a highly negative θ_{218} and a significantly positive θ_{307} (table 1).

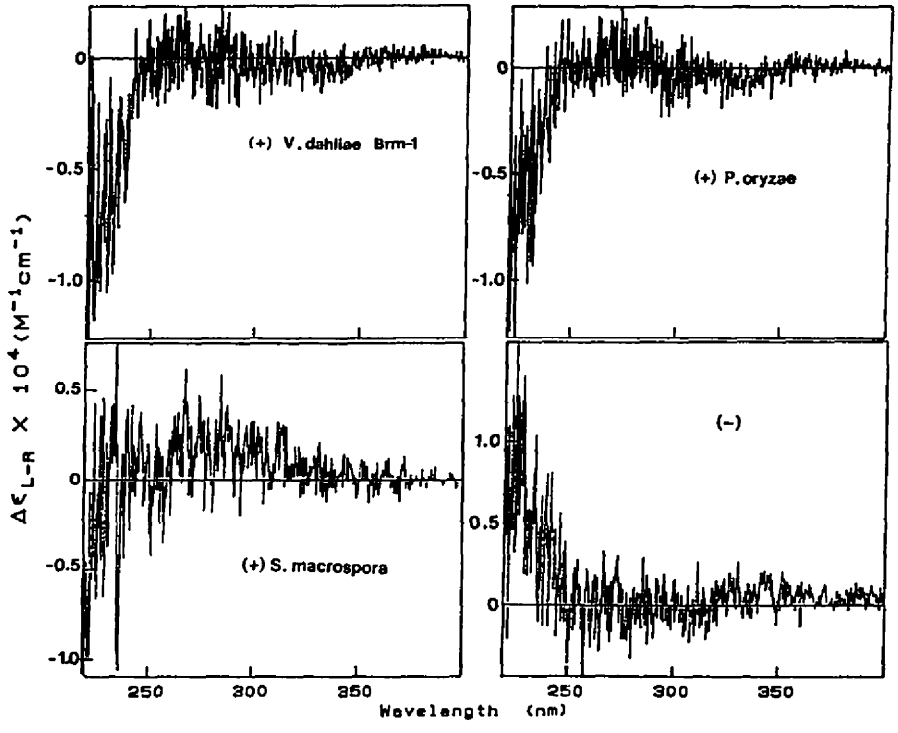


Figure 1 : Circular Dichroism spectra of scytalones from different sources

* : The ε max of the UV spectrum they described differs also significantly from the ε max already published.

The θ_{220} of the scytalone from three different sources being very similar, this suggested that their enantiomeric purities were identical and presumably close to 100 %. This was confirmed by two independant ways.

When treating racemic scytalone (2) with an enzymatic preparation containing the dehydratase from P. oryzae (the enzyme which dehydrates scytalone into 1,3,8-trihydroxynaphthalene (T_3HN) and presumably vermelone into 1,8-dihydroxynaphthalene (DHN)) in the presence of tricyclazole (to avoid further reduction of T_3HN into vermelone which could interfere during the purification) we observed that the dehydration reaction stopped when about 50 % of scytalone was transformed into T_3HN. This established that the dehydratase was enantiospecific. Extraction of the mixture and purification yielded untransformed scytalone with a CD spectrum showing a positive θ_{220} = 4 050, almost equal in absolute value with the θ_{220} of natural (+) scytalone (figure 1, table 2).

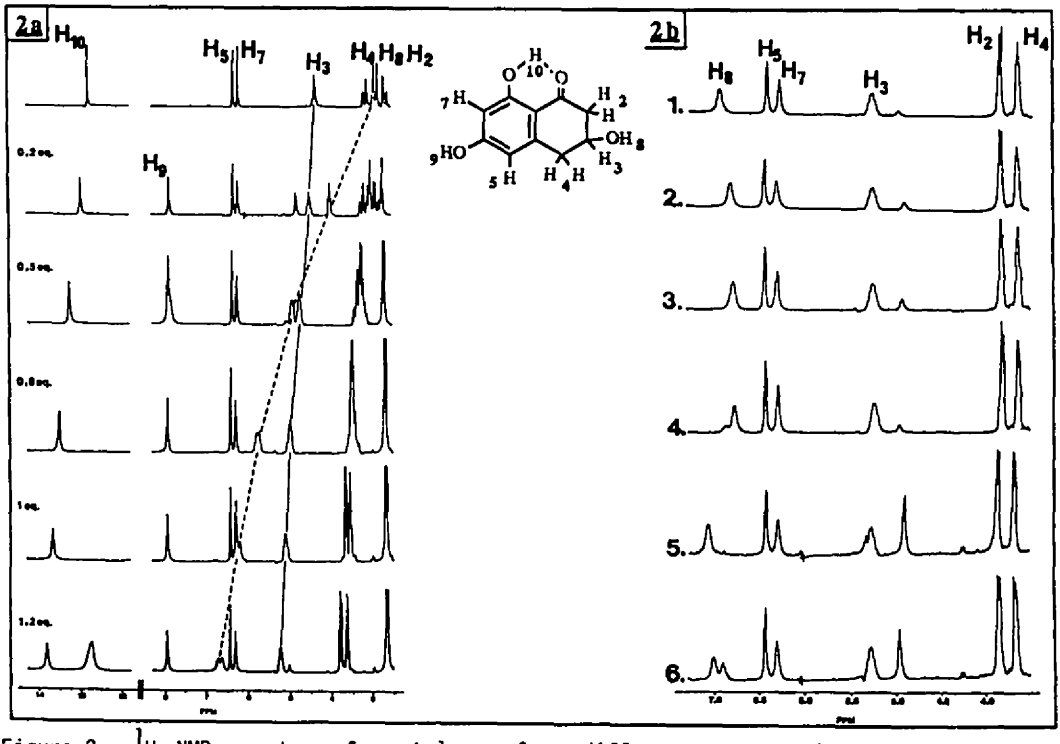


Figure 2 : ^1H NMR spectra of scytalones from different sources in the presence of (+)Eu(hfc)$_3$

a) Racemic. Spectrum in CD_3CN without shift reagent (top). δ : 2.52-3.06 (m, 5H, H_2 + H_4 + OH_8 exchangeable) ; 4.28 (septuplet, 1H, H_3) ; 6.14 (d, 1H, H_7) ; 6.25 (m, 1H, H_5) ; 12.72 (s, 1H, OH_{10}). The OH_9 yields a broad signal which sharpens (δ = 8) upon addition of the shift reagent. The signals appearing at 12.7 ppm and 5 ppm in the 1.2 eq spectrum are due to an impurity of the shift reagent. b) Spectra of scytalones from different sources ($Eu(hfc)_3$ = 1 eq) 1. (+) scytalone from V. dahliae 2. (+) scytalone from P. oryzae 3. (+) scytalone from S. macrospora 4. (+) scytalone from V. dahliae + 0.833 eq of racemic scytalone 5. (-) scytalone (see exp. part) 6. (-) scytalone + 0.6 eq (+) scytalone from P. oryzae. The 5 ppm signal is due to an impurity of the shift reagent.

On the other hand we examined the NMR spectra of the different scytalones in the presence of chiral lanthanide shift reagent (+) $Eu(hfc)_3$. Using this technique in chloroform Wheeler and Stepanovic reported that scytalone from W. dermatitidis was racemic whereas the sample from V. dahliae was optically pure (5). Their conclusion relied on the observation of the aromatic protons of scytalone. Due to the insolubility of scytalone in chloroform and in spite of a very high number of accumulations on a 200 MHz FT NMR we were unable to observe any signal at the chemical shifts described by Wheeler and Stipanovic* (5). To overcome this insolubility problem the chemical shift reagent experiments were run in deuteriated acetonitrile according to Whitesides et al. (13). Figure 2a shows the evolution of the NMR spectrum of racemic scytalone in the presence of increasing quantities of (+) $Eu(hfc)_3$. Chemical shifts of protons of scytalone were only slightly modified ($\Delta\delta < 1$ ppm)** with the exception of the hydroxyl proton signal ($\Delta\delta max \neq 4$ ppm) that readily separated into two signals. Scytalones from V. dahliae, P. oryzae and S. macrospora gave rise to NMR spectra with only one signal corresponding to the hydroxyl proton (figure 2b) suggesting that they were optically pure. Addition of racemic scytalone to the V. dahliae sample yielded two peaks (figure 2b, line 4), establishing that the low field hydroxyl signal was due to the (-) enantiomer. This was confirmed by recording the spectra of (-) scytalone (vide supra) and adding (+) scytalone (figure 2b, lines 5 and 6).

CONCLUSION

This study established that scytalones biosynthesizable by P. oryzae, V. dahliae Brm-1 and S. macrospora are enantiomerically pure. The optical rotation of (+) scytalone is very low and cannot be used as a criterium to analyze scytalones from natural sources. The melting point determination, the circular dichroism and the use of the chemical shift reagent (+) $Eu(hfc)_3$ in acetonitrile are reliable techniques for the enantiomeric analysis of scytalone. The scytalones from Scytalidium or P. lagerbergii (3) are enantiomerically pure and have the same absolute configuration as those produced by P. oryzae, V. dahliae Brm-1 or S. macrospora. No conclusion can be drawn for the scytalone from W. dermatitidis for which only the $[\alpha]_D^{20}$ is available*** (5).

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* : As mentioned previously these signals may correspond to the impurity present in the scytalone samples. These very weak signals (compared to chloroform) can also reflect NMR artefacts such as rotation bands or ^{13}C-H signals.

** : The $\Delta\delta$ of the aromatic protons was small ($\Delta\delta < 0.1$ ppm) and the corresponding signals were slightly broadened.

*** : Surprisingly the negative $[\alpha]_D$ could have suggested that W. dermatitidis biosynthesized (-) scytalone. However the value - 1.37° (C = 0.146) corresponds to an actual reading of 0.002° with a 10 cm path legth and seems to be devoid of significance.

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