

ISOLATION, STRUCTURAL IDENTIFICATION AND  
BIOLOGICAL ACTIVITY OF TWO METABOLITES PRODUCED  
BY *Penicillium olsonii* BAINIER AND SARTORY

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From the culture broth of a fungus, two metabolites have been isolated: bis(2-ethylhexyl) phthalate (DEHP) precedly isolated from *Streptomyces* sp. and 2-(4-hydroxyphenyl)-2-oxoacetaldehyde oxime (PHBA) here reported as a natural compound in the (*E*)-*s-cis* configuration. The producing organism was identified as a strain of *Penicillium olsonii*. Culture growth and chemical identification are discussed in the present work.

In our natural product screening program on marine organisms, a fungus, strain No. 36, identified as *Penicillium olsonii*, was selected. Two metabolites were isolated from the culture filtrate. One of these products was identified as bis(2-ethylhexyl) phthalate (DEHP), known to be a cell aggregation factor and to be produced by a *Streptomyces* sp. strain<sup>1</sup>). The other molecule bearing an oxime function which is unusual in the chemistry of marine natural products was isolated for the first time from a microorganism. This metabolite previously obtained by chemical synthesis and used for its phosphorylated cholinesterase reactivator properties<sup>2</sup>), was identified as 2-(4-hydroxyphenyl)-2-oxoacetaldehyde oxime (PHBA) in the (*E*)-*s-cis* configuration.

### Materials and Methods

#### Taxonomic Study

Among the fungi growing on the vegetation around the edges of the salt pans in Camargue (France), strain No. 36 has been frequently isolated from terminal leaves of *Suaeda fruticosa* and from living twigs of *Arthrocnemum glaucum*<sup>3</sup>). Strain No. 36 was studied using the method described by PITT<sup>4</sup>). The cultural characteristics on Czapeck yeast extract agar (CYA) prepared according to PITT<sup>4</sup>) were described after 7 days of incubation at 25°C. The morphological characteristics of the strain were observed under light microscopy. Strain No. 36 was kept on CYA and stored at 4°C.

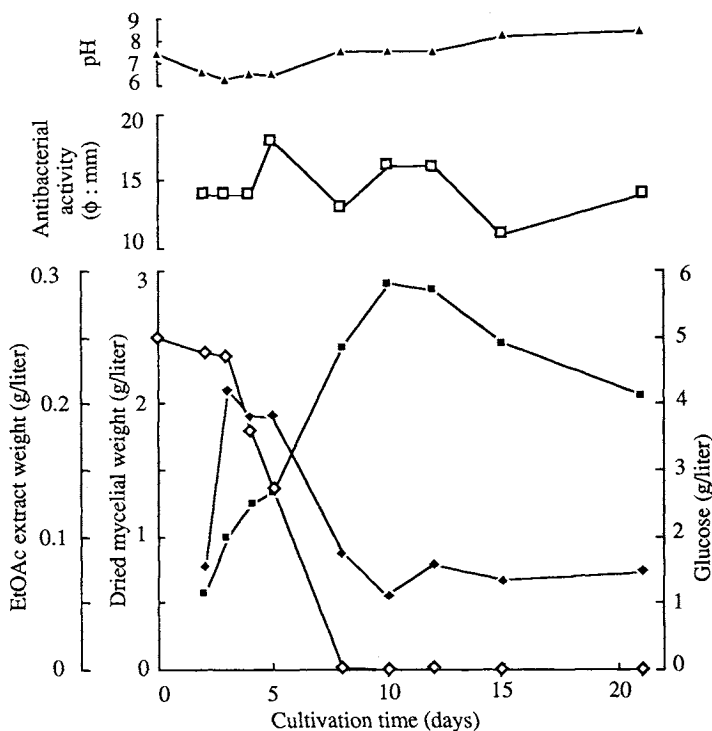
#### Fermentation and Extraction

The fungus was grown in Petri dishes containing CYA medium. A plug of 5 days-culture was used to inoculate 3-liter Erlenmeyer flasks containing one liter of fermentation medium having the following composition: yeast extract 1 g, glucose 5 g in one liter of distilled water. The pH of the medium was adjusted to 8 with 1 M NaOH before sterilization. The fermentation time was determined by performing a series of tests, as shown in Fig. 1. The crude extract production increased up to the third day of fermentation. The maximum of about 200 mg/liter persisted up to the fifth day, by which time the mycelium was on the growth phase and the glucose level was decreasing sharply. The mean pH was 6.4. Ten Erlenmeyer flasks were incubated on a reciprocating shaker for 5 days under low stirring. The extract production was improved by maintaining the temperature at 18°C.

At the end of the fermentation period, the contents of the 10 flasks were filtered to separate the

Fig. 1. Time course of bioactive metabolites.

◆: EtOAc extract weight, ■: dried mycelial weight, ▲: pH, ◇: glucose concentration, □: antibacterial activity.



mycelial cake and the further purification was carried out as shown in Fig. 2.

#### TLC and HPLC Systems

The fractions obtained after each liquid phase chromatography were spotted on HPTLC Merck Kieselgel 60 F<sub>254</sub> or Diol F<sub>254</sub>, developed with hexane-EtOAc (9:1) and CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95:5), respectively and detected with iodine vapor and UV lamp at 254 nm.

A Varian model pump Vista 5000 with injector and CDS 401 control station were used in HPLC. The chromatograph was monitored by a UV detector (Pye Unicam PU 4021 diode array) from which UV spectra could be obtained and by a refractive index detector (Varian RI-3). Separations were carried out using Lichrosorb diol 7 μm (10 i.d. × 250 mm) from Merck Co.

#### Spectroscopy and Chemicals

<sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained from a 200 MHz and a 400 MHz A.M. Bruker spectrometer. IR spectra were obtained from a Bruker IFS 45 TF.

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals Co. All other chemicals were reagent grade.

#### Biological Activity

Extracts 1 and 2 as well as the various other fractions obtained during the isolation process, were tested using the paper disk agar diffusion method against bacteria, yeasts, and filamentous fungi as test organisms, with Mueller-Hinton medium (Pasteur Institute Production) in the case of bacteria and Casitone (Pasteur Institute Production) for yeasts and filamentous fungi<sup>5,6</sup>. The inhibition diameter around the paper disc was measured after incubation at 37°C for 18 hours.

*In vitro* cytotoxicity of PHBA was tested against six strains of carcinoma cells (NSCLC-N6-L16,

P388/DOX, P388, KB and HT29). IC in  $\mu\text{g/ml}$  was determined and compared to 6-mercaptapurine ( $\text{IC}_{50}$ : 0.77, 0.24, 0.70, 0.66 and 0.85  $\mu\text{g/ml}$ , respectively).

## Results

### Cultural and Morphological Characteristics

The fungal colony grew rapidly on CYA. It reached a diameter of about 40 mm after 7 days of incubation at 25°C, with deep velutinous surface. The colony area was white becoming dull green with conidiogenesis; the reverse side was pale yellow; no exsudate was produced; no pigment was present; no growth occurred at 37°C. This fungus can grow rapidly and sporulate abundantly on CYA culture media made up with a range of salinities from distilled water to 12% salinity.

The mycelium was white with conidiophores measuring more than 500  $\mu\text{m}$  length and 6~8  $\mu\text{m}$  width. They were unbranched, smooth-walled, bearing terminal terverticillate appressed penicilli with inflated metulae and rami. The conidia were ellipsoidal, 3~4  $\mu\text{m}$  long, with a finely granulous wall, borne in disordered chains. These characteristics indicate that strain No. 36 is *Penicillium olsonii* Bainier and Sartory based on the description by PITT<sup>4)</sup>.

### Extraction

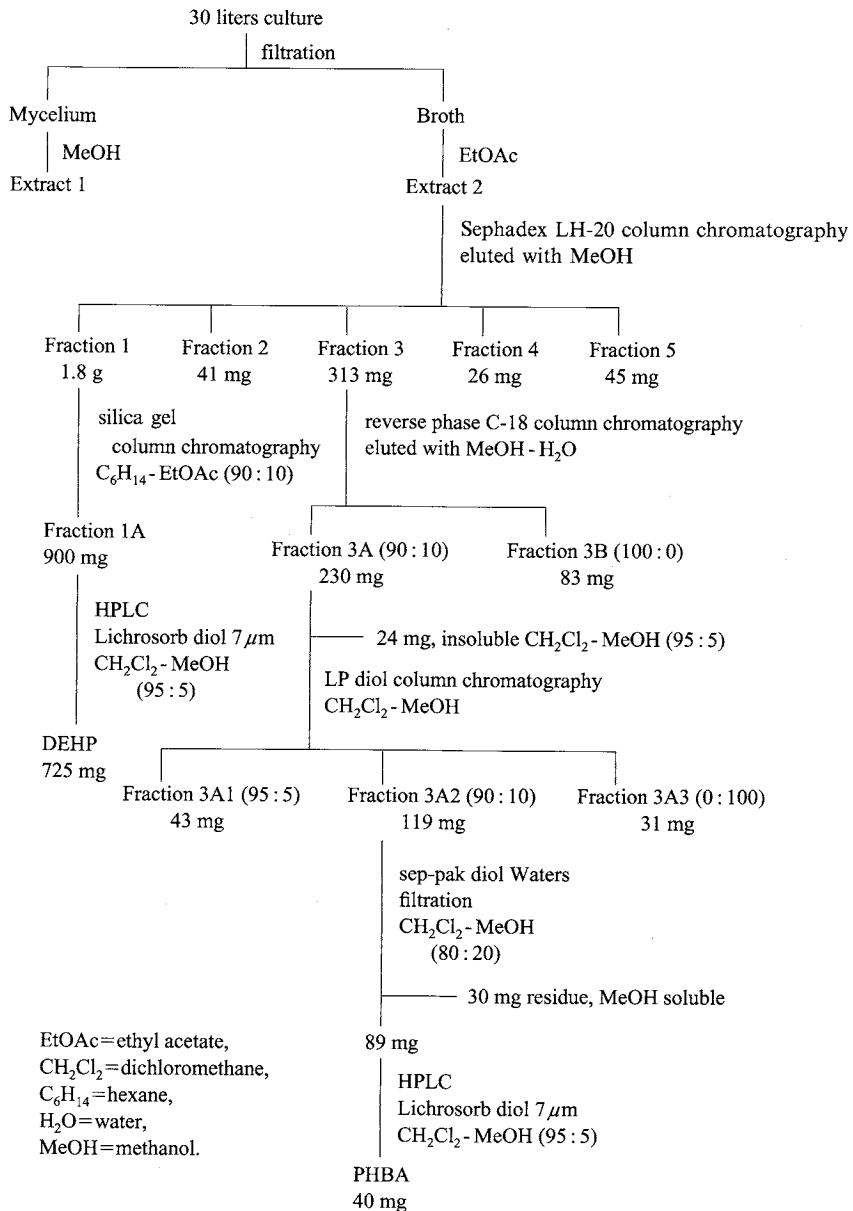
Thirty liters of *P. olsonii* culture was filtered through a fritted disk (porosity No. 3) to separate the mycelium. The broth was extracted three times with EtOAc overnight using 7, 6 then 5 liters of solvent. The EtOAc extract was concentrated to oily residue under reduced pressure at a temperature lower than 40°C. The material was dissolved in MeOH (75 ml) and applied to a Sephadex LH-20 column chromatography (40 i.d.  $\times$  500 mm) and eluted with MeOH 100%. Eight fractions were collected and after regroupments on basis of HPTLC silica plate control (eluted with hexane - EtOAc 9 : 1), five fractions were obtained as shown in Fig. 2, and tested against *Staphylococcus aureus* at 100  $\mu\text{g}$  on paper disc assay. Only the fraction 3 was active: 8 mm of inhibition diameter.

### Isolation of DEHP

The first fraction (1.8 g) was dissolved in hexane and loaded (2 passages) on a silica gel column (28 i.d.  $\times$  235 mm). The fraction eluted with hexane - EtOAc (9 : 1), 900 mg, was purified by HPLC on a Lichrosorb diol column eluted with  $\text{CH}_2\text{Cl}_2$  - MeOH (95 : 5). 725 mg of pure DEHP was obtained: EI-MS  $m/z$  (%) 390.3 ( $\text{M}^+$ , 0.5), 279.2 (25), 167.1 ( $\text{C}_8\text{H}_7\text{O}_4$ , 48), 149.1 ( $\text{C}_8\text{H}_5\text{O}_3$ , 100), 113.2 (9), 83.1 ( $\text{C}_8\text{H}_{17}$ , 9), 71.1 (20), 54.1 (34); UV  $\lambda_{\text{max}}^{\text{hexane-EtOAc } 3\%}$  nm 250, 283 (sh); IR  $\nu_{\text{max}}^{\text{CCl}_4}$   $\text{cm}^{-1}$  2982, 2931, 2862, 1729, 1285, 1275, 1123.

DEHP, a colorless and oily liquid, appears as a single spot  $R_f$  0.62 with hexane - EtOAc (9 : 1) on HPTLC Merck Kieselgel 60 F<sub>254</sub>. The molecular formula  $\text{C}_{24}\text{H}_{38}\text{O}_4$  was established by EI-MS, ( $\text{M}^+$ ): 390 and by elementary analysis (Calcd: C 72.75, H 9.86, O 17.39). The IR spectrum revealed a carbonyl band observed at 1729  $\text{cm}^{-1}$  and strong C-O bands in the range 1120~1200  $\text{cm}^{-1}$ . The presence of a phthalate was inferred from the EI-MS peaks at  $m/z$  167 (48) and  $m/z$  149 (100).  $^1\text{H}$  NMR correlation spectroscopy (COSY) experiments indicated the partial structures of the aromatic portion and the correlations of  $\text{C}_6$ ,  $\text{C}_5$ ,  $\text{C}_4$  and  $\text{C}_8$ ,  $\text{C}_9$ . The  $^{13}\text{C}$  NMR spectrum of DEHP, confirming the symmetry of the molecule, exhibited the expected 12 carbon resonances (see Table 1) assigned by DEPT experiment to two quaternary, three methine and five methylene carbons with two methyl groups. All these data suggested that DEHP<sup>1)</sup> presented the structure illustrated in Fig. 3.

Fig. 2. Purification procedure.



## Isolation of PHBA

The third fraction from the preparative Sephadex LH-20 chromatography, 313 mg, was loaded on a reverse phase C-18 column. The fraction eluted with MeOH-H<sub>2</sub>O (9:1), 230 mg, was concentrated to an oily residue. The material was chromatographed on a LP diol column using a step gradient from CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95:5) to MeOH 100%. The fraction eluted with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (90:10), 119 mg, was filtered on a sep-pak diol column Waters using CH<sub>2</sub>Cl<sub>2</sub>-MeOH (80:20) to separate the material exclusively soluble in MeOH. The filtrate was concentrated then chromatographed by HPLC on a Lichrosorb diol 7 µm column using an isocratic solvent system: CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95:5), 5 ml/minute. The major peak

Table 1.  $^{13}\text{C}$  and  $^1\text{H}$  NMR chemical shifts and DEPT data of DEHP.

Carbon number	$^{13}\text{C}$ NMR <sup>a</sup> $\delta$ , ppm	Carbon type <sup>b</sup>	$^1\text{H}$ NMR <sup>a</sup> $\delta$ , ppm
1	9.91	$\text{CH}_3$	0.82, t, $J=5.3$ Hz
2	21.94	$\text{CH}_2$	1.15~1.30, m
3	22.79	$\text{CH}_2$	1.15~1.30, m
4	27.92	$\text{CH}_2$	1.15~1.30, m
5	37.78	CH	1.59, m
6	67.02	$\text{CH}_2$	4.13, m
7	166.58	OC=O	—
8	29.39	$\text{CH}_2$	1.31, dq, $J=4.3$ Hz
9	12.96	$\text{CH}_3$	0.79, t, $J=4.3$ Hz
10	131.51	=C quat	—
11	129.79	=CH	7.60, dd, $J=6.3\sim 2.2$ Hz
12	127.75	=CH	7.40, dd, $J=6.3\sim 2.2$ Hz

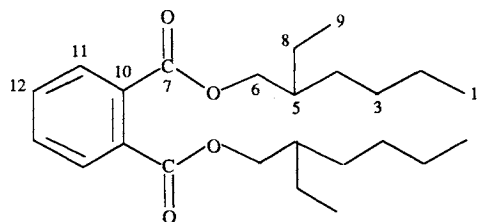
<sup>a</sup> 400 MHz in  $\text{CDCl}_3$ .<sup>b</sup> Based on  $^{13}\text{C}$  DEPT.

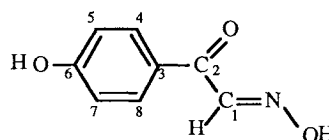
Fig. 3. Bis(2-ethylhexyl) phthalate (DEHP).

Table 2.  $^{13}\text{C}$  and  $^1\text{H}$  NMR chemical shifts and DEPT data of PHBA.

Carbon number	$^{13}\text{C}$ NMR <sup>a</sup> $\delta$ , ppm	Carbon type <sup>b</sup>	$^1\text{H}$ NMR <sup>c</sup> $\delta$ , ppm
1	151.13	=CH	8.08, s
2	191.28	C=O	—
3	131.59	=C quat	—
4	136.28	=CH	8.06, d, $J=2.2$ Hz
5	118.73	=CH	6.89, d, $J=2.2$ Hz
6	166.79	=COH quat	5.06, br

<sup>a</sup> 50 MHz in  $\text{CDCl}_3$ .<sup>b</sup> Based on  $^{13}\text{C}$  DEPT.<sup>c</sup> 50 MHz in  $\text{CD}_3\text{OD}$ .

Fig. 4. 2-(4-Hydroxyphenyl)-2-oxoacetaldehyde oxime (PHBA).



identified with Refractive Index and UV diode array detectors were collected at Rt 5 minutes. The material was concentrated to dryness and 40 mg of PHBA was obtained after repetitive HPLC injections. UV  $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2\text{-MeOH } 5\%}$  nm 241, 311; IR  $\nu_{\text{max}}^{\text{KBr}}$   $\text{cm}^{-1}$  3219,

1602, 1589, 1244. Eight mg of PHBA was recrystallized from  $\text{CH}_2\text{Cl}_2$ -MeOH (50:50) and used for the X-ray experiments. PHBA (mp  $162^\circ\text{C}$ ) was preferably soluble in MeOH and gave a single spot at Rf 0.20 with  $\text{CH}_2\text{Cl}_2$ -MeOH (95:5) on HPTLC diol. During the purification procedure, PHBA was identified by a characteristic yellow spot obtained on HPTLC by spraying Ninhydrine, heating at  $110^\circ\text{C}$  (5 minutes) then spraying of  $\text{SbCl}_5$  in  $\text{CHCl}_3$ . That coloration is probably due to the presence of the aldoxime function. The molecular formula of PHBA was established as  $\text{C}_8\text{H}_7\text{NO}_3$  by using high-resolution electron impact (HREI)-MS ( $\text{M}^+$ :  $m/z$  165.04314; Anal Calcd: 165.04259) related to the fragmentation  $(\text{M}-\text{CHNOH})^+$  ( $m/z$  121.02879 for  $\text{C}_7\text{H}_5\text{O}_2$ ; Anal Calcd: 121.02895) and confirmed by CI-MS (isobutane)  $m/z$  (%) 166 (M+1, 100), 148 (39), 139 (27), 121 (84); and by EI-MS  $m/z$  (%) 165 ( $\text{M}^+$ , 14), 159 (6), 121 (47), 93 (27), 86 (34), 69 (22), 55 (18), 43 (100).  $^1\text{H}$  NMR study confirmed the presence of an aromatic ring substituted at the *para* position with two aromatic protons at  $\delta$  6.89 and 8.06 ( $J=2.2$  Hz). Only six signals were identified in  $^{13}\text{C}$  NMR with a C=O ( $\delta$  191.3), an ethylenic carbon ( $\delta$  151.1) and the phenolic ring as described in Table 2 and in accordance with the MS study ( $m/z$  121). The aldoxime function with a proton signal expected at  $\delta$  12.5 in  $^1\text{H}$  NMR (not observed in  $\text{CD}_3\text{OD}$ ) was attributed to the carbon at  $\delta$  151.1 in  $^{13}\text{C}$  NMR. The structure established as illustrated in Fig. 4 has been confirmed by the X-ray diffraction analysis.

#### X-Ray Diffraction Analysis of PHBA

A crystal of  $0.22 \times 0.40 \times 0.40$  mm of PHBA was selected for the analysis. Crystal data:  $\text{C}_8\text{H}_7\text{NO}_3$ ,

H<sub>2</sub>O:  $M_r=183.2$ , monoclinic,  $P2_1/n$ ,  $a=6.815(1)$ ,  $b=7.382(1)$ ,  $c=16.464(2)$  Å,  $\beta=94.27(1)^\circ$ ,  $V=826.0(3)$  Å<sup>3</sup>,  $Z=4$ ,  $D_x=1.473$  Mg·m<sup>-3</sup>,  $\lambda(\text{MoK}\alpha)=0.70926$  Å,  $\mu=1.12$  cm<sup>-1</sup>,  $F(000)=384$ ,  $T=294$  K, final  $R=0.028$  for 815 observations.

The sample was studied on an automatic diffractometer CAD4 ENRAF-NONIUS with graphite monochromatized MoK $\alpha$  radiation. The cell parameters were obtained by fitting a set of 25 high-theta reflections. The data collection ( $2\theta_{\text{max}}=50^\circ$ , scan  $\omega/2\theta=1$ ,  $t_{\text{max}}=60$  s, range HKL: H 0~8 K 0~8 L 19~19, intensity controls without appreciable decay (0.2%) gives 1717 reflections of which 815 with  $I>3\sigma(I)$ ).

After Lorentz and polarization corrections the structure was solved by Direct Methods which reveal all the non hydrogen atoms of the molecule. After a scale factor refinement and a Fourier Difference map, a water molecule was found. After isotropic ( $R=0.10$ ) then anisotropic refinement ( $R=0.065$ ), the hydrogen atoms were found from a Fourier Difference (between 0.35 and 0.12 e Å<sup>-3</sup>). The whole structure was refined by the full-matrix least-square techniques (use of  $F$  magnitude;  $x, y, z, \beta_{ij}$  for N, O and C atoms and  $x, y, z$  for H atoms; 146 variables and 815 observations;  $w=1/\sigma(F_o)^2=\{\sigma^2(I)+(0.04F_o^2)\}^{-1/2}$ ) with the resulting  $R=0.029$ ,  $R_w=0.028$  and  $S_w=0.84$  (residual  $\Delta\rho\leq 0.11$  e Å<sup>-3</sup>).

Atomic scattering factors were obtained from International Tables for X-Ray Crystallography (1974). All calculations were performed on a Digital Micro VAX 3100 computer with the MOLEN package (DELFT: ENRAF-NONIUS Molecular structure determination package; MolEN Version 1990. Enraf-Nonius, Delft, The Netherlands, 1990).

The configuration of the natural molecule was so established as (*E*)-*s-cis* PHBA illustrated in Fig. 5.

#### Biological Activity

Crude extract 2 (see Fig. 2) had inhibitory activity against Gram-positive bacteria and phytopathogenic fungi but not against moulds, yeasts nor Gram-negative bacteria. This activity was found to occur at the second purification stage in fractions 3 and 3 A, but pure PHBA was not active at 50 ng/ml. Compared to 6-mercaptopurine ( $IC_{50}<1$  µg/ml), PHBA was found to be not cytotoxic ( $IC_{50}>10$  µg/ml) but have an antiproliferative activity (10 µg/ml) against all the treated strains.

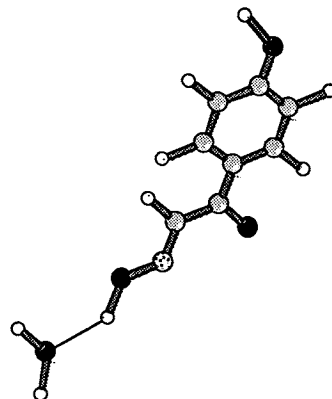
#### Discussion

DEHP was found to be inactive against *Staphylococcus aureus* at 100 µg per disc. This compound is a cell aggregation factor and it is used in mitochondrial oxidative phosphorylation studies as nongenotoxic carcinogen compound<sup>7</sup>.

DEHP were first extracted from the yellow sugar<sup>8</sup>) and have previously been isolated from a *Streptomyces* sp. with a low yield: 2.3% of the ethanolic extract<sup>1</sup>.

In the present work, *Penicillium olsonii* were found to produce DEHP with a high yield representing 23% of the crude EtOAc extract.

Fig. 5. Absolute configuration of natural PHBA.



PHBA produced by *Penicillium olsonii* in the (*E*)-*s-cis* configuration.

PHBA was not active at 50  $\mu\text{g}$  per disc against *Staphylococcus aureus*. So, PHBA was only responsible for a small part of the biological activity detected in the crude EtOAc extract at 100  $\mu\text{g}$ , and unidentified minors metabolites of *Penicillium olsonii* must be involved.

PHBA is an  $\alpha$ -oxoaldoxime obtained in its two isomeric forms (*E*)-*s-cis* and (*E*)-*s-trans* from the corresponding ketone. In this work, PHBA as a natural product from *Penicillium* was obtained in the (*E*)-*s-cis* configuration which is the most active isomer when tested as phosphorylated cholinesterase reactivator to treat poisoning by organophosphates<sup>2,9)</sup>.

Oximes are infrequently found in marine natural product chemistry except for the bastadin, a brominated compound isolated from the marine sponge *Ianthella basta*<sup>10)</sup>.

On the other hand, DEHP and PHBA were never reported from *Penicillium* species. The detection of these two specific metabolites does not allow a correlation between *P. olsonii* and *P. brevicompactum*. Morphologically, *P. olsonii* related to *P. brevicompactum*<sup>11)</sup> although they differ in the complexity of their penicilli<sup>12)</sup>. These two species have been reported to give very similar enzyme electrophoresis pattern<sup>13)</sup> but *P. brevicompactum* produces known compounds such as mycophenolic acid, Raistrick phenols and brevianamide A<sup>12)</sup>.

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