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Microbial decyanation of 1-benzylpyrrolidine-2,5-dicarbonitrile. Mechanistic investigations

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ABSTRACT

Various bacterial and fungal strains were screened for their ability to catalyse the regioselective hydrolysis of 1-benzylpyrrolidine-2,5-dicarbonitrile (**1**). Among the examined strains, *Rhodococcus opacus* sp-lma whole cells transformed both isomers of **1** into 1-benzyl-5-cyano-2-pyrrolidinone (**2**) and *N*-benzylacetamide (**3**). These reactions are difficult to achieve chemically and the synthesis of compound **2** did not compete with microbiological catalysis in terms of efficiency and respect for the guidelines of green chemistry. To distinguish between an oxidative or hydrolytic based-mechanism, the origin of the oxygen atom in **2** was investigated by using $^{18}O_2$ and $^{18}OH_2$ coupled with GC–MS analysis. These experiments confirmed that the oxygen atom in **2** came from water and not from molecular oxygen. The reaction is probably initiated by the dehydrogenation of **1** to generate the iminium ion, which could be trapped by a water molecule to form the cyanohydrin. The cyanohydrin intermediate would spontaneously break down to the γ-lactam product **2**. Conversion of **1** to **2** by induced rat liver microsomes suggests the involvement of a Cyt P-450-type enzyme. A mechanism that accounts for the formation of **3** is also proposed.

1. Introduction

Nitriles are an important group of chemicals with an industrial and environmental significance. They are used as solvents (e.g., acetonitrile), in the fibre and plastic industry (e.g., Nylon and Acrylic) and as pesticides (e.g., Casoron, Bentrol and Bucril). A number of nitriles have been reported to be potent carcinogenic, neurotoxic and environmental pollutants [1,2]. Soil, air and water contaminated with nitriles require remediation [3], and bio-remediation represents a credible alternative to chemical processes.

The enzymatic conversion of nitriles [4] proceeds via different pathways, as summarised in Fig. 1. Pathway **I** corresponds to a direct hydrolysis of a nitrile to the carboxylic acid by a nitrilase [5]. In **II** and **III**, a nitrile is converted by a nitrile hydratase to the corresponding amide, which is subsequently converted to the carboxylic acid by an amidase [6]. The step IV involves the conversion of an aldehyde to hydroxynitrile, that can be further hydrolysed to the corresponding hydroxyacid or hydroxyamide by a nitrilase [7]. In **V**, a nitrogenase catalyses the release of nitrogen to convert the CN to a CH_3 group [8].

The efficiency and selectivity of these enzymes has driven organic chemists to consider their use in biocatalysis to enantio- and regio-selectively access chiral nitriles, carboxylic acids or amides [9,10].

In the search for an environmentally benign and effective method for a regioselective hydrolysis of 1-benzylpyrrolidine-2,5-dicarbonitrile (**1**), we screened various fungi and bacteria. The results confirmed the capacity of *Rhodococcus* strains to degrade nitriles [11] (*R. rhodocrous* ATCC 21197, *R. erythrophilus* DCL14 and *Rhodococcus opacus* sp-lma). This catalysis involves an unusual enzymatic decyanation mechanism consisting of the conversion of the cyanopyrrolidine ring to the corresponding imminium ion, witch leads to the obtained compounds after a series of rearrangement.

2. Experimental

2.1. Chemicals and analytical techniques

1-Benzylpyrrolidine-2,5-dicarbonitrile (**1**) was kindly provided by ORIL-Industrie (Servier-Group, Bolbec, France). Solvents and compounds were obtained from Carlo Erba, and Sigma–Aldrich and were of the highest grade required by their use. Formic acid (98–100%) was obtained from Merck, Germany. Dioxygen $^{18}O_2$

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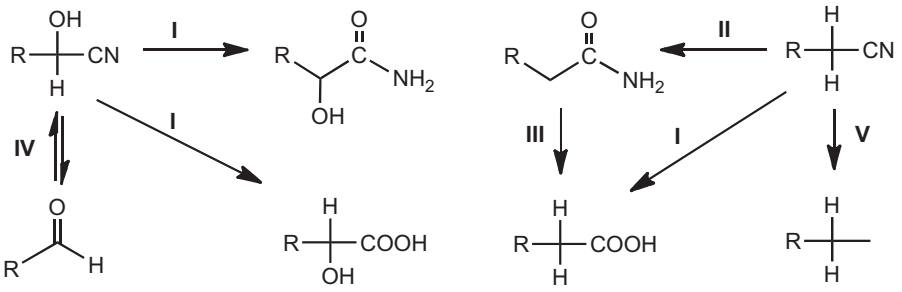

Fig. 1. Enzymatic conversions of nitriles.

(99 atom% ^{18}O) was purchased from Cortecnet, France. $^{18}OH_2$ (97%) was purchased from Cambridge Isotope Laboratories, UK. Bactotryptone, yeast extract and malt extract were purchased from Difco, and soya peptone was obtained from Organotechnie. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on Bruker spectrometers, specifically an Avance 300 NMR or 500 NMR (300 and 500 MHz, respectively).

2.2. Gas chromatography–mass spectrometry (GC–MS) assays

GC–MS data were obtained on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 μm analytical column (HP-5). The capillary column was directly coupled to a mass spectrometer (HP 5972-MS). The analyses were performed under the following conditions: (a) the injector and transfer line were set at 190 °C and 280 °C, respectively; (b) the temperature program was set at 90 °C (2 min) to 230 °C (2 min) at 8 °C min^{-1}; (c) the injection volume was 1 μL; (d) the split ratio was 1:30; (e) the carrier gas flow was 25 cm s^{-1} He; (f) the mass spectra were recorded at 70 eV. Prior to injection, all samples (800 μL) were extracted with ethyl acetate (600 μL), agitated on a vortex mixer and centrifuged at 14,000 rpm for 10 min.The supernatant (300 μL) was diluted with ethyl acetate (800 μL), and the solution (200 μL) was transferred to a GC vial and analysed by GC–MS. Under these conditions, the *m/z* values were 184 [$M^{\bullet+}$−CN] for **1a** and **1b**, 173 [$M^{\bullet+}$−CN] for **2**, and 149 [$M^{\bullet+}$] for **3**.

2.3. Microorganisms and culture conditions

Microorganisms were stored (at 4 °C) on agar slants containing the media reported below supplemented with agar at 20 g L^{-1}. A screen involving 7 fungi and 6 bacteria was conducted. The fungi examined were *Aspergillus ochraceus* ATCC 1009, *Rhizopus arrhizus* ATCC 11145, *Geotrichum candidum* CBS 23376, *Cunningamella echinulata* NRRL 3655, *Curvularia lunata* NRRL 2380, *Fusarium oxysporum* AP68MLPPON and *Beauvaria bassina* ATCC 7159. The fungi were grown at 27 °C on a rotary shaker (200 rpm) in a medium with the following composition (per litre): 0.5 g KH_2PO_4, 1 g K_2HPO_4, 30 g D-glucose, 10 g corn steep, 0.5 g $MgSO_4$, 2 g $NaNO_3$, 0.5 g KCl and 0.02 g $FeSO_4$. The bacteria investigated were *Rhodococcus rhodocrous* ATCC 21197, *Rhodococcus erythrophilus* DCL14, *Pseudomonas putida* CIP.59.19, *Agrobacterium tumefaciens* C58, *Bacillus licheniformis* CIP.52.71.T, and *Rhodococcus opacus* sp-lma and were isolated in our laboratory. Bacteria were cultivated on classical media (LB and YMS).

The culture media were sterilised at 120 °C for 20 min. The microorganisms were cultivated at 30 °C for 72 h in an orbital shaker (200 rpm). Biomass was recovered by centrifugation (4100 rpm, 30 min, 10 °C) and used immediately.

2.4. Bioconversion conditions

Incubations were performed with resting cells. Fresh biomass (2 g) was added to Erlenmeyer flasks (125 mL) containing 1-benzylpyrrolidine-2,5-dicarbonitrile (**1**) (1 mg mL^{-1}) and water (25 mL). The substrate was added as a solution in DMF (0.5%, v/v). Control experiments were also conducted with substrate or biomass alone. The mixtures were stirred in a rotary shaker (30 °C, 150 rpm). The progress of the biotransformation was monitored by GC as previously described.

The preparative incubation was conducted in 500 mL of water containing 1-benzylpyrrolidine-2,5-dicarbonitrile (200 mg) and *Rhodococcus opacus* sp-lma biomass grown on LB medium (20 g wet weight). The reaction was monitored for 5 days. After this time, the biomass was removed by centrifugation (4500 rpm, 20 min), and the supernatant was extracted with AcOEt (3 × 500 mL). The organic layers that contained the target compounds were evaporated under reduced pressure. The residue (160 mg) was purified by flash chromatography on silica gel (60% ethyl acetate: 40% heptane) to give 43 mg of **2** and 36 mg of **3**.

1-Benzyl-5-cyano-2-pyrrolidinone **2**, ^1H NMR (CD_3OD, 300 MHz) δ 7.40–7.28 (m, 5H), 4.93 (d, *J* = 15 Hz, 1H), 4.45 (q, 1H), 4.18 (d, *J* = 15 Hz, 1H), 2.70–2.26 (m, 4H); ^{13}C NMR (CD_3OD, 75.5 MHz): δ 176.7, 136.5, 130.2, 130, 129.5, 129.4, 129.3, 118.8, 49.3, 46.6, 30.2, 24.6; ESI-MS *m/z*: 201 $[M+H]^+$; HRMS calcd for $C_{12}H_{12}N_2O$ $[(M+H)^+]$ 201.1028, found 201.1031; 2D NMR experiments (COSY, HMBC, HSQC) were recorded on 500 MHz and confirms the structure of **2**.

N-Benzylacetamide **3**, ^1H NMR ($CDCl_3$, 300 MHz,) δ 7.35–7.21 (m, 5H), 4.36 (s, 2H), 1.99 (s, 3H,); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.2, 140.1, 129.7, 129.6, 128.7, 128.6, 128.4, 44.4, 22.5; ESI-MS *m/z*: 150 $[M+H]^+$.

2.5. ^{18}O and $^{18}OH_2$ incubations

Rhodococcus opacus sp. was grown in an LB medium (300 mL), harvested and resuspended in H_2O (9 mL). The cell suspension (2 mL) was transferred to 10 mL-vials, which were subsequently sealed with a septum. Air was replaced with argon using three vacuum-purge cycles. The headspace of the vial was then evacuated and replaced with $^{16}O_2$ or pure dioxygen enriched with $^{18}O_2$ (99 atom% ^{18}O). One of the vials was maintained under an argon atmosphere. In a second series of experiments, the cell suspension (1 mL) was transferred to Eppendorf tubes and centrifuged at 14,000 rpm for 5 min. The supernatant was removed, and the biomass was transferred to a 5 mL-vial and resuspended in water enriched with ^{18}O (97 atom% ^{18}O). A control experiment was conducted using the same conditions with unlabelled water. Finally, 10 micromoles of substrate were added by an injection of 5 μL of a solution in DMF (final concentration 1.15 g L^{-1}).

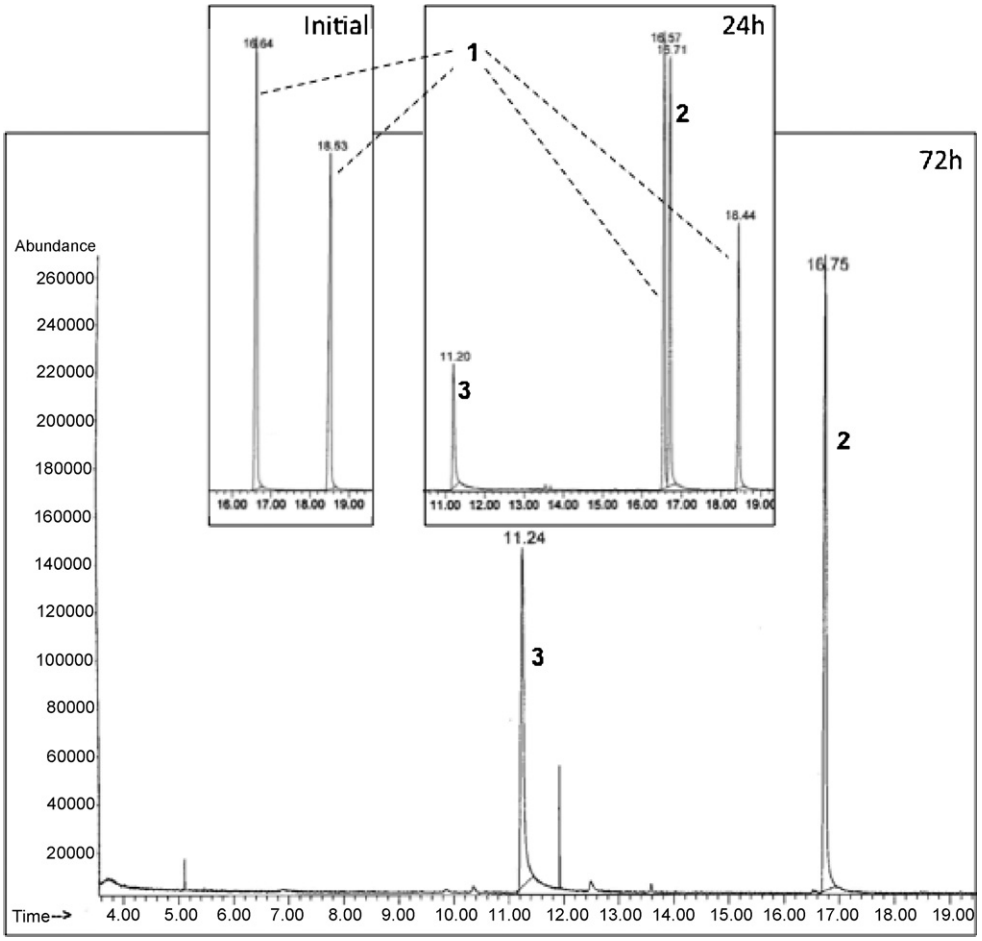

Fig. 2. GC–MS data were obtained on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 μm analytical column (HP-5) coupled to a mass spectrometer (HP 5972-MS). Prior to injection, all samples (800 μL) were extracted with ethyl acetate (200 μL), and then 1 μL of the extracted is injected on the GC column.

A series of experiments were conducted with lyophilised biomass. For this procedure, 0.1 g of dry biomass was resuspended in water enriched with ^{18}O (1 mL, 97 atom% ^{18}O). A solution of **1** in DMF (10 μL) was added to reach a final concentration of 1.15 g L^{-1}. A control experiment with unlabelled water was also conducted.

All the experiments were stirred in a rotary shaker (32 °C, 200 rpm). The progress of the biotransformation was monitored by GC–MS at 24, 48 and 96 h. A separate starting sealed vial was used for each data point to avoid any disturbance due to sample withdrawal.

2.6. Preparation of rat liver microsomes and microsomal incubations

Male Sprague–Dawley rats (175–200 g) were treated with dexamethasone (100 mg/kg, intra-peritoneal in corn oil) once per day for three days. The animals were then killed, and their liver microsomes were prepared according to standard methods [11]. The protein and cytochrome P-450 contents were measured using standard techniques [12].

A stock solution of **1** was prepared in DMSO. Compound **1** was incubated with rat liver microsomes in the presence of NADPH at 37 °C in 1 mL of 50 mM Tris–HCl buffer pH 7.4. The final concentrations of compound **1** and NADPH were 2.13 μM and 4.2 μM, respectively. Microsomes were added to reach a final concentration of 4 μM of cytochrome P-450. The incubations, including all required controls, were terminated after 2 h by the addition of ethyl acetate (500 μL) and extensive extraction. The organic extracts were concentrated to 100 μL, and then 1 μL of each was directly injected onto the GC–MS.

3. Results and discussion

Rhodococcus related strains convert the substrate **1** to two derivatives, as observed in the GC chromatograms. After a series of optimisations investigating the effects of wet biomass weight, medium (LB, YMS), temperature and pH, the strain *Rhodococcus opacus* sp-lma, was found to be the most effective and was able to completely convert the starting substrate in 3 days (Figs. 2 and 3). A preparative incubation was conducted to isolate sufficient amounts of the compounds to elucidate their structures. Thus both trans and cis isomers of **1** were converted to 1-benzyl-5-cyano-2-pyrrolidinone **2** and *N*-benzylacetamide **3**. All products were identified and characterised by Mass and NMR spectroscopy (^1H and ^{13}C, ^1H, ^1H-gCOSY, HSQC and gHMBC).

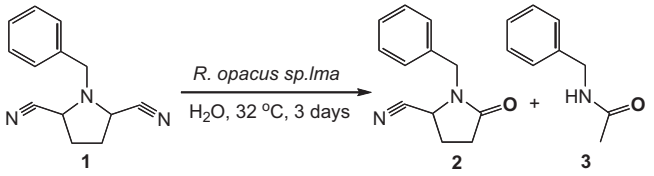

Fig. 3. Bioconversion products of 1-benzylpyrrolidine-2,5-dicarbonitrile **1** by *Rhodococcus opacus* sp-lma.

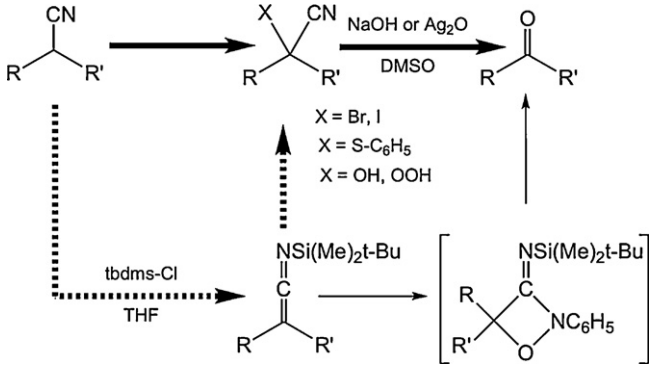

Fig. 4. Chemical decyanation routes.

This unexpected microbial decyanation route encouraged us to compare it to chemical reactions that achieve the same transformation. The catalysis observed with *R. opacus* resembles the oxidative decyanation of secondary nitriles to ketones by a three-step process involving the alpha-halogenation of the nitrile derivative followed by the substitution of the halogen by hydroxide, and the conversion of the resulting cyanohydrin to the corresponding ketone [12] (bold arrows in Fig. 4). In our study, this method proved unsuitable for the halogenation of compound **1**. Kyler and Watt [13] also reported the ineffectiveness of this method and compared five procedures on a large diversity of nitriles (Fig. 4). The authors concluded that none of these routes is universal, and they suggested testing all of them for each specific nitrile compound. Furthermore, these methods have been established for mononitriles, and there is no expectation of selectivity in the case of dinitriles. The alternative route (bold dashed arrows in Fig. 4) was applied to compound **1**; however, the halogenation step was not successful.

Besides this approach, methods for the direct oxidation of alpha-aminonitriles to the corresponding lactams with peracids [14] or through electron transfer photoinduced oxidation [15] were investigated and were effective for acyclic amino-nitriles. We have screened these approaches using a peracid (mCPBA) or a highly reactive oxygen species (hydroxyl radical generated by Fenton oxidation); both were unsuccessful and led only to degradation of the starting material. These results underscore the difficulty in achieving the conversion of **1** to **2** and the synthetic potential of the microbial alternative.

After encountering some initial difficulty with accessing compound **2** from **1** through chemical steps, we achieved the synthesis of **2** by applying previously reported classical reactions [16–19] (Fig. 5). Although this synthesis offered access to **2**, the overall yield was limited to 12% (instead of 22% in non optimized biocatalytic condition), and the reaction catalysts and conditions did not compete with microbiological catalysis in terms of efficiency and respect for the guidelines of green chemistry.

To elucidate the mechanism of this reaction and to discriminate between hydroxylation or deshydrogenation step, we carried out incubation experiments with $^{18}O_2$ and $^{18}OH_2$ under different conditions. The bioconversion and incorporation of the ^{18}O label were monitored using GC–MS analysis. Among the various conditions screened, only the combination of air and $^{18}OH_2$ led to 50% incorporation of ^{18}O in **2** and **3**. No ^{18}O incorporation was observed with $^{18}O_2$, indicating that molecular oxygen is not involved in the reaction. As the oxygen atom in **2** seems to be derived from $^{18}OH_2$, the water contained in the wet biomass may account for the incomplete incorporation of the isotopic label. Therefore, an equivalent incubation was performed with a corresponding quantity of lyophilised biomass. In support of our hypothesis, the use of lyophilized biomass led to 86% and 75% of ^{18}O incorporation in **2** and **3**, respectively. Based on these observations, a mechanism of decyanation of **1** is proposed in Fig. 6. Dehydrogenation of **1** leads to the iminium-carbonium derivative. The carbocation could be trapped by a water molecule to form the cyanohydrin intermediate, that spontaneously breaks down to the γ-lactam product **2**.

Incubation monitoring showed that compound **3** is formed simultaneously with and not subsequently to compound **2**. The incubation of **2**, isolated from previous experiments, with R. opacus did not lead to compound **3**, nor did it form any other derivatives. From day 3 to day 7, the concentration of compound **2** remained constant, whereas compound **3** was further degraded to compounds that were not detected by GC. After one week of incubation, compound **2** was isolated in almost pure form in 40% yield after biomass removal and extraction in ethyl acetate.

Our results obtained with $^{18}OH_2$ and ^{18}O contrast with the proposed mechanism of degradation of pyrrolidines and piperidines by microorganisms [20]. The first step leading to the lactam is probably the formation of an iminium ion rather than monooxgenation. The formation of the benzyl-acetamide **3** could be due to a rearrangement according to Fig. 7.

To obtain more support for the proposed mechanism, we searched the literature for enzymes that may catalyse the conversion of the pyrrolidine ring to the iminium derivative. Ho and Castagnoli [21] reported the mammalian metabolism of benzylpyrrolidines by liver microsome preparations in the presence and absence of a cyanide ion. In the presence of a cyanide ion, the authors identified a cyanohydrin derivative by mass spectrometry

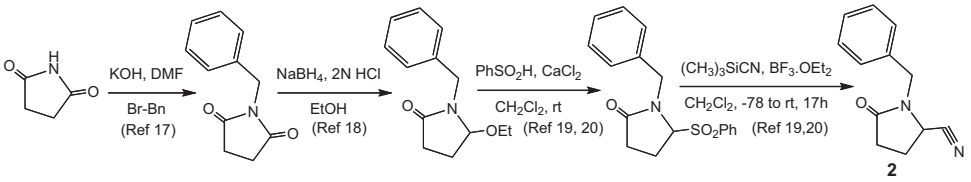

Fig. 5. Synthesis of the 1-benzyl-5-cyano-2-pyrrolidinone 2, according to reported procedures.

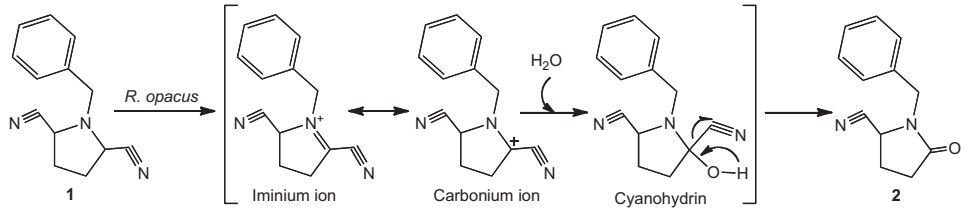

Fig. 6. Proposed mechanism of microbial decyanation supported by the $^{18}O_2$ and $^{18}OH_2$ experiments.

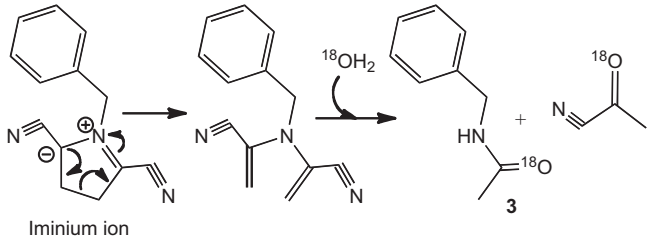

Fig. 7. Proposed mechanism for the formation of compound **3**.

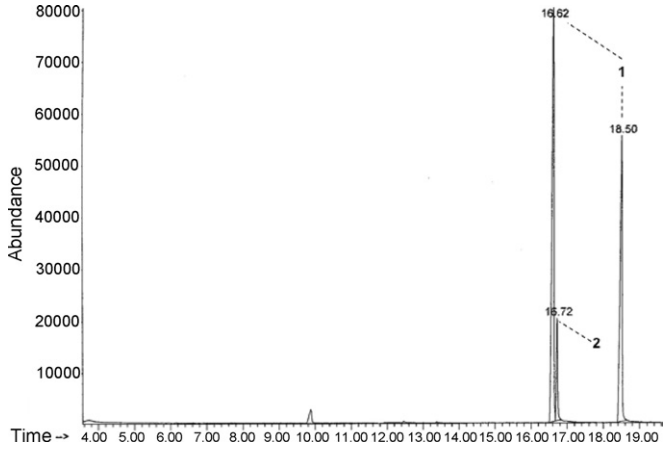

Fig. 8. GC–MS analysis of the incubation of **1** with induced rat liver microsomes.

and implicated microsomal oxidative enzymes such as cytochrome P-450 in its formation. Gorrod and Aislaitner [22] showed that the mammalian toxicity of alicyclic amines is due to the formation of reactive iminium intermediates. Yang and co-workers [23] developed a method to screen for toxic iminium ion formation based on cyanide trapping and analysis by LC–MS–MS. They confirmed that the conversion of alicyclic amines to iminium ions is catalysed by Cyt P-450 type enzymes.

According to these reports, we performed experiments with rat liver microsomes induced with dexamethasone to increase the level of Cyt P-450 [24,25]. Incubation of compound **1** with microsomes for 2 h led to the formation of 7% of compound **2**, as confirmed by GC–MS analysis (Fig. 8), whereas compound **3** was not formed. In the control experiments depleted in NADPH or microsomes, only the starting substrate was detected.

This result strongly supports the involvement of Cyt P-450 type enzymes in the conversion of compound **1** by *Rhodococcus opacus*. This type of enzyme is well-represented in *Rhodococcus* genera and accounts for a variety of oxidative reactions [11,26].

In conclusion, *Rhodococcus opacus* is able to catalyse the conversion of aminonitrile **1** to the corresponding lactam **2** efficiently. Access to compound **2**, is difficult or impossible to achieve by direct oxidation, hydrolysis or independent chemical synthesis. Rat liver microsomes, known to express large quantities of Cyt P-450, are able to catalyse this reaction. This result suggests that this type of enzyme, highly represented in *Rhodococcus genera*, is involved in the conversion of **1** to the iminium intermediate. This reaction will be extended to other aminonitriles to further demonstrate its usefulness as a "green" alternative to harsh chemical procedures for accomplishing the same transformation.

Acknowledgments

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