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ORIGINAL PAPER

Selective activity of *Mucor plumbeus* reductase towards (−)-camphorquinone

Giovanni Gontijo de Souza · Cleber Paulo Andrada Anconi · Sjef Cornelissen · Wagner Batista De Almeida · Hélio Ferreira Dos Santos · Isabel Cristina Pereira Fortes · Jacqueline Aparecida Takahashi

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Abstract The biotransformation of 1*R*-(−)-camphorquinone, achieved by growing cells of four fungi species isolated from soil (*Mucor plumbeus, Lecanicillium muscarium, Thamnostylum* sp. and *Syncephalastrum racemosum*), was investigated in optimized culture media for each species. Fungi were grown aerobically under shaking and their activities with respect to camphorquinone were monitored for 20 days by gas chromatography coupled to mass spectrometry (GCMS). Camphorquinone was found to be stable in control flasks throughout the experiment. The most interesting results were found for *M. plumbeus*, which was only able to perform monoreduction of camphorquinone when cultivated on a glucose–peptone–yeast extract medium. Large-scale experiments were set up and the camphorquinone biotransformation products formed by *M. plumbeus* were purified by column chromatography and identified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Theoretical calculations were employed as a complementary technique to unambiguously identify the biotransformation products. These findings suggest that *M. plumbeus* could be of great use for the selective reduction of camphorquinone and related compounds.

G. G. de Souza · C. P. A. Anconi · S. Cornelissen · W. B. De Almeida · I. C. P. Fortes · J. A. Takahashi (✉)
Departamento de Química, ICEx,
Universidade Federal de Minas Gerais (UFMG),
Campus Universitário, Pampulha, Belo Horizonte,
MG 31270-901, Brazil
e-mail: jat@qui.ufmg.br

C. P. A. Anconi · H. F. Dos Santos
Departamento de Química, ICE,
Universidade Federal de Juiz de Fora (UFJF),
Campus Universitário, Martelos, Juiz de Fora,
MG 36036-330, Brazil

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Introduction

The use of whole microorganisms and enzymes in synthetic chemistry, a process commonly referred to as biotransformation, has shown great applicability mainly due to the enantio- and regioselectivities of biocatalysts, which are often unparalleled in classical chemical reactions. The use of biocatalysts is particularly attractive for the production of fine chemicals such as pharmaceuticals, agrochemicals and chiral molecules [1, 2]. This methodology also has a favorable ecological impact, since reactions using biocatalysts are conducted under mild reaction conditions [3–5] and generate very low volumes of toxic residues for disposal compared to some synthetic procedures. Since biotransformations are usually carried out in water, it is not necessary to use anhydrous solvents—a very common and time-consuming procedure that is used in synthetic chemistry.

Terpene compounds are ubiquitous in nature and are often used as fragrances, flavors or as prototypes of biologically useful substances. They are the subject of many research works because of their importance in metabolism regulation in both plants and animals [6]. Biotransformations have been extensively used to make stereo- and regiospecific modifications of natural occurring terpenes in order to either improve their biological activities or to prepare building blocks and enantiomerically pure derivatives that can be used as intermediate molecules in the synthesis of more complex compounds [7, 8]. In the last decade, one-third of all papers published on terpene production or transformations have made use of fungi [9].

Camphor is a monoterpene that is frequently used as chiral starting material, building block or ligand for a number of asymmetric syntheses [10, 11]. The importance of camphor as a natural source of chirality in asymmetric synthesis is especially reliant on its versatility, since functionalities can be chemically inserted all over its structure [12]. Camphorquinone is a camphor-related compound that can also be selectively reduced to afford many structurally diverse compounds for use in organic syntheses of more complex, biologically active compounds. Camphorquinone reduction can be accomplished by chemical and biotechnological means, the latter being of increasing interest since it offers a different level of selectivity and more environmentally friendly processes.

The selective reduction of the monoterpene camphorquinone is an interesting target for biotransformations, since it is possible to achieve, in many cases, the reduction of only one carbonyl group. This reaction has been widely studied and leads to different and interesting outcomes. Miyazawa and coworkers [13] reported the recovery of six keto alcohols from the biotransformation of (−)- and (+)-camphorquinone (**1** and **2**, respectively) by five fungal species (*Aspergillus niger*, *Fusarium solani*, *Glomerella cingulata*, *Mucor mucedo* and *Rhizoctonia solani*). On the other hand, experiments using *Glomerella cingulata* led to a diastereoisomeric mixture of eight keto alcohols, two of which were dihydroxylated derivatives [14]. When racemic camphorquinone was fed to *Nicotiana tabacum*, selective reduction into three keto alcohols was achieved.

In this article we report the biotransformation of 1*R*-(−)-camphorquinone by *Mucor plumbeus, Lecanicillium muscarium, Thamnostylum* sp. and *Syncephalastrum racemosum.* Experimental characterization techniques and ab initio theoretical calculations have been employed in order to unambiguously identify the products. It was found that *Mucor plumbeus* was the most interesting fungus since its activity with respect to camphorquinone is stereoselective, a feature that can be further exploited for synthetic purposes.

Materials and methods

General

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (Rheinstetten, Germany) DX-200 (200 MHz) Avance spectrometer using tetramethylsilane (TMS) as internal reference. Deuterated chloroform ($CDCl_3$), purchased from Aldrich (Milwaukee, WI, USA), was used as solvent. Gas chromatography coupled to mass spectroscopy using electronic impact (GC–MS EI) spectra were obtained on Shimadzu (Kyoto, Japan) GC-17A/QP-5050 GC-MS equipment using a HP5 silica column containing 5% phenylmethylsiloxane and 95% dimethylsiloxane (25 × 0.2 × 0.33 μm). An aliquot of 1 μL of the sample was injected in split mode (1:10) using helium as carrier gas (0.6 mL/min). The injector temperature was 230°C and the detector temperature was 250°C. The initial temperature was kept at 50°C for 4 min and then raised at a rate of 10°C/min up to the final temperature of 240°C, which was maintained for 4 min. Mass spectra were obtained by EI using the scan mode operating at 70 eV. A quadrupole mass analyzer was used. Data was acquired using Chemstation software. All reagents and solvents were of analytical or chromatographic grade, as purchased from Merck (Darmstadt, Germany) or Grupo Química (São Paulo, Brazil). Silica gel 60 (Merck article 7734) and 60G (Merck article 7731) were used for chromatographic columns and thin-layer chromatography (TLC), respectively. 1*R*-(−)-Camphorquinone was obtained from Aldrich.

Cultivation of fungal species and screening

Mucor plumbeus, Lecanicillium muscarium, Thamnostylum sp. and *Syncephalastrum racemosum*, previously isolated from soil [15], were obtained from the LaBB Collection (UFMG, Brazil) and maintained on potato dextrose agar at 8°C. For the biotransformation experiments, they were transferred to a conical flask containing 200 mL of liquid medium A consisting of (g/L): glucose (1 unit), bacteriological peptone (0.5) and yeast extract (0.5), and medium B consisting of (g/L): glucose (5 units), potassium phosphate monobasic (2), manganase sulfate monohydrate (2), potassium chloride (1), glycine (2) and 2 mL of a trace element solution [16]. Two control (positive and negative) and four experiment conical flasks were prepared for each fungal species. All media were sterilized in an autoclave at 121°C for 15 min before inoculation. Culture media components were purchased from Merck and Biobrás (Montes Claros, Brazil).

Upon fungal growth, 3 mg of camphorquinone in chloroform (1 mg/mL) were added to each conical flask. Two control experiments were run in parallel: one where no camphorquinone was added (cell control or negative control), and a second one containing only the liquid media and camphorquinone (substrate control or positive control) in order to monitor for the presence of endogenous fungal compounds and the stability of camphorquinone in the media, respectively. All flasks were subjected to orbital shake (110 rpm) at 25°C. Every five days, one experimental flask was taken out for each fungus species; the broth was filtered and then extracted three times with 30 mL of dichloromethane. The organic layer was dried at 40°C; the residue was weighed and kept in a sealed flask at 8°C until GC analysis.

Preparative biotransformation conditions and product description

In large-scale experiment, *M. plumbeus* was grown at room temperature on an orbital shaker (180 rpm) for 48 h in a conical flask containing 100 mL of sterilized medium A. This inoculum was used to inoculate, under sterile conditions, 24 erlenmeyer flasks (500 mL) each containing 100 mL of the medium. After three days, 240 mg of compound **1** were dissolved in 24 mL of chloroform and divided equally, under sterile conditions, between the 24 erlenmeyer flasks containing the fully grown fungus. The biotransformation reaction was followed by TLC (hexane:ethylacetate 3:1) using the starting material as the reference compound. The reaction was stopped after five days by adding 30 mL of ethyl acetate to each flask. After overnight extraction under orbital shaking, the broth was collected using vacuum filtration and extracted twice using 250 mL of ethyl acetate. The fungal mycelium was also extracted using 300 mL of ethyl acetate. Broth and mycelium extracts were combined and dried over anhydrous Na_2SO_4. The salt was removed by vacuum filtration and the solvent was evaporated at 35°C under reduced pressure using a rotator evaporator. The residue (238.3 mg) was subjected to silica gel column chromatography using a hexane/ethylacetate/methanol gradient with increasing polarity. After successive chromatography on silica gel columns, 25.4 mg of a pure white crystalline solid, seen as a single spot on TLC (hexane/ethyl acetate 4:1), were obtained. The solid was identified by ^{1}H and ^{13}C NMR spectra with the aid of theoretical evaluations and on the basis of a literature data comparison [17, 18] as a mixture of three isomers of hydroxycamphor: 2α-hydroxycamphor (**9**, 51%), 3α-hydroxycamphor (**11**, 28%) and 3β-hydroxycamphor (**10**, 21%).

Compound **1**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.91 (3H, s, H-10), 1.05 (3H, s, H-8), 1.09 (3H, s, H-9), 1.54–2.27 (4H, m, H-5 and H-6), 2.61 (1H, d, $J = 5.2$ Hz, H-4). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 8.9 (C-10), 17.6 (C-9), 21.2 (C-8), 22.4 and 30.1 (C-5 and C-6), 42.7 (C-7), 58.1 (C-4), 58.8 (C-1), 203.0 and 205.0 (C-2 and C-3).

Compounds **9, 10** and **11**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.88 (3H, s, H-10), 0.93–1.05 (6H, m, H-8 and H-9), 1.25–2.10 (4H, m, H-5 and H-6), 2.27 (1H, d, $J = 4.6$ Hz, H-4), 3.01 (1H, b, H-2 or H-3), 3.75 (1H, s, H-3 of **10**), 3.86 (1H, s, H-2 of **9**), 4.21 (1H, d, $J = 5$ Hz, H-3 of **11**). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 9.21 and 9.52 (C-10 of **10** and **11**), 13.2 (C-10 of **9**), 20.1 and 21.0 (C-8 and C-9 of **10**), 25.2 and 28.6 (C-5 and C-6 of **10**), 49.2 (C-7 of **10**), 74.8 (C-3 of **11**), 79.2 (C-2 of **9**), 219 (C-3 of **9**), 219 (C-2 **10** and **11**).

Theoretical calculations

The four possible structures of the α-keto alcohols derived from 1*R*-(−)-camphorquinone were obtained through full geometry optimization, without any geometrical or symmetry constraints, using the density functional theory (DFT) level (BLYP functional) with the 6-311 + G(2d, p) basis set [19, 20]. In addition, NMR shielding tensors for the four optimized α-keto alcohols and TMS were evaluated at the same level of calculation (BLYP/6-311 + G(2d, p)) in order to determine ^{1}H and ^{13}C NMR shifts. Harmonic frequency calculations were also carried out for all structures, characterizing them as true minima on the potential energy surface (PES) (all frequencies are real). All calculations were performed at the Laboratório de Química Computacional e Modelagem Molecular (LQC-MM), Departamento de Química, ICEx, UFMG, using the Gaussian 03 [21] quantum mechanical software package.

Results and discussion

For feeding experiments, freshly isolated *M. plumbeus, L. muscarium, Thamnostylum* sp. and *S. racemosum* were used. *L. muscarium* showed slow growth on medium A and was therefore only screened on medium B. On the other hand, the other fungal species presented better growth in medium A. In medium B, growth was scarce and stopped upon camphorquinone addition, so experiments using medium B were only performed for *L. muscarium*. Under screening conditions, the species showed different activity patterns in relation to the camphorquinone molecule, which remained stable in control experiments (it did not undergo degradation during the monitored period: 20 days). The formation of a metabolite with *m/z* 168 (a monohydroxylated camphorquinone derivative) was observed by GC–MS after 15 days of incubation of camphorquinone with *L. muscarium*. However, it was further metabolized since its presence was not detected five days later when the experiment was again screened. *Thamnostylum* sp. showed high but unspecific activity towards camphorquinone. A series of metabolites were detected each time the experiment was screened, but no monohydroxylated metabolites (*m/z* 168) were detected by GC–MS. Camphorquinone was detected unchanged up to ten days after the beginning of the incubation period using this species. In contrast, *S. racemosum* was completely inert towards the camphorquinone molecule, which was recovered unaltered at nearly the same concentration (considering the amount initially fed) throughout the 20 days of incubation. Therefore, none of these three species, *L. muscarium, Thamnostylum* sp. and *S. racemosum* showed, under the studied screening conditions, synthetic utility.

In contrast to the other species, *Mucor plumbeus* presented a good biotransformation pattern with respect to camphorquinone. A peak with a retention time of 15.70 min (*m/z* 168) was observed across the experiment

period from the first monitoring day (i.e., after five days of reaction), which was related to the formation of a monohydroxylated camphorquinone derivative, as elucidated from the mass spectrum.

According to the mass spectral analysis, none of the organisms used, including *M. plumbeus*, reduced both ketone groups in the same molecule, a result that would be expected using common chemical reducing reagents. Figure 1 shows all of the camphorquinone reduction products that could potentially be obtained from (−)- and (+)-camphorquinone (**1** and **2**, respectively).

Considering that four different monoreduced products that possess a molecular ion peak at *m*/*z* 168 (**8**–**11**) can be formed from 1*R*-(−)-camphorquinone (**1**), a preparative-scale experiment was set up in order to isolate and identify the *M. plumbeus* monohydroxylated biotransformation product(s). A higher amount of camphorquinone (10 mg/flask) was used in order to get enough product to enable NMR identification. After flash column chromatography, a single TLC spot of product that gave a peak at *m*/*z* 168 was analyzed by ^{1}H and ^{13}C NMR. Theoretical NMR calculations and literature data were also used for correct identification [17, 18]. Therefore, it was possible to identify the product as actually a mixture of three isomers: 2α-hydroxycamphor (**9**, 51%), 3α-hydroxycamphor (**11**, 28%), and 3β-hydroxycamphor (**10**, 21%). Relative yields were determined on the basis of the ^{1}H NMR peak areas of H-2 or H-3 hydrogen signals at δ_H 3.86 (1H, s, H-2 of compound **9**), 3.75 (1H, s, H-3 of compound **10**) and 4.21 (1H, d, $J = 5$ Hz, H-3 of compound **11**). ^{1}H NMR signals expected around δ_H 3.54 corresponding to H-2 for derivative **8** were not present in the spectrum. The corresponding ^{1}H and ^{13}C NMR chemical shifts, evaluated theoretically, are shown in Table 1 for the isomers **8**, **9**, **10** and **11**. It can

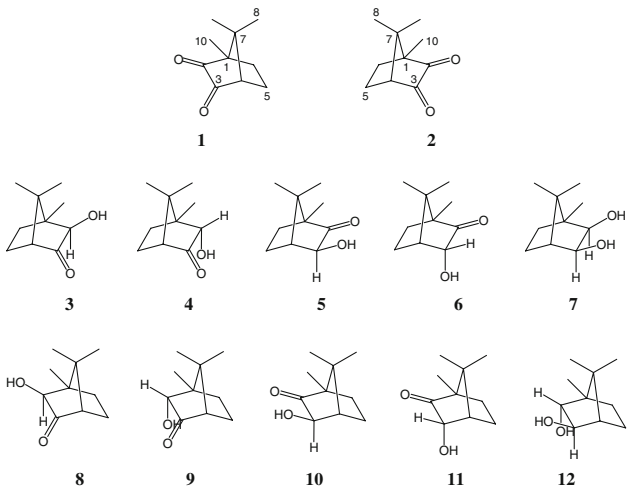

Fig. 1 Structures of 1*R*-(−)-camphorquinone (**1**), 1*S*-(+)-camphorquinone (**2**), and reduced derivatives **3**–**12**

Table 1 ^{13}C NMR chemical shifts for the compounds **8**, **9**, **10** and **11** evaluated at the B3LYP/6-311 + G(2d,p) level of theory. Some experimental values are quoted in parentheses

	Compound			
	8	**9**	**10**	**11**
H2	3.5	3.9 (3.9)	_	_
H3	_	_	3.7 (3.75)	4.2 (4.2)
C1	56.0	57.6	63.6	65.3
C2	85.8	84.7 (79.2)	229.9 (219.0)	230.4 (219.0)
C3	227.9	228.4 (219.0)	83.4	80.9 (74.8)
C4	64.7	65.6	54.7	55.0
C5	24.5	29.5	29.4 (25.2)	21.0
C6	38.2	28.7	32.8 (28.6)	37.7
C7	54.7	51.5	54.9 (49.2)	50.8
C8	20.5	19.6	21.5 (20.1)	20.1
C9	22.7	20.6	22.7 (21.0)	21.5
C10	11.1	14.6 (13.2)	10.5 (9.5)	10.5 (9.2)

be seen from Table 1 that the theoretical values slightly overestimate the ^{13}C NMR chemical shifts. However, these values can still be employed as a reference in future investigations.

Mucor has been shown to be a genus that is very useful for selective structural modification of camphorquinone-related compounds. Miyazawa and coworkers [13] reported that another *Mucor* species, *M. mucedo*, also performed a selective single carbonyl group reduction on that molecule. Nonetheless, the major derivative produced by that species was 3α-hydroxycamphor (**11**) instead of 2α-hydroxycamphor (**9**), the major product formed using *M. plumbeus*. In that study, *M. mucedo* was the only species that was found to be able to produce compound **11** as the major camphorquinone biotransformation product, while three other fungi (*Fusarium solani*, *Glomerela cingulata* and *Rhizoctonia solani*) produced, preferentially, compound **10** [13].

The most interesting aspect of the activity of *M. plumbeus* towards camphorquinone is related to this reaction stereoselectivity. The formation of compound **8** implies a position possessing high hindrance at the active site of the enzyme positioned close to carbon 1, and so compounds **8** and **9** should be the least preferentially formed compounds when chemical means are used. The results show that specific substrate–microorganism interactions are more likely to happen when this fungus is used rather than a broad-action mechanism, showing the great versatility of fungal species that can be exploited in order to achieve chemical modifications at different sites of a given molecule.

In this work, 1*R*-(−)-camphorquinone was reduced by *Mucor plumbeus* to generate a mixture of 2α-, 3α- and 3β-hydroxycamphor isomers. Ab initio theoretical results were employed in order to unambiguously identify the products of

the biotransformation studied, stressing the utility of theoretical studies for complementing experimental spectroscopic analysis. Several reports have extensively described the reduction of camphorquinones by organisms, including plant-cultured cells [17], cyanobacteria [22] and fungi (including *Mucor mucedo* [13, 14] but not *M. plumbeus*). Furthermore, the ratios in which the products were formed here are different from those reported in the mentioned literature, indicating a different stereoselectivity of the carbonyl reductase in *Mucor plumbeus* that can be further exploited, since camphorquinone has been extensively used as a starting material for the synthesis of more complex compounds.

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