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Bioreduction of aldehydes and ketones using *Manihot* species

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

Biocatalysis constitutes an important tool in organic synthesis, especially for the preparation of chiral molecules of biological interest. A series of aliphatic and aromatic aldehydes and two ketones were reduced using plant cell preparations from *Manihot esculenta* and *Manihot dulcis* roots. The reduced products were typically obtained in excellent yields (80–96%), and with excellent enantiomeric excess (94–98%), except for vanillin. Esters, a nitrile, and an amide were also examined, but were not reduced. Preliminary conversion rate studies are reported. This is the first attempt to perform the biotransformation of carbonyl compounds using *Manihot* species.

Keywords: *Manihot esculenta*; *Manihot dulcis*; Euphorbiaceae; Biocatalytic reduction; Aliphatic and aromatic aldehydes and ketones; Ester hydrolysis

1. Introduction

In the past decades, biocatalysis has undergone significant development, and a number of reactions have been introduced and optimized, especially for the synthesis of chiral molecules of industrial and biological interest, such as drugs (Luna, 2004; Sharma et al., 2005), cosmetics (Veit, 2004), and other industrial chemicals (Liu et al., 2004). Several studies have demonstrated the high versatility, efficiency, selectivity, and the important chemical aspects, such as regioselectivity, chemoselectivity, and enantioselectivity of reactions involving biocatalysis (Castro and Knubovets, 2003; Shaw et al., 2003). One of the most important reactions in organic synthesis, and a key step in the manufacture of numerous pharmaceutical and specialty chemicals is the reduction of an aldehyde or a ketone to an alcohol, frequently in the presence of other reducible groups. Additionally, in certain instances, it may be critically important that the reduction product be chiral. Biocatalysis has been studied for the conduct of this reaction (Kroutil et al., 2004), and there are several reports where such reductions with yeasts (Mahima et al., 1993; Anderson et al., 1995; Toneva et al., 2002) and *Geotrichum candidum* (Matsuda et al., 2003) have led to high yields of product and excellent enantioselectivity.

In recent years, plant cell cultures and whole plant cells, in addition to microorganisms and enzymes, have been studied as potential agents for biotransformation reactions. Yadav et al. have investigated the function of *Daucus carota* roots for the asymmetric reduction of aliphatic and aromatic ketones, cyclic ketones, β-ketoesters, and azidoketones in aqueous medium (Yadav et al., 2002a,b). More recently, Comasseto et al. have explored the potential of *Daucus carota* roots for the preparation of chiral organochalcogeno-α-methylbenzyl alcohols (Comasseto et al., 2004). Enantioselective hydrolysis of racemic acetates in appreciable yield has also been conducted using comminuted tissues

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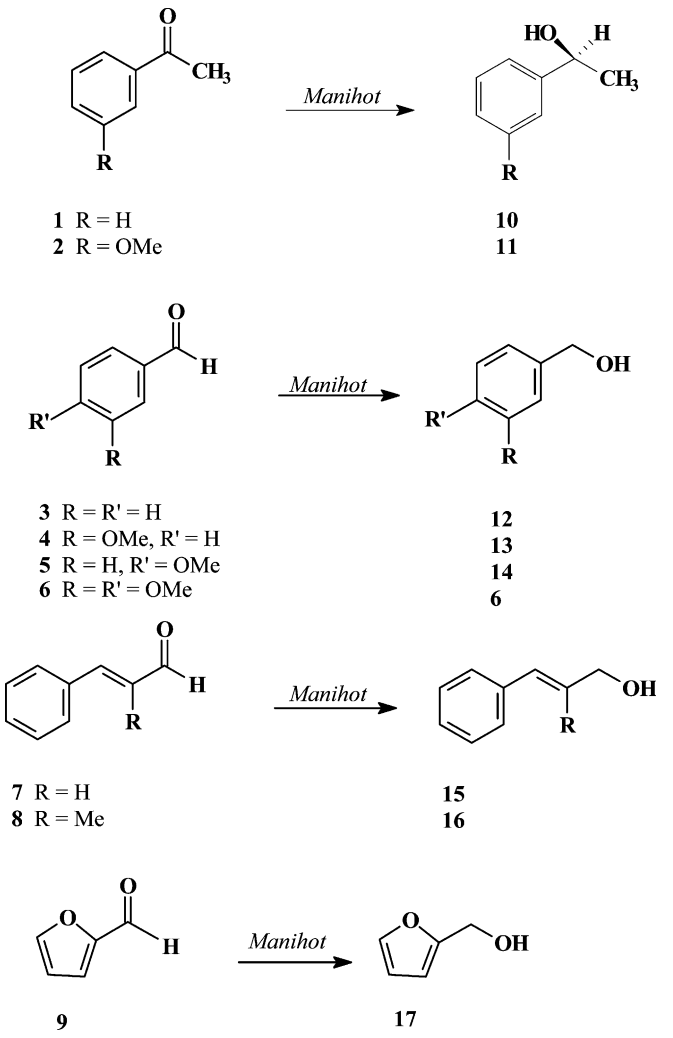
from ripe vegetable roots (Maczka and Mironowicz, 2002). Thus, it is possible that a wide range of plant materials, roots, tubers, seeds, and fruits from the Brazilian northeast used for nutritional and medicinal purposes could be a valuable source of preparations for a variety of biocatalytic reactions.

As part of an evaluation process for the discovery of chemical reactions catalyzed by the roots of local plants, the efficacy of two regional tubers were investigated as potential enzyme sources for the bioreduction of several aromatic aldehydes and ketones to their corresponding alcohols. In particular, this report describes a preliminary exploration of the potential of the roots of two local tubers *Manihot esculenta* Crantz and *Manihot dulcis* (J.F. Gemel) Pax (Euphorbiaceae) to serve as biocatalysts. The roots of *M. esculenta* and *M. dulcis* are valuable for their contemporary economic importance; both are tropical roots used widely in a large variety of traditional food preparations, especially in Brazilian northeast, Latin America, Africa, and Asia (Braga, 1976). In the process of the manufacture of the flour of *M. dulcis,* popularly known as mandioca, several thousand liters of water called "manipueira" are discarded. The potential use as a surfactant has previously been identified (Nitschke et al., 2004). Hydroxynitrile lyases catalyze the stereoselective addition of hydrocyanic acid to aldehydes and ketones (Schmidt and Griengl, 1999), and one of these, from *Manihot esculenta*, which provides an *S*-configured product, has been cloned and over-expressed for potential industrial application (Johnson and Griengl, 1999). The encouraging results obtained here using aqueous extracts for biocatalysis may offer new possibilities for the reduction of selected carbonyl compounds as a critical step in a synthetic organic pathway; and specifically avoiding the use of non-sustainable, hydride reducing agents.



Scheme 1.

2. Results and discussion

As a part of an evaluation process to find reductases from common vegetables, a series of plants used as food in the Brazilian northeast were evaluated, using acetophenone **1** (Scheme 1) as the substrate and a known methodology (Yadav et al., 2002a; Souza, 2003) using selected vegetable species (Table 1). The crude reaction mixtures were initially analyzed by TLC observing the formation of alcohol **10**, which was visualized practically in all of the tested materials. The samples were then analyzed by IR to observe the presence of OH stretching. Finally, the samples were analyzed by ^{1}H NMR spectroscopy to obtain a semi-quantitative measure of the respective yields using the integration area of the methyl ketone singlet at δ_H 2.61, compared to the quartet in the secondary alcohol product **2** δ_H 4.80 (Table 2). The results of this analysis for the selected species are reported in Table 1. The experiments, as well as the ^{1}H NMR spectroscopic analysis were performed in duplicate and the results reflect an arithmetic average.

Table 1
Relative percentage yield using the ^{1}H NMR integration of the methyl group of acetophenone **1** compared with integration of the carbinolic hydrogen of alcohol **10**

Plant material	Yield of **1** (%)	Yield of **10** (%)
Solanum melongena L.	57.8	42.1
Manihot dulcis Crantz	16.2	85.5
Manihot esculenta Crantz	10.7	89.3
Daucus carota L.	53.7	46.3
Colocasia esculenta (L.) Schott	44.2	55.8
Ipomoea batatas (L.) Lam	57.4	42.6

According to the data, the two *Manihot* species yielded the product alcohol in highest yields. Consequently, these *Manihot* species were selected for further development. The initial experiment was to determine the total protein in the enzymatic systems using the Hartree method (Hartree, 1972). For both species, the total protein was about 0.7%, in accordance with literature values of about 1% (Nassar, 1986). A series of simple aromatic aldehydes and ketones (**1**–**9**, Scheme 1) was treated with the freshly cut roots of *M. esculenta* and *M. dulcis* in aqueous solution

Table 2
^{1}H NMR signals used in the integration of selected protons of compounds **1–9**, **18–22**, and **25–27**, compared with integration of the product alcohols **10–17** and **30–37** using *Manihot* species

Reagent/ product	δ_H	
	–C*H*O (**3–9**); –C*H*$_3$ (**1**, **2**, **18–22**), *o*-H-Ph (**26**)	–C*H*ROH (**10–17**, **30–34**, **37**), *o*-H-Ph (**36**)
1/10	2.61, *s*	4.80, *q*
2/11	2.54, *s*	4.87, *q*
3/12	9.61, *s*	4.61, *s*
4/13	9.71, *s*	4.63, *s*
5/14	9.74, *s*	4.60, *s*
7/15	9.42, *s*	4.63, *s*
8/16	9.76, *d*	4.32, *d*
9/17	9.50, *s*	4.10, *s*
18/30	2.20, *m*	4.33, *q*
19/31	1.81, *m*	4.11, *q*
20/32	2.05, *m*	4.28, *q*
21/33	2.51–2.35, *m*	4.32, *m*
22/34	2.47–2.67, *dd*	4.31, *m*
25/35	NC	NC
26/36	8.04, *d*	8.09, *d*
27/37	3.22, *s*	2.23–2.26, *d*

NC: not conclusive.

Table 3
Relative percentage yields of the reduced products of **1–9**, **18–22**, and **27** and the hydrolysis of **25** and **26** using ^{1}H NMR and GC

Product	Bioconversion (%) ^{1}H NMR/GC		ee %[a]	
	M. esculenta	*M. dulcis*	*M. esculenta*	*M. dulcis*
10	84.0/80.0	87.0/83.0	>95.0	>97.0
11	88.0/85.0	89.4/87.0	>94.0	>96.0
12	90.5/91.0	85.2/83.0		
13	100.0/87.6	98.6/94.0		
14	100.0/86.0	100.0/94.5		
15	100.0/94.0	100.0/96.5		
16	94.6/80.0	100.0/85.5		
17	95.1/90.0	93.1/90.0		
30	100.0/97.5	100.0/96.7	>93.0	>96.0
31	100.0/92.3	100.0/93.4		
32	100.0/97.3	100.0/91.7		
33	19.2/12.6	13.8/14.9	>96.0	>98.0
34	13.4/11.5	14.1/12.5	>96.0	>97.0
35	NC/94.1	NC/91.5		
36	30.2/35.3	48.5/35.5		
37	70.4/79.1	99.2/95.4	>97.0	>98.0

NC = not conclusive.
[a] ee: Enantiomeric excess.

at room temperature for 3 days (Machado et al., 2005). The crude reaction mixtures were initially visualized by TLC using the vanillin spray reagent, followed by ^{1}H NMR spectroscopic analysis. The integration of the methyl ketone singlets of ketones **1** and **2** or the aldehydic proton of aldehydes **3–9** were compared with the carbinolic proton resonances of **10** and **11**, or the methylene protons of the primary alcohols of **12–17**. The integration was corrected for proton proportionality. These preliminary results led to a qualitative analysis of the crude reaction mixture using ^{1}H NMR spectroscopy, and the results of this analysis are summarized in Table 3. No significant differences were observed between the two species in their capacity to effect the reduction of the carbonyl group of the aromatic aldehydes or ketones. Vanillin **6** was unaffected by the crude enzyme system present in both of these species.

The reaction mixtures were also analyzed quantitatively by GC–MS and the results are presented in Table 3. The data confirm the preliminary results obtained from the ^{1}H NMR experiments, with little difference in accuracy. In the reduction process using the two *Manihot* species all of the tested aldehydes and ketones yielded alcohols in excellent yield and superior to the reported results for *Daucus carota* (Yadav et al., 2002) and comparable to those of *Zygosaccharomyces rouxii* (Anderson et al., 1995). In the latter case, biocatalytic reduction of a methyl benzyl ketone intermediate in the formation of the 5*H*-2,3-benzodiazepine proceeded in 96% yield and >99.9% ee. An exception was observed for vanillin **6** where only the starting material was obtained, as noted above.

Regioselectivity of the reaction using the two *Manihot* species was observed with the aldehydes **7** and **8**, where the reduction process occurred only at the carbonyl group, without reduction of the double bond (Cantello et al., 1994), a phenomenon observed previously (Van Duersen et al., 2004). Enantioselectivity was observed in the reduction of the ketones **1** and **2,** where the produced alcohols **10** and **11** were formed as the enantiomer with the "*S*" configuration, with ee values of 95% and 97% for *M. esculenta*, and of 94% and 96% for *M. dulcis*, respectively. The enantiomeric excess was measured using polarimetry and the application of an established formula (Carey and Sundberg, 1990).

Maximum bioconversion yield was obtained after 3 days, and was obtained by kinetic study of the selected compounds ketones **1** and **10**, aldehydes **3** and **7**, and ester **15**. The data are presented in graphs A and B (Fig. 1). The data shows the similar kinetic behavior for each of the *Manihot* species. As expected, aldehydes were more reactive than ketones, and benzaldehyde **3** was more reactive than cinnamaldehyde **7**. In addition, the hydrolysis reaction of ethyl butyrate **27** showed a faster reaction rate when compared with the bioreduction rates of ketones and aldehydes. For the ketones **1** and **18**, the bioconversion showed a progressive increase of rate until 48 h, after which it was possible to observe a more dramatic increase in rate until a maximum bioconversion yield was reached at 72 h. In summary, all of the tested compounds showed a maximum bioconversion yield at 72 h.

The enzymatic reaction was then extended to other types of carbonyl-containing compounds, including aliphatic, cyclic, α,β-unsaturated ketones in the steroidal skeleton, and simple carboxylic acid derivatives, including esters, a nitrile, and an amide. These reactions are represented in Scheme 2. The selected compounds having a ketone group were: hexan-3-one **18**, cyclopentanone **19**, cyclohexanone **20**, pulegone **21**, carvone **22**, sitoster-3-one **23**, and cholestan-3-one **24**. The results are shown in Table 3. Excellent yields were obtained with the aliphatic ketones **18** (97.5%;

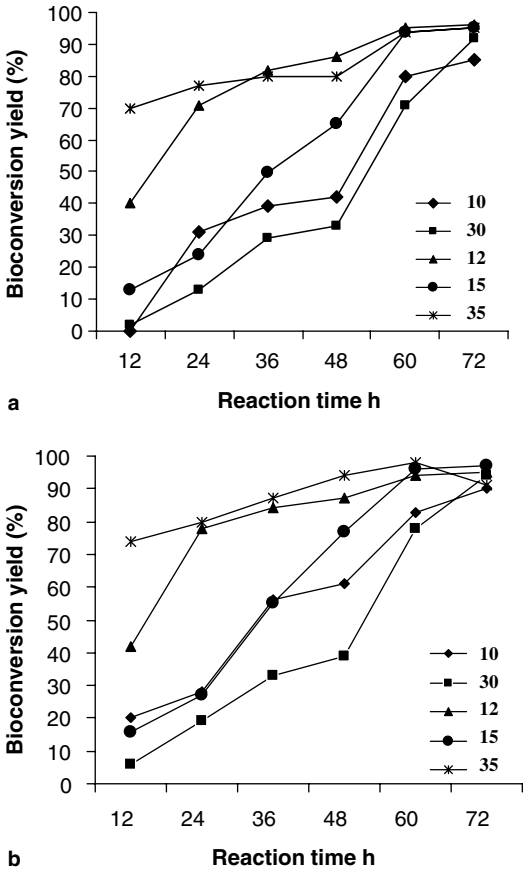
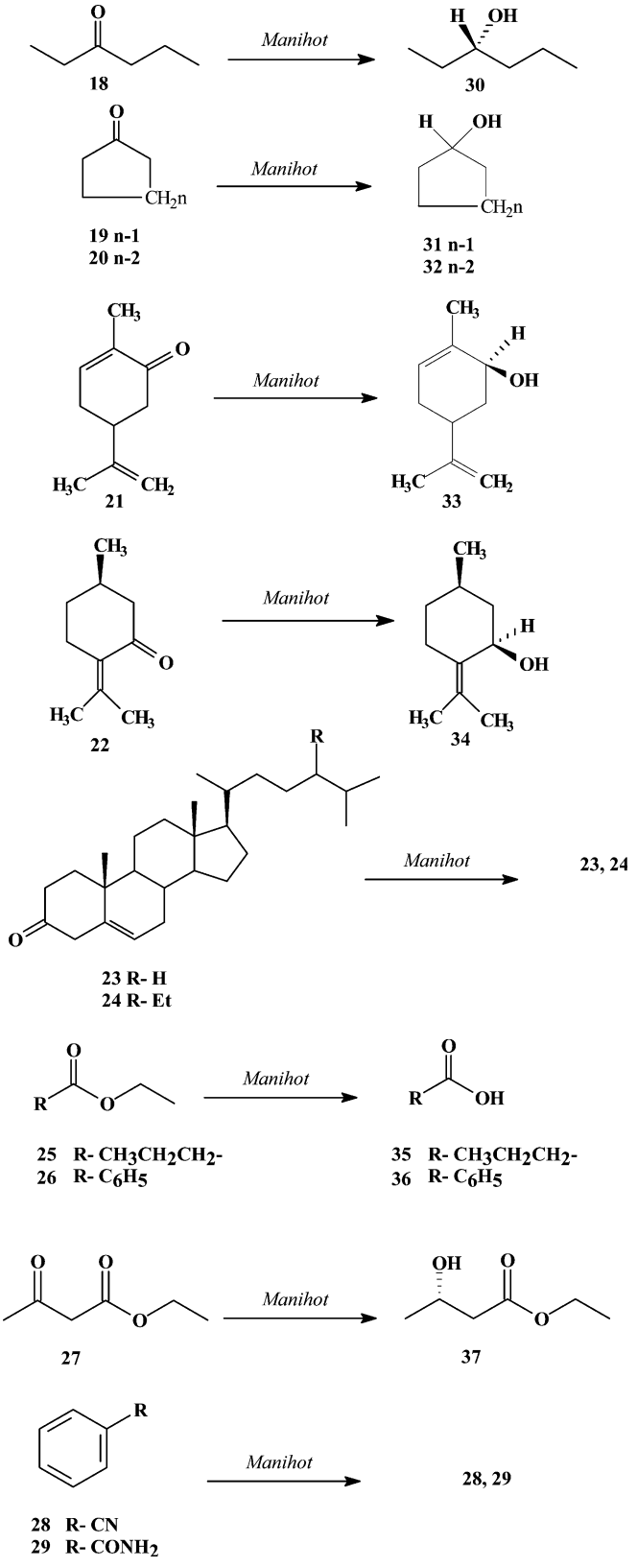


Fig. 1. Bioconversion of ketones and aldehydes: acetophenone **1**, hexan-3-one **18**, benzaldehyde **3** and cinnamaldehyde **7** to the corresponding alcohols (1-phenyl-ethanol **10**), hexan-3-ol **30**, benzyl alcohol **12**, cinnamyl alcohol **15**, and ethyl butyrate **25** to butyric acid **35** using *M. esculenta* (Graph A) and *M. dulcis* (Graph B).

96.7%), **19** (92.30%; 93.4%), and **20** (97.3%; 91.7%), but much lower yields were observed with α,β-unsaturated ketones such as **21** (12.6%; 14.9%), and **22** (11.5%; 12.5%). No reactivity was observed with the two steroidal ketones **23** and **24**. Products formed with the ketones **18**, **21**, **22** and **27** were the alcohols having the "*S*" configuration with an ee of 93/96% (**29**); 96/98% (**21**), and 96/97% (**22**) with the two *Manihot* species (*M. esculenta* and *M. dulcis*), respectively.

The next step was to observe the efficiency of the enzymatic system with other carbonyl groups. The selected compounds included two esters, one aliphatic, ethyl butyrate **25** and one aromatic, ethyl benzoate **26**, an aliphatic β-keto-ester **27**, together with benzonitrile **28** and benzamide **29**.

The products obtained for the two esters were the corresponding carboxylic acids, rather than the reduced alcohols. The enzyme ester hydrolysis by the *Manihot* species is quite effective with an aliphatic ester yielding butyric acid **25** (91.5%; 94.0%). However, it proceeds in low yield with an unsubstituted aromatic ester producing benzoic acid **26** (35.5%; 35.3%). Reaction mixtures were initially quantified by GC-MS, applying the conditions determined for



Scheme 2.

aromatic ketones and aldehydes, and the results are presented in Table 3.

The crude reaction mixtures obtained from the ketones **18**–**20** were also quantified using ^{1}H NMR spectroscopy.

The selected absorptions were the integration of the methylene protons in **18**–**20** compared with the methine proton in the obtained secondary alcohols **29**–**31**. For the two α,β-unsaturated ketones, carvone **21** and **22**, the adjacent methylene resonances were used in comparison with the allylic alcohol methine resonances in **32** and **33**. For compounds**23** and **24**, the selected hydrogen's were H_2 at C-4, alpha to carbonyl (C-3) compared with the CH attached at C-3 around δ_H 4.0–4.5. The crude reaction mixture obtained from ester **25/35** was not determined using this technique because of the overlap of signals in the enzyme hydrolysis product. The reaction mixture obtained from **26/36** was determined by integration, using ^{1}H NMR, of the proton *ortho* to the carbonyl (Table 2).

The *Manihot* reductase enzyme system was also evaluated for a compound, β-keto-ethyl-butyrate **27**, having two different carbonyl groups. Complete regio- and enantio-stereoselectivity was observed through the exclusive reduction of the keto group at C-3 yielding 3*S*(+)-hydroxy-ethyl-butyrate **37** (79.1/95.4% yield) as the major product showing ee values of 97% and 98%, for *M. esculenta* and *M. dulcis*, respectively. Starting material (20.8%) was also recovered from *M. esculenta*. For **27** and **37**, the absorptions of the CH_2 alpha to the ester carbonyl were chosen.

As a result, the *Manihot* reductase system can also be considered as a potential reactant for the hydrolysis of aliphatic systems, and to a lesser extent for aromatic esters. The other tested compounds were the derivatives benzamide **28**, seeking either the carboxylic acid or the amine, and benzonitrile **29** for the respective amine; neither compound was affected by this system, and only starting material was recovered. The selected hydrogen resonances for these two compounds were the 2H, *ortho* to the carbonyl of **28**, and the corresponding hydrogens *ortho* to the nitrile group in **29**.

The observed results using ^{1}H NMR spectroscopy to quantify the effects of the *Manihot* enzyme system on aliphatic ketones, steroidal ketones, esters, an amide, and a nitrile, using selected proton resonances are presented in Table 2. Likewise, for the second set of compounds **18**–**29** to yield products **30**–**37**, ^{1}H NMR spectroscopy was established to be a useful tool for the identification of the yielded products from the enzymatic reactions, and with comparable results to GC–MS. In conclusion, the two *Manihot* species were very effective in selectively reducing aromatic and aliphatic carbonyl groups showing substantial regio- and enantioselectivity in the products. Preliminary studies regarding the path of the reaction have been initiated. Thus the reaction of furfural **9** to the corresponding alcohol **17** has a lag period of approximately 24 h, and then by 36 h has proceeded to form the alcohol **17** in 84% yield, to 95% after 48 h, and to 100% after 72 h. Following this preliminary work, current studies are aimed at expanding the kinetic experiments to other substrates, the purification, stabilization, and cloning and expression of the reductase(s) from *Manihot* species, as well as exploring other plant systems for their potential for regio- and stereoselective synthesis in organic chemistry.

As a result of these and previous studies, it is apparent that an opportunity for developing a new area of synthetic organic chemistry has been established. Many researchers around the world have great difficulty acquiring common reagents for organic synthesis because of local import limitations and high prices. Reagents for stereospecific synthesis are frequently unobtainable. Utilizing the vast potential of natural sources, work in biocatalysis, through its simplicity, employing clean technology and low cost, may become a significant alternative approach for large-scale synthetic chemical transformations.

As Earth's resources are slowly depleted, so the cost of synthetic chemical reagents is expected to rise dramatically in the next few years, thus making synthetic drugs even more expensive. The "green chemistry" movement is, in part, a response to that economic reality, recognizing that the ability to deploy renewable, functionally selective reagents for organic chemical reactions, and also to induce chirality, will become an increasingly important aspect of the synthetic organic chemists' armamentarium (Cordell, 2000; Rathbone et al., 2003).

3. Experimental

3.1. General

IR spectra (film) were recorded using a Perkin–Elmer FT–IR 1000 spectrometer. The optical rotations were measured on a Perkin–Elmer 341 digital polarimeter and chloroform as solvent. The products obtained and the pure starting materials were analyzed by GC–MS on a Hewlett–Packard Model 5971 using a (5%-phenyl)-methylpolysiloxane DB-5 capillary column (30 m × 0.25 mm) with film thickness 0.1 μm; carrier gas helium, flow rate 1 mL/min with split mode. The injector temperature and detector temperature were 250 and 200 °C, respectively. The column temperature was programmed at 4 °C/min from 35 to 180 °C, and then at 10 °C/min from 180 to 250 °C. NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz) using $CDCl_3$ as solvent. Chemical shifts, given on the δ scale, were referenced to the residual, undeuterated portion of the deuterated $CHCl_3$ solvent (δ_H 7.27). Column chromatography was run using silica gel 60 (70-230 mesh, Vetec), while TLC was conducted on precoated silica gel polyester sheets (Kieselgel 60 F_{254}, 0.20 mm, Merck). Compounds were detected by spraying with vanillin/perchloric acid/EtOH solution, followed by heating at 120 °C. All of the aldehydes and ketones were purchased from Across Organics, SA.

3.2. Plant material

The fresh plant materials, including the roots of *Manihot esculenta* and *M. dulcis*, were purchased from a local

market and were identified by botanist Prof. Edson P. Nunes. Vouchers specimens numbers: *M. esculenta* 34.831 and *Manihot dulcis* 34.830 have been deposited at the Herbarium Prisco Bezerra of the Departamento de Biologia, Universidade Federal do Ceará, Brazil.

3.3. Bioreduction of compounds 1–9, 18–24 and hydrolysis of 25–29

3.3.1. Extraction and isolation

The *M. esculenta* and *M. dulcis* roots were rinsed with 5% sodium hypochlorite solution and sterile distilled H_2O, and cut into small pieces (approx. 1 cm long slice) with a sterile knife.

In separate experiments, substrates **1**–**9** (200 mg) were individually added to a suspension of the freshly cut roots of *M. esculenta* and *M. dulcis* roots (20 g) in H_2O (140 mL). The mixtures were incubated in a shaker (160 rpm) at room temperature for 72 h, and the reaction process was monitored by TLC. Each individual suspension was filtered, and the residue was washed with water. The aqueous solutions were then extracted with EtOAc (3×100 mL), and the organic phases were dried with Na_2SO_4 and evaporated under reduced pressure. The residues were filtered on a short silica gel column, using $CHCl_3$ as eluant, to afford the reduced products, for *M. dulcis* and *M. esculenta*, respectively, except for **6** as starting material, yielding: **10** (125 mg/128 mg); **11** (130 mg/135 mg); **12** (150 mg/152 mg); **13** (170 mg/162 mg); **14** (177 mg/170 mg); **6** (120 mg/115 mg); **15** (159 mg/155 mg); **16** (147 mg/140 mg); **17** (138 mg/134 mg); **30** (134 mg/148 mg); **31** (173 mg/131 mg); **32** (140 mg/135 mg); **33** (144 mg/171 mg); **34** (142 mg/ 138 mg); **23** (150 mg/150 mg); **24** (157 mg/159 mg); **35** (157 mg/154 mg); **36** (138 mg/135 mg); **37** (137 mg/140 mg), **28** (130 mg/125 mg); **29** (66 mg/70 mg). The products were analyzed by IR, GC–MS, and ^{1}H NMR spectral data in comparison with literature values (Pouchert, 1981; Pouchert and Behnke, 1993).

In the kinetics experiments, the same procedure was used as for the bioreduction, and samples were analyzed by GC with reaction times that varied from 12, 24, 36, 48, 60, to 72 h. Experiments were performed in duplicate.

Cholestan-3-one **23** and sitoster-3-one **24** were obtained from an oxidation process using PCC (Ma et al., 2005) and compounds **18**–**22** and **25**–**28** were purchased from Across Organics S.A. Cholestan-3-ol was obtained from bile stones of a patient, recrystallized with $CHCl_3$. *S*(-)1-phenyl-ethanol **10**, *S*(-)3-methoxy-phenyl-ethanol **11**, benzyl alcohol **12**, 4-methoxy-benzyl alcohol **13**, 3-methoxybenzyl alcohol **14**, cinnamyl alcohol **15**, 1-methyl-3-phenylallyl alcohol **16**, furfuryl alcohol **17**, *S*(−)-hexan-3-ol **29**, cyclopentanol **30**, cyclohexanol **31**, *S*(−)-carveol **32**, *R*(+)-pulegol **33**, cholestan-3-one **23**, sitoster-3-one **24**, butyric acid **34**, benzoic acid **35**, 3*S*(+)-hydroxy-ethyl-butyrate **37**, benzamide **28**, and benzonitrile **29** were fully characterized according to their spectral data (Pouchert, 1981; Pouchert and Behnke, 1993; Lee et al., 1996).

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