Ene-Reductase Catalyzed Regio- and Stereoselective 1,4-Mono-Reduction of Pseudoionone to Geranylacetone

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The regio- and stereoselective mono-reduction of a particular C=C bond of conjugated C=C double bonds is a very challenging task. Here the regio- and stereoselective 1,4-reduction of pseudoionone, an α,β,γ,δ-bisunsaturated ketone, was demonstrated to give geranylacetone, an industrially relevant molecule. OYE1 from Saccharomycetes pastorianus was identified as the most suitable biocatalyst for this reaction. Elevated substrate concentrations of up to 200 mM were tolerated allowing still to reach excellent conversions (>99% and 80% for 100 or 200 mM pseudoionone concentration, respectively). Interestingly, the organic cosolvent often required for substrate solubilization in aqueous buffer can be avoided for pseudoionone when using permeabilized E. coli cells containing the overexpressed enzyme instead of purified enzyme, reaching still >99% conversion at 100 mM (19.2 g/L) substrate concentration. Performing this reaction at a 0.5 g scale allowed to run the reaction to completion (>99%) and pure product was isolated with 80% yield. Finally, the stereoselective reduction of the (E,E)- over the (E,Z)-pseudoionone isomer was enabled by the ene-reductase from Zymomonas mobilis (NCR). Thus, both (E)-geranylacetone and (E,Z)-pseudoionone were obtained with isomeric excess above 60%.

Introduction

Geranylacetone (6,10-dimethylundeca-5,9-dien-2-one) is known for its rose/floral/green odor (Scheme 1).[1] Additionally, recent studies suggested that this compound could display potential antitrypanosomal and mosquito repellent properties.[2] Theoretically, this valuable compound could be obtained in a very straightforward fashion by reduction of the 3,4-double bond of pseudoionone. Actually, the (asymmetric) reduction of isolated C=C bonds is a widely employed reaction in organic synthesis.[3] The reduction may be achieved for instance by using metallo-organic complexes or organocatalysts, the latter at the expense of e.g. a nicotinamide-mimic (‘Hantzsch ester’) as reductant.[4]

While the unpredictably changing costs of precious metals as well as environmental concerns may disadvantage metallo-catalyst, the lower catalytic efficiency and/or poor atom economy of organocatalysts require alternatives. Especially, the chemo- and regioselective hydrogenation of a specific single C=C bond as part of conjugated alkenes or in conjugation with alkyne groups within a compound is a very challenging task to achieve with these methods. Only a few examples employing chiral rhodium-, copper- or palladium-complexes have been described.[5] Alternatively, biocatalysts may be considered due...

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Scheme 1. Ene-reductase catalyzed regioselective 1,4-reduction of pseudoionone [isomer mixture (E,E)/(E,Z), 60:40] to geranylacetone [isomer mixture (E)/(Z)].
to their high chemos-, regio- and stereoselectivity as well as their intrinsic environmental compatibility concerning biodegradation. For the reduction of electronically activated alkenes, flavin-dependent ene-reductases (EREDs) can be applied. A broad variety of successfully transformed substrates including α,β-unsaturated ketones/alddehydes/carboxylic acids and nitroalkanes has been reported. Despite the many papers published, studies describing the mono-reduction of conjugated double bonds in a regioselective fashion are scarce. Only five reports can be found in literature: namely (i) the use of *Penicillium citrinum* hyphae in a biphasic system, (ii) whole-cell biocatalysis with *Rhodotorula mucilaginosa* used as submerged culture, (iii) the utilization of F420-dependent reductases (FDRs) and EREDs belonging either (iv) to the MDR-superfamily like the ones from the fungus *Cyclopitys aegerita* or (v) the Old Yellow Enzyme (OYE) family. Among these, the first four describe the regioselective 1,4-reduction in a α,β,γ,δ-double bond conjugated system, while the fifth one showed the chemoselective reduction of α,β-unsaturated aldehydes with a conjugated C=C triple bond at the γ position.

Concerning the specific reduction of pseudoionone to geranylacetone, previous attempts to perform this reaction, using either chemical or biocatalytic methods, did not give satisfying results. For instance, the selective 1,4-hydration of pseudoionone using Raney-nickel was described by Meyer. Unfortunately, when this method was repeated, results showed that instead of the formation of geranylacetone, the main product was the 1,6-hydrogenated compound (data not published). This disagreement could be explained by the fact that the position of the double bond after the hydrogenation in the earlier study was postulated, but not proven for lack of appropriate analytical methods. Using microorganisms, an *in vivo* process involving *Rhodotorula mucilaginosa* demonstrated the formation of geranylacetone starting from pseudoionone. However, the desired product was obtained only after 14 days at low substrate loadings (120 mg/L) and with poor analytical yield (24%). Consequently, there is a need to achieve the regioselective mono-reduction (1,4- vs. 1,6-) of pseudoionone to geranylacetone at preparative relevant conditions (Scheme 1).

Results and Discussion

Evaluation of pseudoionone as substrate for EREDs

Performing an initial screening to evaluate the biocatalytic regioselective reduction of pseudoionone [isomer mixture (E,E)(E,Z), 60:40] to geranylacetone (Scheme 1 and Scheme S1), a set of ene-reductases was tested. The chosen EREDs included OYE1 from *Saccharomyces pastorianus*, OYE2 and OYE3 from *Saccharomyces cerevisiae*, 12-oxophytodienoate reductase isoenzyme OPR3 from *Lycopersicon esculentum*, cyclohexene reductase (NCR) from *Zymomonas mobilis*, estrogen binding protein EBP1 from *Candida albicans*, and the xeno-biotic reductases XenA and XenB from *Pseudomonas putida* and *Pseudomonas fluorescens*, respectively. Purified enzyme and stoichiometric amounts of cofactor were used for these initial experiments. From the enzymes tested, OYE1, OYE2, OYE3, NCR and EBP1 led to geranylacetone formation, while no pseudoionone was transformed when using OPR3, XenA or XenB (Table S1). It is worth to note, that under the conditions tested, no isomerization was observed, thus the ratio of (E,E) to (E,Z) did not change in the blank reactions nor in the experiments with enzymes which did not accept the substrate. Among the enzymes that accepted the substrate, OYE1–3 showed the highest GC conversions, ranging between 47 and 59%. In all the cases, the only product detected was the one resulting from the 1,4-selective mono-reduction, neither double reduction nor 1,6-reduction was detected. Interestingly, most enzymes displayed a preference towards (E,E)-pseudoionone over the (E,Z)-isomer (Table S1). The highest selectivity was found with NCR, which under these conditions transformed exclusively the (E,E)-isomer leading to the formation of (E)-geranylacetone only.

Freeze-dried *E. coli*/ERED, a simpler catalyst preparation

Employing lyophilized *E. coli* cells containing the recombinant protein may offer advantages over the use of the purified enzyme, such as circumventing tedious purification steps of the biocatalyst, which translates to easier access to the catalyst and lower cost of the overall process. Hence, pseudoionone reduction was tested utilizing lyophilized *E. coli* BL21(DE3) cells bearing the most promising EREDs, *i.e.* OYE1 and OYE2 (Figure S1A). In this experiment *E. coli*/OYE1 showed the best conversion (68%) (Table 1). Regardless of the *E. coli* preparation studied, the preferred substrate was again the (E,E)-isomer, and no side products were detected.

Reaction conditions

Due to the results described above, OYE1 was chosen as the best suitable biocatalyst for further identification of the reaction conditions to reach best conversion at a high initial substrate concentration.

Evaluation of the most suitable cofactor regeneration system

As ene-reductases in general rely on flavin mononucleotide and nicotinamide cofactors to perform the reduction, recycling of the cofactors is required to achieve an economic feasible process. Although several recycling systems for NAD(P)H, which then reduces the FMN cofactor, have been described, identifying the most suitable one must be done on a case-to-case basis. Consequently, several cofactor regeneration methods were tested (Table S2). Best conversions were achieved using glucose-6-phosphate dehydrogenase/glucose-6-phosphate (G6PDH/Gluc6P) using NADPH as cofactor (>99% geranylacetone formation). Similarly, recycling by GDH/glucose led to
conversions of pseudoionone in the range of 83–85% when using both NAD$^+$ or NADP$^+$ as cofactor (Table S2). NADH recycling using formate dehydrogenase (FDH) or alcohol dehydrogenase (ADH-A from Rhodococcus ruber) led to lower conversions ranging from 15 to 65%, depending on the amount of recycling enzyme (FDH) or the cosolvent used (ADH-A) (Table S2). It is noteworthy that carbonyl reduction was detected as side product (12–13%) in the case of ADH-A with 10% of isopropanol as cosubstrate/cosolvent and reducing agent. Since high conversions were observed in presence of GDH/NADH/glucose as well as G6PDH/NADPH/glucose-6-phosphate and given that NAD$^+$ and glucose are less expensive and more stable than their phosphorylated counterparts, GDH/glucose was chosen as the most suitable approach. Consequently, all further reactions were performed with this cofactor recycling system.

**Influence of the solvent**

The solubility of the substrate in the reaction medium is an important factor to be considered.$^{[22]}$ As pseudoionone is immiscible with water, the addition of an organic solvent to the reaction mixture to improve its solubilisation seems to be a logic step. Consequently, the compatibility of the OYE1/GDH system was evaluated with selected organic solvents in monophasic and biphasic systems. For this purpose, eight different solvents were tested at 10% v/v: four of them completely miscible in water, *i.e.* DMSO, MeOH, 1,2-dimethoxyethane [1,2-(MeO)Et], and 2-propanol (2-ProOH), and four solvents virtually immiscible in water, *i.e.* n-heptane, 2,2-dimethoxypropane [2,2-(MeO)2Prop], and 2,2,4-trimethylpentane (2,2,4-Me3Pent). These solvents were selected due to the capability of the OYE1 to maintain a high activity in their presence.$^{[23]}$

In general, better results were observed when water soluble cosolvents were used (Figure 1). Highest conversions were reached with DMSO (81%), 1,2-(MeO)2Et (78%) and MeOH (67%). On the other hand, 2-propanol was not well tolerated as cosolvent, translating in a drop of geranylacetone formation (25%). Concerning the biphasic systems, the percentage of geranylacetone formed was below 45% in all the cases. These lower conversions could be due to a lower availability of pseudoionone in the aqueous phase for the enzyme to act upon. Nevertheless, depending on the solvent used the conversions obtained were quite different. Thus, when the substrate was dissolved in solvents with higher log $P$, such as n-heptane (log $P$ 4.7) or 2,2,4-trimethylpentane (log $P$ 4.4), the conversion reached approximately 44%, while in the presence of solvents with lower log $P$, such as 2,2-dimethoxypropane (log $P$ 1.4) just 16% conversion was reached and no product was detected for EtOAc (log $P$ 0.7).

Since the aim is to attain good conversions when using initial pseudoionone concentrations above 100 mM, a higher cosolvent concentration could help to enhance the solubility of the concentrated substrate and its accessibility by the enzyme. Therefore, the tolerance of the system at increasing concentrations (10–50%) of the three preferred solvents was investigated (Figure S2). With the only exception of DMSO 20%, conversions sharply dropped when the cosolvent concentration was higher than 10%. It is likely that this is due to the instability of the cofactor regeneration system under these conditions, since it was described the near-quantitative reduction of cyclohex-2-enone catalyzed by OYE1, even at higher concentrations of these cosolvents, but without a cofactor regeneration system.$^{[23]}$ Hence, DMSO was selected as solvent not only because it allowed the best conversion, but also because it was the only one tolerated at concentrations higher than 10%.

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**Table 1. Substrate and product distribution of the reduction of pseudoionone catalyzed by lyophilized cells containing ene-reductase. Isomers were identified via comparison of the MS-fragmentation pattern with the NIST-library and the retention indices described in literature.$^{[20,21]}$**

| Enzyme | Pseudoionone [%] (E,E) | Geranylacetone [%] (Z,Z) | Conversion [%] | Pseudoionone isomeric excess [%]$^{[2]}$ | Geranylacetone isomeric excess [%]$^{[3]}$ |
|--------|------------------------|--------------------------|---------------|----------------------------------------|----------------------------------------|
| OYE1   | 28                     | 4                        | 11            | 57                                     | 68                                     |
|        | (E,Z)                  | (Z,E)                    | 68            | 75                                     | (E,Z)                                  |
| OYE2   | 38                     | 7                        | 4             | 51                                     | 54                                     |
|        | (E,Z)                  | (Z,E)                    | 54            | 69                                     | (E,Z)                                  |
| Blank  | 36                     | 64                       | n. c.$^{[4]}$ | n. c.$^{[4]}$                          | n. c.$^{[4]}$                          |

Reaction conditions: phosphate buffer (100 mM, pH 7.0), NADH (15 mM), pseudoionone (10 mM), DMSO (10% v/v) and the corresponding lyophilized E. coli cells (6.25 mg/mL), 30 °C, 24 h. [a] i.e (%)$=([\text{PI/GA main isomer (%)}] – \text{PI/GA minor isomer (%)})/[\Sigma \text{PI/GA isomers in the reaction (%)}] $^{[100]}$. [b] n.c. = no conversion. [c] n.a. = not applicable.

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Figure 1. Biocatalytic pseudoionone (■) reduction to geranylacetone (▲) in the presence of different solvents (10% v/v). Reaction conditions: phosphate buffer (100 mM, pH 7.0), NAD$^+$ (100 μM), pseudoionone (10 mM), cosolvent (10% v/v), GDH (10 U), glucose (20 mM), lyophilized E. coli/OYE1 cells (6.25 mg/mL), 30 °C, 24 h.
Evaluation of NCR for comparison due to the observed high stereoselectivity

As already observed in the initial tests using purified enzymes, the ene-reductase NCR displayed a high preference for the transformation of the (E,E)-isomer into (E)-geranylacetone, thus, the (Z)-product isomer was not detectable under the conditions employed and at the conversion achieved (Table S1). Consequently, freeze-dried E. coli/NCR whole cells (Figure S1B) were investigated in a similar fashion as reported above for OYE1, confirming the high preference observed at the onset of the reaction. For a more detailed evaluation, the conditions were tuned (Table S3 and S4) to achieve close to 90% conversion and the composition of the substrate and product isomers was followed over time (Figure 2A). It became clear that although NCR preferred to reduce the (E,E)-pseudoionone over the (E,Z)-isomer, the enzyme was also able to act on the latter. At the start of the reaction only (E)-geranylacetone was formed, also resulting in the accumulation of (E,Z)-substrate. However, as the reaction continued (Z)-geranylacetone was also detected and the percentage of this isomer steadily increased as the reaction advanced. As the reaction progressed and the amount of the faster-reacting isomer was consumed, the enzyme was exposed to an increasing amount of the slower reacting (E,Z)-isomer. Consequently, the product mixture increased in the content of the non-preferred (Z)-isomer, gradually reaching the initial (E)/(Z) isomer ratio of the substrates (Figure 2B). In this sense, starting from a 60:40 (E,E)/(E,Z) isomeric mixture of the substrate, the (E)-product isomeric excess dropped from >99% (0.5 h, 6% conversion) to 34% after 8 h of reaction (88% conversion). Simultaneously, the isomer composition of the substrate changed from an initial 20% (E,E)-pseudoionone isomeric excess to 87% (E,Z)-pseudoionone isomeric excess (12% of substrate remaining) after 8 h. The observed stereoselectivity can be estimated to be about 10 times faster for the (E,E)- over the (E,Z)-isomer. Interestingly, although the use of ERED for the stereoselective reduction of double bonds is well documented, to the best of our knowledge this is the first example of employing ene-reductases to discriminate between stereoisomers of the non-reduced conjugated double bond. Only another very recent work described the possibility to apply a previously engineered ERED, OYE3 W116A/S296F, for the production of (R)-citronellal, discriminating (E)-citral and (Z)-citral isomers. However in this case the selectivity of the variant depends on the steric configuration of the double bond to be reduced instead of the non-reduced conjugated double bond.

This feature could be applied for the kinetic resolution of conjugated dienes based on the (E/Z) isomer configuration of the γ,δ-double bond. As OYE1 was more active than NCR and since NCR did not display outstanding stereoselectivity, OYE1 was investigated further.

Increasing substrate pseudoionone concentration

Employing high substrate concentration in biocatalytic processes is mandatory from an economic point of view since it improves catalyst and volumetric productivities (kg of product per kg of enzyme, and kg of product per L per h, respectively). Therefore, biotransformations at substrate concentrations from 10 mM (1.9 g/L) to 200 mM (38.5 g/L) were carried out using OYE1 and the GDH/glucose cofactor recycling system with 10% or 20% v/v DMSO. As it is shown in Figure 3, the highest concentration of product formed (5.4 mM) was obtained at 25 mM initial pseudoionone concentration and then decreased with increasing substrate concentration. Amounts of geranylacetone formed at 20% v/v DMSO were lower in comparison, although it has to be stressed, that at 100 mM and even 200 mM of substrate concentration product formation was observed, indicating that this high concentration of substrate was feasible.

Consequently, the catalyst loading was increased for the experiments at 100 and 200 mM substrate concentration. To our delight, using ten times more freeze-dried E. coli/OYE1 cells than in the previous attempt enabled to reach >99% of conversion at 100 mM, and a respective value of 75–97% conversion at 200 mM of pseudoionone depending on the...
activity of the E. coli preparation used (Table 2, entries 1–2). This means, that actually both substrate isomers (E,E) and (E,Z), which were present in a 60:40 ratio, were transformed.

Organic solvent-free pseudoionone bioreduction

Although DMSO appeared to be a rather suitable solvent, it is increasing the waste due to difficulties to remove it during downstream process and has therefore a significant environmental impact. Consequently, it is desired to minimize solvent use ("the best solvent is no solvent"). Although initial attempts to reduce pseudoionone in absence of any cosolvent at the beginning of this study failed (data not shown), it has to be pointed out that those initial trials were performed using purified enzymes. Now, performing the reduction with lyophilized cells implies the addition of all components belonging to the cell. This includes the cell membranes that could act as a solubilizing agent for the substrate. Therefore, the reduction was repeated as described before for 100 and 200 mM but omitting the organic solvent. Gratifyingly, also in the absence of DMSO, at 100 mM initial substrate concentration the reaction went to completion and exclusively the product belonging to the cell. This includes the cell membranes that could act as a solubilizing agent for the substrate. Therefore, the reaction was repeated as described before for 100 and 200 mM but omitting the organic solvent. Gratifyingly, also in the absence of DMSO, at 100 mM initial substrate concentration the reaction went to completion and exclusively the product was detected after 24 h (>99% conv.). After extraction and flash chromatography, pure geranylacetone was obtained in high yields (390 mg, 80% yield). Moreover, in order to prove the possibility to apply this strategy to other compounds containing two conjugated double bonds, a similar reaction was performed using 6-methyl-3,5-heptadien-2-one as substrate. In this case, the conversion reached 97% giving sulcatone as product. Interestingly, here the reduction of the carbonyl group to the corresponding alcohol was observed as a second reduction step after the reduction of the α,β-double bond (3%). Purification after extraction translated into very good yield (77%).

Hence, these results clearly indicate the suitability of using this method for the biocatalytic reduction of α,β,δ,γ-unsaturated ketones in the mmol scale and avoiding the use of organic solvents or toxic compounds.

Conclusion

Geranylacetone is an industrially relevant molecule mainly due to its use as fragrance and flavouring compound. In this study geranylacetone was prepared from pseudoionone, which required the regioselective mono-reduction of a single C=C bond of the two conjugated double bonds. This reaction was not achieved before in a selective fashion using established chemical approaches. Thus, the reduction of the double bond in α-position of pseudoionone [isomer mixture (E,Z)/(E,E), 40:60] was attained with exquisite regioselectivity (1,4- vs 1,6-). This was accomplished by using recombinant ene-reductases like OYE1 from S. pastorianus or NCR from Zymomonas mobilis together with glucose as reducing agent and glucose dehydrogenase as cofactor recycling enzyme. While NCR showed a preference for the (E,E)-substrate isomer, this selectivity was less pronounced for OYE1. The biocatalytic reduction was performed at 30 °C, in buffer. It is especially important to underline, that pseudoionone is a challenging substrate for a transformation in buffer due to its insolubility. Actually, when using purified enzyme in the absence of cosolvent, no conversion was observed. Conversion was only observed for purified enzymes using water miscible organic cosolvents (e.g. DMSO 10% v/v). Interestingly, when using freeze-dried E. coli/ERED cells, the organic solvent can be omitted, which represents a significant improvement to eliminate the organic solvent and thereby reduce the production of unnecessary organic waste, additionally simplifying the work up. For the reaction investigated here, it means avoiding the use of 7.1 kg of DMSO per 1 kg of pure product, produced at 100 mM initial substrate concentration. Furthermore, it was shown that using the conditions employed preparative relevant substrate concentrations of 200 mM were

Table 2. Bioreduction of pseudoionone to geranylacetone catalyzed by lyophilized whole E. coli cells containing OYE1 at high initial substrate concentration in presence and absence of cosolvent.

| Entry | [Pseudoionone]_{init} [mM] | Cosolvent [v/v] | Geranylacetone [%] |
|-------|-----------------------------|-----------------|-------------------|
| 1     | 100                         | DMSO (10%)      | > 99              |
| 2     | 200                         | DMSO (10%)      | 75–97[a]          |
| 3     | 100                         | –               | > 99              |
| 4     | 200                         | –               | 61                |

Reaction conditions: phosphate buffer (300 mM, pH 7.0), NAD\(^+\) (1 mM), pseudoionone (100 or 200 mM), DMSO (none or 10% v/v), GDH (100 U), Gluc (200 or 400 mM), lyophilized E. coli/OYE1 (62.5 mg/mL), 30 °C, 24 h.

[a] Depending on the activity of the E. coli preparation.

OYE1 stereo and regioselective reduction at 100 mM substrate concentration and 25 ml scale

After identification of a suitable biocatalyst (OYE1), reaction conditions and initial substrate loading (100 mM) for the stereo- and regioselective reduction of pseudoionone, the reaction was performed with 481 mg (2.5 mmol) of substrate. As observed for the small-scale reactions, all substrate was converted after 24 h (>99% conv.). After extraction and flash chromatography, pure geranylacetone was obtained in high yields (390 mg, 80% yield). Moreover, in order to prove the possibility to apply this strategy to other compounds containing two conjugated double bonds, a similar reaction was performed using 6-methyl-3,5-heptadien-2-one as substrate. In this case, the conversion reached 97% giving sulcatone as product. Interestingly, here the reduction of the carbonyl group to the corresponding alcohol was observed as a second reduction step after the reduction of the α,β-double bond (3%). Purification after extraction translated into very good yield (77%).

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[a] Depending on the activity of the E. coli preparation.
reached. At a substrate concentration of 100 mM, the reaction was completed within 24 hours and 80 % yield of pure isolated product was achieved corresponding to 15.6 g/L. Moreover, this advantageous strategy can be applied to obtain other interesting compounds when starting from the corresponding α,β,δ,γ-unaturated precursor. For example, the floral compound sulcatone (6-methyl-5-heptadien-2-one), was also produced at 100 mM initial substrate concentration and 77 % yield.

**Experimental Section**

**Materials**

Pseudoionone and geranylacetone were provided by BASF SE (Ludwigshafen), while 6-methyl-3,5-heptadien-2-one and 6-methyl-5-hepten-2-one were obtained from Alfa Aesar and Sigma-Aldrich, respectively. Dodecane, DMSO, ethanol, n-heptane, 1,2-(MeO)2Et, 2,2-(MeO)2Pr, Gluc, Gluc6P, HLDH and sodium phosphate, were bought from Sigma-Aldrich. Ammonium formate (NH4HCO3) and NADH were purchased from Roth, while NAD+, NADPH and NADP+ were obtained from Codecx, PanReac AppliChem and BioCatalytic, respectively. MeOH and EtOAc were ordered from ChemLab, 2,2,4-trimethylpentane (tert-Butanol) from Acros Organics, G6PDH, FDH, GDH and ADH-A were obtained from Biochemica, Evocatal, DMS and from our own lab, respectively.

**Methods**

**Ene-reductase obtaining and purification**

The following ene-reductases were overexpressed and purified as recently published: OYE1 from *Saccharomyces pastorianus*, OYE2 and OYE3 from *Saccharomyces cerevisiae*,[18] 12-oxophytodienoate reductase isoenzym OPR3 from *Lycopersicon esculentum*,[19] cyclo-hexenone reductase (NCR) from *Zymomonas mobilis*,[16] estrogen binding protein EB1 from *Candida albicans,*[17] and the xenobiotic reductases XenA and XenB from *Pseudomonas putida* and *Pseudomonas fluorescens,* respectively.[16](Table S5).

When lyophilized cells were required for the biotransformations, the culture was centrifuged (3200 g, 20 min, 4 °C). Then, the supernatant was discarded, while the pellet was washed with 10–20 mL of buffer phosphate (50 mM, pH 7.5). After another centrifugation (3200 g, 20 min, 4 °C), the washed pellet was resuspended in a small amount of buffer phosphate (50 mM, pH 7.5), transferred to round bottom flask and frozen using liquid nitrogen prior lyophilization. The lyophilized cells were stored at −20 °C until their use.

**SDS-PAGE**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast ExpressPlus PAGE gels (10% or 12.5% acrylamide) purchased from GenScript. Gels were run on a Mini-PROTEAN Tetra System–Mark apparatus (Bio-Rad) at 100 V. Molecular weight marker was purchased from ThermoScientific (Prestained protein ladder, 10–180 kDa). Proteins were stained with Coomassie Brilliant Blue G-250 (Bradford).

**Biotransformations**

All biocatalysis reactions were performed in a similar manner, although with slight modifications depending on the parameter studied. Additionally, the corresponding reaction blanks in absence of enzyme were carried out. In all the cases, reactions were incubated at 30 °C and 1400 rpm in triplicate. A summary of the reaction conditions is given in Table S6. Analysis of the samples was performed as described in the corresponding subsequent section.

**Ene-reductase screening towards the acceptance of pseudoionone as substrate**

The corresponding purified enzyme (protein final concentration: 75–125 μg/mL) was added into a 1.5 mL microcentrifuge tube containing sodium phosphate buffer solution (0.8 mL, 100 mM, pH 7), and the cofactor (NADH, 15 mM). The substrate was added diluted in DMSO (10 % v/v, final concentration: 10 mM) After 24 h the products were extracted with EtOAc (2 × 0.5 mL). The combined organic phases were dried over Na2SO4 and analysed as described below to determine the conversion.

**Initial evaluation of the pseudoionone reduction using lyophilized cells**

To test the possibility of using lyophilized cells to perform the 1,4-selective reduction of pseudoionone, reactions were performed containing NADH (15 mM), pseudoionone (10 mM), DMSO (10 %, v/v) and 6.25 mg/mL of lyophilized E.coli cells with the desired overexpressed ene-reductase (OYE1, OYE2, EB1 or NCR) in sodium phosphate buffer (100 mM, pH 7.0, 0.8 mL). In order to avoid solubilisation problems, the substrate was dissolved in DMSO prior its addition to the reaction. After 24 h the products were extracted with EtOAc (2 × 0.5 mL). The combined organic phases were dried over Na2SO4 and analysed as described below to determine the conversion.

**Evaluation of the cofactor regeneration systems**

Influence of the cofactor regeneration system on the reduction of pseudoionone catalyzed by OYE1 was studied by measuring the conversion of pseudoionone to geranylacetone when using different combinations of NAD+/NADP+ as biocatalyst was tested. For this purpose, similar reactions as described above were carried out. These reactions included sodium phosphate buffer (100 mM, pH 7.0, 0.8 mL), GDH (10–100 U), Gluc (20 mM), NAD+ or NADP+, 100 μM), pseudoionone (10 mM), DMSO (10 %, v/v) and 6.25 mg/mL of OYE1 overexpressing lyophilized E.coli cells. To avoid solubilisation problems, the substrate was dissolved in DMSO prior its addition to the reaction. In those cases where the cosubstrate could act also as cosolvent, i.e. 2-propanol and ethanol, additional reactions were carried out replacing the DMSO with the aforementioned cosolvents (2–10 %).

Additionally, the utility of the GDH/Gluc cofactor regeneration system in reactions containing NCR as biocatalyst was tested. For this purpose, similar reactions as described above were carried out. These reactions included sodium phosphate buffer (100 mM, pH 7.0, 0.8 mL), GDH (10–100 U), Gluc (20 mM), NAD+ (100 μM), pseudoionone (10 mM), DMSO (10 %, v/v) and 6.25 mg/mL of NCR overexpressing lyophilized E.coli cells. In order to minimise solubilisation problems, the substrate was dissolved in DMSO prior to its addition to the reaction.
To quantify the amount of substrate and product present in the reaction crude, reactions were first extracted with 400 μL of EtOAc containing 20 mM of dodecane as internal standard and subsequently extracted with another 600 μL of EtOAc. The combined organic phases were dried over Na₂SO₄ and lyophilized as described below to determine the conversion.

Evaluation of the influence of different solvents on the OYE1/GDH system

Investigation of the suitability of different solvents was performed by lyophilising the percentage of geranylocitrate produced in the presence of the following solvents: DMSO, MeOH, 1,2-dimethoxyethane (1,2-(MeO)₂Et), 2-propanol (2-PrOH), n-heptane, EtOAc, 2,2-dimethoxypropane (2,2-(MeO)₂Pr) and 2,2,4-trimethylpentane (2,2,4-Me₃-Pent). Thus, pseudoionone reduction reactions were carried out in phosphate buffer (100 mM, pH 7.0, 0.8 mL) containing 6-methyl-3,5-heptadien-2-one as substrate were also performed at this scale and substrate concentration. For this purpose, reactions as those described previously at high substrate concentration and in absence of cosolvent were performed in duplicate. Reaction conditions were as follows: buffer phosphate (300 mM, pH 7.0, 25 mM), substrate (pseudoionone or 6-methyl-3,5-heptadien-2-one, 100 mM), GDH (125 U), glucose (200 mM), NAD⁺ (1 mM), OYE1 (62.5 mg/ml reacted or mL reaction), and pseudoionone (10 mM) dissolved in the corresponding solvent (10 % final reaction volume).

Additionally, tolerance towards increasing concentrations of DMSO, MeOH and 1,2-(MeO)₂Et was tested. To achieve this, similar biotransformations as described above were carried out. The only difference was the increasing solvent concentration, which ranged between 10 and 50 %.

In order to quantify the amount of substrate and product present in the reaction, reactions were first extracted with 400 μL of EtOAc containing 20 mM of dodecane as standard and subsequently extracted with another 600 μL of EtOAc. The combined organic phases were dried over Na₂SO₄ and lyophilized as described below to determine the conversion.

Study of the effect of increasing concentrations of pseudoionone using the OYE1/GDH system

A first set of reactions was carried out at increasing concentration of pseudoionone (10–200 mM). In addition to the substrate, each reaction contained buffer phosphate (250 mM, pH 7.0, 0.8 mL), GDH (10 U), glucose (20 mM), NAD⁺ (100 μM), OYE1 (6.25 mg/ml reacted or ml reaction), and pseudoionone (10 mM) dissolved in the corresponding solvent (10 % final reaction volume).

To check the ability of NCR to carry to completion the reduction of pseudoionone increasing amounts of the enzyme were used during the biotransformation. These reactions comprised lyophilized cells enclosing the overexpressed NCR (12.5, 31.25 or 62.5 mg/mL) in sodium phosphate buffer (100 mM, pH 7.0, 0.8 mL), Gluc (20 mM), NAD⁺ (0.1 mM) and pseudoionone (10 mM) dissolved in DMSO (10 % final reaction volume). Reactions were stopped after 24 h.

Stereo preference was then studied by monitoring the formation of (E)- and (Z)-geranylacetone produced over time. The reaction mixtures contained lyophilized cells enclosing the overexpressed NCR (31.25 mg/mL) in sodium phosphate buffer (100 mM, pH 7.0, 0.8 mL), Gluc (20 mM), NAD⁺ (0.1 mM), pseudoionone (10 mM) and DMSO (10 % v/v). Reactions were stopped at the corresponding time (0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h) by freezing them at −20 °C. In all the cases, as negative controls were carried out lyophilized E. coli cells lacking the recombinant reductase with empty vector.

In order to quantify the amount of substrate and product present in the reaction crude, reactions were first extracted with 400 μL of EtOAc containing 20 mM of dodecane as standard and subsequently extracted with another 600 μL of EtOAc. The combined organic phases were dried over Na₂SO₄ and lyophilized as described below to determine the conversion.
**Analytical Procedures**

**Sample analysis by gas chromatography**

After extraction, samples were analyzed by gas chromatography. GC-MS analysis were performed on an Agilent 7890A GC-system, equipped with a 5975 C mass selective detector, using a HP-5MS column (30 m x 0.25 mm x 0.25 μm) using hydrogen as carrier gas (flow = 0.5 mL/min). Pseudoionone and geranylacetone isomers were identified by comparison of the MS-segmentation fragment pattern with the NIST-library and the retention indices described in literature.[20]

Retention times: (Z)-geranylacetone, 7.7 min; (E)-geranylacetone, 7.9 min; (E,Z)-pseudoionone, 8.9 min; (E,E)-pseudoionone, 9.5 min. Injector and detector temperature 250 °C, split ratio 90:1. Temperature program: 100 °C hold 0.5 min, and 10 °C/min to 300 °C (Figure S4).

In order to determine the conversions, GC-FID spectra were recorded with an Agilent 7890A GC-system, equipped with a HP-5 column (30 m x 0.25 mm x 0.25 μm) using hydrogen as carrier gas (flow = 0.5 mL/min). Pseudoionone and geranylacetone isomers were identified by comparison of the MS-segmentation fragment pattern with the NIST-library and the retention indices described in literature.[20]

Retention times: (Z)-geranylacetone, 7.7 min; (E)-geranylacetone, 7.9 min; (E,Z)-pseudoionone, 8.9 min; (E,E)-pseudoionone, 9.5 min. Injector and detector temperature 250 °C, split ratio 90:1. Temperature program: 100 °C hold 0.5 min, and 10 °C/min to 300 °C (Figure S4).

**Calibration curves**

Calibration curves of pseudoionone (substrate) and geranylacetone (product) were obtained by plotting the peak area ratio measured at increasing concentrations of each compound (0.5–250 mM). Samples were dissolved in EtOAc and measured in triplicates. Analysis of the samples was performed by gas chromatography (Figure S5A).

**Response factors and compound quantification**

Response factors of both, pseudoionone and geranylacetone, using dodecane (10 mM) as internal standard.[29] For that purpose, samples of dodecane (10 mM) dissolved in EtOAc containing increasing concentrations (from 0.5 to 250 mM) of either pseudoionone or geranylacetone were analysed by gas chromatography as described above (Figure S5B). Response factors were deduced by plotting the ratio of the peak areas as a function of the concentration of the analyte present in the sample [(Equation 1)](1)

\[ RF = \frac{\sum A_{\text{Comp}} / A_{\text{IS}}}{(\text{Comp})/(\text{IS})} \]

\[ A = \text{Area}; \text{Comp} = \text{Compound (pseudoionone or geranylacetone)}; \]
\[ \text{IS} = \text{Internal standard (dodecane)}. \]

Therefore, to quantify the pseudoionone and geranylacetone present in the reaction mixture, their peak areas were normalized to the internal standard, dodecane, and treated with their corresponding response factor. Each compound percentage was determined by comparing the concentration previously calculated with the total concentration of pseudoionone and geranylacetone measured [Equation (2)](2)

\[ %\text{Comp} = \frac{\text{Comp}}{(\text{PI}) + (\text{GA})} \]

\[ \text{Comp} = \text{Compound (pseudoionone or geranylacetone)}; \]
\[ \text{PI} = \text{Pseudoionone}; \]
\[ \text{GA} = \text{Geranylacetone} \]

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**Conflict of Interest**

The authors declare no conflict of interest.

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