Enzymatic Asymmetric Reduction of Unfunctionalized C=C Bonds with Archaeal Geranylgeranyl Reductases

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The asymmetric reduction of activated C=C bonds such as enones is well established for non-enzymatic methods as well as in biocatalysis. However, the asymmetric reduction of unfunctionalized C=C bonds is mainly performed with transition metal catalysts whereas biocatalytic approaches are lacking. We have tested two FAD-dependent archaeal geranylgeranyl reductases (GGR) for the asymmetric reduction of isolated C=C bonds. The reduction of up to four double bonds in terpene chains with different chain lengths and head groups was confirmed. Methyl-branched *E*-alkenes were chemoselectively reduced in the presence of cyclic, terminal or activated alkenes. Using a removable succinate "spacer", farnesol and geraniol could be quantitatively reduced (>99%). The reduction is strictly (*R*)-selective (enantiomeric excess >99%). Hence, GGRs are promising biocatalysts for the asymmetric reduction of unactivated isolated C=C bonds, opening new opportunities for the synthesis of enantiopure branched alkyl chains.

In 2001, R. Noyori was awarded the Nobel prize in chemistry for his work on asymmetric olefin reductions, which highlights the general importance of this strategy for the synthesis of enantioenriched chemicals.[1] The scope was further expanded to unfunctionalized, substituted olefins by the Pfaltz group.[2] In parallel, biocatalysts have evolved as valuable tools for asymmetric reactions.[3–5] Within this field, oxidoreductases account for over one quarter of the established enzymatic reactions.[4] In addition to carbonyl and imine reduction, the reduction of alkenes has been well investigated for activated C=C bonds conjugated to an electron-withdrawing group.[5] Such transformations are, e.g., performed by old yellow enzymes via hydride transfer in a Michael-type addition. Therefore, these enzymes cannot reduce electron-rich C=C bonds by the same mechanism.

We therefore focused on the enzymatic asymmetric reduction of non-activated C=C bonds. For this purpose, we applied archaeal geranylgeranyl reductases (GGRs), FAD-dependent enzymes that are known to hydrogenate the double bonds of membrane terpene units in archaea.[6] GGRs from *Sulfolobus acidocaldarius* (UniProt: Sa_0986, SaGGR) and *Archaeoglobus fulgidus* (UniProt: Af0464, AfGGR) reduce a variety of digeranylgeranylglycerol ($2\times C_{20}$) derivatives to fully reduced products.[7,8] The enzymatic (partial) reduction of GeranylGeranyl (di) Phosphate (C_{20}) [**GGP(P)**] results in all-(*R*)-configured phytyl (di) phosphate (cf. Figure 1).[9,10] Moreover, Meadows *et al.* showed the conversion of farnesol (C_{15}) which yielded a mixture of partially reduced products (cf. Scheme 1).[11] We hypothesized that GGRs might be suitable catalysts for the asymmetric reduction of alkenes of various chain length. To verify this hypothesis, we overproduced GGRs heterologously in *E. coli*

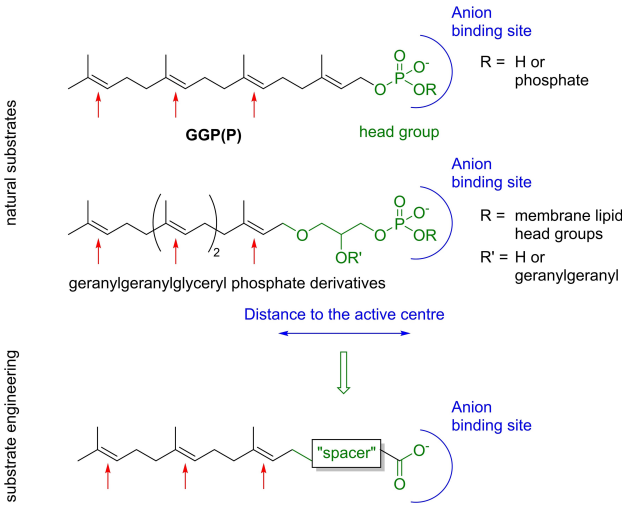

Figure 1. The spacer hypothesis. GGRs only reduce the three distal C=C bonds of the natural substrate geranylgeranyl (di)phosphate [**GGP(P)**, top], while phosphorylated glycerol ether derivatives (middle) are fully reduced (red arrows).[9] This is explained by the distance of the anion binding site to the active center.[8] A substrate elongated by a spacer might still bind to the anion binding site while presenting the proximal double-bond to the active site, thus allowing full reduction of the substrate (bottom).

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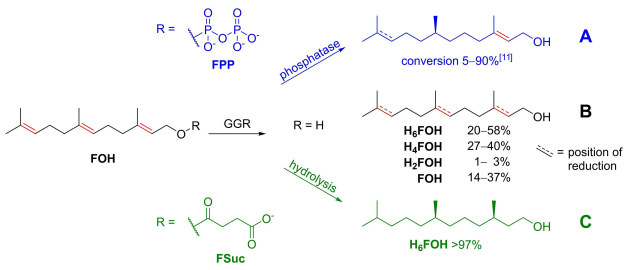


Scheme 1. Enzymatic reduction of farnesol derivatives by GGR. **A**: Reduction of **FP**(**P**) and subsequent dephosphorylation by phosphatases mainly leads to tetrahydrofarnesol (**H_4FOH**) leaving the proximal C=C bond intact. **B**: Reduction of farnesol (**FOH**) leads to a mixture of reduction products (dihydro **H_2**, hexahydro **H_6**, for detailed information cf. Figure S3 and Table S1). **C**: The use of farnesyl succinate (**FSuc**) leads to fully reduced product **H_6FOH** and almost quantitative conversion. Conditions for **B** and **C**: sodium dithionite (425 mM), potassium phosphate buffer (100 mM, pH 6.5), FAD (200 μM), substrate (1 mM), GGR (1.5 mg mL^{-1}) under nitrogen atmosphere at 55 °C for 16–18 h; hydrolysis was performed in NaOH (5 M) at 55 °C for 3 h.

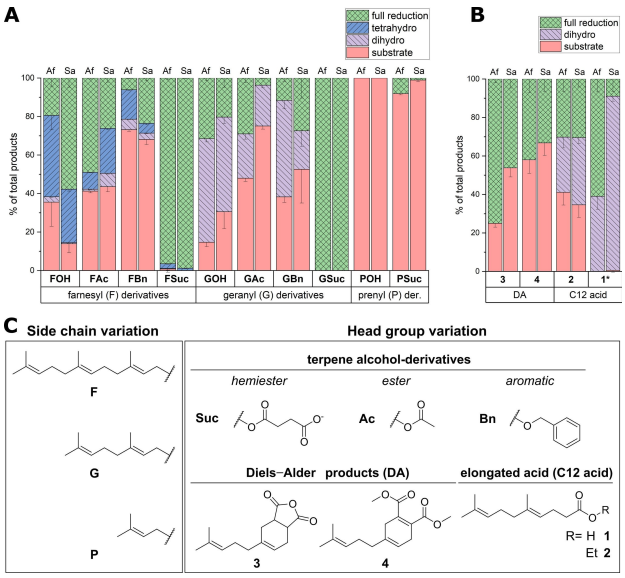


Figure 2. A and **B**: Column diagrams of substrate conversion with AfGGR (Af, left column) and SaGGR (Sa, right column) (conditions described in Scheme 1, quantification by GC-MS analysis: isomers of the same number of double bonds are combined for clarity). Comparison of terpene alcohol derivatives (**A**) and Diels-Alder (**3**, **4**) and carboxylic acid (**1**, **2**) derivatives (**B**). Dihydroprenyl- and tetrahydrogeranyl derivatives are named as "full reduction". Reduction equivalents relate to the side chain C=C bonds as cyclic C=C bonds were not reduced. *: Analyzed as the methyl ester. **C**: Structures of tested substrates.

with a His tag for purification and tested the enzymatic reduction of terpenoids of different chain length carrying various head groups with different physicochemical properties.

A physiological substrate of GGRs is digeranylgeranylglycerol, which proves the reduction of alcohols as head groups in addition to (di)phosphate derivatives.[7] We observed the reduction of the smaller terpene alcohols geranylgeraniol (**GGOH**, C_{20}), farnesol (**FOH**, C_{15}), and geraniol (**GOH**, C_{10}), which yielded mixtures of fully and partially reduced products: the major product was either fully reduced or the proximal C=C bond was retained (Scheme 1B). Additionally, the tertiary allyl alcohol geranyllinalool was accepted by GGRs as a substrate without the terminal double bond being affected, thus finally leading to the formation of isophytol (Figure S1). A reduction of prenol (**POH**, C_5) could not be detected.

It is known that the (di)phosphate of the substrate **GGP**(**P**) binds to the anion binding site of GGRs, thus keeping the proximal C=C bond away from the reduction site (Figure 1, top). In contrast, the longer, phosphorylated glycerol derivatives are fully reduced (Figure 1, middle).[7,9] Therefore, we hypothesized that a "spacer" would enable a substrate to bind to the anion binding site and to place the C=C bonds of interest into the active site (Figure 1, bottom).

Initial experiments with farnesyl (di)phosphate [**FP**(**P**)] showed high conversion and, most importantly, the fully reduced product which indicated hydrolysis of the (di) phosphate to the alcohol under the tested conditions (55 °C, pH 6.5, 18 hours) followed by an additional reduction step (Figure S2). This is supported by Meadows *et al.* who postulated a phosphatase activity in GGRs.[11] Further to the hydrolysis, **FP**(**P**) exhibited detergent properties at concentrations >400 μM which led to reduced conversions. Therefore, instead of phosphates, we used carboxylates as head groups, which were more convenient and are abundant in various possible compounds as putative enzymatic substrates.

The formation of a hemiester is a straightforward option to generate a reversible spacer with a negative charge under standard reaction conditions. Succinate hemiesters of alcohols are easily prepared, stable, and can be hydrolyzed without difficulty after reduction. Enzymatic conversions of farnesyl succinate (**FSuc**) led to almost quantitative conversion and, as well important, to the fully reduced product hexahydrofarnesol (**H_6FOH**) after hydrolysis (Scheme 1C). Similarly, quantitative conversion was obtained with geranyl succinate (**GSuc**) leading to tetrahydrogeraniol (**H_4GOH**). Prenyl succinate (**PSuc**) was reduced (8% conversion) to dihydroprenyl succinate (**H_2PSuc**). This shows that the "spacer" actually allowed hydrogenation of a compound that is otherwise not a substrate (Figure 2A).

Hence, the succinate hemiester is a valuable means to increase – or even to enable – the enzymatic reduction of acyclic terpene alcohols. To determine the reduction of terpenoid carboxylic acids, we elongated the chain of **GOH** by an acetate unit to provide carboxylic acid **1** (Figure 2C). The latter was fully converted in enzymatic conversions; however, a mixture of reduction products was obtained (Figure 2B, right). This indicates that the interaction of **1** with the anion binding site leads to quantitative reduction of one double bond but is too weak to prevent (partial) reduction of the second.

In addition to the (negatively charged) carboxylate head groups, we tested the (neutral) ester derivatives **GAc**, **FAc**, and **2** which confirmed that esters are accepted by GGRs as substrates, too. A tolerance for aromatic head groups was shown by enzymatic reduction of the benzyl ethers **GBn** and **FBn**. Reduction of a short chain (C_6) in proximity to a bulky head group was observed for the Diels-Alder products of myrcene, **3** and **4** (Figure 2C). GGRs selectively reduced the double bond of the methyl-branched side chain without

attacking the cyclic or the activated C=C bond(s) (Figure 2B, left).

The natural product phytol, common to all green plants, is all-(*R*)-configured.[10] To ascertain if this selectivity is retained in the case of substrates with other head groups or shorter chain length, we determined the diastereomeric ratio (*dr*) and enantiomeric excess (*ee*) of the reduction products of the succinates **FSuc** and **GSuc**, and of the alcohol **GOH**. The *ee* (> 99 %) of the reduction products (**H_4GOH**) of **GOH**, as well as of **GSuc** after hydrolysis, were analyzed by chiral-phase GC-FID (Figure 3). Absolute configuration was determined by comparison with authentic (*R*)-**H_4GOH** derived from non-enzymatic hydrogenation of commercial (*R*)-citronellol, with subsequent confirmation via VCD analysis (Figure S7).

The configuration of **H_6FOH** was elucidated according to Matsueda *et al.* and by comparison to an authentic reference, which was obtained by Noyori-type Ru-catalyzed asymmetric hydrogenation in the context of the total synthesis of several vitamin E homologues and stereoisomers as described in literature.[12] The *dr* was determined by ^{13}C NMR analysis of the alcohol **H_6FOH** which matches (3*R*,7*R*)- or (3*S*,7*S*)-**H_6FOH** (Figure 4A). The *ee* was determined by derivatization with (*S*)-2-methoxy-2-(1-naphthyl)propionic acid [(*S*)-**MαNP**] and ^{1}H NMR analysis of the shifted methyl group at position 3 of the farnesyl chain, which indicated (3*R*,7*R*,2′*S*)- or (3*R*,7*S*,2′*S*)-configuration (Figure 4B). The authentic reference of (3*R*,7*R*)-**H_6FOH** (DSM) contained 0.75 % (3*S*,7*R*)-isomer. This peak, referring to the corresponding (3*S*,7*R*,2′*S*)-derivative, is not present in the enzymatic assay product. Thus, the *ee* of the GGR-catalyzed reduction products is > 98.5 %.

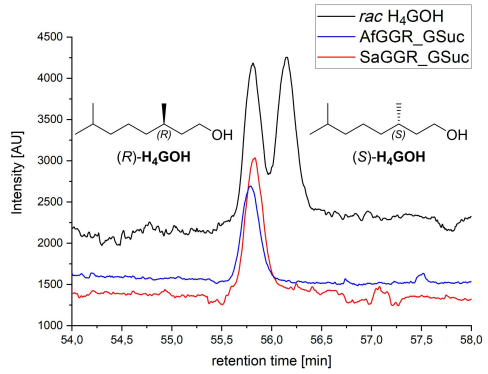


Figure 3. Chiral-phase GC-FID analyses. Upper chromatograph: authentic *rac*-**H_4GOH**. Lower two chromatographs: derived from the reduction of **GSuc** with AfGGR and SaGGR, followed by hydrolysis to (*R*)-**H_4GOH**.

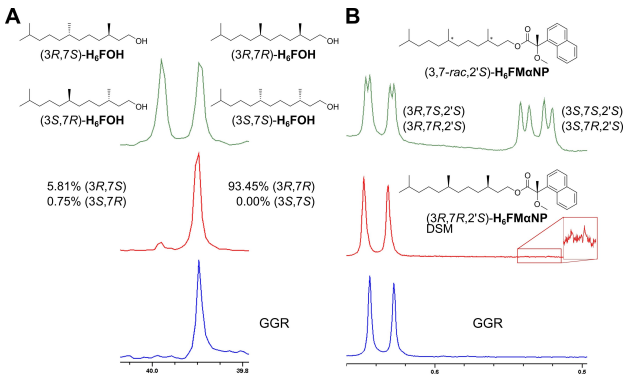
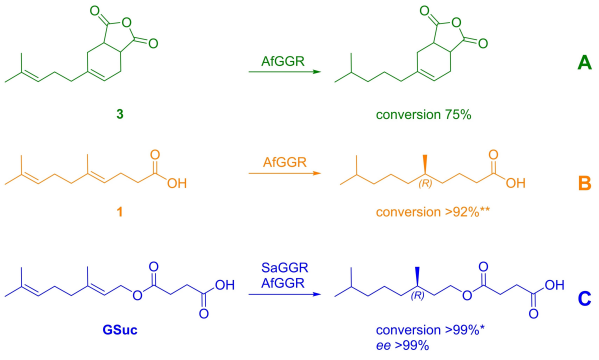


Figure 4. A: ^{13}C NMR analysis (100 MHz, $CDCl_3$) of racemic **H_6FOH** (green), (3*R*,7*R*)-**H_6FOH** (red), and the enzymatic assay product of **FSuc** with AfGGR after hydrolysis (blue); SaGGR showed similar results (Figure S3). **B**: ^{1}H NMR spectra (400 MHz, $CDCl_3$) of the (*S*)-**MαNP** esters of the respective **H_6FOH** measured in **A**.

In conclusion, archaeal GGRs reduce isolated C=C bonds chemo-, diastereo-, and enantioselectively. They tolerate ionic, aromatic, and hydrophobic head groups. The reduction products are (*R*)-configured with *ee* > 99 %. In addition, we found succinate hemiesters to be conveniently cleavable spacer head groups increasing the conversion of acyclic terpene alcohols, to make accessible fully reduced products (Scheme 2C) and, as shown for **PSuc**, to enable enzymatic reduction of a short alkene which is otherwise not a substrate. Reduction occurs exclusively at methyl-branched C=C bonds without affecting cyclic, terminal or activated double bonds (Scheme 2A, Figure S1, Figure 2). The tolerance for different head groups can be exploited for the reduction of acyclic terpenoids at several stages within a putative synthetic scheme.

The two enzymes tested act differently on smaller molecules. While SaGGR is more effective in reducing longer terpene chains, AfGGR reduces shorter substrates more efficiently: **1** is fully reduced by AfGGR (Scheme 2B), while SaGGR mainly reduces one of the double bonds. Such variations in substrate spectrum and selectivity may provide a starting point for the development of further useful enzymes or variants of this family.



Scheme 2. Diversity of reductions with GGRs. **A**: Selective reduction of the side chain of the Diels-Alder product **3** leaving the cyclic C=C bond intact. **B**: Reduction of the methyl-branched acid **1** with AfGGR leads to fully reduced product. Configuration assumed according to **GOH. C**: Reduction of **GSuc** leads to full conversion. (*: Conditions described in Scheme 1, **: substrate concentration changed to 500 μM.)

An important bioactive compound containing all-(*R*)-configured terpene side chains is vitamin E (α-tocopherol), which is mostly used as a racemate, while the all-(*R*)-isomer is the preferred bioactive form.[13] Terpenoids can be used for chiral derivatizations on polymers,[14] lipids for liposomes,[15] and with other bioactive compounds as repellents.[16] The enzymatic asymmetric reduction of isolated non-activated C=C bonds, as demonstrated herein by GGRs, creates new opportunities for the synthesis of enantiopure branched alkyls and provides a new reaction to add to the biocatalytic toolbox, which will stimulate further steps towards stream-lined biocatalytic synthesis of chiral building blocks such as enzyme and substrate engineering, assay development,[17] cofactor regeneration, and host engineering.

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Conflict of Interest

The authors declare no conflict of interest.

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