Pergamon

Tetrahedron 58 (2002) 7291–7293

TETRAHEDRON

Efficient cyclization of squalene epoxide to lanosterol with immobilized cells of baker's yeast

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Received 4 April 2002; revised 14 June 2002; accepted 28 June 2002

Abstract—The cyclization of squalene epoxide to lanosterol with baker's yeast (*Saccharomyces cerevisiae*) can conveniently be carried out in aqueous solution with glass cored immobilisates of cells in calcium alginate. This enables the manifold use of the microorganism to obtain lanosterol in a single biocatalytic step using the immobilisates repeatedly.

1. Introduction

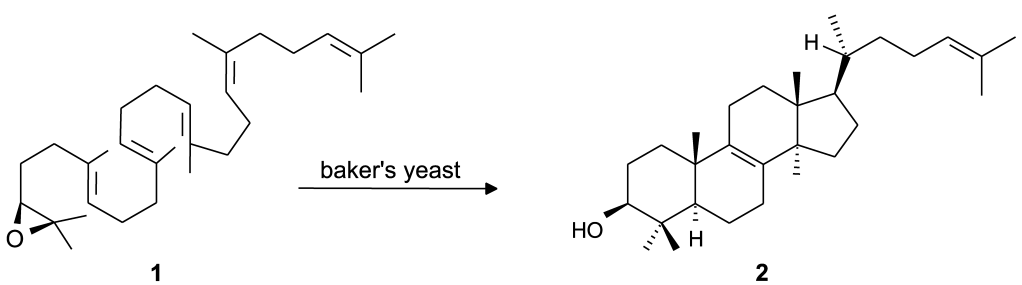
In recent years baker's yeast (*Saccharomyces cerevisiae*) gained increasing importance as a biocatalyst in asymmetric synthesis due to good yields and high stereoselectivity shown in many examples.[1–6] Yeast reactions have also successfully been applied in natural product synthesis.[7–10] Although most of the known applications are carried out with whole cell systems, examples are known where the microorganisms have been immobilized prior to their use.[7,11–13]

Among the numerous yeast enzymes lanosterol synthase is doubtless the most remarkable one because of its ability to build up four rings and to introduce seven asymmetric centers selectively during the conversion of squalene epoxide (**1**) to lanosterol (**2**, see Scheme 1; for mechanistic aspects of this and related transformations, see Corey et al.[14–16]).

2. Results and discussion

Whole cells of baker's yeast deliver upon ultrasonic pretreatment in aqueous suspension **2** with 42% yield starting with racemic **1**. With respect to the highly hydrophobic substrate the addition of a detergent (here: Triton X 100) is necessary.[17] However, the detergent affects the cell membranes and leads to an inactivation of the cells within a single conversion; a re-use of the cells is therefore not possible.[18] Herein we report on investigations to achieve re-usable immobilisates of yeast cells by different techniques protecting the microorganisms from decomposition.

A common method to entrap cells constitutes the immobilization in polyacrylic amide that has been applied for different organisms and also for the yeast-mediated transformation of sekoketone to sekoketol.[7] In our case this procedure could not be used successfully as the number of cells that could be immobilized in the polymer was far too low.



1 **2**

Scheme 1.

Keywords: baker's yeast; squalene epoxide; immobilization; calcium alginate; lanosterol synthase.

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0040–4020/02/$ - see front matter
PII: S0040-4020(02)00755-X

In a second attempt to encapsulate baker's yeast cells in polyamide microcapsules, a procedure by Green et al.[17] for the reduction of 2-phenyl-1,2-propanedione to the corresponding 2-hydroxy compound, was adopted and modified in this work. Due to the fact that the microcapsules contain the cells in an aqueous environment, but are applied in organic solvents—in the present case in octane and decane—the achievement of this method could have been to carry out the reaction directly in a lipophilic medium with no need of a detergent at all. But incapsulated yeast cells showed no cyclization activity which is most likely related to the well known fragility of the enzyme lanosterol synthase that seems to be inactivated even by traces of organic solvents present in the capsule cores. However, the permeability of the capsule shells for the substrates was experimentally proved.

Thirdly, calcium alginate was used as a polymer for the immobilization. In this case, highly stable gel beads were obtained that were capable of carrying out the cyclization and could be used in five consecutive conversions. Even thereafter active cells could still be detected in the suspension as shown in Table 1 (first part). However, the overall yield of 42% was still low and we assumed that the substrate did not reach the center of the beads by diffusion and therefore, only cells just beneath the surface were reactive at all. To overcome this problem a second type of alginate immobilisates was prepared. This type consisted of a glass core being coated with a thin layer of alginate containing the cells to make them more easily accessible for the substrate. With these immobilisates the total yield of lanosterol formation could be more than twice up to 89% upon a fivefold use of the glass-cored beads, however, with decreasing yields within the experimental series (see Table 1, second part). Notably, for each run of this and the previously described experimental set a fresh solution of squalene epoxide was employed.

With these experiments we have shown that a manifold use of yeast cells for the cyclization of squalene epoxide to lanosterol is possible in spite of the necessity of Triton X 100 as a detergent to dissolve the polyene substrate if the cells are protected by immobilization. With calcium alginate coated glass beads higher overall yields could be obtained than with a single use of free cells. This is a crucial step in the development of a multi-usable biocatalytic system for one-step steroid synthesis. With respect to the still limited repeated use of the beads and the relatively large amount of cells needed for the immobilisates these investigations will still have to be continued. A further increase of the immobilisate surface seems to be a promising goal in view of the results that have been obtained with glass-cored versus solid beads.

Table 1. Cyclization of squalene epoxide with immobilized cells of baker's yeast

Number of conversions[a]	Cells in calcium alginate		Cells in calcium alginate on glass beads	
	Yield (%)	cfu/g[b]	Yield (%)	cfu/g[b]
1	12	1.4×10^8	28	1.6×10^8
2	11	4.9×10^7	24	5.1×10^7
3	8	3.4×10^7	19	3.2×10^7
4	7	9.3×10^7	11	9.1×10^7
5	4	8.2×10^7	7	7.9×10^7
Added yields	42		89	

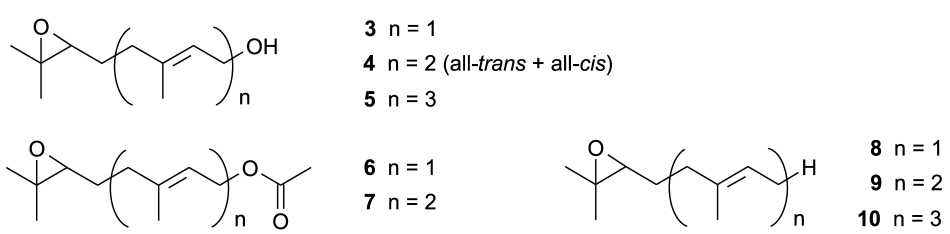
[a] Each with a fresh solution of squalene epoxide.
[b] cfu/g, cell forming units per gram of immobilisate (excluded the weight of the glass beads) counted on agar plates. Fresh cells: 9.8×10^8 cfu/g. Immobilisates have been stored in nutrition medium over night between the third and fourth conversion.

In addition to these investigations we tested if the high chemoselectivity of lanosterol synthase could also be applied to cyclizations of smaller polyene epoxides. Therefore, conversions of the epoxides **3**–**10** (see Scheme 2) were investigated using again immobilized cells of baker's yeast under conditions that were successful for the cyclization of squalene epoxide. However, substrates **3**, **4**, **6** and **8** were converted to vicinal diols instead upon hydrolysis of the epoxides in 6–23% yields (spectroscopic data of these products are available from Ref. 18 or upon request from the corresponding author). Cyclic products could not be detected in these cases and notably, the epoxides **5**, **7**, **9** and **10** were unreactive at all. According to these results we have to assume that either the selectivity of the lanosterol synthase is too high to accept non-native epoxides or another enzyme—an epoxide hydrolase—reacts faster than the cyclase with these shorter chain substrates.

3. Experimental

3.1. General

All reactions were carried out with racemic 2,3-epoxy squalene. The cyclization product, lanosterol, was identified by thin layer chromatography on Merck 60 F_{254} precoated silica gel plates and comparison with commercial available material. Yeast cells were purchased from Sigma (baker's Yeast, Type I), sodium alginate (BioChemika) from Fluka. Water was always used tridistilled with a Millipore Q system. Reactions with alginate immobilisates were carried out in tris-(hydroxymethyl)-aminomethane (TRIS) buffered solution at a pH of 7.4.



Scheme 2.

3.2. Immobilization of yeast cells in calcium alginate

2.0 g sodium alginate were dissolved in 60 mL of water and combined with a suspension of 16 g dried yeast cells in another 60 mL of water. Using a syringe, this suspension was now dropwise given into 800 mL of 2% aqueous solution of calcium chloride. During this procedure the droplets stiffened immediately when they had contact with the calcium chloride solution and formed rigid beads with a diameter of 2–3 mm in average. The mixture was stirred for further 30 min to harden the immobilisates, then they were collected with a sieve and washed three times with each 800 mL of water.

3.3. Immobilization of yeast cells in calcium alginate on glass beads

300 g of glass beads with a diameter of 1.5–2 mm were added to the solution of 16 g yeast cells and 2.0 g sodium alginate in 60 mL of water and mixed thoroughly. During this procedure the glass beads were covered with a thin layer of the highly viscid alginate solution. The coated glass beads were slowly poured into 800 mL of an aqueous solution containing 2% of calcium chloride and left in this environment for 30 min. Then the glass-cored beads were collected and washed like the solid alginate beads described in the previous procedure.

3.4. Cyclization of squalene epoxide with alginate beads (solid or with glass core)

The immobilisates that have been prepared by one of the above described procedures were given into a solution of 80 mg squalene epoxide and 1.0 g Triton X 100 in 80 mL of Tris buffer and stirred for 20 h at a temperature of 30°C. Then the beads were separated from the solution, washed three times with 40 mL portions of water and the collected aqueous solutions were dried by lyophilization. The resulting dry powder was extracted with three portions (each 60 mL) of ether, evaporated and the reaction product was isolated by column chromatography on 200 g silica gel (grain size: 0.200–0.063 mm, Merck).

The recovered alginate beads (solid or with glass core) were used for five consecutive runs, each with a fresh solution of squalene epoxide.

3.5. Determination of the cell forming units per gram of immobilisates (cfu/g) on agar plates

After every conversion samples of approximately 1 g of the immobilisates were taken and their weight was determined precisely. They were then dissolved in a 1% sodium ethylendiamine tetraacetate (NaEDTA) solution and diluted to exactly 10 mL. 1 mL of this solution was a second time diluted to 10 mL and this procedure was repeated two more times to yield a 10^{-4} fold diluted sample. 100 μL of this sample were distributed on an agar plate that was stored for 48 h at 30°C. Then the cell forming units were counted microscopically and the cfu/g values were calculated.

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