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Effects of medium sterilization on the production of zaragozic acids by the fungus *Leptodontidium elatius*

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The production of zaragozic acids by fermentation of the fungus *Leptodontidium elatius* was examined at the 800-L fermentor scale under two different production medium batch sterilization conditions. Low production-medium heat input (R_0 = 33.4 min) resulted in a 4′-desacetoxy zaragozic acid C : 4′-*O*-desacetyl zaragozic acid C : zaragozic acid C ratio of 0.53 : 0.60 : 1.0. At a higher heat input (R_0 = 50.5 min), the ratio shifted to 1.0 : 0.66 : 1.0 with a corresponding 26% increase in total zaragozic acid production. This higher total zaragozic acid titer resulted from an increase in the amount of 4′-desacetoxy zaragozic acid C produced while the levels of the other two analogues remained unchanged. Batch sterilization conditions also resulted in differences in growth, carbon substrate consumption, and oxygen uptake rates. The structures of the zaragozic acids produced suggest a precursor/end product relationship. A biosynthetic model describing the synthesis of the three zaragozic acids listed above is postulated and used to explain the effects of production-medium heat input during sterilization.

Keywords: medium sterilization; fermentation development; squalene synthase inhibitors; *Leptodontidium elatius*; zaragozic acids

Introduction

Zaragozic acid C and its 4′-desacetoxy and 4′-*O*-desacetyl analogues are produced by the fungus *Leptodontidium elatius* and belong to a class of natural products characterized by a novel 2,8-dioxobicyclo [3.2.1] octane-4,6,7-trihydroxyl-3,4,5-tricarboxylic acid core (Figure 1) [3,11,13]. A variety of zaragozic acids has been isolated from fungi with the individual chemical species differing from each other in the structures of the 1-alkyl and 6-acyl side chains (Figure 1) [2–4]. Zaragozic acids are potent inhibitors of squalene synthase—a key enzyme involved in the synthesis of cholesterol. As a result zaragozic acids hold promise for the treatment of atherosclerosis. In addition, the zaragozic acids possess broad spectrum fungicidal activity at low minimum inhibitory concentrations [3].

The primary goal of heat sterilization, whether accomplished by batch or continuous methods, is to eliminate the indigenous population of viable microorganisms in the medium prior to inoculation with the culture of interest. The lethality of a sterilization process can be quantified using the F_0 method where an F_0 value of one is equal to a 1-min hold time at 121° C [10]. The equation used to calculate F_0 values takes into account the relative sterilizing effects of temperatures above and below 121° C [5].

In addition to the reduction of the viable microbial population, heat sterilization accelerates chemical reactions in the medium. Vital nutrients (eg, vitamins, amino acids, and sugars) may be destroyed while the hydrolysis of complex substrates (eg, proteins and polysaccharides) may be enhanced. The formation of insoluble compounds may eliminate the availability of a nutrient while inhibitory compounds resulting from the interaction of medium components may result in a growth medium which is not conducive to growth and/or product formation [1,15]. Chemical reactions which occur during medium sterilization can be quantified using the R_0 method where an R_0 value of one equals a 1-min hold time at 121° C [6]. Since the equations used to calculate R_0 and F_0 are different, sterilization time and temperature profiles which result in constant R_0 values may result in different F_0 values, suggesting that both values must be taken into account in order to achieve a medium which is nutritionally rich and free of viable microbes [6].

Sterilization conditions have been shown previously to affect fermentation performance. Efrotomycin production by *Nocardia lactamdurans* was greatly improved by sterilizing glucose together with the rest of the medium components [12]. It was suggested that complexation of glucose with proteins in the medium regulated protein metabolism and allowed for a controlled shift from glucose to oil metabolism. This controlled shift from glucose to oil as the carbon source was critical to good efrotomycin production. In the case of zaragozic acid production by *Leptodontidium elatius*, production-medium heat input seems to affect both the levels and the spectrum of the zaragozic acids produced, making this fermentation an interesting scale-up challenge.

Materials and methods

Zaragozic acid C fermentation

Leptodontidium elatius ATCC 70411 was isolated from wood from the Joyce Kilmer Forest in North Carolina, USA. The culture was maintained as aliquots of a mycelial suspension in 5% (w/v) glycerol stored at −70° C. The zaragozic acid fermentation process consisted of three stages of inoculum development and a 500-L production stage (pilot scale). The production medium consisted of (in g L^{-1}): lac-

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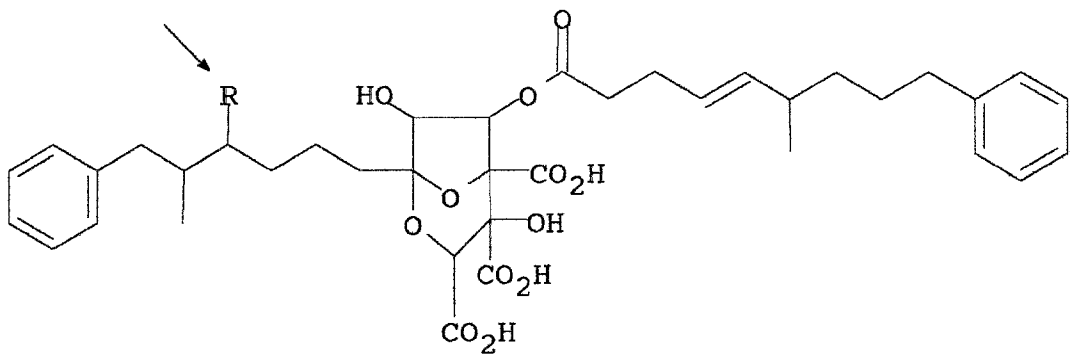

	R (C-4′)
Desacetoxy ZAC	H
Desacetyl ZAC	OH
Zaragozic Acid C	CH_3COO

Figure 1 Structures of desacetoxy ZAC, desacetyl ZAC, and zaragozic acid C

tose, 50; glycerol, 100; primatone HS (Quest, Norwich, NY, USA), 22.5; yeast extract (Fidco, Minneapolis, MN, USA), 7.5; sodium citrate, 11; and $MgSO_4$, 0.5. The pH was adjusted to 6.0 prior to sterilization.

Medium sterilization and fermentation parameters were controlled automatically by a Honeywell TDC 3000 distributed control system (Phoenix, AZ, USA) [7]. Sterilization of production medium was achieved by increasing medium temperature rapidly to 121° C, maintaining that temperature for 25 min (low heat input) or 40 min (high heat input) followed by rapid cooling to 28° C. On-line calculation of R_0 was performed using the equation and constants described in the literature [6]. Using the above sterilization conditions, R_0 values of 33.4 min and 50.5 min were achieved. Process conditions for the production fermentation were: temperature, 28° C; agitation, 160 rpm; aeration, 0.4 vvm; backpressure, 0.7 kg cm^{-2}, and pH maintained below 7.2. Off-gas analyses were performed using a Perkin-Elmer MGA 1200 mass spectrometer (Perkin-Elmer, Emeryville, CA, USA) and a Hewlett Packard model HP 1000 computer as previously described [8,12]. The data presented are representative of two sets of batches run under the above conditions.

For experimentation at the shake-flask scale, 40 ml of production medium in 250-ml Erlenmeyer flasks were sterilized in an autoclave at 121° C for 20, 40, or 60 min. These production cultures were inoculated with 2 ml of inoculum and incubated for 20 days at 28° C with 220 rpm rotary shaking. Data presented are the average of two flasks and are representative of duplicate experiments. The titers for individual flasks were within ±5% of the average.

Analytical methods

Zaragozic acids were extracted from fermentation whole-broth samples by adjusting broth pH to 2.0–2.2 with concentrated sulfuric acid followed by vigorous shaking with two volumes of methyl ethyl ketone. The organic and aqueous phases were separated by centrifugation and an aliquot of the organic layer was evaporated to dryness in a chemical fume hood. The dried extracts were dissolved in methanol (proportional to twice the original broth volume) and analyzed by high pressure liquid chromatography. Twenty microliters of sample were chromatographed on a Zorbax C8-silica column (5-μm spherical particle, 4.6 mm ID × 25 cm, Mac Mod Analytical Inc, Chadds Ford, PA, USA) employing a mobile phase of acetonitrile : 0.1% (v/v) phosphoric acid in water (65 : 35) at a flow rate of 1 ml min^{-1}. Ultraviolet absorption of the eluant at 205 nm was monitored and zaragozic acids were identified and quantified by comparison to pure standards.

Carbohydrate analyses were performed by high pressure liquid chromatography. Fermentation broth samples were prepared by diluting samples 1 : 10 with 0.1 N sulfuric acid followed by filtration to remove mycelia and precipitates. A 20-μl aliquot of filtrate was chromatographed on an Aminex HPX-87H column (Bio-Rad Inc, Richmond, CA, USA) with a mobile phase of 0.0089 N sulfuric acid at a flow rate of 0.6 ml min^{-1}. Changes in the refractive index of the eluant were monitored and carbohydrates were identified and quantified by comparison to standards. Growth of the cultures was monitored by measuring dry cell weight.

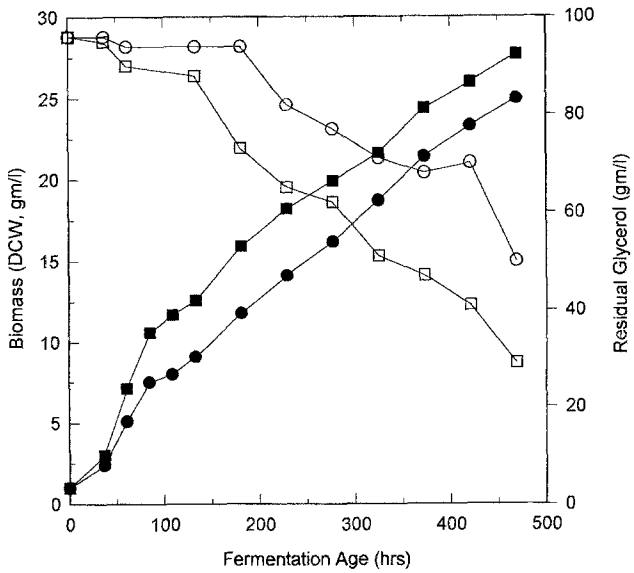

Figure 2 Effect of production-medium heat input on growth and glycerol utilization. Low heat input (R_0 = 33.4 min): growth (●), and glycerol (○). High heat input (R_0 = 50.5 min): growth (■), and glycerol (□)

Results

Low (R_0 = 33.4 min) *vs* high (R_0 = 50.5 min) production-medium heat input resulted in differences in growth, carbon substrate consumption, and off-gas profiles. The high heat input batch attained a slightly higher level of biomass due to an improved growth rate during the first 100 h of the fermentation (Figure 2). Around 150 h the utilization of glycerol became apparent and the higher level of biomass in the high heat input batch resulted in an increased rate of glycerol consumption. However, the specific glycerol consumption rate for the two batches was equivalent at approximately 15 mg g DCW^{-1} h^{-1} (Figure 2). Corresponding to biomass and carbon substrate consumption, fermentor off-gas analysis also revealed differences between the two sterilization conditions (Figure 3). The oxygen uptake rate (OUR) for the low heat input batch reached a value of 3.0–3.5 mmol L^{-1} h^{-1} within the first 50 h of fermentation and then slowly increased to 4.5 mmol over the balance of the cycle. The differences in the OUR profiles is consistent with the levels of biomass attained for the two batches. In contrast, the high heat input batch reached an OUR of 6.0 during the first 50 h of fermentation and then slowly dropped to 3.0 over the balance of the cycle. The respiratory quotients (RQ) for both batches were comparable throughout the cycle remaining between 1.0 and 1.1 (data not shown) even when glycerol consumption began. The slightly higher level of biomass observed for the high heat-input batch may have resulted from an increase in the hydrolysis of complex substrates (eg, proteins) resulting in a richer medium.

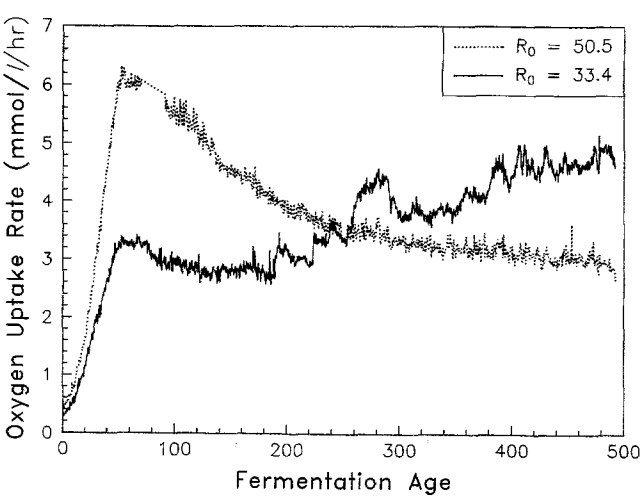

Figure 3 Oxygen uptake rate profiles for low (R_0 = 33.4 min, ——) and high (R_0 = 50.5 min, ·····) heat input batches

The biosynthesis of zaragozic acids was initiated after 150 h of fermentation for both sterilization conditions and increased linearly through the end of the cycle (Figure 4a,b). The initiation of product synthesis corresponded with the beginning of glycerol utilization. A comparison of the production profiles for the individual zaragozic acid species reveals that the rate of accumulation of 4′-desacetoxy zaragozic acid C (desacetoxy ZAC) is increased with a higher heat input (Figure 4a,b). By comparison, sterilization conditions had no effect on the accumulation rates of 4′-*O*-desacetyl zaragozic acid C (desacetyl ZAC) or zaragozic acid C (Figure 4a,b). The ratio of desacetoxy ZAC : desacetyl ZAC : zaragozic acid C remained constant throughout the time course of both fermentations but shifted from 0.53 : 0.60 : 1.0 at low-heat input to 1.0 : 0.66 : 1.0 at high heat input. The increase in desacetoxy ZAC titer (from higher heat input) resulted in a 26% increase in total zaragozic acids produced over the low heat-input batch (Figure 4a,b).

When the fermentation was scaled-down to shake flasks, a pattern of results similar to those achieved at the pilot scale was obtained (Table 1). Employing a 20-min autoclave cycle at 121° C, a significant amount of desacetoxy ZAC was produced. The ratio of the zaragozic acid species produced is 0.68 : 0.14 : 1.0. At a higher heat input (40-min autoclave cycle), the zaragozic acid species ratio shifted to 1.20 : 0.15 : 1.0. The desacetoxy ZAC titer increased two-fold while the level of zaragozic acid C increased by a modest 15% (compared to the 20-min control). The desacetyl ZAC titer was not affected at this level of heat input. A further increase in heat input (60-min autoclave cycle) resulted in no further significant increase in the level of desacetoxy ZAC produced (compared to the 40-min cycle). However, a 32% drop in the titer of zaragozic acid C (compared to the 20-min control) was observed.

Discussion

Recently, the biosynthetic precursors for zaragozic acids were established through ^{14}C-and ^{13}C-labelled substrate feeding (Figure 1) [2,4,9]. The acetoxy group at the C-4′ position is thought to be derived from molecular oxygen and acetate as is the case for the synthesis of the squalestatins (zaragozic acids) produced by *Phoma* sp C2932 [13]. Zaragozic acid labelling studies definitively demonstrated that the carbons of the acetoxy group are derived from acetate [2,4,9].

Figure 5 illustrates a postulated biochemical scheme for

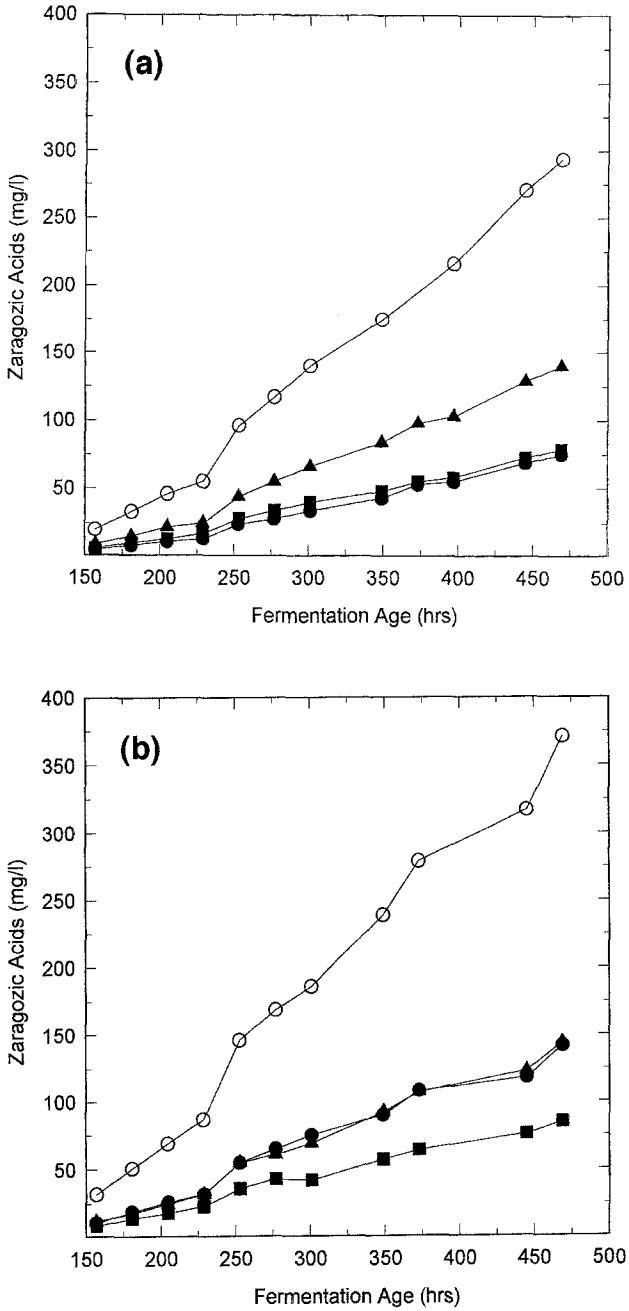

Figure 4 Effect of (a) low (R_0 = 33.4 min) and (b) high (R_0 = 50.5 min) production-medium heat input on the production of total zaragozic acids (○), desacetyl ZAC (■), desacetoxy ZAC (●), and zaragozic acid C (▲)

Table 1 Effect of sterilization time on zaragozic acid production by *Leptodontidium elatius* at the shake-flask scale

Sterilization time (min)	Desacetoxy ZAC (mg L^{-1})	Desacetyl ZAC (mg L^{-1})	Zaragozic acid C (mg L^{-1})	Total zaragozic acids (mg L^{-1})
20	259	53	382	694
40	524	64	438	1026
60	557	55	291	903

Autoclave temperature was 121° C. Titers are the average of duplicate flasks given in mg L^{-1} for 20 days of incubation

the enzymatic conversions of desacetoxy ZAC to desacetyl ZAC and from desacetyl ZAC to zaragozic acid C. From the results of studies with labelled substrates, it appears plausible that the acetoxy group at the C-4′ position is added by a two-step process: 1) an enzymatic monooxygenation reaction (eg cytochrome P-450) is responsible for the conversion of desacetoxy ZAC to desacetyl ZAC (secondary alcohol at the C-4′ position); and 2) acetylation of the hydroxy group at the C-4′ position of desacetyl ZAC yielding zaragozic acid C.

While the formation of desacetyl ZAC and zaragozic acid C is unaffected by heat input, the synthesis of these two analogues clearly does not keep pace with the increase in the synthesis of desacetoxy ZAC (Figure 4a,b). Even under relatively mild sterilization conditions at the shake-flask scale (20 min at 121° C, Table 1), a sizeable amount of desacetoxy ZAC is produced indicating that the rate-limiting steps in the formation of zaragozic acid C are the penultimate and ultimate steps in this biosynthetic pathway.

A possible explanation for this 'uncoupling' phenomenon may be that the enzyme(s) responsible for the conversion of desacetoxy ZAC to zaragozic acid C are regulated differently from those enzymes which synthesize desacetoxy ZAC. If the levels of enzyme(s) required for the synthesis of desacetoxy ZAC are in excess of those necessary for its conversion to zaragozic acid C, then an increase in the flux of precursors (ie, acetate, succinate, and phenylalanine) would result in an increase in the amount of desacetoxy ZAC produced. The increased rate of glycerol utilization observed for the high heat-input batch would result in a greater flux of carbon into acetate and succinate. Similarly, an increase in the hydrolysis of protein substrates (as a result of heat input) could result in an increased supply of phenylalanine. While the OUR profiles for the two batches are different, the RQs are similar indicating that both batches are metabolically similar (Figure 3). Studies with labelled precursors indicate that molecular oxygen and acetate would be required for the two-step conversion of desacetoxy ZAC to zaragozic acid C (Figure 5). The availability of molecular oxygen and acetate for the conversion of desacetoxy ZAC to zaragozic acid C appears to be adequate since the dissolved oxygen in the fermentation broth remained above 80% of saturation (data not shown) and an adequate supply of acetate has already been demonstrated for the increase in desacetoxy ZAC synthesis.

Other explanations may also exist which describe the observed results. It should be noted that with the medium formulation employed for the zaragozic acid fermentation, the presence of lactose in the medium during sterilization will result in Maillard-type browning reactions between lactose and free amino acids along with the conversion of lactose to lactulose [14]. How or if Maillard-type compounds or lactulose affect the level and/or spectrum of the zaragozic acids produced is unclear.

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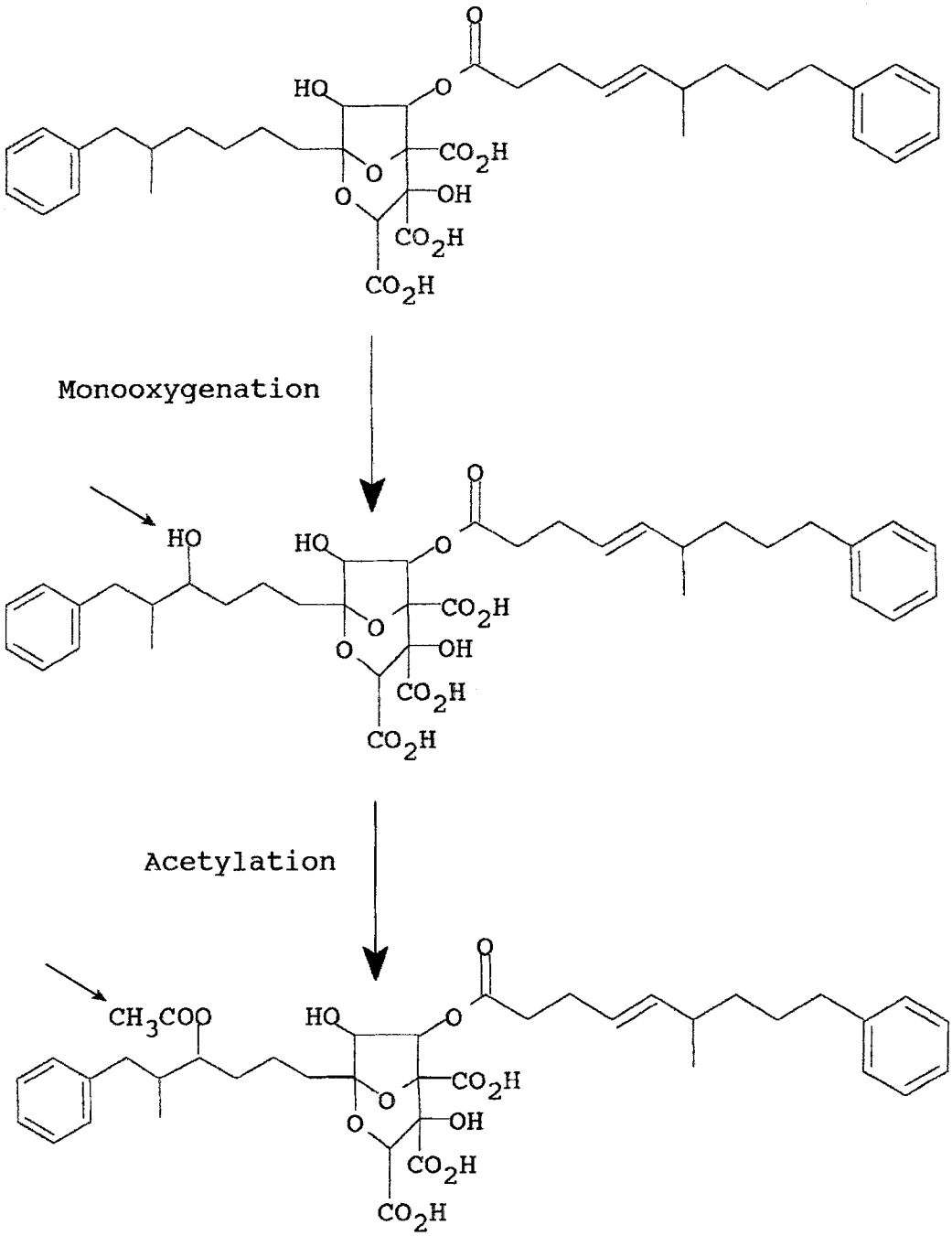

Figure 5 Postulated 2-step enzymatic sequence (at the C-4′ position) for the conversion of desacetoxy ZAC to desacetyl ZAC (monooxygenation) and desacetyl ZAC to zaragozic acid C (acetylation)

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