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# Closure of the Oxide Bridge in Morphine Biosynthesis

**Rainer Lenz and Meinhart H. Zenk***

Lehrstuhl für Pharmazeutische Biologie, Universität München
Karlstrasse 29, 80333 München, Germany

**Abstract:** A highly substrate specific enzyme has been discovered and purified to homogeneity, which transfers the acetyl-moiety from acetyl-coenzyme A to the 7-OH group of salutaridinol. The formed 7-O-acetyl-salutaridinol spontaneously closes the oxide bridge at pH 8-9 by allylic elimination furnishing the morphine precursor thebaine.

The morphine skeleton consists of five ring systems housing five asymmetric centers. Thebaine is the first biogenetic precursor of morphine containing already this pentacyclic ring system. The immediate precursor of thebaine is the tetracyclic salutaridinol.[1,2] The transition of salutaridinol to thebaine involves the closure of the oxide bridge between C-4 and C-5 of the biogenetic precursor (7S)-salutaridinol. Salutaridinol has the correct (7S)-configuration for an allylic *syn*-displacement of the activated hydroxyl group by the phenolic hydroxyl group which follows the precedented stereocontrol for $S_N2'$ substitutions at cyclohexene rings.[2] The oxide bridge closure was first achieved in studies on the chemical synthesis of morphine by using dibrominated precursors.[3,4] Later, with the biogenetic precursor, salutaridinol, in hand, it was shown, that under acid catalysis the pentacycle was easily formed both with the (7R)- and (7S)-epimer[1] yielding in both cases thebaine (Fig. 1).

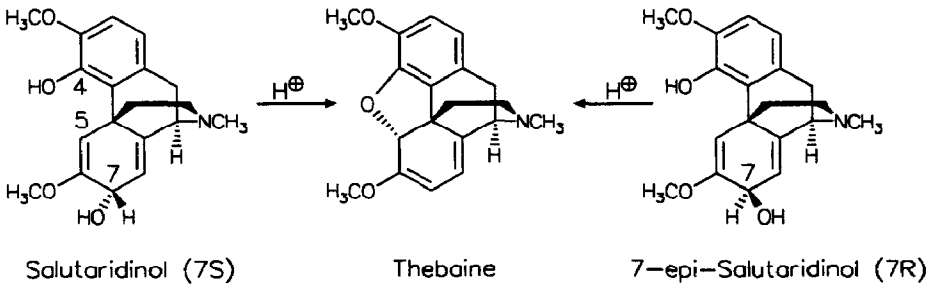


Fig. 1. Acid catalyzed transformation of both salutaridinol epimers into the morphine precursor thebaine.

In our attempt to solve the complete sequence of biosynthetic events at the enzyme level, leading from the primary metabolite *L*-tyrosine to the end product morphine, we want to detect targets for the gene technological modification of the genus *Papaver* at the alkaloid level. After establishing the cytochrome P-450 catalyzed oxidative phenol coupling of (R)-reticuline to yield salutaridine[5,6] and the discovery of salutaridine: NADPH 7-oxidoreductase,[7] reducing stereoselectively salutaridine to (7S)-salutaridinol, we now want to explore the way in

which salutaridinol is transformed to thebaine in the poppy plant. In the presence of $H^+$ (pH 1) at 30°C, salutaridinol is chemically transformed with a first order reaction velocity constant of 0.00073 x $s^{-1}$ and 7-epi-salutaridinol with that of 0.00033 x $s^{-1}$ into thebaine. This reaction was even observed[1] to occur at pH 5, a pH value which can easily be present in vacuoles of plant cells. The non-enzymatic transformation of these epimers was, however, ruled out by the fact that only salutaridinol and not its (7R)-epimer was incorporated into the morphinan alkaloids of the differentiated *Papaver* plant *in vivo*,[1,2] indicating a strictly enzyme catalyzed reaction. Feeding [7-$^3$H]salutaridinol and separately [7-$^3$H] 7-epi-salutaridinol (each 10 nmol; 2x$10^5$ cpm) to *Papaver* cell suspension cultures showed again the exclusive conversion of salutaridinol into thebaine (95% incorporation), while the 7-epimer was metabolically inert. This experiment proves that our poppy cell culture strain[6] contains an enzyme system capable of thebaine formation.

In an attempt to discover this enzyme, cell-free enzyme extracts from suspension cells were prepared,[7] but showed absolutely no activity towards the expected reaction. It was therefore assumed that the enzyme under consideration might be housed in a cellular organelle. Sucrose density centrifugation[8] of a crude organelle preparation[9] from cultured poppy cells yielded three organellar fractions: mitochondria, proplastids and glyoxisomes. Separate incubation of the three fractions (each 0.6 mg protein) with [7-$^3$H]salutaridinol (1 nmol; 2x$10^5$ cpm) in 100 mM $KPO_4^{2-}$ buffer, pH 7.0, at 30°C for 20 hrs showed that the main enzyme activity converting salutaridinol to thebaine was found in the mitochondrial fraction, but also the proplastid and glyoxisomal fractions showed minor enzyme activity. Further purification of the mitochondrial fraction by percoll gradient centrifugation[10] showed that the alkaloid transformation activity was correlated with the presence of intact mitochondria. Since intact mitochondria are known to generate ATP it was speculated that possibly an intermediate 7-O-phosphate ester of salutaridinol was produced, as previously suggested by Battersby,[11] which could subsequently undergo allylic elimination to give the complete pentacyclic system. Fortification of the enzyme system with ATP/$Mg^{2+}$, or other nucleotide triphosphates, did not enhance the enzyme catalyzed conversion of salutaridinol to thebaine, indicating that there is no involvement of energy rich phosphates in this alkaloid transformation. However, addition of a cofactor mix[8] consisting of pyruvate, thiamine pyrophosphate, ADP, $Mg^{2+}$, $NAD^+$, $NADP^+$ and coenzyme A enhanced salutaridinol conversion to thebaine by 100%. Careful analysis of the possible mitochondrial intermediates formed gave the clue that the mitochondria generated acetyl-coenzyme A (Ac-CoA) in the presence of these factors and indeed all of the cofactors could be replaced by addition of Ac-CoA. The enzyme catalyzing the transition of salutaridinol to thebaine in the presence of Ac-CoA as the only cofactor is probably a cytosolic enzyme, adhering also in small amounts to organelles. Since $^3$H-labelled salutaridinol of high specific activity (17 μCi/μmol) was used for these experiments, even extremely low levels of enzyme activity could be detected.

This soluble Ac-CoA dependent enzyme was purified by ammonium sulfate precipitation and four subsequent chromatographic steps to apparent homogeneity. The purification was 3000-fold at a yield of 2.7%. The enzyme has a broad pH optimum between pH 7 and 9, a temperature optimum at 47°C, an isoelectric point at pH 4.8 and a molecular weight of 50 ± 1 kD. The enzyme catalyzes the stoichiometric transfer of an acetyl group from one mole of Ac-CoA to one mole of salutaridinol. 7-epi-Salutaridinol as a substrate is inactive, thus demonstrating the *in vivo* observed specificity. At pH 9, monoacetyl-salutaridinol spontaneously closes the oxide bridge thus yielding thebaine which fact was verified by MS and NMR. The question remained, which of the two OH-groups, that at C-4 or C-7 of the salutaridinol molecule, is acetylated by action of the enzyme. Incubation of salutaridinol (40 μM) and [2-$^{14}$C]Ac-CoA (40 μM; 5x$10^5$ cpm) in 200 mM phosphate buffer, pH 7.0 together

with 192 pkat of the homogeneous enzyme in a final volume of 50 µl yielded after 5 min at 30°C a sole radioactive compound at Rf 0.8 (Fig. 2).

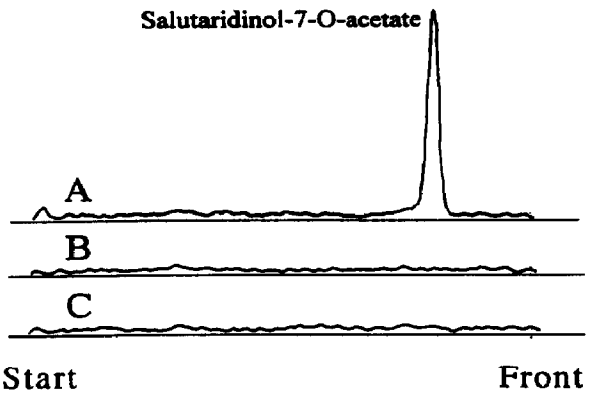


Fig. 2. Radio-TLC of an incubation mixture consisting of A: salutaridinol, [2-$^{14}$C]Ac-CoA and enzyme, showing the formation of salutaridinol-7-O-acetate; B: control without salutaridinol; C: control without enzyme. Solvent system - $CHCl_3$: acetone: diethylamine (5:4:1).

Omission of either enzyme or salutaridinol resulted in no reaction. Salutaridinol-7-O-acetate was then synthesized by incubation of salutaridinol (30 µmol) and acetic anhydride (30 µmol) in 50 µl pyridine at room temperature for 24 hrs. TLC resolution ($CHCl_3$: EtOH: EtAc: acetone = 6:2:1:1) yielded a compound (Rf 0.3) showing the following spectral characteristics: **$^1$H-NMR ($CDCl_3$)**: δ (ppm) = 6.72 (d, 1 H, $C_2$-H), 6.63 (d, 1 H, $C_1$-H), 6.48 (s, 1 H, $C_5$-H), 5.83 (dd, 1 H, $C_8$-H), 3.87 (s, 3 H, $C_3$-$OCH_3$), 3.66 (s, 3 H, $C_6$-$OCH_3$), 2.48 (s, 3 H, $NCH_3$), 2.02 (s, 3 H, $C_7$-OAc). This chemically synthesized salutaridinol-7-O-acetate was identical to the enzymatically formed metabolite and in aqueous phase at pH 9 it was converted to thebaine. The enzyme catalyzed the stoichiometric transfer of the acetyl-moiety of Ac-CoA to the 7-OH group of salutaridinol, yielding 7-O-acetyl-salutaridinol. The enzyme is named therefore: acetyl-coenzyme A: salutaridinol-7-O-acetyltransferase. This enzyme catalyzes the reaction depicted in Fig. 3. 7-O-Acetyl-salutaridinol is a new metabolite in the biosynthesis of morphine-type alkaloids and has previously not been recognized as such. The enzyme is present in *Papaver somniferum* cell suspension cultures, as well as in seedlings and mature plants of this species. The most interesting fact seems to be that 7-O-acetyl-salutaridinol, at slightly alkaline pH values, spontaneously rearranges to thebaine by closing the oxide bridge. We firmly believe that this reaction is spontaneous, since this reaction both in crude *Papaver* homogenates and with the homogeneous acetyltransferase yielded at pH 8 to 9 almost exclusively thebaine together with small amounts of breakdown products. A search for a specific enzyme catalyzing the formation of thebaine from 7-O-acetyl-salutaridinol was not successful. It is therefore most likely that the biosynthetic pathway from *L*-tyrosine to morphine will consist of 16 enzymes, 15 of which have been discovered in this laboratory to date. In addition, two of the rarely occurring spontaneous reactions, the isomerization of neopinone to codeinone[12] and the formation of thebaine from 7-O-acetyl-salutaridinol, make the biosynthetic pathway complete.

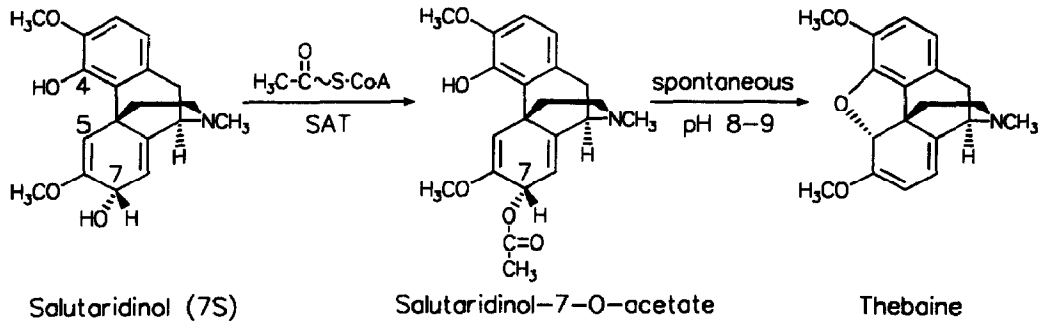


Fig. 3. Reaction catalyzed by acetyl-coenzyme A: salutaridinol-7-O-acetyltransferase (SAT) and subsequent spontaneous allylic elimination at pH 8-9 to thebaine.

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# Dedicated to Professor Arnold Brossi on the occasion of his 70th birthday.

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