Pergamon

Tetrahedron Letters, Vol. 37, No. 13, pp. 2221-2224, 1996

0040-4039/96 $15.00 + 0.00

S0040-4039(96)00276-6

Does the Malonyl-coenzyme A:21-Hydroxypregnane 21-Hydroxy-malonyltransferase Catalyze the First Step in the Formation of the Butenolide Ring of Cardenolides?

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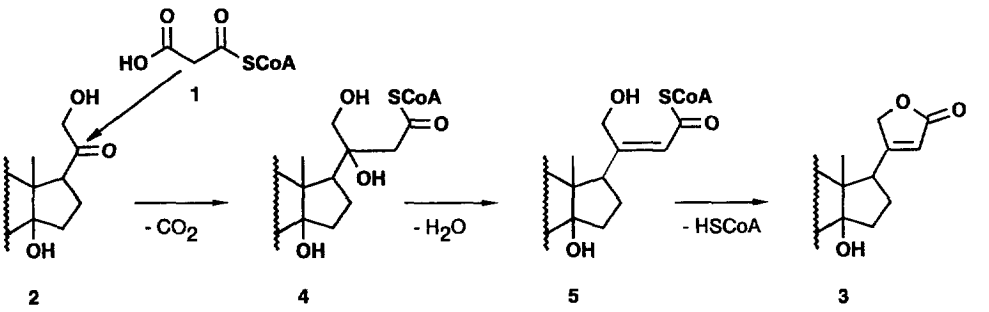
Abstract: An enzyme catalyzing the transfer of the malonyl moiety from malonyl-coenzyme A **1** to the 21-hydroxy group of 3β,14β,21-trihydroxy-5β-pregnane-20-one **2** was isolated from *Digitalis lanata* leaves and characterized. The role of this particular enzyme in cardenolide biosynthesis is discussed.

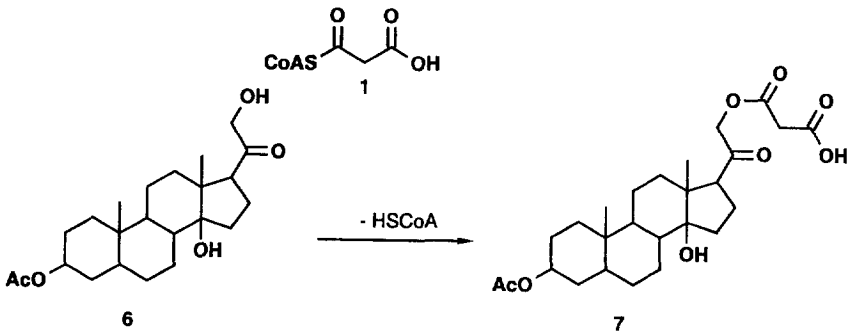
Cardenolides are thought to be formed via a series of pregnane intermediates[1,2]. As far as the formation of the butenolide ring is concerned, it is supposed that the condensation of 3β,14β,21-trihydroxy-5β-pregnane-20-one **2** with a dicarbon unit yields the final product of the hypothetical sequence, namely digitoxigenin **3**. One putative mechanism of the butenolide formation using malonyl-coenzyme A **1** as the cosubstrate is depicted in scheme 1. The first step of this mechanism is the nucleophilic attack of **1** at the carbonyl group (C-20) of the pregnane. Loss of CO_2 accompanies the attack at the carbonyl group and in this way the CoA ester of a norcholanoic acid **4** is formed. Dehydration of this intermediate leads to the $\Delta^{20(22)}$E-derivative **5** and finally, intramolecular ester formation yields the complete butenolide ring.



Scheme 1: Hypothetical mechanism of butenolide ring formation

Feeding experiments with *Digitalis lanata* shoot cultures showed that the addition of 3β-O-acetyl-14β,21-dihydroxy-5β-pregnane-20-one **6** to the bathing medium resulted in a 4-fold increase in the cardenolide content in the shoots, indicating that this substance may indeed be a good substrate for the putative butenolide ring-forming enzyme[3].

Cell-free extracts from *Digitalis lanata* leaves were prepared in an attempt to discover the enzyme catalyzing this reaction. When **6** was incubated together with **1** in an enzyme preparation of *Digitalis lanata* leaves, a product was formed which was identified as the malonyl hemi-ester **7** of the substrate (scheme 2). The enzyme catalyzing this reaction was termed malonyl-coenzyme A:21-hydroxypregnane 21-hydroxy-malonyltransferase (MHPMT).



Scheme 2: Reaction catalyzed by the malonyl-coenzyme A:21-hydroxypregnane 21-hydroxy-malonyltransferase

At the beginning of our studies the leaves were homogenized in 100 mM Hepes-KOH buffer (pH 7.0, with 250 mM sucrose, 20 mM mercaptoethanol and 2 % polyvinylpolypyrrolidone). The homogenate was centrifugated for 20 min at 20,000 x *g*, the supernatant was filtered through Miracloth and subsequently submitted to gel filtration chromatography in order to remove cardenolides and other substances with low molecular weights. This method was also used to exchange buffers when necessary. In a total volume of 250 μl the incubation mixture contained: 5 μl dimethylsulfoxide with **6** (final concentration 0.5 mM), 20 μl buffer with **1** (final concentration 4 mM) and 225 μl protein extract. The assay was incubated for 2 h at 37 °C and then terminated by the addition of 1 ml CH_2Cl_2. Prior to extraction, 10 μl of a testosterone solution (1 mg ml^{-1} EtOH) were added as the internal standard. The cups were shaken vigorously for 5 sec and phase separation was facilitated by centrifugation (5 min at 12,000 x *g*); the organic phase was removed and evaporated at 40 °C. The residue was dissolved in 60 μl CH_2Cl_2 and the amount of malonylated pregnane formed was determined as its decarboxylated derivative by capillary GC.

Enzyme activity was quite similar in most of the buffers examined (Hepes-KOH, Tris-HCl, Mops-KOH, phosphate and McIlvaine's citrate-phosphate, each 100 mM and adjusted to pH 7.0), but in Hepes-KOH buffer only 50% of the maximum activity was obtained.

At 37 °C the malonylation rate was linear up to 1 hour, when the protein concentration in the incubation mixture lay between 0.3 and 0.8 mg per ml.

Thiol reagents stimulated the activity of the MHPMT. When 1,4-dithiothreitol (DTT) was substituted for mercaptoethanol, enzyme activity increased by a factor of 4 (5 mM DTT) to almost 5 (10 mM DTT).

Optimal enzyme activity was observed at pH 6.5, with about half the maximum activities each at pH 5.8 and 7.0. The malonyl hemi-ester was saponified above pH 8.0.

The temperature optimum lay at around 50 °C. The energy of activation, as taken from an Arrhenius plot, was 59.5 kJ mol^{-1}.

No loss of enzyme activity over 24 h was observed when the enzyme extract was kept at -20 °C, +4 °C or even 22 °C.

The major part, almost 70%, of the MHPMT activity was found to be soluble. Only 30 % of the total activity was found to be associated with the microsomes. The product was demalonylated by isolated microsomes but not by the soluble protein fraction.

Only **1** and acetoacetyl-CoA, which has a quite similar structure to **1**, were accepted as cosubstrates. No reaction was observed when acetyl-CoA or succinyl-CoA were added to the incubation mixture as the cosubstrates. CoA inhibited the malonylation reaction.

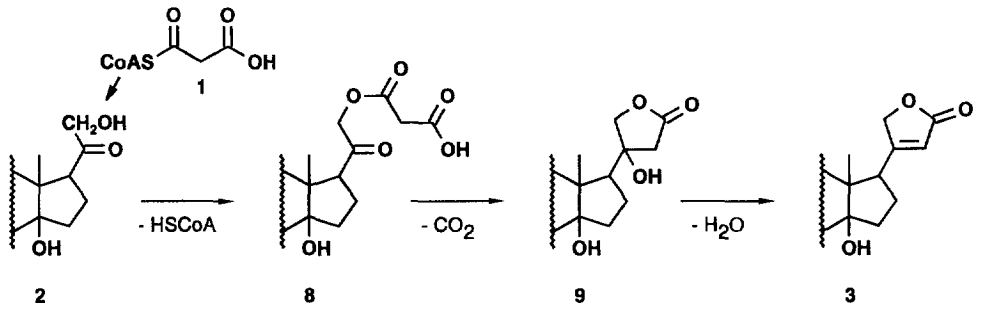
Investigating the substrate specifity, different steroids with 21-hydroxy groups, i.e., 3β,21-dihydroxy-pregn(5)en-20-one (21-hydroxy-pregnenolone), 21-hydroxy-pregn(4)ene-3,20-dione (21-hydroxy-progesterone), 21-hydroxy-5β-pregnane-3,20-dione and 3β,21-dihydroxy-5β-pregnane-20-one were tested. The products were identified by GC-MS[4] by comparing the spectra with those of 21-O-acetylated steroids, since the malonyl hemi-esters are decarboxylated to the corresponding acetyl esters at the high temperatures used in GC-analysis. **6** and **2** were the most suitable substrates for the transferase reaction. All other 21-hydroxylated steroids tested were malonylated only to a very small extent. Since only 14β-hydroxylated substances are good substrates for the enzyme, it may also be assumed that the rings C and D of the steroid skeleton have to be cis-connected.

Different tissues of *Digitalis lanata* were examined to correlate between the cardenolide content and MHPMT activity. The highest enzyme activities were detected in the young leaves (58 μkat kg^{-1} protein), whereas no activity was found in cardenolide-free suspension cultures. In dark-grown shoot cultures, which do not accumulate cardenolides but are able to regain their biosynthetical capacity when transferred into light[5], an enzyme activity of 16 μkat kg^{-1} protein was found. A good correlation between the cardenolide content and the MHPMT activity was also observed in various strains of light-grown shoot cultures, which differed in their cardenolide content. Strain C with the lowest cardenolide content of approximately 50 nmol per g dry weight exhibited with 23 μkat kg^{-1} protein only weak MHPMT activity, whereas in strain E with almost 500 nmol cardenolides per g dry weight an activity of 53 μkat kg^{-1} protein was determined. Hence, it may be supposed that this enzyme plays a regulatory role in the biosynthetic pathway.

Tissues of other cardenolide-free and cardenolide-containing species were also examined for MHPMT activity. Malonylation was not observed in protein extracts of either *Equisetum arvense*, *Lamium album*, *Urginea maritima* or *Helleborus niger*. The MHPMT activity in *Digitalis lanata* (100%) was quite similar to that in *Nerium oleander* (116%) and *Isoplexis canariensis* (94%), and activity was also found in *Convallaria majalis* (32%).

Since malonyl hemi-esters are known to decarboxylate at temperatures above 140 °C, the assay mixture was incubated at 90 °C for 1 hour and 120 °C for 20 min. The malonylated pregnane decarboxylates under these conditions and forms two products, namely 3β,21-di-O-acetyl-14β-hydroxy-5β-pregnane-20-one and the 3β-O-acetyl derivative of **3**. The butenolide ring is formed spontaneously, when **7** is stored at room temperature for

2 to 3 weeks. Scheme 3 presents the hypothesis drawn from these experiments. In contrast to the earlier hypothesis of butenolide ring formation we postulate that the nucleophilic attack of **1** at C-20-carbonyl of **2** (Claisen condensation) is not the first step in butenolide ring formation, but that a malonyl hemi-ester **8** is formed prior to this condensation, which decarboxylates and yields the butenolide ring **3** under dehydratation of **9**.



Scheme 3: Proposed pathway of butenolide ring formation

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft.

References and Notes

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4. MS data of 3β,21-di-O-acetyl-14β-hydroxy-5β-pregnane-20-one: 434 (M^+), 416 (M^+-H_2O), 406 (M^+-C_2H_4), 392 (M^+-C_2H_2O), 374 (M^+-C_2H_2O-H_2O), 356 (M^+-C_2H_2O-H_2O-H_2O), 346 (M^+-C_2H_2O-CH_2OH-CH_3), 331 (M^+-C_2H_4-CO_2-CH_2OH), 314 (M^+-C_2H_2O-C_2H_2O-H_2O-H_2O), 301 (M^+-C_2H_2O-C_2H_2O-CH_2OH-H_2O).
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(*Received in Germany* 7 *December* 1995; *revised* 26 *January* 1996; *accepted* 7 *February* 1996)