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Journal of Chromatography B, 860 (2007) 195–201
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Synthesis of a putative substrate for malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase and development of an HPLC method for the quantification of the enzyme reaction

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Received 29 July 2007; accepted 22 October 2007
Available online 1 November 2007

Abstract

The butenolide ring is the main common characteristic of all cardenolides. Its formation is supposed to be initiated by the transfer of a malonyl moiety from malonyl-coenzyme A to an appropriate 21-hydroxypregnane. A new, reliable, fast and sensitive method to determine malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase activity had to be developed since previous attempts employing HPLC, TLC or GC did not prove successful. A surrogate substrate was synthesized containing a side chain resembling the sugar side chain attached to C-3 of putative cardenolide precursors and containing a chromophor allowing UV detection. 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one and its 21-*O*-malonylated derivative were synthesized, the latter being the expected product of the enzyme reaction. The new substrate was well accepted by the enzyme. An HPLC method has been established to detect and quantify 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one and its 21-*O*-malonylated derivative, 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-one 21-*O*-malonylhemiester. The method was validated.

Keywords: HPLC method; Method validation; Benzoylation; Malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase; Cardenolides; Cardiac glycosides

1. Introduction

Cardenolides, such as digitoxin and digoxin, belong to the cardiac glycosides. These molecules have remained clinically important for the treatment of congestive heart failure and supraventricular arrhythmias since the effects of *Digitalis purpurea* were first described in 1785 [1]. More than two centuries later, digoxin and other representatives of cardiac glycosides still have their place in therapy despite the introduction of newer drugs like ACE inhibitors and β-blockers [2–4]. In the late1960s, antitumor activity of cardiac glycosides was postulated [5] and subsequently confirmed [6–8]. More recently, the effects of cardenolides were tested on patients with cystic fibrosis [9,10]. Digitoxin mimics the effects of gene therapy with cystic fibrosis transmembrane conductance regulator (CFTR) and suppresses hypersecretion of interleukin-8 (IL-8) from cystic fibrosis lung epithelial cells. Currently, cardenolides can only be extracted from plant materials and clarifying their biosynthesis would diversify their production, e.g., by metabolic engineering. With this in mind, a number of enzymes have been isolated, characterized and the responsible genes have been cloned [11,12].

The butenolide ring is the main characteristic of all cardenolides and its formation is believed to be initiated by the transfer of a malonyl moiety from malonyl-coenzyme A to suitable 21-hydroxypregnanes [13]. Direct investigation of this reaction and purification of the enzyme involved in the formation of 21-malonyloxy pregnanes is difficult because quantification of the malonylated product proved impossible and TLC techniques [14] have been used to detect the reaction product. Gas chromatography has also been used, but due to the decarboxylation of the product 3β-acetoxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester (**2**) at high temperatures, only the acetylated derivative (**7**) is identified (Fig. 1) [15]. The synthesis of a new putative substrate, incorporating a chromophor, and its corresponding reaction product,

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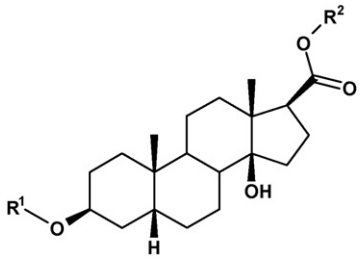
doi:10.1016/j.jchromb.2007.10.038



	R¹	R²
1:	Acetyl	H
2:	Acetyl	Malonyl
5:	Benzoyl	H
6:	Benzoyl	Malonyl
7:	Acetyl	Acetyl

Fig. 1. Structures of compounds cited in the text.

allowed the development of a simple and reliable HPLC method.

2. Materials and method

2.1. Reagents and materials

Digitoxigenin, benzoyl chloride, pyridine, ethylacetate, acetonitrile, sulfuric acid, TLC F_{254} aluminium and glass plates were from Merck (Darmstadt, Germany). Testosterone was from Sigma (Taufkirchen, Germany), zinc dust was from Aldrich (Steinheim, Germany), TLC glass plates RP 18 were from Machery-Nagel (Düren, Germany) and malonyl dichloride was obtained from Fluka (Buchs, Switzerland). Malonyl-coenzyme A was purchased from Sigma (Deisenhofen, Germany) and dimethylsulfoxide from Serva (Heildelberg, Germany).

2.2. Preparation of 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester

2.2.1. Synthesis of benzoyldigitoxigenin (*4*)

The synthesis of **4** was carried out following the method of Elber et al. [16], with some modifications. Five hundred milligram of digitoxigenin (**3**) was dissolved under agitation in dried pyridine (2.5 ml) and subsequently treated with an equal volume of benzoyl chloride for 30 min at room temperature (25–30 °C). The reaction was stopped adding 8.5 ml water hydrolysing the remaining benzoyl chloride. Afterwards the solution was extracted three times with CH_2Cl_2 and the benzoylated product was isolated on preparative TLC plates in dichloromethane/ethylacetate (4:1). The R_f was 0.45 with a yield of 72.6%. Its melting point was determined with Büchi 535 equipment using capillary tubes (80 mm × 1.0 mm) at 257.3–258.7 °C (uncorrected).

2.2.2. Synthesis of 3β-benzoyloxy-5β-pregnane-14β, 21-dihydroxy-20-one (*5*)

In order to synthesize compound **5** 464 mg of **4** was subjected to ozonolysis, following the protocol described by Rabitzsch [17] with minor modifications only. The reaction was carried out at −70 °C. The main product **5** was isolated by preparative TLC using CH_2Cl_2/CH_3OH (92.5:7.5) as mobile phase. The R_f was 0.92, the yield 31.3% and the melting point 156.7–161.4 °C (uncorrected).

2.2.3. Synthesis of 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester (*6*)

Compound **6** was synthesized using the method of Padua et al. (manuscript submitted). As recommended, the malonylation (achieved by dissolving the educt in malonylchloride) was carried out at −70 °C. The reaction was started adding 47 mg of the powdery compound **5** to the pre-chilled malonyl dichloride. The reaction was stopped by adding ice, and the resulting precipitate was collected by suction filtration and dissolved in chloroform. The organic phase was washed and evaporated completely. The reaction product was isolated by preparative TLC on RP 18 plates using dichloromethane: acetone (9:1 v/v) as the developing solvent. The R_f was 0.22, the yield 35.75% and the melting point 143.8–170.3 °C (uncorrected). Fig. 2 shows the main compounds involved in the synthesis.

2.3. Characterization of synthesized products

NMR spectra were recorded either on a Varian Unity 300 or a Varian Inova 500 spectrometer (Varian, Darmstadt, Germany). ESI MS were recorded on a Finnigan LCQ (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with quaternary pump Rheos 4000 (Ercatech, Bern, Switzerland). High-resolution electrospray ionization mass spectrometry (HRESI) mass spectra were recorded on A Bruker FTICR 4.7 T mass spectrometer (Bruker Biospin, Rheinstetten, Germany). Electron ionization mass spectroscopy (EI MS) spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorokerosene as the reference substance for HRESI MS. Compound **6** was characterized by comparing the chromatographic patterns of both chemical and enzymatic products by means of TLC and HPLC.

2.4. Enzyme test

In a total volume of 250 μl the incubation mixture contained 10 μl ethanol (96%) with **5** (final concentration 0.2 mM), 10 μl dimethylsulfoxide with malonyl-coA (final concentration 0.2 mM) and 230 μl protein extract prepared from young fresh leaves of *D. purpurea* L. The assay was incubated for 1 h at 42 °C (Thermomixer 5437; Eppendorf, Hamburg, Germany). The reaction was terminated adding 1 ml of CH_2Cl_2, but before terminating the reaction, the medium was acidified with 250 μl citrate buffer (50 mM, pH 3.5). Prior to extraction, 15 μl of a testosterone solution (10 mM in MeOH) was added as the internal standard. The eppendorf tubes were shaken thoroughly for 5 s and phase separation was facilitated by centrifugation (2 min, 13,000 × *g*) (Centriguge Biofuge 13; Heraeus, Nuremberg, Germany). The organic phase was removed and evaporated overnight in a dry block (Dry-Block DB 2A; Techne, Cambridge, U.K.). The residue was dissolved in 60 μl MeOH and the yield of malonylated pregnane was determined by HPLC.