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Journal of Chromatography B, 860 (2007) 195–201
JOURNAL OF CHROMATOGRAPHY B
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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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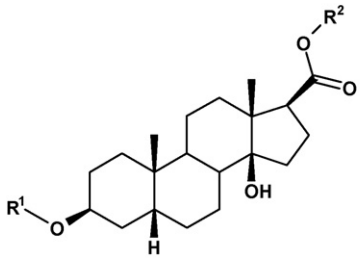
doi:10.1016/j.jchromb.2007.10.038



	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.

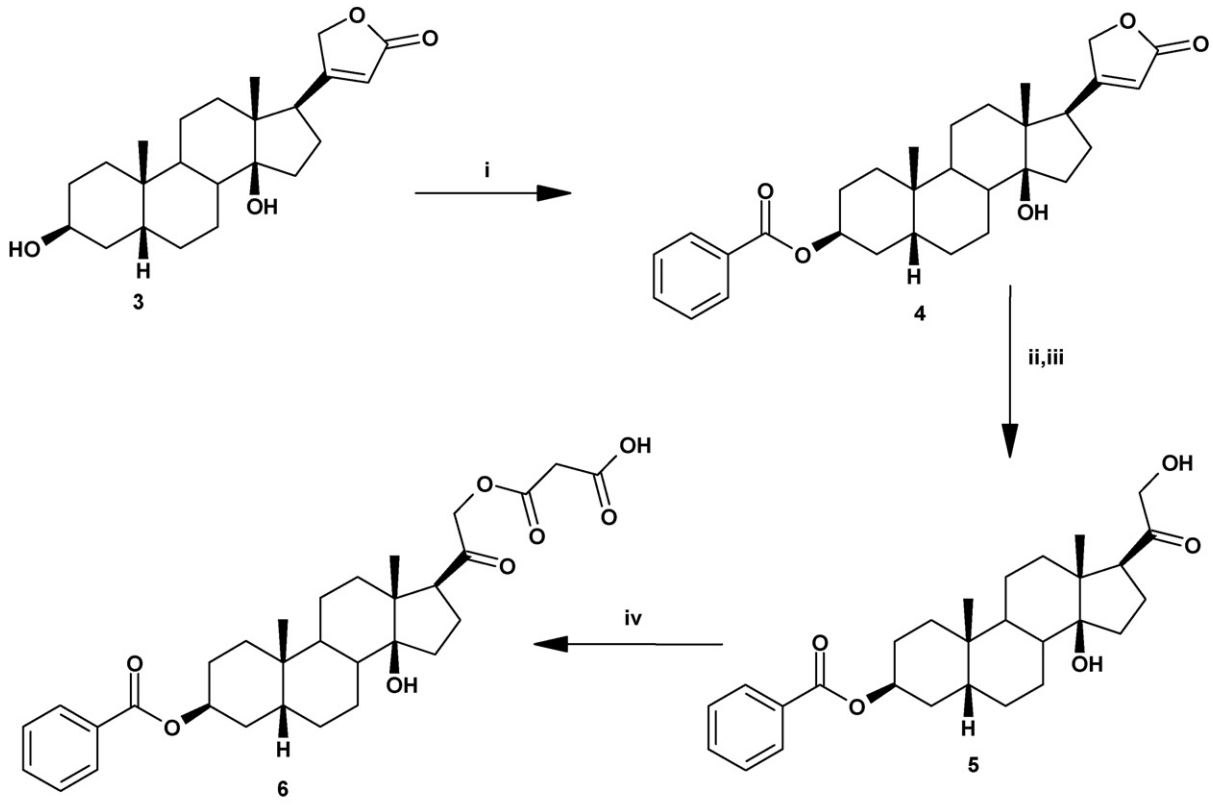


Fig. 2. Synthesis of 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-one-21-malonylhemiester (**6**) (34 mg, 35.75%) from 500 mg of digitoxigenin (**3**). Benzoyldigitoxigenin (**4**) (464 mg, 72.6%), 3β-benzoyloxy-5β-pregnane-14β, 21-dihydroxy-20-one (**5**) (80 mg, 31.3%). Reagents and conditions: (i) C_6H_5COCl, pyridine, RT, 30 min; (ii) O_3-pyridine-EtOAc, −70 °C, 3.6 h; (iii) AcH, H_2O, Zn-dust, 4 °C, 20 h; (iv) $CH_2(COCl)_2$, −70 °C, 5 min..

2.5. Development of the quantification method

2.5.1. Instrumentation and operating conditions

The HPLC system consisted of a 1525 Binary HPLC Pump system with a Waters 2487 Dual wavelength (λ) absorbance detector and an automatic sampler Waters 717 plus (Waters, Eschborn, Germany). The separation of substrate and product was achieved on a Reprosil-Pur 120-C_{18} column (particle size 5 μm, 4.6 mm × 250 mm). UV absorbance was measured at 195 and 228 nm. An autosampler (10 °C) was used. Methanolic solution (15 μl) was injected and analyzed using double-distilled water (pH 2.5, adjusted with H_3PO_4) (solvent A) and acetonitrile (solvent B). The gradient used for the separation of compounds **5** and **6** was as follows: initial = 65% acetonitrile, 22 min = 85% acetonitrile, 23 min = 100% acetonitrile isocratic until 25 min, 26 min = 65% acetonitrile, 28 min = 65% acetonitrile. UV spectra were recorded between 190 and 295 nm.

2.5.2. Calibration curves

For constructing a calibration curve solutions of **6** in methanol were used at the following concentrations: 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM ($n = 3$ for each concentration).

2.5.3. Sensibility and variability of the method

Intra-assay precision was calculated within a single run as the coefficient of variation (CV%) for five determinations at one concentration. Inter-assay precision was established assaying all seven concentrations twice in triplicate. The lower limit of quantification (LLOQ) was defined as the lowest amount of **6** that could not be accurately determined with the HPLC procedure used. Compound **6** was checked for its detection limit in HPLC and TLC.

2.5.4. Application

The HPLC method developed has been implemented with a view to quantifying the reaction catalyzed by the malonyl-coA: 21-hydroxypregnane 21-*O*-malonyltransferase from *D. purpurea* (*Dp*21MaT) as well as its characterization [18].

3. Results and discussion

3.1. Characterization of the synthesized compounds

In an earlier study products of malonyl-coA: 21-hydroxypregnane 21-*O*-malonyltransferase action were identified by GC-MS and their spectra compared to those of pregnane acetyl esters as the malonylhemiesters were decarboxylated to the corresponding acetyl esters at the high temperatures used in GC analysis [15]. Alternative methods identifying the putative substrates and products, such as HPLC and TLC, were also flawed. Therefore, a reliable, fast and sensitive method had to be developed. For practical reasons HPLC/UV without mass detection is the method of choice, although MS-hyphenated methods would have allowed the use of ^{13}C-labelled

Table 1
^{13}C NMR chemical shifts (ppm) of **6** and ^{1}H NMR chemical shifts (ppm) of **6** and **3** in $CDCl_3$ solution

Carbon	δ (ppm) (**6**)	Hydrogen	δ (ppm) (**6**)	δ (ppm) (**3**)
1[a]	30.9	3α	5.28	4.18
1[a]	30.9			
2[a]	25.3			
3	71.1	1α, 1β,	2.40–1.20	2.20–1.20
4[a]	30.8	2α, 2β,		
5	37.3	4α, 4β,		
6	26.4	5β,		
7	21.5	6α, 6β,		
8/23	40.18	7α, 7β,		
9/10	35.3	8β,		
11	20.9	9α,		
12	39.3	11α, 11β,		
13	49.9	12α, 12β,		
14	85.1	15α, 15β,		
15	34.0	16α, 16β,		
16[b]	24.9			
17	57.9			
18	15.3			
19	23.9	17α	2.81	2.79
20	210.5	18	0.92	0.88
21	69.9	19	1	0.98
22	166.3	21a	4.82	5.01
24	168	21b	4.78	4.90
1′	165.9	22	–	5.86
2′	132.7	23	3.54	–
5′	131.2	3′/7′	8.06	–
4′/6′	129.5	4′/6′	7.44	–
3′/7′	128.3	5′	7.55	–

[a] Interchangeable.

natural substrate(s) or product(s), thus increasing the sensitivity of the assay or providing an optimal internal standard, respectively.

A surrogate substrate was synthesized containing a side chain resembling the sugar side chain attached to C-3 of putative cardenolide precursors and a chromophor, making UV/HPLC detection more sensitive than a method employing possible natural substrates, namely 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one (**3**) and its 21-*O*-malonylated derivative were synthesized, the latter being the expected product of the enzyme reaction.

For a comparison the educt digitoxigenin **3** was also characterized spectroscopically. The HRESI mass spectrum exhibited a pseudo molecular ion of compound **3** at *m/z* 375.253234 $[M+H]^+$, corresponding to the molecular formula $C_{23}H_{35}O_4$. The peaks at *m/z* 357 and 339 correspond to the loss of two molecules of water, which confirms the presence of two hydroxy groups in compound **3**. The ^{1}H NMR data (Table 1) indicate that compound **3** is a steroid with two methyl groups at δ 0.88 and 0.98 (s, 3H each), 21 protons between 1.20 and 2.20 ppm, a methine at δ 2.79 down field due to the fact that it is close to the double bond. A methine signal at δ 4.18 (m) was attributed to the proton in position C-3 and 2H signal at δ 4.90 (*dd*, 1H, *J* = 1.6 and 18.1 Hz) and 5.01 (*dd*, 1H, *J* = 1.6 and 18.2 Hz.) This indicates that these two protons are carried by the same carbon and are different due to their position in the space, the existence of a broad singlet at 5.86 down field due to the double bond and the presence of the cyclic carbonyl ester.

EI MS (70 eV): *m/z* (%) = 374 $[M]^+$ (7), 356 $[M-H_2O]^+$ (34), 338 $[M-2H_2O]^+$ (6), 246 (16), 203 (100), 195 (7), 162 (14), 147 (15), 124 (11), 111 (17), 85 (14).

After benzoylation of **3**, compound **4** was obtained as a white powder. Compound **4** was formed spontaneously following the addition of benzoyl chloride for 5 min. Adding an equal volume of pyridine to the reaction medium shifted the reaction quite rapidly towards the formation of the benzoylated product. Under these conditions complete benzoylation was achieved after 30 min of incubation. The HRESI mass spectrum exhibited a pseudo molecular ion of compound **4** at *m/z* 479.279164 $[M+H]^+$, corresponding to the molecular formula $C_{30}H_{39}O_5$.

HRESI MS: *m/z* = 479.279164 $[M+H]^+$, 501.261095 $[M+Na]^+$. The ^{1}H NMR spectra of **4** and **3** were nearly identical, the only difference being the presence of a phenyl group with five down field protons at δ 8.03 (dd, 2H, *J* = 1.5 and 7.0 Hz), 7.55 (t, 1H, *J* = 7.4 Hz), 7.44 (t, 2H, *J* = 7.8 Hz) in **4**. The shift of the H-3α from 4.18 to 5.34 ppm is due to the new ester linkage at C-3. Benzoylation of another cardenolide genin, namely uzarigenin, was reported by Elber et al. [16]; however, NMR data were not provided.

The target enzyme substrate **5** was obtained after ozonolysis of **4** and isolated as a pure white product directly from the ozonolysis reaction mixture. Performing ozonolysis at −70 °C allowed the benzene ring to stay intact since at low temperatures only C=C bonds in alkenes, but not in aromatic cycles and alkynes, are attacked [19]. The HRESI mass spectrum exhibited a pseudo molecular ion of compound **5** at *m/z* 477.26115 $[M+Na]^+$, corresponding to the molecular formula $C_{28}H_{38}O_5Na$. The peaks at *m/z* 437 and 419 correspond to the loss of two molecules of water, which confirms the presence of two OH in compound **5** (δ 4.15 and δ 3.08 for 14β-OH and 21-OH, respectively).

The proton spectra of compounds **5** and **4** were quite similar, the only difference being in the range 5.00–4.00 ppm with the presence of a doublet at δ 4.30 down field in compound **5** due to a carbonyl and alcohol group and the doublet of doublets in compound **4** which was not present anymore in compound **5**.

EI MS (70 eV): *m/z* (%) = 454 $[M]^+$ (18), 436 $[M-H_2O]^+$ (4), 427 (8), 426 (29), 423 $[M-CH_3O]^+$ (100), 408 (11), 378 (9), 354 (9). **HRESI MS**: *m/z* = 477.26115 $[M+Na]^+$, 493.23508 $[M+K]^+$.

Compound **5** was accepted as substrate by malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase. The enzyme reaction yielded **6**. The enzymatic product was checked for its purity by TLC and HPLC and was further compared to the synthetic compound **6**. The (+)-HRESI/MS exhibited a *pseudo*molecular ion of compound **6** at *m/z* 563.261297 $[M+Na]^+$, corresponding to the molecular formula $C_{31}H_{40}O_8Na$. CI MS mass spectrum exhibits a pseudo molecular ion at *m/z* 496, which is consistent with the loss of carbon dioxide (CO_2).

The ^{1}H NMR data indicated that compound **6** is a tetracyclic triterpenoid [20] with two methyl groups at δ 0.92 and 1.00 (s, 3H each), 21 protons between 1.20 and 2.32 and a doublet of doublets at δ 2.85. A methylene group at δ 3.52 (s, 2H) is down

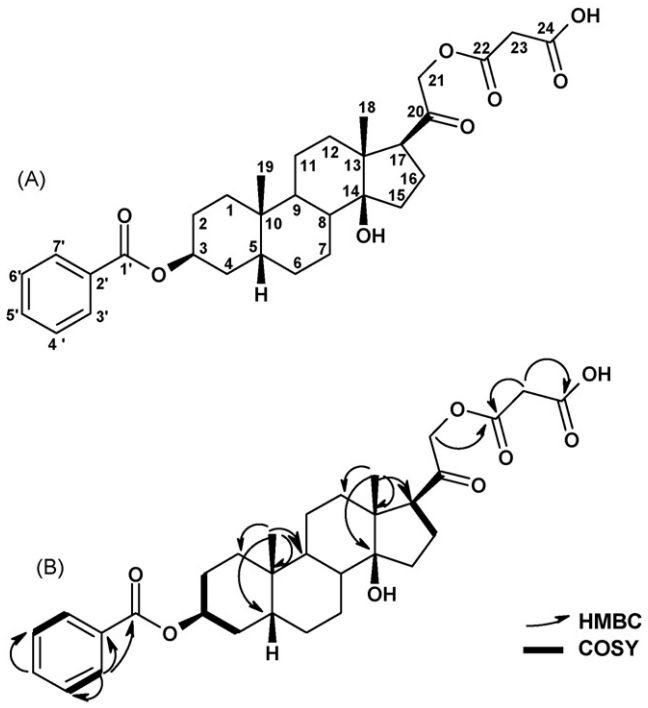


Fig. 3. (A) 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester; (B) attributed HMBC correlations in compound 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester and 1H-1H COSY.

field due to the fact that it is close to two carbonyl carbons and the proton at the position 3 appears at δ 5.36. The presence of a substituted phenyl group is characterized by five down field protons at δ 7.44 (t, 2H, J = 7.6 Hz), 7.56 (t, 1H, J = 7.5 Hz), 8.06 (d, 2H, J = 7.2 Hz).

The ^{13}C NMR including COSY, DEPT, HMBC and HSQC showed 31 carbon signals that were sorted by DEPT into 2 methyl, 11 methylene, 10 methine and 7 quaternary carbon signals, 5 of which are carbonyl carbons. Correlation is seen between the H-3 signal and protons H-2 and H-4, the H-23 signal and carbons 22 and 24 in the ^{1}H-^{1}H COSY and HMBC spectra (Fig. 3B). This contributes to the conclusion that compound **6** is 3β-benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester (Fig. 3A), described here for the first time.

CI MS: m/z (%) = 514 $[M-CO_2+NH_4]^+$ (48), 496 $[M-CO_2]^+$ (70), 479 $[M-CO_2-H_2O+H]^+$ (100), 437 (20), 421 (24), 301 (62), 193 (19), 176 (53), 161 (62), 144 (70), 134 (35), 122 (17), 97 (16), 80 (23).

(+)- **HRESI MS**: m/z = 563.26130 $[M+Na]^+$, 523 $[M-H_2O+H^+]$ (calcd. 563.26154 for $C_{31}H_{40}O_8Na$).

The NMR data (Table 1) obtained for the skeletons are consistent with those described for other pregnane derivatives [21].

After benzoylation, ozonolysis and malonylation of **3** the chemical synthesis of compound **6** was achieved with an overall yield of 4.8%.

3.2. Quantification method

3.2.1. UV spectrum

The UV spectrum of benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester (**6**) exhibited two absorption peaks at 195 and 228 nm, whereas compound **2** did not show any specific maximum. The UV spectrum of **6** is specific for compounds with benzoyloxy substituents, as also noted by Reichelt et al. [22]. Furthermore, these results are consistent with those recorded for free and glucose-conjugated benzoic acid. They showed UV spectra from 200 nm with maxima at 233 and 229 and shoulders at 275 and 272 nm, respectively [23]. At a wavelength of 195 nm compound **6** displays absorption 10 times that of compound **2**; at 228 nm absorption is 50 times higher in compound **6** compared to compound **2**. This fact can be attributed to the benzoyl substitute at C-3. Wavelengths of 195 and 228 nm were subsequently used in the development of the quantification method.

3.2.2. Chromatographic separation and linearity

Conditions for chromatography were chosen investigating several different mobile phases and internal standards. The aqueous solvent had to be acidified to maintain the protonated status of **6**. The auto sampler temperature of 10 °C guaranteed the chemical stability of **6**. The peaks of all analytes were well separated. Calibration curves were established at 195 and 228 nm resulting in the regression equations $y = 963247x - 38382$ and $y = 292228x - 11433$, respectively. Excellent linearity (r^2 = 0.9997) was observed at both wave-

Table 2
Summary of validation results

Concentration (μM)	Area 195 nm			Area 228 nm		
	Mean	S.D.[a]	Precision (CV%)	Mean	S.D.	Precision (CV%)
200	3827719	77781	2	1159034	21927	1.2
100	1883402	11590	0.6	576787	3954	0.6
50	880660	20206	2.3	267712	7234	2.7
25	443178	19323	4.3	134254	4925	3.6
12.5	190190	688	0.3	56969	1429	2.5
6.25	102231	2441	2.3	29956	742	2.4
3.125	49719	1124	2.2	14691	295	2
r^2	0.9997			0.9997		
Slope	963247			292228		

[a] S.D.: standard deviation.

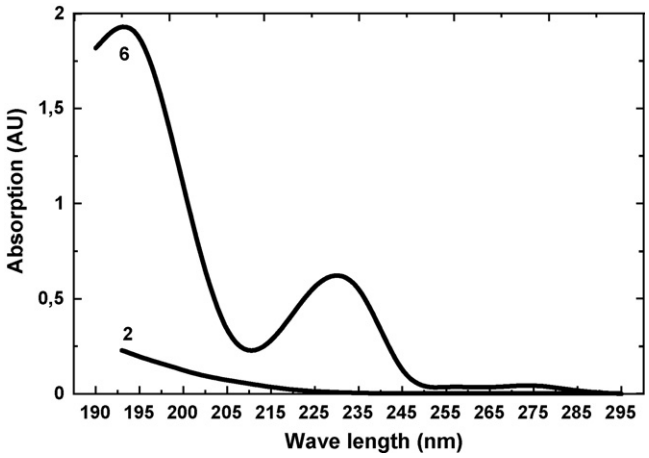


Fig. 4. UV spectrum of compounds **2** and **6**.

lengths. Testosterone was chosen as the internal standard because of its structural similarity to the educts and products analyzed.

3.2.3. Precision and sensitivity

The precision of the method expressed as the coefficient of variation (%) is shown in Table 2. Variations for intra-assay precision range from 0.3 to 4.3% at 195 nm and from 0.6 to 3.6% at 228 nm; inter-assay precision (results not shown) varies from 0.8 to 15.7%. Developing a method to determine oxcarbazepine and its main metabolites in human plasma and cerebrospinal fluid, Kimiskidis et al. [24] reported coefficients of variations ranging from 2.3 to 7.1% and 3.4 to 8.2% for intra- and inter-assay precision assays, respectively. Boudra and Morgavi [25] obtained values between 2.9 and 35% while developing a method for the quantitation of ochratoxins in plasma and raw milk.

The lower limit of quantification was defined as the lowest concentration measured with satisfactory accuracy and precision. For **6** the LLOQ was measured as a concentration of 3.125 μM, or 62.5 pmoles, at 195 as well as at 228 nm. Thus, the described method can be successfully used at 195 and 228 nm. The HPLC method used proved to be more sensitive than the TLC system, which can be attributed to the benzoyl group attached to the product being analyzed.

Fig. 4 illustrates the suitability of the described HPLC method comparing detection of the natural substrate and product to the newly synthesized surrogate substrate **5** and its product **6**. The separation of both substrate and product, which had been a real challenge in the past, can be achieved. The retention times were 15.3 min for the substrate and 14.2 min for the product (Fig. 5).

The benzoyloxy-5β-pregnane-14β-hydroxy-20-on-21-malonylhemiester stored as a dry powder was stable for more than 1 month.

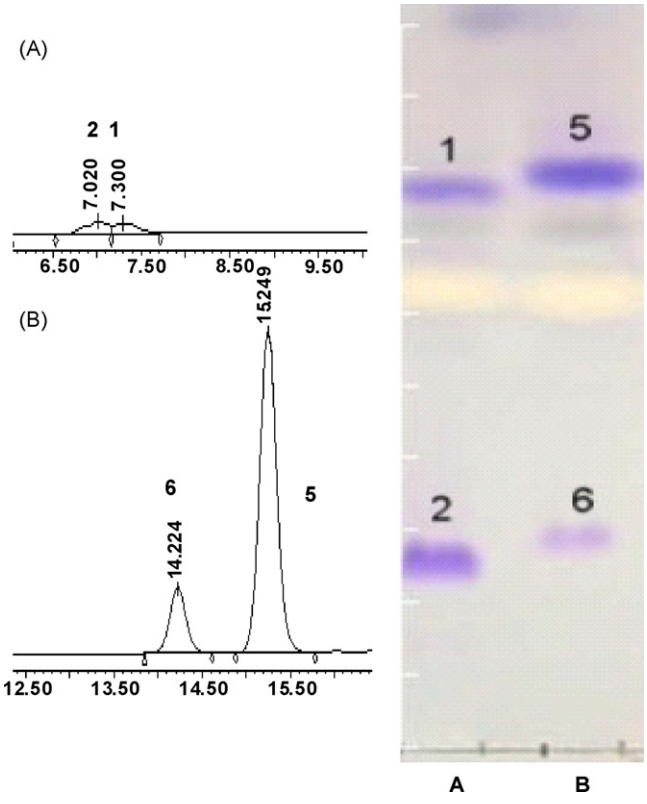


Fig. 5. HPL and TL chromatograms. (A) Assay with non-benzoylated substrate; substrate (**1**), product (**2**). (B) Assay with benzoylated substrate; substrate (**5**), product 19 (**6**).

Acknowledgements

The authors wish to thank the German Academic Exchange Service (DAAD) for granting doctoral fellowships to S.P. Kuate and R.M. Padua. We are grateful to Ms. Barbara White for linguistic advice.

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