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Norcholanic acids as substrates for recombinant 3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase, enzymes of the 5β-cardenolide biosynthesis

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ABSTRACT

The conversion of 23-nor-5,20(22)*E*-choladienic acid-3β-ol and other intermediates of the putative norcholanic acid pathway of cardenolide biosynthesis by recombinant 3β-hydroxysteroid dehydrogenase from *Digitalis lanata* in dehydrogenation and reduction reactions was investigated. 23-nor-4,20(22)*E*-choladienic acid-3-one was found to be a substrate of recombinant progesterone 5β-reductases from *D. lanata* and *Arabidopsis thaliana*. The role of various substrates in cardenolide biosynthesis is discussed.

Cardenolides are plant-derived drugs widely used for the treatment of cardiac insufficiency in humans. Recent findings have indicated potential new therapeutic roles for these compounds in various other diseases including cancer.[1,2] Cardiac glycosides possess a steroid skeleton and most of the *Digitalis* cardenolides are 5β-configured. The structural elements responsible for the biological activity of cardenolides are a 14β-hydroxy group and an unsaturated five-membered lactone-ring at C17β. The biosynthesis of the butenolide ring of cardenolides still needs to be investigated. Two possible routes are discussed, but text books still only opt for the so-called 'pregnane pathway' including intermediates such as pregnenolone **10** and progesterone **11** although an alternative 'norcholanic acid pathway' was already introduced by Maier et al. in 1986 (Scheme 1).[3] Feeding experiments employing labelled norcholanic acids indicated that some norcholanic acids, and in particular those with a pregnenolone structure, serve as precursors of cardenolides.[3,4] Kreis et al. proposed a 'norcholanic acid pathway' starting with 23-nor-5,20(22)*E*-choladienic acid-3β-ol **3**, the norcholanic acid equivalent of pregnenolone **10**, and discussed that the norcholanic acid pathway may exist alongside the well-accepted pregnane pathway. If the norcholanic acid pathway is operative in cardenolide formation, putative intermediates of that pathway should be substrates of enzymes, such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and progesterone 5β-reductase (5β-POR), supposedly involved in the cardenolide biosynthesis.[5] The objective of this study was to investigate this issue. Moreover, the suitability of plant enzymes with rather broad substrate preferences for the selective conversion of steroid compounds should be demonstrated.

The 3-dehydrogenation of norcholanic acids in the presence of NAD as the co-substrate was investigated using recombinant 3β-HSD.[6] The following educts were reduced to their respective 3-keto compounds under standard incubation conditions: 23-nor-5α-chol-20(22)*E*-enic acid-3β-ol **1**, 23-nor-5β-chol-20(22)*E*-enic acid-3β-ol **2**, 23-nor-5,20(22)*E*-choladienic acid-3β-ol **3** and 3β-hydroxy-5β-cardenolide **9**. 5α-pregnane-3β-ol-20-one and 5β-pregnane-3β-ol-20-one, that is, pregnanes corresponding to **1** and **2**, respectively, were previously found to be dehydrogenated by recombinant 3β-HSD.[6] In contrast, 23-nor-5-cholenic acid-3β,20ξ-diol **7** was not accepted as substrate. This corroborates previous findings where the administration of this particular norcholanic acid did not increase the cardenolide pool in *Digitalis lanata* shoot cultures.[4] In the same study, administration of the norcholadienic acid **3** increased the cardenolide pool by about 80%.[4] Maier et al. also reported that **7** is a poor cardenolide precursor compared to **3**.[3] The enzyme recombinant 3β-HSD also catalyzed the 3-dehydrogenation of 3β-hydroxy-5-ene-cardenolide **14**, a compound possessing a butenolide ring in position C17, to its 3-keto derivative **15** (Scheme 1).

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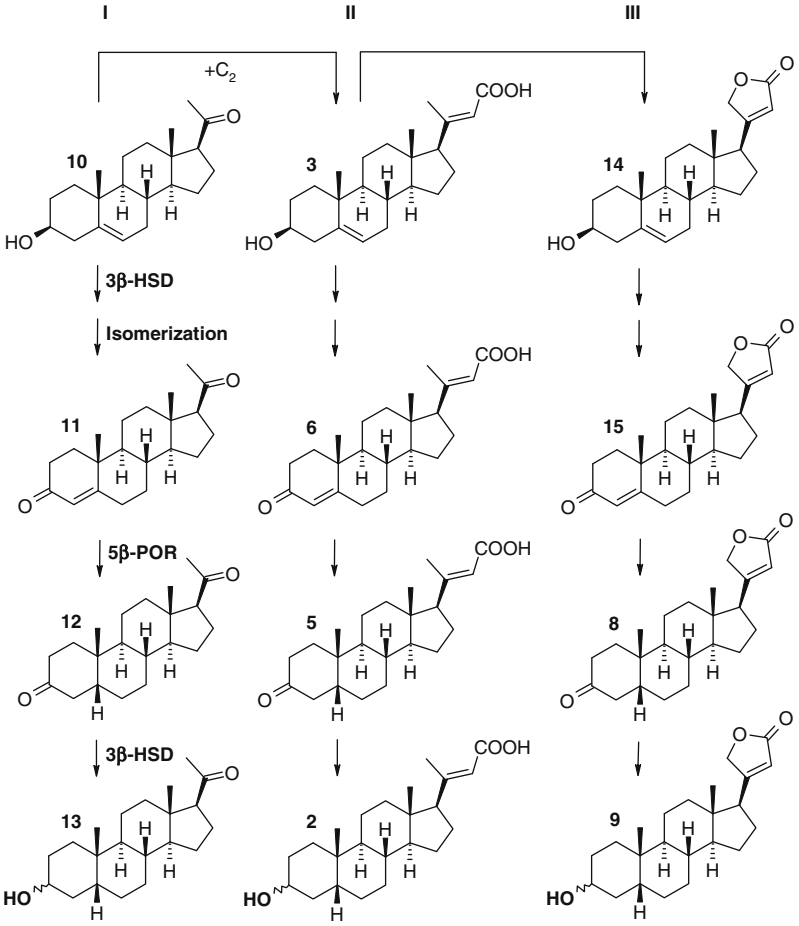


Scheme 1. Possible cardenolide pathways: routes including pregnane intermediates (I), norcholanic acids (II) or cardenolides (III).

The K_m and V_{max} values for the tested norcholanic acids **1**, **2** and **3** varied from 326 to 581 μM and 227 to 440 pkat/mg protein, respectively, for dehydrogenation (1 mM NAD as co-substrate) (Table 1). The K_m value of pregnenolone **10** was 56 μM with a V_{max} of 337 pkat/mg protein. Interestingly, as deduced from the V_{max}/K_m ratios, the cardenolide **9** (K_m = 254 μM; V_{max} = 7074 pkat/mg protein) was the best substrate being about 30 and 5 times more converted than the norcholanic acids and pregnenolone **10**, respectively. This indicates that 3β-dehydrogenation and 5β-reduction may be further down the pathway towards the 5β-cardenolides than previously assumed and that the biosynthesis might even start with a butenolide ring formation.

The conversion of **1** and **2** to their corresponding 3-keto compounds was simultaneously confirmed in experiments where these compounds were fed to suspension-cultured *D. lanata* cells (see Supplementary data). The 3-keto products were already detectable in the spent medium, but not in the cells after 3 h of incubation.

Using NADH as a co-substrate recombinant 3β-HSD did not only reduce 5β-pregnane-3,20-dione **12**, the putative natural substrate,[6] but also 23-nor-5α-chol-20(22)*E*-enic acid-3-one **4** and 23-nor-5β-chol-20(22)*E*-enic acid-3-one **5**. The corresponding pregnane-3,20-diones had already been described to be substrates of recombinant 3β-HSD.[6] Since no 3α-configured norcholanic acids references were available to us we can not exclude that mixtures of the respective 3α and 3β isomers **13** were formed. It is very likely that this was the case since 5β-pregnane-3,20-dione was shown to be reduced to its two isomeric 3-ols.[6]

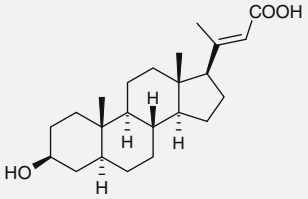
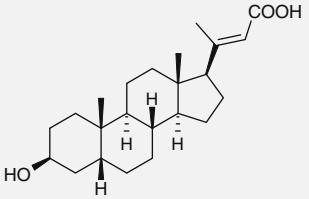
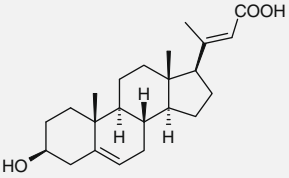
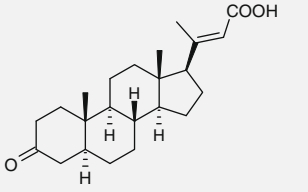
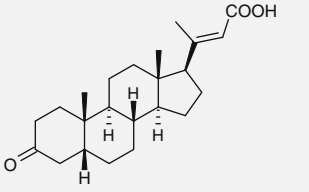
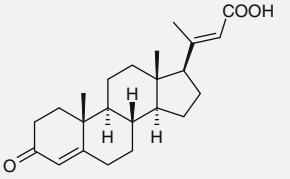
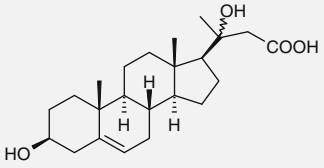
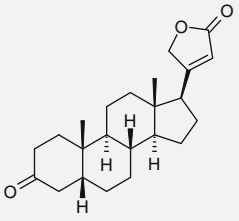
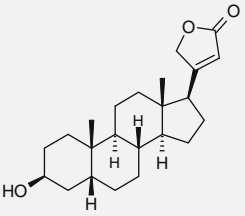
The K_m and V_{max} values for the tested substrates **4**, **5** and **8** varied between 359–1516 μM and 1146–10860 pkat/mg protein, respectively, for reduction (2 mM NADH as co-substrate) (Table 1). The K_m value for the putative natural substrate pregnane-3,20-dione **12** was 526 μM, and V_{max} was 444 pkat/mg protein. Hence, the norcholanic acid **5** was converted less efficiently whereas 3-oxo-5β-cardenolide **8** and the norcholanic acid **4** were converted with V_{max}/K_m ratios about 10 times and 7 times, respectively, higher than that for pregnane **12** (Table 1).

The formation of **1** and **2** from **4** and **5**, respectively, by the native 3β-HSD was also observed in feeding experiments with suspension-cultured *D. lanata* cells. The products were detected in the spent medium, but not in the cells after 24 h of incubation.

Under the incubation conditions chosen subsequent chemical isomerization of the Δ5 double bond yields the respective Δ4 compounds which are potential substrates for other enzymes, namely steroid 5β-reductases. The crucial step in the biosynthesis of 5β-cardenolides is catalyzed by the stereospecific progesterone 5β-reductase (5β-POR). This short chain dehydrogenase/reductase (SDR) requires NADPH as a co-substrate and on the basis of structural data it was proposed that 5β-POR reduces a conjugated double bond in a steroid substrate via a 1–4 addition mechanism.[7] A carbonyl group at position 3 in conjugation with a Δ4-double bond is reported to be an essential structure in steroid substrates for 5β-POR.[8] Here, the 5β-reduction of norcholanic acids was studied using recombinant 5β-POR, a recombinant form of the native *D. lanata* progesterone 5β-reductase.[8] Besides its putative natural substrate progesterone **11** the enzyme accepted various pregnanes but no sterols.[8] The rather large 3-oxo cholanoic acid **6** also contains the structural features mentioned above.

Actually, the enzyme accepted 23-nor-4,20(22)*E*-choladienic acid-3-one **6** yielding the 3-keto compound 23-nor-5β-chol-20(22)*E*-enic acid-3-one **5**. Side chain and carboxyl group seem

Table 1
Compounds tested as substrates for recombinant 3β-HSD and 5β-POR. Reaction conditions and kinetic parameters are shown below the respective structure.[a,b] The expression and purification of the recombinant forms of 3β-HSD (GenBank AJ345026) and 5β-POR (GenBank AAS93804) were achieved as described previously (see Supplementary data)[6,8]

No.	Structure and kinetic data	No.	Structure and kinetic data	No.	Structure and kinetic data
1	 23-Nor-5α-chol-20(22)*E*-enic acid-3β-ol 3β-HSD + NAD Relative activity 80% K_m 326 μM V_{max} 227 pkat/mg protein V_{max}/K_m 0.7	**2**	 23-Nor-5β-chol-20(22)*E*-enic acid-3β-ol 3β-HSD + NAD Relative activity 141% K_m 581 μM V_{max} 440 pkat/mg protein V_{max}/K_m 0.8	**3**	 23-Nor-5,20(22)*E*-choladienic acid-3β-ol 3β-HSD + NAD Relative activity 32% K_m 376 μM V_{max} 417 pkat/mg protein V_{max}/K_m 1.1
4	 23-Nor-5α-chol-20(22)*E*-enic acid-3-one 3β-HSD + NADH Relative activity 28% K_m 359 μM V_{max} 2047 pkat/mg protein V_{max}/K_m 5.7	**5**	 23-Nor-5β-chol-20(22)*E*-enic acid-3-one 3β-HSD + NADH Relative activity 80% K_m 501 μM V_{max} 1146 pkat/mg protein V_{max}/K_m 0.4	**6**	 23-Nor-4,20(22)*E*-choladienic acid-3-one 5β-POR + NADPH Relative activity 27% K_m 250 μM V_{max} 845 pkat/mg protein V_{max}/K_m 3.4
7	 23-Nor-5-cholenic acid-3β,20ξ-diol 3β-HSD + NAD Relative activity 0	**8**	 3-Oxo-5β-cardenolide 3β-HSD + NADH Relative activity 140% K_m 1516 μM V_{max} 10860 pkat/mg protein V_{max}/K_m 7.2	**9**	 3β-Hydroxy-5β-cardenolide 3β-HSD + NAD Relative activity 116% K_m 254 μM V_{max} 7074 pkat/mg protein V_{max}/K_m 28

[a] Reference substrate of recombinant 3β-HSD + NAD: pregnenolone: K_m 56 μM, V_{max} 337 pkat/mg protein, V_{max}/K_m 6.1; reference substrate of recombinant 3β-HSD + NADH: 5β-Pregnane-3,20-dione K_m 526 μM, V_{max} 444 pkat/mg protein, V_{max}/K_m 0.8; reference substrate of recombinant 5β-POR + NADPH: Progesterone K_m 86 μM, V_{max} 40 pkat/mg protein, V_{max}/K_m 0.5.

[b] For synthesis of the norcholanic acids the methods described by Maier et al. were used with minor modifications. Identity was confirmed by comparing the mass spectra of synthesized compounds seen here with published data (See also Supplementary data).[13–15]

to not have a clear limiting or stimulating effect on acceptance as a substrate. It has recently been demonstrated that the enzyme also reduces small molecules with activated C=C double bonds.[9] This demonstrates that very low substrate specificities are possible even for enzymes previously assumed to be 'key enzymes' of a certain pathway.[5,10]

The K_m value for 23-nor-4,20(22)*E*-choladienic acid-3-one **6** was 250 μM, and V_{max} was 845 pkat/mg protein. The kinetic constants determined for progesterone **11** were 86 μM and 40 pkat/mg protein for K_m and V_{max}, respectively.

The recombinant steroid 5β-reductases derived from *Arabidopsis thaliana* and *Digitalis* (formely *Isoplexis*) *canariensis* (data not shown) also accepted **6** as substrate.[11,12] The recombinant *A. thaliana* enzyme converted **6** more efficiently than the recombinant 5β-POR of *D. lanata* which is in accordance with previous findings showing that the *A. thaliana* enzyme is a very active dehydrogenase/reductase, and that it differs considerably from recombinant *D. lanata* 5β-POR with respect to its substrate preferences although it has a very similar structure.[9,11]

The 5β-reduction of **6** was also seen in vivo using suspension-cultured *D. lanata* cells. The product could be detected in the spent medium, but not in the cells after an incubation time of 48 h.

The results presented here support the feasibility of a 'norcholanic acid pathway' of cardenolide formation with the provision that norcholanic acids have so far not been detected in cardenolide-producing plants. Our conclusion is mainly based on the relative conversion rates seen in a standard enzyme assay and the kinetic constants determined. Compared to the 'pregnane route' (Scheme 1), where the conversion rates were set to equal 100% for each individual step, the dehydrogenation of **3** was only 32%, the 5β-reduction of **6** was only 27% and the reduction of **5** only 80%. On the other hand, the dehydrogenation of **2** to **5** had a relative activity of 141%, indicating that in vivo the equilibrium might shift to the left, that is, to compound **5**. Hence, formation of the probable intermediate **2** of the norcholanic acid pathway appears not to be favoured. This assumption is also supported by the enzyme kinetic data. Moreover, it was found that 5β-pregnane-3,20-dione is only a poor substrate for reduction by recombinant

3β-HSD,[6] indicating that in vivo the equilibrium might shift to the right, that is, to 5β-pregnane-3β-ol-20-one. Due to the fact that the putative natural substrates were converted more efficiently than the norcholanic acids the pregnane pathway is probably the main pathway, whereas the 'norcholanic acid pathway' functions as a possible shunt—provided that norcholanic acids are formed and channelled into such a pathway.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.11.029.

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15. *23-Nor-5α-chol-20(22)E-enic acid-3β-ol* (**1**):. ^{1}H NMR ($CDCl_3$, TMS): 0.50 (3H, s, C-18), 0.73 (3H, s, C-19), 2.08 (3H, s, C-21), 5.08 (1H, s, C-22). *23-Nor-5β-chol-20(22)E-enic acid-3β-ol* (**2**): ^{1}H NMR ($CDCl_3$, TMS): 0.51 (3H, s, C-18), 0.88 (3H, s, C-19), 2.07 (3H, s, C-21), 3.87 (1H, m, C-3), 5.6 (1H, s, C-22). *23-Nor-5,20(22)E-choladienic acid-3β-ol* (**3**): ^{1}H NMR ($CDCl_3$, TMS): 0.53 (3H, s, C-18), 0.93 (3H, s, C-19), 2.07 (3H, s, C-21), 3.25 (1H, m, C-3), 5.27 (1H, s, C-6), 5.60 (1H, s, C-22). *23-Nor-5α-chol-20(22)E-enic acid-3-one* (**4**): GC–MS (EI, 70 eV): 314 (M^+-CO_2), 299 ($M^+-CO_2-CH_3$), 281 ($M^+-CO_2-CH_3-H_2O$). *23-Nor-5β-chol-20(22)E-enic acid-3-one* (**5**): GC–MS (EI, 70 eV): 314 (M^+-CO_2), 299 ($M^+-CO_2-CH_3$), 281 ($M^+-CO_2-CH_3-H_2O$). *23-Nor-4,20(22)E-choladienic acid-3-one* (**6**): GC–MS (EI, 70 eV): 338 (M^+-H_2O), 312 (M^+-CO_2), 294 ($M^+-CO_2-H_2O$). *23-Nor-5-cholenic acid-3β,20ξ-diol* (**7**): GC–MS (EI, 70 eV): 314 ($M^+-H_2O-CO_2$), 299 ($M^+-H_2O-CO_2-CH_3$), 296 ($M^+-H_2O-CO_2-H_2O$), 281 ($M^+-H_2O-CO_2-H_2O-CH_3$). *3-Oxo-5β-cardenolide* (**8**): GC–MS (EI, 70 eV): 356 (M^+), 341 (M^+-CH_3), 323 ($M^+-CH_3-H_2O$), 246 ($M^+-C_6H_6O_2$). *3β-Hydroxy-5β-cardenolide* (**9**): GC–MS (EI, 70 eV): 358 (M^+), 343 (M^+-CH_3), 340 (M^+-H_2O), 325 ($M^+-CH_3-H_2O$), 248 ($M^+-C_6H_6O_2$).