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The nitration of canrenone with acetic anhydride/nitric acid

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3-Oxo-17α-pregna-4,6-diene-21,17-carbolactone (canrenone, II) is produced from the potassium salt of 17-hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid (I) by acid catalyzed lactonization. II reacts with acetic anhydride/nitric acid to give one main product (III) and some minor products. The structure of III was determined by chemical and spectral analysis to be the 4-nitro derivative of canrenone. This result is in contrast to the known reactions of II with most other reagents that were found to add at Δ^6, and also in contrast to the reactions of acetic anhydride/nitric acid with alkenes. Electrophilic substitution at the ambident C4 is discussed as the reaction path. The 4-nitro group enhances the inhibitory activity of II against Na^+/K^+-ATPase, the target enzyme of the cardioactive digitalis glycosides, which appears to indicate increased cardioactivity. (*Steroids* **62**:762–766, 1997) © *1997 by Elsevier Science Inc.*

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Introduction

Within our program of C/D-trans steroid modification and the impact of structural elements on the inhibition of Na^+/K^+-ATPase (cf.[1]), we have found that 3-oxo-17α-pregna-4,6-diene-21,17-carbolactone (canrenone, II, Scheme 1) reacts with acetic anhydride/nitric acid with formation of one main product (III) and at least nine minor products. This paper describes the elucidation of III by chemical and spectral methods. It reveals that III is the 4-nitro derivative of II. A pathway for the formation of III, and the influence of the 4-nitro group on the inhibition of Na^+/K^+-ATPase are discussed.

Mixtures of acetic anhydride/nitric acid are well known as mild reagents for electrophilic nitration[2] of reactive aromatic compounds[2,3] such as phenols or phenol ethers etc. Alkenes with appropriate structure react by an addition/elimination mechanism to give mainly β-nitro alkenes in addition to β-nitro-acetates or -nitrates.[3] Conjugated dienes react by 1,4-addition.[3,4] Enol esters of saturated cyclic ketones react to give α-nitro ketones,[5] but acetone enol acetate is unreactive.[3] Formation to a greater extent of side products of different natures is reported in many papers, partially restricting the preparative usefulness of the acetic anhydride/nitric acid reagent (e.g.,[3,6,7]).

We found acetic anhydride/nitric acid to be a very useful reagent for the synthesis of nitric acid esters (nitrates) of the sensitive (because of easy loss of the 2-deoxy sugar side group and/or the tertiary 14β-OH) cardenolide and bufadienolide glycosides in high yields with little or no side products, despite the presence of double bonds as potential points of attack in ring B (Δ^4) and/or in the butenolide or bufadienolide (VI) side group.[8] Saturated or 4-ene-3-ketosteroids with primary and/or secondary hydroxy groups react in the same manner to give nitrates, whereas the nitration of the tertiary hydroxy group of 17α-hydroxy-pregn-4-ene-3,20-dione[1] produces more side products that are not found in the absence of the 4-ene bond (cf.[9]).

Experimental

General procedures

Reactions and separations are monitored by thin-layer chromatography (TLC) on precoated TLC aluminum sheets silica gel 60 F_{254} (Merck, Darmstadt, Germany). If necessary, development is repeated several times (nx). Detection is achieved by spraying with methanol/phosphoric acid (85%; 85:15 v/v), 4 min 140°C, UV 366 nm (in brackets: fluorescence color). Preparative TLC: Same sheets loaded with up to 25 mg mixture/18 cm starting line. Detection: UV 254 nm. Melting points (m.p.) (corrected): Boetius micro hot stage apparatus (VEB Analytik, Dresden, Germany). To check for decomposition during melting, the melt is investigated by TLC. UV spectra (ethanol): λ_{max} (nm), (ε). Beckman Spectro-

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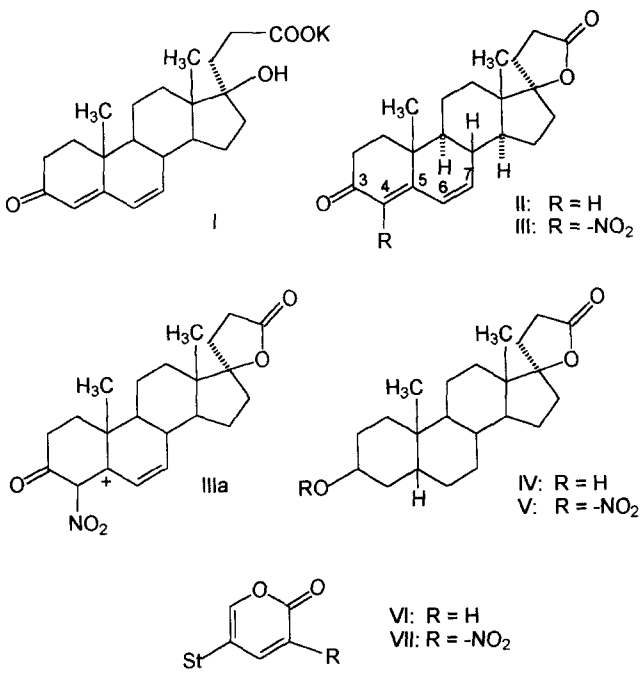


Scheme 1

photometer DU 7500. Infrared (IR) spectra (KBr): cm^{-1} (intensity): very strong (vs), strong (s), medium (m). Genesis FTIR (ATI, Mattson, Madison, Wisconsin, USA). Low resolution EI mass spectra (MS): m/z (relative intensity). Kratos MS80RFA mass spectrometer (Kratos Ltd., Manchester, GB) coupled with Maspec Data System (Data Version: 1.23, Software Version: 10.1, Mass Spectrometry Services, Ltd., Manchester, GB). Source temperature was 170°C. NMR spectra (^{1}H-, ^{13}C-) in $CDCl_3$ (UVASOL, Merck): δ (ppm), singulet (s), doublet (d). Varian Gemini, 300 MHz ^{1}H resonance frequency or 75.4 MHz ^{13}C resonance frequency, internal standard: TMS (UVASOL, Merck). Differentiation of C-atoms according to the number of attached H-atoms in ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra is reached by automated distortionless enhancement by polarization transfer. Its results, in combination with chemical shift comparisons, are used for the assignment of signals (cf.[10–12]). Potassium canrenoate (I) (Sigma Chemical Co., St. Louis, Missouri, USA) is used as starting material for the synthesis of canrenone (II), which is not commercially available. IR of I: 1550 (vs, -COOH); 1660 (vs, 3-C=O); 1640 (s, 4,6-diene), 1605 (m, 4,6-diene). Absolute HNO_3 is distilled from a mixture of ground KNO_3 (101.4 g, 1 mol, dried at 120°C for 5 h) and concentrated H_2SO_4 (100 mL, 1.8 mol) at 15 mm Hg.

Canrenone (II) from potassium canrenoate (I)

NH_2SO_4 (20 mL; 10 mmol) is added to a boiling solution of I (5 g; 12.6 mmol) in methanol (80 mL). The mixture is boiled for 5 min under reflux and cooled to 4°C overnight. Recrystallization of the filtered deposit from ethyl acetate yields 3.95 g II (92%; not optimized) pale yellow crystals, m.p. 160–162°C[13–15] without decomposition. TLC (ethyl acetate), R_f: II 0.52 (yellow-brown), I 0.02 (yellow-brown). MS: 340.4 (100, M^+, $C_{22}H_{28}O_3$); 325 (17, $M^+ - CH_3$); 267 (68, M^+ − lactone ring + H); 227.2 (19, M^+ − ring D − lactone ring + H); 162 (17, rings A, B + 2H); 136.2 (39, M^+ − rings C, D − lactone ring + 2H); 69.9 (54). UV: 283 (28,000). IR: 1770 (vs, lactone C=O); 1650 (vs, 3-C=O); 1614 (vs, 4,6-diene); 1583 (m, 4,6-diene). ^{13}C-NMR: quaternary C atoms: 199.3 C3, 176.5 C22, 163.0 C5, 95.3 C17, 46.5 C13, 36.0 C10. CH groups: 139.4 C7, 128.3 C6, 124.0 C4, 50.4 C14, 47.0 C9, 37.8 C8. CH_2 groups: 35.5 C12, 33.9 C1, 33.9 C2, 31.6 C16?, 31.2 C15?, 29.2 C21, 22.5 C20, 20.1 C11. CH_3 groups: 16.3 C19, 14.4 C18. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra: 5.69 (s, 1H, H4); 6.09 (d, 1H, J=10.2 Hz, broad lines, H6); 6.15 (dd, 1H, J=10.2, 2.5 Hz, H7); 1.04 (s, 3H, $18H_3$); 1.14 (s, 3H, $19H_3$).

4-nitrocanrenone (III) from canrenone (II)

Absolute nitric acid (0.1 mL; 2.4 mmol) is added to acetic anhydride (0.3 mL; 3.2 mmol) with cooling in ice. To this mixture, the solution of II (100 mg; 0.3 mmol) in methylene chloride (1.25 mL) is added while cooling in ice. After 1 h at room temperature, chloroform (10 mL) is added, and the mixture is washed with water, aqueous $KHCO_3$ solution (10%; just until the beginning of brown coloration) and water again, dried with Na_2SO_4, and evaporated. Preparative TLC of the residue on four TLC sheets (10 cm height × 20 cm) with butyl acetate/toluene (40:60 v/v, 3×). Elution of the main band (R_f 0.57) in front of II (R_f 0.30) with acetone yields 59 mg III (52%; not optimized). After recrystallization from acetone-ether-petroleum ether (boiling point 60–80°C) a m.p. of 146–149°C is found with about 50% decomposition to three more polar compounds. Fluorescence after TLC detection: II, yellow-brown; III, orange. Spraying the TLC with 1% diphenylamine in ethanol and irradiation with the UV lamp at 254 nm for 10 min[16] shows no colour in the case of III in contrast to the nitrate V (dark gray spot, indicating $-O-NO_2$).

MS: 385.2 (14, M^+, $C_{22}H_{27}NO_5$); 281.9 (28); 173.3 (42); 120.0 (54); 83 (100). UV: 286 (25,600). IR: 1770 (m, lactone C=O); 1681 (vs, 3-C=O); 1614 (vs, 4,6-diene); 1587 (m, 4,6-diene); 1537 (vs, ν_{as}=C−NO_2); 1371 (s, ν_s=C−NO_2). ^{13}C-NMR: quaternary C atoms: 187.5 C3, 176.4 C22, 151.6 C5, 144.2 C4, 95.0 C17, 46.4 C13, 36.6 C10. CH groups: 146.7 C6, 120.7 C7, 50.1 C14, 46.4 C9, 38.0 C8. CH_2 groups: 35.3 C12, 33.1 C1?, 32.5 C2?, 31.4 C16?, 31.0 C15, 29.2 C21, 22.3 C20, 19.9 C11. CH_3 groups: 16.1 C19, 14.4 C18. ^{1}H-NMR: 6.44 (dd, 1H, J = 10.2 Hz, 2.0 Hz, H6); 6.16 (dd, 1H, J = 10.2, 3.0 Hz, H7); 1.05 (s, 3H, $18H_3$); 1.25 (s, 3H, $19H_3$).

4-nitrocanrenone (III): Reversible reaction with base

The solution of 10 mg (0.026 mmol) of III in chloroform (10 mL) is extracted with aqueous $KHCO_3$ solution (10%; 5 mL; 5 mmol). The brown aqueous phase is carefully acidified with 0.1n H_2SO_4 and re-extracted with chloroform. This yields a product (8.4 mg), identical with the starting material III in TLC (butyl acetate/toluene 50:50 v/v, 2×). R_f: III 0.46 (orange); II 0.34 (yellow-brown).

Attempted reduction of 4-nitrocanrenone (III) with zinc

1 mg (0.0026 mmol) of III is dissolved in methanol (0.8 mL). After the addition of zinc dust (100 mg; 1.5 mmol), acetic acid (0.1 mL; 1.6 mmol) and water (0.1 mL; 5.6 mmol) the mixture is stirred for 5 min at room temperature. After the addition of chloroform (5 mL), filtration, washing with water, and drying with Na_2SO_4, TLC, as above, shows the presence of the starting material III, but no formation of II. After analogous treatment of V, almost pure IV is found, R_f IV 0.29 (grey-green), V 0.74 (grey-green).

Results

Synthesis

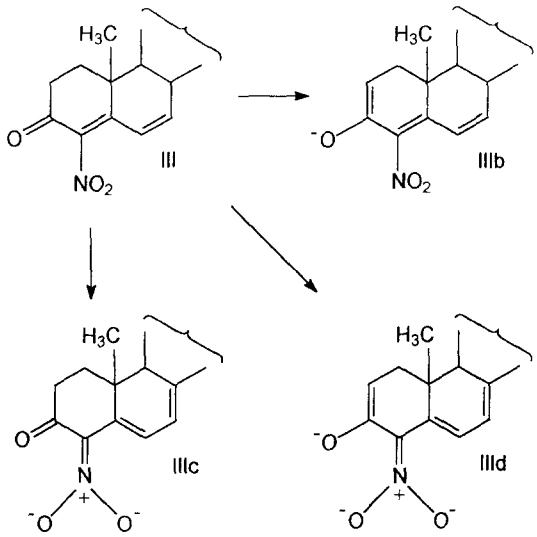
Tal[14] has produced II from I by HCl treatment (8 h; pH 1) in refluxing $CHCl_3/H_2O$. Change of solvent (MeOH) and catalyst (H_2SO_4) shortens the reaction time to 5 min of reflux. With the equimolar amount of HCl instead of H_2SO_4, only traces of II are formed, possibly because of differences in acid strength. Furthermore, our procedure demonstrates the remarkable ease of lactonization under smooth conditions despite the fact that the -OH group involved is a tertiary one. In many cases, these are very reluctant to esterification and tend toward elimination. The product of acid catalyzed reaction of I, obtained here, however, is identical to the known canrenone,[13–15] as proven by spectral evidence (see below).

The formation of III by reaction of canrenone (II) with acetic anhydride/nitric acid is connected with its 3-keto-4,6-diene system, because its hydrogenation to the hexahydro derivative IV (Scheme 1, 3β-hydroxy-5β,17α-pregnane-21,17β-carbolactone[17]) strongly changes the reaction path with acetic anhydride/nitric acid, leading to the 3β-O-nitrate (V)[1] almost without side products. III and V differ in their TLC mobility relative to their parent compounds (see experimental part) and in their chemical behavior: On TLC, V but not III produced a positive test for nitric acid esters with diphenylamine/UV.[16] V but not III is convertible to its parent compound by reduction with zinc/acetic acid.[8] These results exclude for III the structure of a 3-*O*-enol nitrate derivative of II.

The introduction of an o-NO_2 group in phenol increases its acidity from $pk_a = 9.98$ to $pk_a = 7.23$.[18] The acidity of II is also increased by introduction of the 4-NO_2 group: III (in contrast to II and V) can be extracted from its chloroform solution by aqueous $KHCO_3$ solution, with the characteristic brown color as is found with other nitrosteroids.[4,19,20] As proved by TLC, III is reformed after acidification of the basic aqueous solution. In similar cases of base treatment, recovery of the starting nitro compound has already been found,[21] but other reactions are also possible, such as epimerization,[19,20] double bond shift,[3,19] or C-C splitting.[21] Whether III on base treatment forms the 3-enolate (IIIb, Scheme 2) and/or the 4-nitronate (IIIc, cf.[19,21]) with shift of the double bonds and/or the 3-enolate-4-nitronate (IIId), demands further investigation.

Spectral comparison of canrenone (II) and its main nitration product (III)

MS. The MS of the lactonization product (II) from I is in agreement with literature data for canrenone.[14] M^+ as the base peak demonstrates the high stability of II under these strong conditions. Identification of other main peaks beside fragments without -CH_3 or the lactone ring is tentative. The M^+ peak of III is comparatively weak but proves clearly that III is a mono nitro derivative of II. As the intense fragmentation of III shows, the $-NO_2$ substitution strongly decreases the stability of the molecule. Moreover, the course of fragmentation of III differs markedly from that of II, making an interpretation difficult.



Scheme 2

UV spectra. The UV of II (produced from I) agrees with literature data for canrenone.[13,14] The influence of the nitration of II to III on λ_{max} (increase) and ε (decrease) is small. This demonstrates the preservation of the 3-keto-4,6-diene system of II in III. Furthermore, it proves that III is neither an addition product at the Δ^6 bond with 3-keto-4-ene structure (for which λ_{max} around 240 nm is characteristic[10,13,14,22]), nor an 1,4-addition product[3,4] to the 4,6-diene system. In UV, shifts of similar magnitude and direction as obtained for III are found for the structurally similar 2-nitro-3-keto-4-ene steroids.[21] The structure of an allylic nitration product of II is excluded for III by the fact that allylic 6-nitro groups shift λ_{max} of 3-keto-4-ene steroids to lower wavelengths.[19,20]

IR spectra. For the conversion of I to II (22-COO^- → 22−C=O) the expected IR spectral change[10,22] is found. The IR of II is in agreement with literature data for canrenone.[14,15] The IR of its nitration product III excludes the presence of a $-O-NO_2$ group (no signal around 1640 cm^{-1}; cf.[4,22]) and demonstrates the presence of a vinylic $-NO_2$ group.[4,19,23] Little or no influence of the nitration is found on the signals of =C-H, lactone ring −C=O, and 4,6-diene-C=C. The signal of 3-C=O, however, is strongly shifted to higher frequencies, indicating its neighborhood to the $-NO_2$ group. Shifts of similar magnitude and direction are found for saturated (5α/β-H) or unsaturated (4-ene) 2- or 4-nitro-3-keto steroids[21] or a 20-keto-21-nitrosteroid,[24] whereas nearly no influence on the 3-C=O-4-ene frequencies is found for allylic 6α/β-nitro groups.[19]

^{13}C-NMR. The ^{13}C-NMR of II (not found in literature) shows signals of all 22 C atoms, as does the spectrum of III. Distortionless enhancement by polarization transfer (DEPT)[10,11,12] differentiates them according to the number of attached H-atoms. The strongest shift (III-II) as a result of nitration was found for C4 with smaller ones for C3, C6, and C7. One H is found attached to C4 in II but not in III. This result proves the position of the $-NO_2$ group in III at C4.

^{1}H-NMR. The ^{1}H-NMR of II is in agreement with literature data for canrenone.[14,15] The proton at C4, which is present in ^{1}H-NMR of II, is absent in III. This confirms the conclusion from ^{13}C-NMR with respect to the position of the $-NO_2$ group in III at C4. The AB system of H6 and H7 is preserved after nitration with a strong shift of H6, excluding $-NO_2$ substitution at C6 or C7. Consistent with these results, the signal of 19-CH_3 is shifted in III compared to II, whereas that of the more distant 18-CH_3, is not.

Discussion

The reaction course

Canrenone (II) contains the polar 3-keto-4,6-diene system with the positive end at C7 and the negative at the ambident[25] part C4-C3=O. Nucleophilic reagents attack this system at C7 as many examples prove (CH_3^-,[26] RS^-,[13,27] CN^-,[28] etc.). Electrophilic reagents should attack the ambident, negative end of the system at 3=O or at C4, depending on the nature of the reagent and the reaction conditions.[25,29,30] Contrary to this, the electrophiles OsO_4 and RCO_3H add to the Δ^6 bond, leading to 6,7-disubstitution.[14] But according to theory, the hard [in terms of the hard and soft acids and bases (HSAB) Principle[31]] electrophilic reagent acetyl chloride and similar ones attack the harder part of the ambident negative end, the 3=O, with formation of the isomeric 3-O-enol acetates.[32] The electrophilic attack of the comparatively softer acetyl nitrate or NO_2^+ (for discussion of the nature of the reagent, see references 2, 3, and 23) takes place at the softer C4, leading to the 4-nitro derivative of II, possibly via the intermediate IIIa (Scheme 1). The cation at C5 may be stabilized by the Δ^6 bond, in this way facilitating the nitration of a 3-keto-4,6-diene system in comparison to a 3-keto-4-ene system, which has not such a possibility of stabilization. (Similar relations were found for the nitration of steroidal enamides[6,7]). 3-Keto-4-ene steroids, however, may be nitrated at C2 with amyl nitrate with base catalysis,[21] contrary to the reaction of their 3-O-enol acetates with nitric acid leading to the 6-nitro-3-keto-4-ene derivatives.[20] Saturated 3-ketosteroids were nitrated by amyl nitrate and base at C2 or C4 depending on their A/B-*cis* or -*trans* connection, but this may be inverted by change of the cation (K^+/Li^+) of the base used.[21] These examples demonstrate the great influence of the reaction conditions on the regioselectivity of the nitration of steroid ketones (for a review of the complex relations in substitution reactions in steroid ketones, see reference 33). Despite the structural similarity of the bufadienolide ring (VI, Scheme 1) in the bufadienolides with the 3-keto-4,6-diene system in II, analogous C-nitration in the bufadienolide ring (VII) was not found.[8] Obviously, the additional oxygen decreases the reactivity of VI.

Biological activity

The successful improvement of the biological activity of many steroid hormones by the introduction of fluorine to the molecule has initiated trials to investigate whether the $-NO_2$ group may cause similar effects as fluorine[19,20,24] because of its high electronegativity and its ability to form donor-acceptor complexes.[23] With one exception (6α-nitro-17α-acetoxyprogesterone[20]), however, the results were discouraging. This may be caused, at least partially, by the limited stability of many nitro derivatives,[4,6,7,19–21,34] which, together with their questionable metabolic stability, limits their attractiveness for drug use.

The potency of II to inhibit Na^+/K^+-ATPase (the target enzyme of cardiac action of the therapeutically used digitalis glycosides (cf.[35]) is increased by about 30% on introduction of the 4-NO_2 group. This results from a favorable influence on receptor kinetics, i.e., increase of the velocity of the effector-receptor complex formation (k_{on}) and decrease of its decay (k_{off}).[1] This effect is not a general one: the inhibitory activity was found to be decreased by -NO_2 groups in positions 17 or 20 or in the case of 16-nitromethyl substitution or by the 3β-ONO_2 group in V; all in C/D-trans steroids.[1] In the C/D-*cis* series, however, high activities were found for 20- and 21-NO_2 derivatives in another test.[36,37]

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