Structure elucidation of two isomeric steroids: photolytical and thermal reaction products from norethisterone studied by two-dimensional nuclear magnetic resonance

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Introduction

As a part of our research into the light-induced side effects of the oral contraceptives, the photodecomposition of norethisterone (NE; *1*, Fig. 1), a commonly used progestogenic steroid, was studied.[1,2] One of the major components (15%) among the isolated photoproducts appeared to have a mass of m/e = 316 and, moreover, its mass spectral fragmentation pattern indicated the addition of a water molecule to ring A of NE (M = 298). In addition, a second component (5%) was isolated, which showed an identical m/e value. However, according to the photochemistry of analogues of NE at least eight structures can be postulated for a steroid having m/e = 316.[3-6] To facilitate the structure elucidation of the hydroxylated steroids isolated from a photoreaction mixture containing more than twenty products,[2] one of the products expected, *i.e.* the 5β-hydroxylated compound, has been synthesized.

We attached importance to the exact identification of particularly these hydroxyketone steroids. One of the components might well be a decomposition product of NE-4β,5β-epoxide (*2*; Fig. 1). If this will be the case, the initial amount of the epoxide *2* in the total yield of photoproducts of NE will be even higher than originally determined (12%).[1] This previously identified photoproduct is a reactive and cytotoxic epoxide, formed in incubations of NE with rat liver microsomes.[1] The generation of an α-C-hydroxylactone has been reported before in a photoreaction of steroidal α,β-unsaturated lactone.[3] The former product is thought to be important since the incubation of albumin with steroids possessing an α-C-hydroxyketone moiety results in the formation of covalently bound steroid–protein adducts.[7] In the case of NE it was found that irreversible binding of steroid to albumin did occur during and after irradiation with UV-B light (280-320 nm).[8] The cytotoxic effect of the photoproducts of NE towards the micro-organism *Salmonella typhimurium* TA98 could not be ascribed to the epoxide product *2* (unpublished observations). Nevertheless, irreversible binding of steroid type molecules to protein was observed in the micro-organism (unpublished observations). The isolated NE derivatives with a hydroxy-ketone moiety might (partly) be responsible for the irreversible binding to albumin and the observed effects towards the bacterium.

In this paper we present the identification of both isomeric products of NE (obtained by photochemical and/or thermal means) using two-dimensional (2D) NMR techniques. 2D NMR proved to be superior to other usually applied spectrometric techniques by virtue of the detailed structural information the NMR techniques disclose.

Methods

MATERIALS

Norethisterone (norethindrone; NE; *1*) was purchased from Sigma (St. Louis, USA) and used without further purification. All solvents ('Bakergrade', Baker, Deventer, The Netherlands) were distilled before use. H_2O_2, NaOH,

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Abstract

Two-dimensional nuclear magnetic resonance was used for the structure elucidation of two isomeric photoproducts of norethisterone, a commonly used progestogen in oral contraceptives. The predominant one of the two isolated products derived from photochemical decomposition of norethisterone upon irradiation with UV-B light (280-320 nm) was 5α,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one. The minor photoproduct appeared to be the analogous 5β-isomer, *i.e.* 5β,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one. The latter compound was also obtained from the thermal reduction of norethisterone-4β,5β-epoxide using aluminium amalgam in isopropanol. Two-dimensional NMR appeared to be superior to mass and IR spectrometry in identifying the isomers.

aluminium amalgam and $NaHCO_3$ were obtained from Brocacef (Amsterdam, The Netherlands), and CD_3OD from Merck (Darmstadt, FRG).

ISOLATION OF THE PHOTOPRODUCTS

A solution of $0.167 \times 10^{-3} M$ NE in phosphate buffer (0.04 *M*, pH 7.4) containing 10% ethanol was divided in quantities of about 13 ml over 16 quartz tubes (15 × 1 × 1 cm). The tubes were placed in a carousel and irradiated for 40 min at ambient temperature in a Rayonet Photochemical Reactor (RPR-208, Southern New England Ultraviolet Co., Middletown, USA; two UV lamps, RUL 3000 Å, UV-B region). The distance between the lamps and the tubes was 8 cm. In order to obtain a sufficient amount of photoproducts this procedure was repeated fifteen times. Following irradiation, the photoproducts were extracted with dichloromethane and the products were isolated by means of column and thin layer chromatography.[2]

SYNTHESIS OF 5β,17β-DIHYDROXY-19-NOR-17α-PREGN-20-YN-3-ONE

A quantity of 100 mg NE, dissolved in 20 ml methanol, was converted into its 4β,5β-epoxide derivative (2) by adding 700 µl 30% H_2O_2 and 480 µl 4 N NaOH at 0°C.[9] After extraction with dichloromethane the product was found to be pure (> 98%) on TLC. Virtually no α-epoxide (which can be separated from the β-isomer by developing [DC-plastikfolie Kieselgel 60F254 (Merck, Darmstadt, FRG)] three times with the eluent hexane–acetone–diethylether, 4+1+1, vol/vol) could be detected. After separation, the epoxide was redissolved in isopropanol (5 ml) and reduced using aluminium amalgam (900 mg) and $NaHCO_3$ (90 µl, 10% solution in water) at 0°C under nitrogen atmosphere.[10] Addition of water to the reaction mixture and subsequent extraction with dichloromethane gave the desired product (3, Fig. 1) which was purified by column chromatography (Si60 Lobar column Grosse C, Merck, using hexane–acetone–diethylether, 1+1+1, vol/vol, as the mobile phase); yield 70%.

SPECTROSCOPIC METHODS

NMR samples were prepared by dissolving about 15 mg of steroid in 1 ml CD_3OD. Spectra were recorded on a Bruker (Karlsruhe, FRG) WM-500 or WM-200 spectrometer as described previously,[11] except that instead of SECSY the analogous correlated spectroscopy (COSY) was used for obtaining the chemical shift correlation spectra.[12,13] IR spectra (KBr) were recorded on a Beckman (Mijdrecht, The Netherlands) IR-10, mass spectra on a (Manchester, UK) Kratos MS 9/50 (20 eV, source temperature 100°C) and circular dichroism spectra (ethanol) on a Dichrograph III CNRS-Roussel-Jouan (Longjumeau, France).

Results and discussion

Since our aim is to study the light-induced side effects of oral contraceptives, the photodecomposition of NE was performed in an aqueous medium buffered at pH=7.4, at ambient temperature, using UV wavelengths that are part of the sunlight spectrum.

The low solubility of NE in the water–ethanol solution (9+1, vol/vol) together with the arduous isolation procedure, possibly accompanied by dehydration of the products, impedes a precise determination of the amount of photoproducts formed.[14] On TLC the two photoproducts with m/e = 316 could not be sufficiently separated from other products to allow an exact densitometric determination.[1] However, densitometric data as well as visual perception after TLC and column chromatography indicated that one product is a major photodecomposition product (15%) and that the other is formed in a yield of approximately 5%.

SYNTHESES OF 5β,17β-DIHYDROXY-19-NOR-17α-PREGN-20-YN-3-ONE

Thermally, NE-4β,5β-epoxide (2) is easily synthesized in quantitative yield and, if the concentration of starting material is not too high, virtually without any α-epoxide.[9] The choice of the reducing agent has been limited since reduction of the ketone moiety in the epoxide has to be avoided. Chromous ions,[15] palladium on barium sulfate with cyclohexene,[16] electrochemical reduction[17] and aluminium amalgam[10] have been applied as reducing agents for the generation of a hydroxyketone from an epoxyketone. The aluminium amalgam approach seemed the most promising one because of its simplicity and high yields. This conversion method was reported to be quantitative without introducing a by-product having a double bond conjugated to the ketone function.[10]

However, following the prescribed procedure with ethanol as solvent it appeared that during the reduction of NE-4β,5β-epoxide a number of side products were formed including NE. An explanation

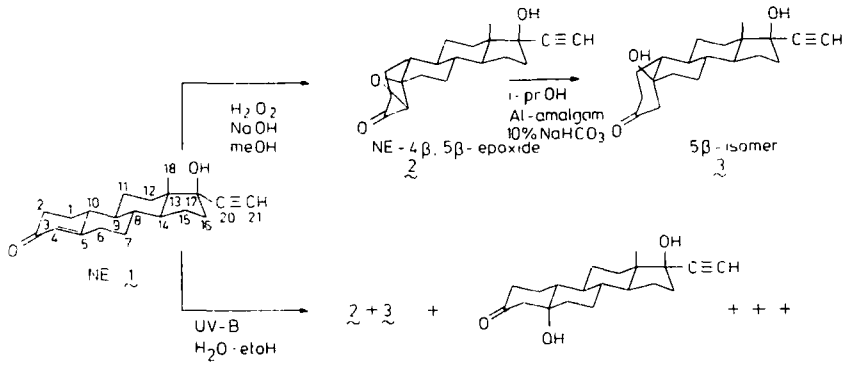

FIGURE 1
Reaction scheme

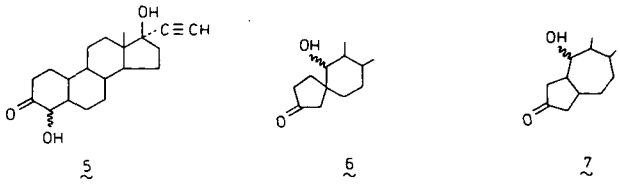

FIGURE 2
Other products with m/e = 316 expected to be formed according to the photochemistry of analogues of NE

for the generation of NE and other, not further identified, by-products might be that in the present case a tertiary alcohol should be the end-product, whereas in former experiments only secondary β-C-hydroxyketone steroids were generated with this reagent.[10 18]

The notion that secondary alcohols are far less reactive than primary alcohols in addition reactions to conjugated ketones,[19] evoked a change in solvent in order to reduce the number and/or quantity of the above mentioned side products. Indeed, the number and quantity were noticeably decreased by using isopropanol instead of ethanol as the reaction medium.

IDENTIFICATION OF THE PRODUCTS WITH M/E = 316

Both the photoproducts and the thermally reduced epoxide displayed a mass parent peak at m/e = 316, which is an indication for addition of a water molecule to the starting component NE (m/e = 298). According to the literature hydroxylation at C5 is likely, both in the thermal and in the photochemical reaction.[4 10] However, addition at C4 as well as addition at C10 accompanied by a rearrangement are known to occur in UV-light induced addition reactions involving conjugated ketone steroids.[3 5 6] In the latter cases *5*, *6* and *7* will be formed (Fig. 2). The mass spectral fragmentation patterns were inconclusive with respect to the site of hydroxylation. For example, fission of the C10–C1 bond and the C4–C5 bond should give a base peak at m/e = 246 in the case of hydroxylation at C5.[4] The latter peak was not detected; instead a base peak at m/e = 247 was observed. Moreover, fragments m/e = 230/231 were observed indicating substitution at C4, but dehydrogenation and fragmentation in ring D will also give a base peak at m/e = 231 as is observed in the mass spectrum of NE.

The C17 hydroxyl group, already giving rise to a

TABLE I
Chemical shift data obtained for the steroids 3, 4, 8 and 9

Carbon	*3*	*4*	*8**	*9**	Proton	*3*†	*4*†	*8**	*9**
1	23.2	26.9	29.2	32.1	1α	2.08	1.72	1.69	1.33
					1β	2.28	2.15	2.32	2.38
2	37.3	42.1	37.5	42.3	2α	2.40	2.37	2.44	2.39
					2β	2.16	2.45	2.16	2.48
3	214.9	206.1	216.0	214.9					
4	49.9	56.4	44.1	50.0	4α	3.01	2.30	2.82	2.27
					4β	2.08	2.59	2.07	2.27
5	78.0	75.9	40.4	45.5	5			2.26	1.50
6	41.7	40.4	32.1	35.3	6α	~1.73	~1.72	~1.59	1.75
					6β	1.66	~1.72	1.83	1.30
7	29.6	26.9	26.5	31.8	7α	1.15	1.38	1.26	1.05
					7β	~1.73	1.56	~1.59	1.79
8	43.4	43.4	43.9	43.3	8β	1.33	1.29	1.27	1.29
9	42.6	43.6	39.6	49.6	9α	1.42	1.21	1.52	0.79
10	48.0	48.5	41.7	47.2	10β	1.54	~1.65	1.69	1.33
11	27.5	27.3	27.1	27.3	11α	1.97	1.86	1.91	1.96
					11β	1.33	1.31	1.29	1.31
12	34.1	34.3	34.3	34.2	12α	1.91	1.86	1.93	1.84
					12β	~1.73	~1.67	1.74	1.69
13	‡	‡	‡	‡					
14	50.9	50.8	50.9	51.0	14α	~1.73	1.70	1.78	1.66
15	24.2	24.1	24.2	24.2	15α	~1.73	1.74	1.76	1.74
					15β	1.42	1.39	1.41	1.40
16	40.1	40.1	40.2	40.2	16α	2.30	2.29	2.29	2.28
					16β	2.04	2.01	2.02	2.01
17	80.6	80.7	80.7	80.7					
18	13.6	13.6	13.6	13.6	18	0.94	0.94	0.94	0.94
20	89.1	89.5	89.2	89.1					
21	75.1	75.0	75.0	75.0	21	2.96	2.93	2.96	2.94

*See ref. 2.
†Distinction between α and β protons is made in analogy to the previously analysed steroids *8* and *9*.[20]
‡Not measured.

tertiary alcohol absorption, interfered with an unambiguous interpretation of the IR-spectra of the products; in fact, an absorption band at 1300 cm^{-1} again pointed to the presence of a secondary alcohol function in the isolated photoproducts. TLC experiments and circular dichroism spectroscopy proved that the minor photoproduct was identical with the synthesized product, thus facilitating its identification, also because enough material was at hand.

Using a recently developed strategy to interpret the complex NMR spectra of 19-norsteroidal compounds by means of 2D NMR techniques,[11] the site of substitution and the stereochemistry of both products could be elucidated. In this case the 1D 500 MHz ^{1}H-NMR spectra, the COSY spectra and the 2D J-resolved spectra of the isomers were analysed. Complementary information was needed from a ^{1}H-^{13}C hetero shift correlation experiment. After plotting cross sections along the vertical (f_1) axis in the ^{13}C-^{1}H hetero shift correlation spectra in order to obtain the precise correlation between a C-atom and its (geminal) proton(s), an unambiguous assignment of the spectra was reached. The chemical shift data obtained for products *3* and *4* are given in Table 1 together with data observed previously for closely related steroids.[20] In Figure 3 the conventional 1D 500 MHz ^{1}H-NMR spectra of both products and cross sections through the 2D J spectra at the resonance positions of the protons at C1 and C4 are presented. An isolated AX-pattern, albeit with some small long-range couplings superimposed at 3.01 and 2.08 ppm in the photoproduct *3* points to the presence of a C5 hydroxy-substituted steroid. A similar pattern with resonances at 2.59 and 2.30 ppm is recognized in the spectrum of product *4*.

The other possible structures (*5*, *6* and *7*) for the isolated photoproducts can be excluded. A C4 hydroxy-substituted steroid would display only one doublet at low field for H4; the latter resonance would be connected in the COSY spectrum to a multiplet arising from H5. Each proton at C1 in the spirocompound *6* has to give rise to an eight line multiplet (or simplified pattern) caused by three couplings (namely to its geminal and C2 protons). However, the resonances in the NMR spectrum of the photoproduct *3* ascribed to 1α-H and 1β-H show a more complicated pattern (Fig. 3), caused by a fourth coupling to 10β-H. Concerning the last possible structure (*7*), none of the protons would give rise to a doublet like the ones observed for the C4 protons in *3* and *4*, whereas the proton at C10 in *7* should resonate at relatively low field manifested as a quartet (or triplet).

Having established the site of substitution we have only the stereochemistry at C5 left to deal with. The conformation of the product can be derived from a perusal of the ^{13}C chemical shifts of a product in relation to either the shifts of 17β-hydroxy-19-nor-5β,17α-pregn-20-yn-3-one (Fig. 4; *8*; Table 1) or of its 5α isomer (Fig. 4; *9*; Table 1). The differences in

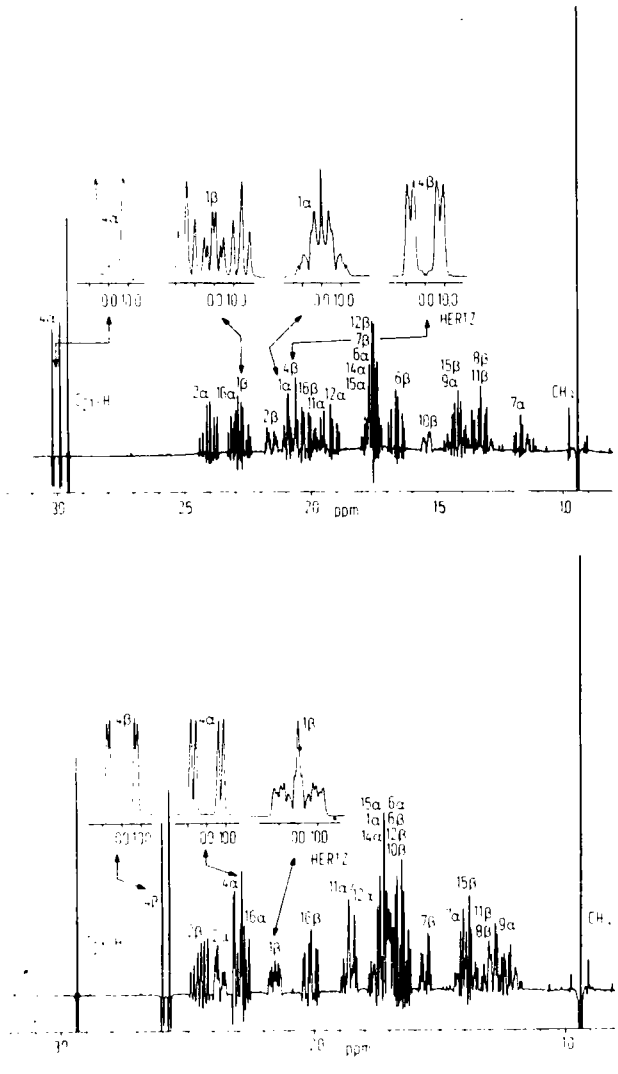

FIGURE 3
Top: 1D 500 MHz ^{1}H-NMR spectrum of product *3*. The inserts show cross sections obtained from the 2D J spectrum at the resonance positions of the protons at C1 and C4. Bottom: Idem of photoproduct *4*

the chemical shifts of the C atoms in ring A and ring B between for example the photoproduct *4* and steroid *9* (Table 1) are in accordance with those one would expect upon hydroxylation at C5 (see also *143* and *179* in ref. 21). We conclude that the photoproduct *4* is 5α,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one (= 5α,17β-dihydroxyestran-20-yn-3-one). The synthesized product *3* is its 5β isomer: 5β,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one (= 5β,17β-dihydroxyestran-20-yn-3-one). The circular dichroism data (for *3*: λ_{max}^{MeOH} = 286 nm, $\Delta\varepsilon$ is negative, and for *4*: λ_{max}^{MeOH} = 287 nm, $\Delta\varepsilon$ is positive) confirmed the assigned stereochemistry.

It is noted that the isolated 5α isomer *4* is probably not a (photo)decomposition product of the NE-4β,5β-epoxide photoproduct *2*, since the configur-

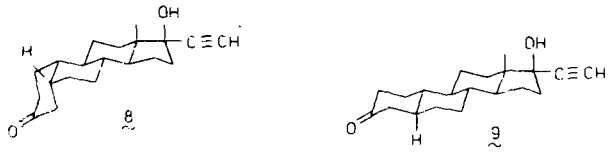

FIGURE 4
Structures of *8* and *9*

ation about C5 is reversed in product *4* with respect to the epoxide *2* and it is reported that β-diketones are formed upon irradiation of α,β-epoxyketones.[14] Instead, product *4* seems to originate from a direct addition of a water molecule to the 4=5 double bond in NE.[4] It is conjectured that the photoproduct *3* might be a secondary product formed by reduction of the epoxide *2* either during the isolation procedure or during the irradiation. In that case even more epoxide will be formed photochemically than originally determined.[1] However, this conjecture has not yet been confirmed. With respect to the earlier mentioned formation of covalently bound steroid–protein adducts (*Introduction*),[8,9] it should be mentioned that the photoproducts are not likely to be responsible for (a part of) the formation of such adducts since they are not steroids with an α-C-hydroxyketone function.[7]

In summary, 5α,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one (*4*) is one of the main products of the decomposition of NE by UV-B light in aqueous medium. The corresponding 5β-isomer is also formed in a detectable quantity. The 4β,5β-epoxide can be reduced to this tertiary alcohol derivative, 5β,17β-dihydroxy-19-nor-17α-pregn-20-yn-3-one (*3*), using aluminium amalgam. In the latter reaction isopropanol used as solvent instead of ethanol reduces the formation of side products.

Two-dimensional NMR proved to be the method of choice for the identification of the two isomeric steroids *3* and *4*. The NMR data allowed for an unambiguous structure elucidation of these stereoisomers in contrast to the mass and IR spectrometric data.

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