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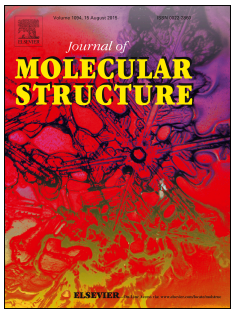


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PII: S0022-2860(16)30050-3

DOI: 10.1016/j.molstruc.2016.01.050

Reference: MOLSTR 22166

To appear in: *Journal of Molecular Structure*

Received Date: 2 November 2015

Revised Date: 19 January 2016

Accepted Date: 20 January 2016

Please cite this article as: G. Mótyán, Á. Baji, I. Zupkó, É. Frank, Regio- and stereoselective synthesis of pregnane-fused isoxazolines by nitril-oxide/alkene 1,3-dipolar cycloaddition and an evaluation of their cell-growth inhibitory effect *in vitro*, *Journal of Molecular Structure* (2016), doi: 10.1016/j.molstruc.2016.01.050.

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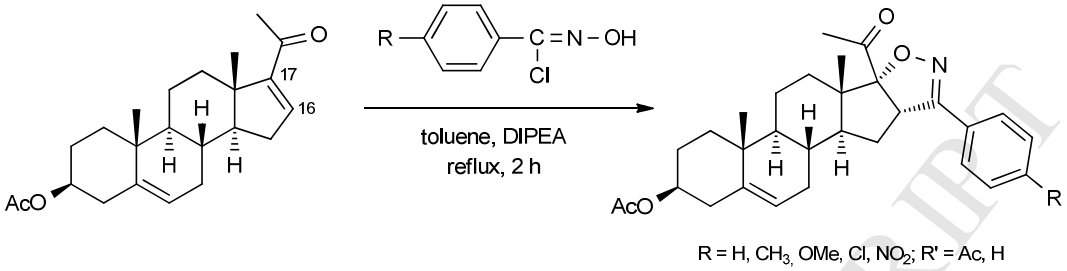

# Regio- and stereoselective synthesis of pregnane-fused isoxazolines by nitril-oxide/alkene 1,3-dipolar cycloaddition and an evaluation of their cell-growth inhibitory effect *in vitro*

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**Abstract:**

Efficient syntheses of some pregnane-fused isoxazolines from 16-dehydropregnenolone acetate with different arylnitrile oxides were carried out by 1,3-dipolar cycloadditions. The intermolecular ring-closures occurred in a highly regio- and stereoselective manner permitting the formation of a single 16α,17α-condensed diastereomer in which the *O* terminus of the nitrile oxide dipole is attached to C-17 of the sterane core. The conversions were found to be affected significantly by the electronic character of the substituents on the aromatic moiety of the 1,3-dipoles. Deacetylation of the primary products resulted in the corresponding 3β-OH analogs. All of the synthesized compounds were subjected to *in vitro* pharmacological studies for the determination of their antiproliferative effects on four breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361).



**Keywords:** steroids, 1,3-dipolar cycloaddition, nitrile oxides, isoxazoline, stereostructure, antiproliferative effect

## 1. Introduction

1,3-Dipolar cycloadditions of nitrile oxides with alkene or alkyne dipolarophiles leading to 2-isoxazoline or isoxazole derivatives [1, 2] have been extensively studied and have found general application in organic synthesis owing to the great importance of these heterocycles as structural building blocks of several biologically active molecules [3, 4] and versatile intermediates in the synthesis of numerous bifunctional compounds [5].

The propargyl/allenyl-type nitrile oxide 1,3-dipoles, containing a *N* atom in the center can readily undergo cyclization with multiple-bond systems under thermal or catalytic conditions [6]. The reactivity of the dipolarophile as well as the regio- and stereoselectivity of the thermally-induced process depend on certain structural features. Thus, monosubstituted alkenes are generally more reactive than disubstituted olefins or alkynes, and the reactions with nitrile oxides occur with almost complete regiospecificity to furnish exclusively 5-substituted isoxazolines. However, mixtures of regioisomers are usually obtained with 1,2-disubstituted dipolarophiles, although steric effects may have an influence on the regioisomeric ratio, especially in case of polycyclic systems [1, 6]. π-Conjugation of the double bond has a strong promoting effect on the reactivity of the alkene and site-selectivity often predominates when different olefinic moieties compete for the dipole. Ring-strain also destabilizes dipolarophiles and enhances their activity toward nitrile oxides. The stereochemical outcome of the cycloadditions is affected by steric and geometric features of the dipolarophile.

Attention to the incorporation of five-membered *N*,*O*-heterocyclic moiety into steroids either connected to or condensed with ring A or D of the sterane core is caused by the fact that some of these modified compounds have been reported to exhibit various biological activities, including anabolic, antibacterial, antiproliferative, hypocholesteremic and anti-inflammatory properties [5, 7−10]. Moreover, steroids are attractive chiral models with relatively rigid

framework for controlling the regio- and/or stereoselectivity of nitrile oxide/alkene cycloadditions [11]. However, sterane-based dipolarophiles, containing an unactivated and/or sterically less hindered double bond are usually not reactive enough necessitating elevated temperature and/or prolonged reaction time for sufficient conversion and the reactions often suffer from a lack of selectivity [5, 12, 13]. The introduction of an enone moiety into the vicinity of sterically interfering groups has been found to have a strong driving effect on the reactivity of such alkenes and also on the regio- and stereochemical outcome of the thermally-induced ring-closures [1]. In this regard, we recently reported the regio- and stereoselective synthesis of some novel 5α-androstanes containing an 2-isoxazoline moiety condensed to ring A or D by intermolecular 1,3-dipolar cycloaddition of aryl nitrile oxides to steroidal α,β-unsaturated ketones [14]. We have demonstrated that the cyclic enone moiety of the five-membered ring D is more reactive than that of the six-membered ring A presumably due to ring strain and conformation effects.

As a continuation of our research for the construction of steroidal ring-condensed heterocycles [10, 14–18], our aim was to extend the ring closure reactions to the pregnane skeleton to make available some additional ring D-fused isoxazolines. The present work describes the transformations of 16-dehydropregnenolone acetate (16-DPA) containing a reactive dipolarophilic enone moiety, with nitrile oxides via thermal 1,3-dipolar cycloaddition. The regio- and stereoselectivity of the process and the influence of steric and electronic factors on the ring-closure reactions were also investigated. Since several steroidal isoxazoles and their saturated analogs have been reported to possess cell-growth-inhibitory effect on malignant cell lines of diverse origins [7, 9–11], our further goal was to investigate our compounds for such activities against four human adherent breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361).

## 2. Experimental

### *2.1. General*

Melting points (mp-s) were determined on an SMS Optimelt digital apparatus. Elemental analysis data were obtained with a Perkin Elmer CHN analyzer model 2400 and FT-IR spectra were recorded on a FT/IR-4700 spectrometer (Jasco) using ATR. Infrared absorbance is reported in reciprocal centimeters ($cm^{-1}$). $^{1}H$ NMR spectra were obtained at room temperature in $CDCl_3$ solution at 500 MHz (Brucker DRX 500) and the $^{13}C$ NMR spectra at 125 MHz with the same instrument. Chemical shifts are reported in ppm ($\delta$ scale) relative to TMS; coupling constants (*J*) are given in Hz. Multiplicity of the $^{1}H$ resonance peaks are indicated as singlet (s), doublet (d), triplet (t) and multiplet (m). $^{13}C$ NMR spectra are $^{1}H$-decoupled. For the determination of multiplicities, the *J*-MOD pulse sequence was used. Automated flow injection analyses were performed by using an HPLC/MSD system. The system comprised an Agilent 1100 micro vacuum degasser, a quaternary pump, a micro-well plate autoinjector and a 1946A MSD equipped with an electrospray ion (ESI) source operated in positive ion mode. The ESI parameters: nebulizing gas $N_2$, at 35 psi; drying gas $N_2$, at 350 °C and 12 L $min^{-1}$; capillary voltage 3000 V; and fragmentor voltage 70 V. The MSD was operated in scan mode with the mass range *m/z* 60−620. Samples (0.2 μL) were injected with an automated needle wash directly into the solvent flow (0.3 mL $min^{-1}$) of MeCN/$H_2O$ 70:30 (v/v) supplemented with 0.1% formic acid. The system was controlled by Agilent LC/MSD Chemstation software. All solvents were distilled immediately prior to use. Reagents and materials were obtained from reliable commercial suppliers and were used without purification. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. The $R_f$ values were determined for the spots observed by

illumination at 254 and 365 nm. Solvent systems (ss): $CH_2Cl_2$ (A); EtOAc/$CH_2Cl_2$ (10/80, v/v) (B). Flash chromatography: Merck silica gel 60, 40–63 μm.

*2.2. General procedure for the preparation of steroidal isoxazolines (**6a–e**)*

16-DPA (**1**, 357 mg, 1.00 mmol) and the appropriate aromatic hydroximidoyl chloride (**4a–e**, 1.50 mmol) were dissolved in toluene (15 mL), and DIPEA (0.52 mL, 3.00 mmol) was added dropwise to the reaction mixture at room temperature, with subsequent refluxing for 2 h. The solvent was then evaporated off *in vacuo* and the resulting crude product was purified by column chromatography with $CH_2Cl_2$.

*2.2.1. 3β-Acetoxy-3′-phenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**6a**)*

In accordance with the general procedure, *N*-hydroxybenzenecarboximidoyl chloride (**4a**, 233 mg) was used for the synthesis. The product **6a** (452 mg) was obtained as a white precipitate. Mp 201–204 °C (Mp 196–198 °C [22]); $R_f$ = 0.25 (ss A); IR: 1730, 1708, 1593, 1442, 1193 $cm^{-1}$, $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 0.79 (s, 3H, 18-$H_3$), 1.01 (s, 3H, 19-$H_3$), 1.04 (m, 1H, 9α-H), 1.14 (m, 1H, 1α-H), 1.45-1.61 (overlapping m, 5H, 2α-H, 8β-H, 11β-H, 14α-H, 15α-H ), 1.69-1.77 (overlapping m, 4H, 7β-H, 15β-H, 11α-H, 12β-H), 1.87 (m, 3H, 1β-H, 2β-H, 7α-H), 2.02 (s, 3H Ac-$CH_3$), 2.08 (m, 1H, 12α-H), 2.28 (m, 2H, 4-$H_2$), 2.31 (s, 3H, 21-$H_3$), 4.42 (t-like m, 1H, 16-H), 4.58 (m, 1H, 3-H), 5.30 (m, 1H, 6-H), 7.39 (m, 3H, 3″-H, 4″-H and 5″-H), 7.66 (m, 2H, 2″-H and 6″-H); $^{13}$C NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.5 (C-11), 21.4 (Ac-$CH_3$), 27.4 (C-21), 27.6 (C-2), 31.2 (C-8), 31.6 (2C, C-7 and C-12), 31.8 (C-15), 35.8 (C-10), 36.9 (C-1), 38.0 (C-4), 48.3 (C-13), 49.1 (C-9), 50.4 (C-14), 51.0 (C-16), 73.7 (C-3), 106.1 (C-17), 121.8 (C-6), 127.1 (2C, C-2″ and C-3″), 128.6 (C-1″), 128.7 (2C, C-3″ and C-5″), 130.1 (C-4″), 139.6 (C-5), 159.9 (C-3′), 170.4 (Ac-CO), 206.4 (C-

20); ESI-MS: 476 $[M+H]^+$; Anal. Calcd for $C_{30}H_{37}NO_4$ C, 75.76; H, 7.84; Found: C, 75.92; H, 7.69.

*2.2.2. 3β-Acetoxy-3′-4″-tolyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**6b**)*

In accordance with the general procedure, *N*-hydroxy-4-methylbenzenecarboximidoyl chloride (**4b**, 255 mg) was used for the synthesis. The product **6b** (480 mg) was obtained as a white precipitate. Mp 164–166 °C (Mp 121–123 °C [22]); $R_f$ = 0.26 (ss A); IR: 1727, 1712, 1593, 1456, 1197 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.78 (s, 3H, 18-$H_3$), 1.02 (s, 3H, 19-$H_3$), 1.04 (m, 1H, 9α-H), 1.14 (m, 1H, 1α-H), 1.44-1.61 (overlapping m, 5H), 1.68-1.75 (overlapping m, 4H), 1.87 (m, 3H), 2.02 (s, 3H Ac-$CH_3$), 2.07 (m, 1H), 2.28 (m, 2H, 4-$H_2$), 2.30 (s, 3H, 21-$H_3$), 2.36 (s, 3H, 4″-$CH_3$), 4.40 (t-like m, 1H, 16-H), 4.58 (m, 1H, 3-H), 5.30 (m, 1H, 6-H), 7.19 (d, 2H, *J* = 7.8 Hz, 3″-H and 5″-H), 7.55 (d, 2H, *J* = 7.8 Hz, 2″-H and 6″-H); $^{13}C$ NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.5 (C-11), 21.4 (2C, Ac-$CH_3$ and 4″-$CH_3$), 27.4 (C-21), 27.6 (C-2), 31.2 (C-8), 31.6 (2C, C-7 and C-12), 31.8 (C-15), 36.5 (C-10), 36.9 (C-1), 38.0 (C-4), 48.3 (C-13), 49.1 (C-9), 50.4 (C-14), 51.1 (C-16), 73.7 (C-3), 105.9 (C-17), 121.9 (C-6), 125.7 (C-1″), 127.0 (2C, C-2″ and C-6″), 129.4 (2C, C-3″ and C-5″), 139.6 (C-5), 140.4 (C-4″), 159.9 (C-3′), 170.4 (Ac-CO), 206.6 (C-20); ESI-MS: 490 $[M+H]^+$; Anal. Calcd for $C_{31}H_{39}NO_4$ C, 76.04; H, 8.03; Found: C, 76.16; H, 7.86.

*2.2.3. 3β-Acetoxy-3′-4″-methoxyphenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**6c**)*

In accordance with the general procedure, *N*-hydroxy-4-methoxybenzenecarboximidoyl chloride (**4c**, 279 mg) was used for the synthesis. The product **6c** (490 mg) was obtained as a white precipitate. Mp 187–189 °C (Mp 119–121 °C [22]); $R_f$ = 0.20 (ss A); IR: 1722, 1712, 1609, 1465, 1190 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.78 (s, 3H, 18-$H_3$), 1.01 (s, 3H, 19-

$H_3$), 1.04 (m, 1H, 9α-H), 1.13 (m, 1H, 1α-H), 1.44-1.61 (overlapping m, 5H), 1.67-1.75 (overlapping m, 4H), 1.86 (m, 3H), 2.01 (s, 3H Ac-$CH_3$), 2.07 (m, 1H), 2.28 (m, 2H, 4-$H_2$), 2.30 (s, 3H, 21-$H_3$), 3.82 (s, 3H, 4″-OMe), 4.39 (t-like m, 1H, 16-H), 4.57 (m, 1H, 3-H), 5.29 (m, 1H, 6-H), 6.90 (d, 2H, $J$ = 7.2 Hz, 3″-H and 5″-H), 7.59 (d, 2H, $J$ = 7.2 Hz, 2″-H and 6″-H); $^{13}$C NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.5 (C-11), 21.4 (Ac-$CH_3$), 27.4 (C-21), 27.6 (C-2), 31.2 (C-8), 31.6 (2C, C-7 and C-12), 31.8 (C-15), 36.5 (C-10), 36.9 (C-1), 38.0 (C-4), 48.2 (C-13), 49.1 (C-9), 50.4 (C-14), 51.2 (C-16), 55.3 (4″-OMe), 73.7 (C-3), 105.7 (C-17), 114.2 (2C, C-3″ and C-5″), 121.0 (C-1″), 121.9 (C-6), 128.6 (2C, C-2″ and C-6″), 139.6 (C-5), 159.9 (C-3′), 161.0 (C-4″), 170.4 (Ac-CO), 206.7 (C-20); ESI-MS: 506 $[M+H]^+$; Anal. Calcd for $C_{31}H_{39}NO_5$ C, 73.63; H, 7.77; Found: C, 73.45; H, 7.59.

*2.2.4. 3β-Acetoxy-3′-4″-chlorophenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**6d**)*

In accordance with the general procedure, *N*-hydroxy-4-chlorobenzenecarboximidoyl chloride (**4d**, 292 mg) was used for the synthesis. The product **6d** (383 mg) was obtained as a white precipitate. Mp 164–167 °C (Mp 114–116 °C [22]); $R_f$ = 0.48 (ss A); IR: 1730, 1707, 1594, 1455, 1195 $cm^{-1}$; $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 0.78 (s, 3H, 18-$H_3$), 1.01 (s, 3H, 19-$H_3$), 1.04 (m, 1H, 9α-H), 1.14 (m, 1H, 1α-H), 1.45-1.61 (overlapping m, 5H), 1.68-1.76 (overlapping m, 4H), 1.86 (m, 3H), 2.02 (s, 3H Ac-$CH_3$), 2.06 (m, 1H), 2.28 (m, 2H, 4-$H_2$), 2.30 (s, 3H, 21-$H_3$), 4.42 (dd, 1H, $J$ = 8.5 Hz, $J$ = 1.5 Hz, 16-H), 4.58 (m, 1H, 3-H), 5.29 (m, 1H, 6-H), 7.35 (d, 2H, $J$ = 8.5 Hz, 3″-H and 5″-H), 7.59 (d, 2H, $J$ = 8.5 Hz, 2″-H and 6″-H); $^{13}$C NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.5 (C-11), 21.4 (Ac-$CH_3$), 27.3 (C-21), 27.6 (C-2), 31.2 (C-8), 31.5 (2C, C-7 and C-12), 31.8 (C-15), 36.5 (C-10), 36.9 (C-1), 37.9 (C-4), 48.3 (C-13), 49.1 (C-9), 50.5 (C-14), 50.8 (C-16), 73.6 (C-3), 106.4 (C-17), 121.8 (C-6), 127.1 (C-1″), 128.3 (2C, C-3″ and C-5″), 129.0 (2C, C-2″ and C-6″), 136.1 (C-4″),

139.6 (C-5), 159.1 (C-3′), 170.4 (Ac-CO), 206.1 (C-20); ESI-MS: 510 $[M+H]^+$; Anal. Calcd for $C_{30}H_{36}ClNO_4$ C, 70.64; H, 7.11; Found: C, 70.81; H, 6.98.

*2.2.5. 3β-Acetoxy-3′-4″-nitrophenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**6e**)*

In accordance with the general procedure, *N*-hydroxy-4-nitrobenzenecarboximidoyl chloride (**4e**, 300 mg) was used for the synthesis. The product **6e** (312 mg) was obtained as a white precipitate. Mp 226–228 °C (Mp 109–111 °C [22]); $R_f$ = 0.42 (ss A); IR: 1730, 1709, 1596, 1515, 1455, 1351, 1235, 852 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.79 (s, 3H, 18-$H_3$), 1.02 (s, 3H, 19-$H_3$), 1.04 (m, 1H, 9α-H), 1.13 (m, 1H, 1α-H), 1.46-1.63 (overlapping m, 5H), 1.69-1.81 (overlapping m, 4H), 1.86 (m, 3H), 2.01 (s, 3H Ac-$CH_3$), 2.06 (m, 1H), 2.28 (m, 2H, 4-$H_2$), 2.32 (s, 3H, 21-$H_3$), 4.43 (d, 1H, *J* = 9.2 Hz, 16-H), 4.57 (m, 1H, 3-H), 5.29 (m, 1H, 6-H), 7.83 (d, 2H, *J* = 8.6 Hz, 2″-H and 6″-H), 8.24 (d, 2H, *J* = 8.6 Hz, 3″-H and 5″-H); $^{13}C$ NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.4 (C-11), 21.4 (Ac-$CH_3$), 27.3 (C-21), 27.6 (C-2), 31.2 (C-8), 31.5 (2C, C-7 and C-12), 31.8 (C-15), 36.5 (C-10), 36.9 (C-1), 37.9 (C-4), 48.3 (C-13), 49.1 (C-9), 50.3 (C-14), 50.6 (C-16), 73.6 (C-3), 107.4 (C-17), 121.6 (C-6), 124.0 (2C, C-3″ and C-5″), 127.8 (2C, C-2″ and C-6″), 134.7 (C-1″), 139.7 (C-5), 148.4 (C-4″), 158.5 (C-3′), 170.4 (Ac-CO), 205.4 (C-20); ESI-MS: 521 $[M+H]^+$; Anal. Calcd for $C_{30}H_{36}N_2O_6$ C, 69.21; H, 6.97; Found: C, 69.07; H, 7.08.

*2.3. General procedure for the preparation of 3-deacetylated steroidal isoxazolines (**10a–e**)*

The 3β-acetoxy derivative (**6a–e**) (0.50 mmol) was dissolved in MeOH (10 mL) and KOH (56 mg, 1.00 mmol) was added. The mixture was stirred for 2 h at room temperature, and then diluted with water. The resulting precipitate was filtered off, washed with water and dried.

*2.3.1. 3β-Hydroxy-3′-phenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**10a**)*

Compound **6a** (238 mg) was used for the synthesis as described in the General procedure. Yield (**10a**): 199 mg (92%, white solid); mp 189–192 °C; $R_f$ = 0.38 (ss B); IR: 3322, 1709, 1593, 1456, 1356, 1194 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.79 (s, 3H, 18-$H_3$), 1.00 (m, 1H and s, 3H, 19-$H_3$), 1.08 (m, 1H), 1.46-1.60 (overlapping m, 5H), 1.66-1.76 (overlapping m, 5H), 1.86 (m, 3H), 2.07 (m, 1H), 2.18-2.28 (overlapping m, 2H), 2.31 (s, 3H, 21-$H_3$), 3.51 (m, 1H, 3-H), 4.42 (t-like m, 1H, 16-H), 5.27 (m, 1H, 6-H), 7.39 (m, 3H, 3″-H, 4″-H and 5″-H), 7.67 (m, 2H, 2″-H and 6″-H); $^{13}C$ NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.2 (C-19), 20.5 (C-11), 27.4 (C-21), 31.2 (C-8), 31.5 ($CH_2$), 31.6 (2C, 2 × $CH_2$), 31.9 (C-15), 36.4 (C-10), 37.2 (C-1), 42.1 (C-4), 48.3 (C-13), 49.2 (C-9), 50.5 (C-14), 51.0 (C-16), 71.5 (C-3), 106.1 (C-17), 120.9 (C-6), 127.1 (2C, C-3″ and C-5″), 128.6 (C-1″), 128.7 (2C, C-2″ and C-6″), 130.1 (C-4″), 140.7 (C-5), 160.0 (C-3′), 206.4 (C-20); ESI-MS: 434 $[M+H]^+$; Anal. Calcd for $C_{28}H_{35}NO_3$ C, 77.56; H, 8.14; Found: C, 77.72; H, 8.22.

*2.3.2. 3β-Hydroxy-3′-4″-tolyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**10b**)*

Compound **6b** (245 mg) was used for the synthesis as described in the General procedure. Yield (**10b**): 199 mg (89%, white solid); mp 217–220 °C; $R_f$ = 0.37 (ss B); IR: 3325, 1708, 1592, 1456, 1192 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.78 (s, 3H, 18-$H_3$), 1.00 (s, 3H, 19-$H_3$), 1.03 (m, 1H), 1.08 (m, 1H), 1.45-1.59 (overlapping m, 5H), 1.66-1.75 (overlapping m, 4H), 1.87 (m, 3H), 2.06 (m, 1H), 2.25 (m, 2H), 2.30 (s, 3H, 21-$H_3$), 2.36 (s, 3H, 4″-$CH_3$), 3.51 (m, 1H, 3-H), 4.40 (t-like m, 1H, 16-H), 5.26 (m, 1H, 6-H), 7.19 (d, 2H, $J$ = 7.9 Hz, 3″-H and 5″-H), 7.55 (d, 2H, $J$ = 7.9 Hz, 2″-H and 6″-H); $^{13}C$ NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.3 (C-19), 20.5 (C-11), 21.4 (4″-$CH_3$), 27.4 (C-21), 31.2 (C-8), 31.5 ($CH_2$), 31.6 ($CH_2$, 2 × $CH_2$), 31.8 (C-15), 36.4 (C-10), 37.2 (C-1), 42.1 (C-4), 48.3 (C-13), 49.2 (C-9), 50.4 (C-14), 51.1 (C-16), 71.5 (C-3), 105.9 (C-17), 120.9 (C-6), 125.7 (C-1″), 127.0 (2C, C-2″ and C-6″),

129.4 (2C, C-3″ and C-5″), 140.4 (C-4″), 140.7 (C-5), 160.0 (C-3′), 206.6 (C-20); ESI-MS: 448 $[M+H]^+$; Anal. Calcd for $C_{29}H_{37}NO_3$ C, 77.82; H, 8.33; Found: C, 77.65; H, 8.19.

*2.3.3. 3β-Hydroxy-3′-4″-methoxyphenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**10c**)*

Compound **6c** (253 mg) was used for the synthesis as described in the General procedure. Yield (**10c**): 202 mg (87%, white solid); mp 191–192 °C; $R_f$ = 0.33 (ss B); IR: 3333, 1709, 1609, 1594, 1455, 1354, 1193 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 0.78 (s, 3H, 18-$H_3$), 1.00 (s, 3H, 19-$H_3$), 1.04 (m, 1H), 1.08 (m, 1H), 1.44-1.60 (overlapping m, 5H), 1.68-1.75 (overlapping m, 4H), 1.85 (m, 3H), 2.06 (m, 1H), 2.28 (m, 2H), 2.30 (s, 3H, 21-$H_3$), 3.50 (m, 1H, 3-H), 3.82 (s, 3H, 4″-OMe), 4.39 (t-like m, 1H, 16-H), 5.27 (m, 1H, 6-H), 6.90 (d, 2H, *J* = 8.6 Hz, 3″-H and 5″-H), 7.59 (d, 2H, *J* = 8.6 Hz, 2″-H and 6″-H); $^{13}C$ NMR ($CDCl_3$, 125 MHz): $\delta$15.2 (C-18), 19.3 (C-19), 20.5 (C-11), 27.3 (C-21), 31.0 (C-8), 31.4 ($CH_2$), 31.5 (2C, 2×$CH_2$), 31.8 (C-15), 36.4 (C-10), 37.2 (C-1), 42.1 (C-4), 48.2 (C-13), 49.2 (C-9), 50.4 (C-14), 51.2 (C-16), 55.3 (4″-OMe), 71.5 (C-3), 105.8 (C-17), 114.2 (2C, C-3″ and C-5″), 120.9 (C-6), 121.0 (C-1″), 128.6 (2C, C-2″ and C-6″), 140.7 (C-5), 159.6 (C-3′), 161.0 (C-4″), 206.7 (C-20); ESI-MS: 464 $[M+H]^+$; Anal. Calcd for $C_{29}H_{37}NO_4$ C, 75.13; H, 8.04; Found: C, 75.20; H, 7.92.

*2.3.4. 3β-Hydroxy-3′-4″-chlorophenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**10d**)*

Compound **6d** (255 mg) was used for the synthesis as described in the General procedure. Yield (**10d**): 199 mg (85%, white solid); mp 205–207 °C; $R_f$ = 0.40 (ss B); IR: 3331, 1709, 1593, 1455, 1193 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$0.78 (s, 3H, 18-$H_3$), 1.00 (m, 1H és s, 3H, 19-$H_3$), 1.08 (m, 1H), 1.45-1.61 (overlapping m, 5H), 1.66-1.77 (overlapping m, 5H),

1.86 (m, 3H), 2.06 (m, 1H), 2.18-2.28 (overlapping m, 2H), 2.30 (s, 3H, 21-$H_3$), 3.51 (m, 1H, 3-H), 4.38 (dd, 1H, *J* = 8.7 Hz, *J* = 1.4 Hz, 16-H), 5.27 (m, 1H, 6-H), 7.36 (d, 2H, *J* = 8.6 Hz, 3″-H and 5″-H), 7.59 (d, 2H, *J* = 8.6 Hz, 2″-H and 6″-H); $^{13}$C NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.3 (C-19), 20.5 (C-11), 27.3 (C-21), 31.2 (C-8), 31.5 ($CH_2$), 31.6 ($CH_2$), 31.8 ($CH_2$), 36.4 (C-10), 37.2 (C-1), 42.1 (C-4), 48.3 (C-13), 49.2 (C-9), 50.5 (C-14), 50.8 (C-16), 71.5 (C-3), 106.4 (C-17), 120.8 (C-6), 127.1 (C-1″), 128.3 (2C, C-3″ and C-5″), 129.0 (2C, C-2″ and C-6″), 136.1 (C-4″), 140.7 (C-5), 159.1 (C-3′), 206.2 (C-20); ESI-MS: 468 $[M+H]^+$; Anal. Calcd for $C_{28}H_{34}ClNO_3$ C, 71.85; H, 7.32; Found: C, 72.02; H, 7.23.

*2.3.5. 3β-Hydroxy-3′-4″-nitrophenyl-2′-isoxazolino[4′,5′-d:16α,17α]-pregn-5-en-20-one (**10e**)*

Compound **6e** (260 mg) was used for the synthesis as described in the General procedure. Yield (**10e**): 215 mg (90%, white solid); mp 250–253 °C; $R_f$ = 0.38 (ss B); IR: 3341, 1711, 1607, 1568, 1455, 1349, 1198, 851 $cm^{-1}$; $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 0.79 (s, 3H, 18-$H_3$), 1.00 (s, 3H, 19-$H_3$), 1.04 (m, 1H), 1.08 (m, 1H), 1.45-1.63 (overlapping m, 5H), 1.70-1.81 (overlapping m, 4H), 1.86 (m, 3H), 2.05 (m, 1H), 2.26 (m, 2H), 2.32 (s, 3H, 21-$H_3$), 3.48 (m, 1H, 3-H), 4.44 (d, 1H, *J* = 9.1 Hz, 16-H), 5.26 (m, 1H, 6-H), 7.84 (d, 2H, *J* = 8.4 Hz, 2″-H and 6″-H), 8.24 (d, 2H, *J* = 8.4 Hz, 3″-H and 5″-H); $^{13}$C NMR ($CDCl_3$, 125 MHz): $\delta$ 15.2 (C-18), 19.3 (C-19), 20.5 (C-11), 27.3 (C-21), 31.2 (C-8), 31.5 (2C, 2×$CH_2$), 31.8 ($CH_2$), 36.4 (C-10), 37.1 (C-1), 42.0 (C-4), 48.3 (C-13), 49.1 (C-9), 50.3 (C-14), 50.7 (C-16), 71.5 (C-3), 107.4 (C-17), 120.7 (C-6), 124.0 (2C, C-3″ and C-5″), 127.7 (2C, C-2″ and C-6″), 134.7 (C-1″), 140.8 (C-5), 148.4 (C-4″), 158.5 (C-3′), 205.5 (C-20); ESI-MS: 479 $[M+H]^+$; Anal. Calcd for $C_{28}H_{34}N_2O_5$ C, 70.27; H, 7.16; Found: C, 70.42; H, 7.02.

*2.4. Determination of antiproliferative activities*

Human breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361) were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential aminoacids and an antibiotic-antimycotic mixture. All cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were grown in a humidified atmosphere of 5% $CO_2$ at 37 °C. For pharmacological investigations, 10 mM stock solutions of the tested compounds were prepared with DMSO. The highest applied DMSO concentration of the medium (0.3%) did not have a substantial effect on the proliferation of the cells. All the chemicals, if otherwise not specified, were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary). Cells were seeded onto 96-well plates at a density of 5,000/well, except for MDA-MB-361 cells which was seeded 10000/well and allowed to stand overnight, after which the medium containing the tested compound was added. After a 72-h incubation, viability was determined by the addition of 20 μL of MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) solution (5 mg/mL). The precipitated formazan crystals were solubilized in DMSO and the absorbance was determined at 545 nm with an ELISA reader [19]. Two independent experiments were performed with 5 parallel wells; cisplatin, an agent administered clinically in the treatment of certain gynecological malignancies, was used as a positive control. All tested agents were screened at 10 and 30 μM.

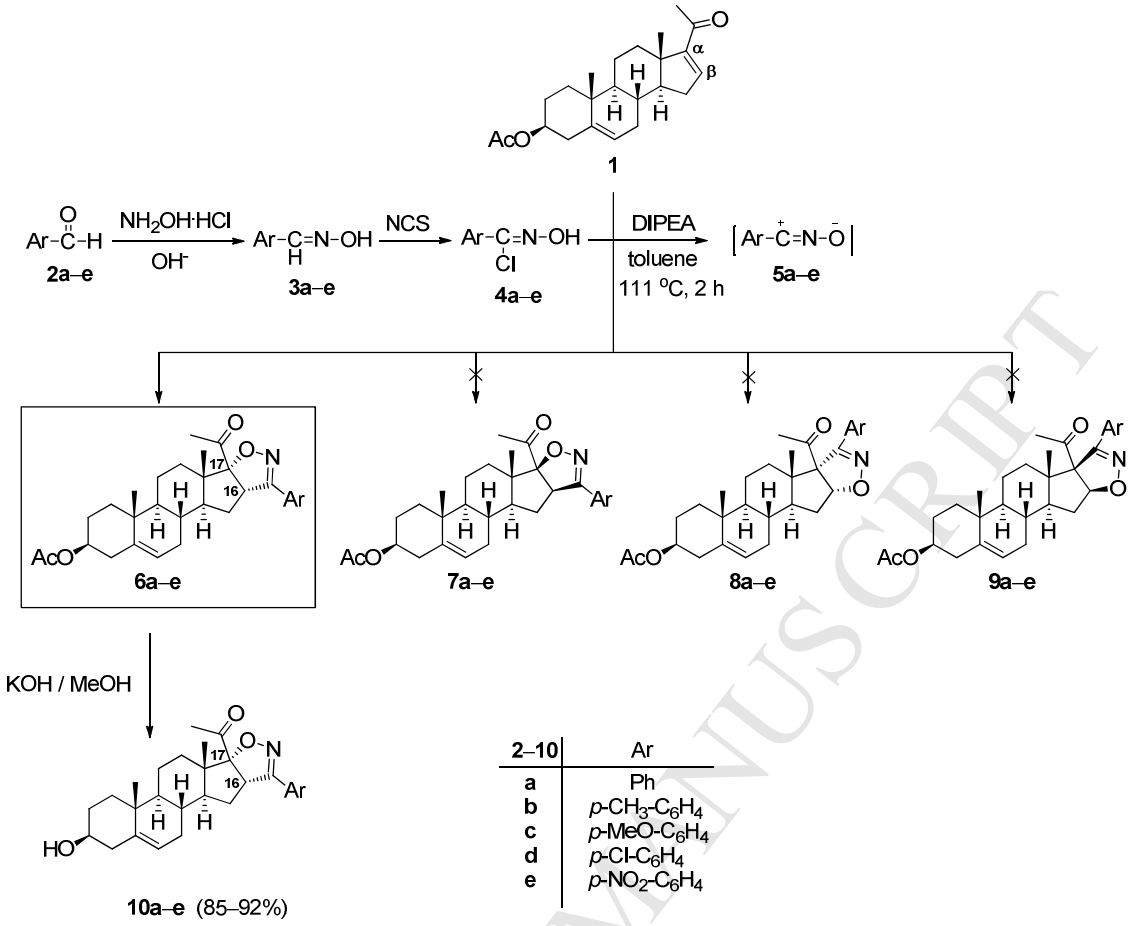
## 3. Results and discussion

### *3.1. Synthesis*

For the transformations, 16-DPA (**1**) was used as steroidal dipolarophile (Scheme 1). The relatively stable aromatic hydroximidoyl chloride precursors (**4a**–**e**) of nitrile oxide 1,3-dipoles (**5a**–**e**) [20] were prepared in two steps by the condensation of benzaldehyde (**2a**) or

its *p*-substituted derivatives (**2b**–**e**) with hydroxylamine hydrochloride in alkaline medium and subsequent chlorination of the aldoximes **3a**–**e** with *N*-chlorosuccinimide (NCS) [21].

The 1,3-dipolar cycloaddition of the steroidal enone **1** was first carried out in toluene using an excess of *N*-hydroxybenzenecarboximidoyl chloride (**4a**), which underwent almost immediate dehydrohalogenation by the slow addition of *N*,*N*-diisopropylethylamine (DIPEA), leading to the *in situ* formation of benzonitrile oxide 1,3-dipole (**5a**). After stirring of the mixture for 5 min at room temperature, the solution was refluxed to achieve complete conversion for compound **1** within 2 h to afford a single product **6a** in a yield of 95% after chromatographic purification (Table 1, entry 1). Similar intermolecular ring closures of **1** with different benzonitrile oxides (**5b**–**e**), obtained from the corresponding aryl aldehydes (**2b**–**e**) by the general protocol mentioned above, were then performed under the same conditions to furnish ring-condensed isoxazolines (**6b**–**e**) in moderate to excellent yields (Table 1, entries 2-5). The yields of the cycloadducts **6b–e** were found to depend on the electronic character of the substituents on the aromatic moiety of the dipoles **5b**–**e**. The electron-donating $CH_3$ and OMe groups in **5b** and **5c** favored cycloaddition to **1**, in consequence of the higher resistance of these dipoles to undergo dimerization to furoxanes [1, 2], while the yields of the desired products **6d** and **6e** were decreased in case of electron-withdrawing Cl and $NO_2$ substituents on the aromatic ring in **5d** and **5e**. Although the synthesis of **6a**–**e** was reported earlier in moderate-yields by a single step procedure from **1** with aldoximes **3a**–**e** in the presence of chloramine-T in refluxing ethanol [22], the exact stereostructure of the products was not supported unambiguously. Moreover, neither the spectral data nor the melting points determined by the authors for the ring D-fused isoxazolines **6a**–**e** are in agreement with those obtained by us.




**Scheme 1.** Regio- and stereoselective formation of ring D-fused 2-isoxazolines from 16-DPA (**1**) and their deacetylations

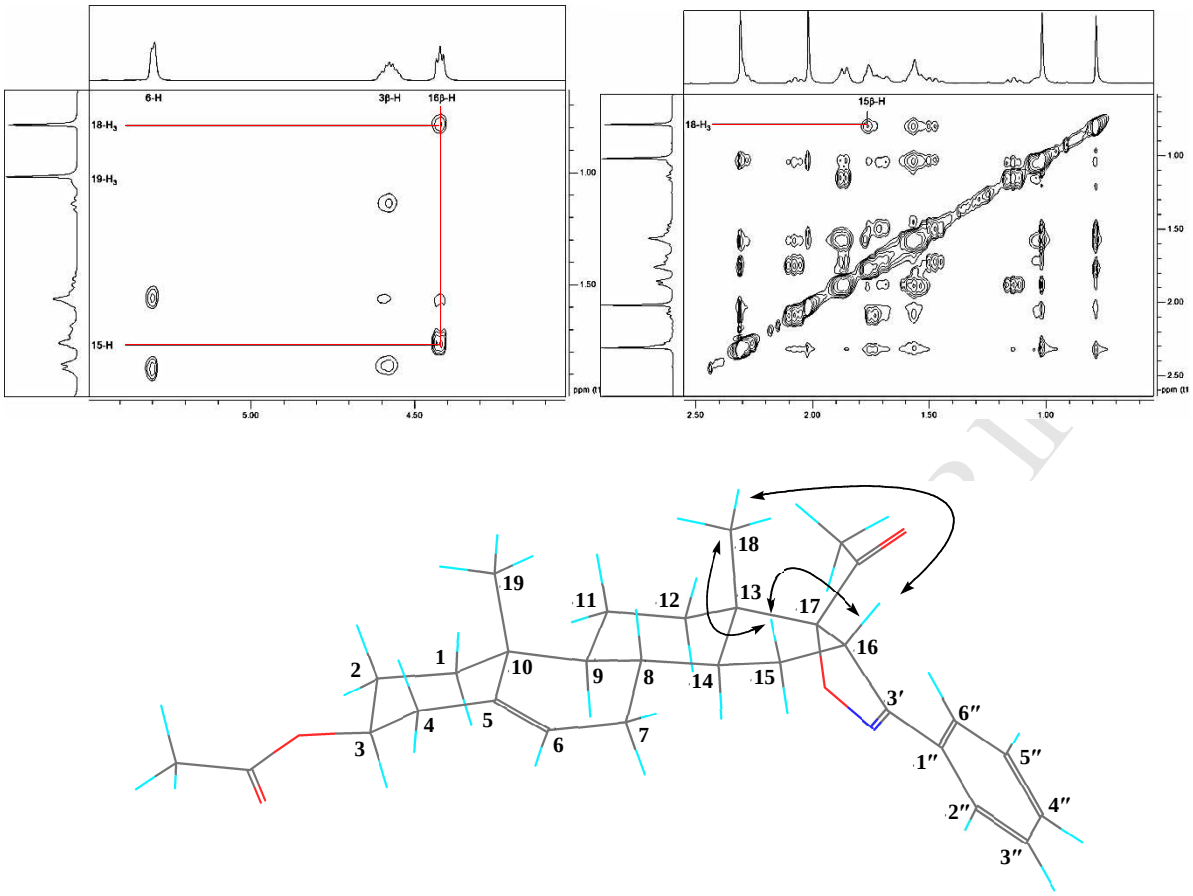
**Table 1.** Cycloaddition products of **1** with different aromatic nitrile oxides

| Entry | Hydroximidoyl chloride/Nitrile oxide | Ar | Product | Yield[a] (%) |
|---|---|---|---|---|
| 1 | **4a**/**5a** | Ph | **6a** | 95 |
| 2 | **4b**/**5b** | *p*-$CH_3$-$C_6H_4$ | **6b** | 98 |
| 3 | **4c**/**5c** | *p*-MeO-$C_6H_4$ | **6c** | 97 |
| 4 | **4d**/**5d** | *p*-Cl-$C_6H_4$ | **6d** | 75 |
| 5 | **4e**/**5e** | *p*-$NO_2$-$C_6H_4$ | **6e** | 60 |

[a]Determined after purification by column chromatography

In principle, the formation of four isoxazolines (**6**–**9**) can be conceived as possible products in the cycloadditions of **1** with aromatic nitrile oxides (**5a**–**e**), as depicted in Scheme 1. The orientation of the 1,3-dipole relative to the double bond of the enone moiety can be of two kinds; the negatively charged O terminus may interact with either the α or the β carbon of

the dipolarophile **1** leading to two regioisomeric pairs, each involving two diastereomers (**6** and **7** or **8** and **9**). The regiochemical mode of addition could easily be determined on the basis of the $^{13}$C NMR spectra of **6a**–**e** indicating the presence of the C-17 signal at around 106 ppm, which is consistent with its linkage to an oxygen. Similar regioselectivity was found to predominate during the thermal cycloaddition of α-aryl-*N*-phenylnitrones to the C16-C17 π-bond of **1** [23]. The attack of the nitrile oxide dipole **5** from above the general plane of the sterane framework (the β side) is unfavorable in **1** in consequence of the same spatial orientation of the 18-$CH_3$. Therefore, the most facilitated isomer is undoubtedly **6**, in which the isoxazoline ring is located in the 16α,17α position opposite to the angular methyl group on C-13. In the $^{1}$H NMR spectra of the products, the coupling pattern of 16-H ($\delta$ ~ 4.41 ppm) is informative in respect of the connection mode of the heteroring. The triplet-like signal ($^{3}J$ ~ 5.2 Hz) for **6a**–**c** and **10a**–**c**, the double doublet ($^{3}J$ ~ 8.6 Hz, 1.5 Hz) for **6d** and **10d** or the doublet ($^{3}J$ ~ 9.2 Hz) for **6e** and **10e** all indicate dihedral angles of about 40° and 110° between 16-H and the 15-protons, respectively. This is in good agreement with the torsion angles calculated for the 16α,17α-isoxazolines. In the case of the 16β,17β-fused heteroring (**7**), the θ(H16,C16,C15,H15$^{\alpha}$) = ~150° and θ(H16,C16,C15,H15$^{\beta}$) = ~ 20° would manifest in two large (~ 10 Hz) coupling constants. The stereochemistry was supported with the aid of homonuclear 2D NMR (COSY and NOESY) and heteronuclear 2D NMR (HSQC and HMBC) measurements. The NOESY spectrum of **6a** showed cross-peaks between the C-18 methyl protons and 16β-H and 15β-H, respectively, while the spatial vicinity of 15β-H and 16β-H was also supported (Scheme 2).




**Scheme 2.** Partial NOESY spectra and 3D representation of **6a**

For the enlargement of the compound library suitable for pharmacological studies and in the hope of finding structure-activity relationships, further derivatives of **6a**–**e** were synthesized by simple deacetylations to furnish the corresponding 3β-OH analogs (**10a**–**e**) (Scheme 1).

### *3.2. Pharmacological studies*

Since a number of compounds containing isoxazoline moiety have been reported to exert noteworthy antiproliferative activities [24, 25] and some sterane-based *N*,*O*-heterocycles have also been demonstrated to inhibit cell proliferation [11, 26], the synthesized isoxazolines (**6a**–**e** and **10a**–**e**) were subjected to *in vitro* pharmacological studies in order to characterize

their cell-growth inhibitory effects on four malignant human breast cell lines, MCF7, T47D, MDA-MB-231 and MDA-MB-361 (Table 2). Their antiproliferative activities were determined by a microplate-based MTT colorimetric assay [19], in comparison with cisplatin as reference agent. The cell-proliferation inhibitory potencies, expressed as growth inhibition, revealed that some of the 3β-OH analogs exhibited marked effects on cell proliferation, especially at 30 μM (Table 2).

The cell growth-inhibitory potencies of the 3-acetate analogs (**6a**–**e**) were generally found to be lower than those of their 3β-hydroxy counterparts (**10a**–**e**). Hence, the esters (**6a**–**e**) may be considered to be practically ineffective. Among the hydroxylated compounds, *p*-substitution of the aromatic ring with a $CH_3$ (**10b**), Cl (**10d**) or $NO_2$ group (**10e**) caused relevant changes in the biological effects as compared with the unsubstituted derivative **10a**. The introduction of OMe substituent in **10c** was, however, less favorable. The *p*-nitro-substituted derivative (**10e**) proved to be the most promising compound in the currently presented set.

**Table 2** Antiproliferative effects of the synthesized compounds

| Entry | Product | Growth inhibition % ± (SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MCF7 | | T47D | | MDA-MB-231 | | MDA-MB-361 | |
| | | 10 μM | 30 μM | 10 μM | 30 μM | 10 μM | 30 μM | 10 μM | 30 μM |
| 1 | **6a** | <25[a] | <25 | <25 | <25 | <25 | <25 | <25 | <25 |
| 2 | **6b** | <25 | <25 | <25 | 28.7 (1.1) | <25 | 58.9 (1.7) | <25 | 28.3 (2.3) |
| 3 | **6c** | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 |
| 4 | **6d** | <25 | <25 | <25 | <25 | <25 | <25 | <25 | <25 |
| 5 | **6e** | <25 | 75.4 (1.4) | <25 | 29.0 (1.5) | <25 | 45.4 (2.2) | <25 | 32.3 (0.8) |
| 6 | **10a** | <25 | 40.6 (1.0) | <25 | 45.2 (0.8) | <25 | 70.5 (2.4) | <25 | 49.0 (2.2) |
| 7 | **10b** | <25 | 71.3 (0.6) | <25 | 70.6 (1.4) | 41.8 (0.4) | 84.1 (1.0) | <25 | 67.5 (0.8) |
| 8 | **10c** | <25 | <25 | <25 | 36.1 (2.4) | <25 | <25 | <25 | <25 |
| 9 | **10d** | <25 | 72.8 (0.6) | <25 | 62.7 (1.3) | <25 | 85.8 (1.2) | <25 | 76.7 (1.4) |
| 10 | **10e** | 59.8 (2.1) | 82.5 (1.4) | 72.6 (2.15) | 93.3 (0.6) | 60.3 (0.9) | 90.7 (2.0) | 70.5 (0.7) | 85.4 (0.9) |
| | **cisplatin** | 66.9 (1.8) | 96.8 (0.4) | 51.0 (2.0) | 57.9 (1.4) | <25 | 71.7 (1.2) | 67.5 (1.0) | 87.7 (1.1) |

[a]Compounds eliciting less than 25% inhibition of proliferation were considered ineffective and the exact results are not given, for simplicity.

## 4. Conclusions

In summary, an improved method for the efficient synthesis of steroidal ring D-fused 2-isoxazolines in the pregnane series by thermally-induced 1,3-dipolar cycloaddition of a steroidal enone with nitrile oxide 1,3-dipoles was developed. The intermolecular ring-closures afforded the heterocyclic products regio- and stereoselectively in good to excellent yields. The conversions were observed to increase by the presence of electron-donating groups on the aromatic ring of the 1,3-dipole in consequence of the lower tendency of these species to undergo dimerization. The stereostructure of the synthesized compounds was established by NMR measurements. The library of compounds was expanded by further deacetylations to furnish the 3-OH analogs of the primarily formed cycloadducts. The antiproliferative activities of the synthesized derivatives on breast cancer cell lines depended mainly on both

the substitution pattern of the incorporated heteroring and the nature of substituents on C-3 of the sterane core. Although the effects of the tested compounds on cell division are moderate, the results suggest that steroidal isoxazolines can be promising candidates motivating the search for further derivatives with better activities.

**Acknowledgements**

The financial support by the Hungarian Scientific Research Fund (OTKA K-109107 and K-109293) is gratefully acknowledged. The research by G. Mótyán was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11/1-2012-0001 ″National Excellence Program″. The work of Á. Baji was supported by a PhD Fellowship of the Talentum Fund of Richter Gedeon Plc.

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**Highlights**

> Isoxazoline hetero rings were introduced into ring D of the sterane core.
> Intermolecular alkene–nitrile oxide cycloadditions were carried out.
> The stereo- and regioselectivity of the ring-closures were investigated.
> The structures of all novel compounds were confirmed by NMR measurements.
> Antiproliferative effects were determined *in vitro* on 4 malignant human cell lines.