Steroids xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series

Erzsébet Mernyák [a,*], Judit Huber [a,e], Johanna Szabó [a], Gyula Schneider [a], Anasztázia Hetényi [b], László Márk [c,d], Gábor Maász [c,d], Ágnes Berényi [e], Ida Kovács [e], Renáta Minorics [e], István Zupkó [e,*], János Wölfling [a]

[a] Department of Organic Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged, Hungary
[b] Department of Medical Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged, Hungary
[c] Department of Biochemistry and Medical Chemistry, University of Pécs, Szigeti út 12, H-7624 Pécs, Hungary
[d] János Szentágothai Research Centre, University of Pécs, Szigeti út 12, H-7624 Pécs, Hungary
[e] Department of Pharmacodynamics and Biopharmacy, University of Szeged, Eötvös u. 6., H-6720 Szeged, Hungary

ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 10 June 2013
Accepted 25 June 2013
Available online xxxx

Keywords:
Estrone
Oxadiazolidinone
Dipolar cycloaddition
Microwave irradiation
Steroid MALDI-TOF
Antiproliferative effect

ABSTRACT

Cyclic nitrones of estrone 3-methyl or 3-benzyl ether were reacted with phenyl isocyanate or nonsubstituted phenyl isocyanates as reactive C=N dipolarophiles, yielding condensed homosteroidal oxadiazolidinones. These dipolar cycloadditions were carried out under conventional heating or microwave irradiation. The chemo- and stereoselectivities of the reactions and the effects of the aromatic substituents on the reaction rates and yields were investigated and compared. The structures of the new products were determined by NMR (one- and two-dimensional) and MALDI-MS techniques, with C_{70} fullerenes as matrix in the latter case. The antiproliferative properties of the synthetized compounds were determined on a panel of human adherent cell lines (HeLa, MCF7, A2780 and A431) by means of MTT assays. Some of them exhibited activities comparable to that of the reference agent cisplatin. Flow cytometry indicated that one of the most potent agents (**11a**) resulted in a cell cycle blockade.

1. Introduction

The formation of nitrone dipoles is usually induced by Lewis-acid catalysts, but literature also provides examples of the electrophile-induced synthesis of nitrones [1–5]. We recently described the halogen and phenylselenyl bromide-induced formation of cyclic nitrones derived from δ-alkenyl D-seco-oximes of estrone and 13α-estrone 3-methyl ether [6,7]. The 13β-oxime behaved as an ambident nucleophile. The trapping of the intermediate halonium or seleniranium ion proceeded via the *O* atom in the case of the *Z*-oxime, and via the *N* atom in the case of the *E*-oxime. The oxazepine derivative (as a steroidal C=N dipolarophile) and the cyclic nitrone (a 1,3-dipole) reacted with each other in an intermolecular 1,3-dipolar cycloaddition, stereoselectively furnishing a nonsymmetrical steroid dimer. The oxime *O*-benzyl ether of the estrone 3-methyl ether behaved similarly to the corresponding oxime, leading to the steroid dimer. In the 13α-estrone series, no intermolecular cycloaddition was observed; only the reduced counterparts of the corresponding cyclic oxyiminium salts were obtained. Moreover, steroidal nitrone 1,3-dipoles reacted stereoselectively with *N*-phenylmaleimide as a C=C dipolarophile, stereoselectively yielding condensed cycloadducts containing six-membered piperidino D rings and isoxazolidine E rings [6].

We now describe the 1,3-dipolar cycloadditions of cyclic nitrones of estrone 3-methyl or 3-benzyl ether with phenyl isocyanate or its substituted derivatives as C=N dipolarophiles. Novel steroidal oxadiazolidinones were primarily prepared in order to investigate the chemoselectivities of the reactions and to observe the effects of the aromatic substituents on the dipolar cycloadditions. We were additionally interested in the investigation of the stereoselectivities of the reactions in comparison with our previous findings [6,7]. The cycloadditions were carried out under conventional heating or were promoted by microwave irradiation. Microwave heating is a very effective and nonpolluting method of activation. The key features of microwave-assisted reactions

* Corresponding authors. Tel.: +36 205236959 (E. Mernyak); fax: +36 62 544199. (I. Zupkó)
E-mail addresses: bobe@chem.u-szeged.hu (E. Mernyák), zupko@pharm.u-szeged.hu (I. Zupkó).

0039-128X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.steroids.2013.06.009

are enhanced selectivity, improved reaction rates, milder reactions, and the formation of cleaner products in higher yields and with less waste relative to conventional heating.

Synthetic steroidal compounds are crucial lead molecules for anticancer drug discovery and development. Analogs of naturally occurring estrogens are particularly important anticancer drug candidates. 2-Methoxyestradiol, a currently investigated metabolite produced by catechol-*O*-methyltransferase (EC number: 2.1.1.6.), inhibits the proliferation of a broad range of cancer cell lines [8]. A new set of estrone 16-oxime ethers with considerable antiproliferative activities were synthetized recently. *In vitro* experiments on the most promising estrone analogs with the aim of elucidation of the mechanism of action revealed apoptosis induction and blockade of the G1–S phase transition in the cell cycle [9]. Several lines of evidence support the crucial role of ring D as a molecular moiety determining the cell growth-inhibitory capacities of estrone-based molecules [10,11]. These previous data prompted us to investigate the possible antiproliferative properties of these novel steroids.

2. Experimental

Melting points (mps) were determined with a Kofler hot-stage apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer CHN analyzer model 2400. Thin-layer chromatography: silica gel 60 F_{254}; layer thickness 0.2 mm (Merck); solvent: 2% ethyl acetate/dichloromethane; detection with iodine or UV (365 nm) after spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid and heating at 100–120 °C for 10 min. Flash chromatography: silica gel 60, 40–63 μm (Merck). The reactions under microwave irradiation were carried out with a CEM Corporation Focused Microwave System, Model Discover SP. ^{1}H NMR spectra were recorded in $CDCl_3$ solution (if not otherwise stated) with a Bruker DRX-500 instrument at 500 MHz, with Me_4Si as internal standard. ^{13}C NMR spectra were recorded with the same instrument at 125 MHz under the same conditions. The mass spectrometer used was an Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in reflector mode. The ions were accelerated under delayed extraction conditions (80 ns) in positive ion modes, with an acceleration voltage of 20.00 kV. The instrument uses a 337 nm pulsed (50 Hz) nitrogen laser. 1 μl aliquots of the standard solutions were loaded onto the target plate (MTP 384 target plate ground steel TF, Bruker Daltonics, Bremen, Germany) by mixing with the same volume of a saturated matrix solution prepared by dissolving C_{70} fullerenes in toluene.

2.1. General procedure for the synthesis of cyclic nitrones (3, 4, 5 and 6) and their subsequent cycloaddition with phenyl isocyanates (7)

Oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was dissolved in dry acetonitrile (5 ml), the solution was cooled in an ice-water bath (0–5 °C) and NBS (0.50 mmol) or NIS (0.50 mmol) was added under a nitrogen atmosphere. The mixture was stirred for 0.5 h. The solvent was evaporated off, and toluene (5 ml) and phenyl isocyanate (0.50 mmol) or a substituted phenyl isocyanate (0.50 mmol) were added.

A: Conventional heating. The reaction mixture was refluxed for the time indicated in Table 1 and then poured onto water and extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated. The crude product was subjected to flash chromatography with dichloromethane as eluent.

B: Microwave irradiation. The reaction mixture was placed into a pressure tube equipped with a stirrer bar and was inserted into the cavity of the microwave apparatus. The mixture was heated at 100 °C for 1 min and then poured onto water and extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate, filtered off, and evaporated. The crude product was subjected to flash chromatography with dichloromethane as eluent.

Table 1
Synthesis of steroidal oxadiazolidinones **8**–**11**.

Entry	Starting oxime	Electrophilic reagent	4-Substituent of phenyl isocyanate	Time (h)	Product	Yield (%)[a]
1	1	NBS	H	3	8a	89 (93)
2	1	NIS	H	3	10a	84 (90)
3	1	NBS	OMe	0.5	8b	92 (93)
4	1	NIS	OMe	0.5	10b	95 (96)
5	1	NBS	Cl	2	8c	96 (96)
6	1	NIS	Cl	2	10c	90 (93)
7	2	NBS	H	3	9a	89 (93)
8	2	NIS	H	3	11a	85 (89)
9	2	NBS	OMe	0.5	9b	95 (96)
10	2	NIS	OMe	0.5	11b	97 (98)
11	2	NBS	Cl	2	9c	90 (92)
12	2	NIS	Cl	2	11c	91 (93)

[a] The yields in brackets are those on the use of microwave irradiation at 100 °C for 1 min.

2.1.1. Reaction of 16-bromomethyl nitrone 3 or 4 with phenyl isocyanate 7a

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was reacted with NBS (89 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml) and phenyl isocyanate (0.06 ml, 0.50 mmol) were added.

8a was obtained as a white solid (method **A**: 227 mg, 89%, method **B**: 238 mg, 93%). Mp 124–127 °C; R_f = 0.55. Anal. Calcd. for $C_{27}H_{31}BrN_2O_3$: C, 63.41; H, 6.11. Found: C, 63.58; H, 6.27%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.86 (m, 2H, 6-H_2), 3.45 (m, 1H, 16-H), 3.66–3.70 (overlapping multiplets, 2H, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 5.17 (s, 1H, 17a-H), 6.61 (d, 1H, J = 2.3 Hz, 4-H), 6.65 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz, 2-H), 7.03 (d, 1H, J = 8.6 Hz, 1-H), 7.29 (d, 2H, J = 7.3 Hz, 2′-H and 6′-H), 7.42 (t, 1H, J = 7.3 Hz, 4′-H), 7.47 (t, 2H, J = 7.3 Hz, 3′-H and 5′-H). ^{13}C NMR (δ, ppm): 18.6 (C-18), 25.0, 26.5, 28.3, 29.8, 35.5, 35.6, 38.8, 38.9 (C-13), 39.7, 42.7, 55.2 (3-OCH_3), 63.2 (C-16), 86.0 (C-17a), 111.7 (C-2), 113.5 (C-4), 126.0 (C-1), 128.0 (2C, C-2′ and C-6′), 128.8 (C-4′), 129.9 (2C: C-3′and C-5′), 131.5 (C-10), 137.5 and 138.9 (C-5 and C-1′), 157.7 (C-3), 160.6 (NCO). MS positive mode: 467 (12%, $[M-CO_2]^+$), 392 (63%, $[M-CO_2-C_6H_5]^+$).

9a was obtained as a white solid (method **A**: 262 mg, 89%, method **B**: 274 mg, 93%). Mp 165–169 °C; R_f = 0.60. Anal. Calcd. for $C_{33}H_{35}BrN_2O_3$: C, 67.46; H, 6.00. Found: C, 67.58; H, 5.92%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.45 (m, 1H, 16-H), 3.65–3.72 (overlapping multiplets, 2H, 16a-H_2), 5.02 (s, 2H, 3-OCH_2), 5.17 (s, 1H, 17a-H), 6.71 (s, 1H, 4H), 6.73 (d, 1H, J = 8.5 Hz, 2-H), 7.03 (d, 1H, J = 8.5 Hz, 1-H), 7.30–7.33 (overlapping multiplets, 3H, 2′-H, 6′-H and 4″-H), 7.35–7.43 (overlapping multiplets, 5H, 2″-H, 3″-H, 5″-H, 6″-H, 4′-H), 7.47 (m, 2H, 3′-H and 5′-H). ^{13}C NMR (δ, ppm): 18.6 (C-18), 24.6, 26.1, 27.9, 29.4, 35.1, 35.2, 38.4, 38.5 (C-13), 39.3, 42.3, 62.8 (C-16), 69.5 (OCH_2), 85.5 (C-17a), 112.1 (C-2), 114.2 (C-4), 126.0 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 128.8 (C-4′), 129.9 (2C: C-3′ and C-5′), 131.8 (C-10), 137.2 (C-1″), 137.5 (C-5), 138.8 (C-1′), 156.9 (C-3), 160.6 (NCO). MS positive mode: 543 (20%, $[M-CO_2]^+$), 429 (80%, $[M-Br-C_6H_5]^+$).

2.1.2. Reaction of 16-bromomethyl nitrone 3 or 4 with 4-methoxyphenyl isocyanate 7b

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was reacted with NBS (89 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml)

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009

and 4-methoxyphenyl isocyanate (0.07 ml, 0.50 mmol) were added.

8b was obtained as a white solid (method **A**: 250 mg, 92%, method **B**: 253 mg, 93%). Mp 130–133 °C; R_f = 0.55. Anal. Calcd. for $C_{28}H_{33}BrN_2O_4$: C, 62.11; H, 6.14. Found: C, 62.34; H, 6.25%. ^{1}H NMR (δ, ppm): 1.15 (s, 3H, 18-H_3), 2.86 (m, 2H, 6-H_2), 3.42 (m, 1H, 16-H), 3.65–3.72 (overlapping multiplets, 2H, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 3.84 (s, 3H, 4′-OCH_3), 5.07 (s, 1H, 17a-H), 6.61 (d, 1H, J = 2.6 Hz, 4-H), 6.66 (dd, 1H, J = 8.6 Hz, J = 2.6 Hz, 2-H), 6.96 (d, 2H, J = 8.9 Hz, 3′-H and 5′-H), 7.05 (d, 1H, J = 8.6 Hz, 1-H), 7.20 (d, 2H, J = 8.9 Hz, 2′-H and 6′-H). ^{13}C NMR (δ, ppm): 18.7 (C-18), 25.0, 26.5, 28.4, 29.8, 35.2, 35.6, 38.8, 38.9 (C-13), 39.8, 42.8, 55.2 (3-OCH_3), 55.5 (4′-OCH_3), 63.1 (C-16), 85.9 (C-17a), 111.7 (C-2), 113.6 (C-4), 115.2 (2C: C-3′and C-5′), 126.0 (C-1), 131.3 and 131.6 (C-1′ and C-10), 137.5 (C-5), 157.7 (C-3), 159.7 (C-4′), 160.8 (NCO). MS positive mode: 497 (7%, [M−CO_2]$^+$), 417 (17%, [M−CO_2−Br]$^+$), 392 (56%, [M−CO_2−C_7H_7]$^+$), 296 (100%, [M−CO_2−CH_2Br−C_7H_7]$^+$).

9b was obtained as a white solid (method **A**: 293 mg, 95%, method **B**: 296 mg, 96%). Mp 165–167 °C; R_f = 0.50. Anal. Calcd. for $C_{34}H_{37}BrN_2O_4$: C, 66.12; H, 6.04. Found: C, 66.31; H, 6.15%. ^{1}H NMR (δ, ppm): 1.15 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.43 (m, 1H, 16-H), 3.64–3.71 (overlapping multiplets, 2H, 16a-H_2), 3.84 (s, 3H, 4′-OCH_3). 5.02 (s, 2H, 3-OCH_2), 5.07 (s, 1H, 17a-H), 6.71 (s, 1H, 4-H), 6.74 (d, 1H, J = 8.4 Hz, 2-H), 6.97 (d, 2H, J = 8.6 Hz, 3′-H and 5′-H), 7.05 (d, 1H, J = 8.5 Hz, 1-H), 7.20 (m, 2H, 2′-H and 6′-H), 7.32 (m, 1H, 4″-H), 7.38 (m, 2H, 3″-H and 5″-H), 7.41 (m, 2H, 2″-H and 6″-H. ^{13}C NMR (δ, ppm): 18.7 (C-18), 24.6, 26.1, 28.0, 29.4, 34.8, 35.2, 38.4 (2C), 39.3, 42.4, 55.1 (4′-OCH_3), 62.7 (C-16), 69.5 (OCH_2), 85.5 (C-17a), 112.5 (C-2), 114.6 (C-4), 115.2 (2C: C-3′ and C-5′), 126.0 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 131.2 (C-1′), 131.8 (C-10), 137.2 (C-1″), 137.6 (C-5), 156.9 (C-3), 159.6 (C-4′), 160.8 (NCO). MS positive mode: 573 (10%, [M−CO_2]$^+$), 429 (100%, [M−Br−C_7H_7O]$^+$).

2.1.3. Reaction of 16-bromomethyl nitrone 3 or 4 with 4-chlorophenyl isocyanate 7c

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was reacted with NBS (89 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml) and 4-chlorophenyl isocyanate (77 mg, 0.50 mmol) were added.

8c was obtained as a white solid (method **A**: 262 mg, 96%, method **B**: 262 mg, 96%). Mp 127–130 °C; R_f = 0.77. Anal. Calcd. for $C_{27}H_{30}BrClN_2O_3$: C, 62.11; H, 6.14. Found: C, 62.38; H, 6.05%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.86 (m, 2H, 6-H_2), 3.41 (m, 1H, 16-H), 3.63–3.70 (overlapping multiplets, 2H, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 5.14 (s, 1H, 17a-H), 6.62 (d, 1H, J = 2.2 Hz, 4-H), 6.67 (dd, 1H, J = 8.4 Hz, J = 2.2 Hz, 2-H), 7.06 (d, 1H, J = 8.4 Hz, 1-H), 7.24 (d, 2H, J = 8.4 Hz, 3′-H and 5′-H), 7.45 (d, 2H, J = 8.4 Hz, 2′-H and 6′-H). ^{13}C NMR (δ, ppm): 18.6 (C-18), 24.9. 26.5. 28.3. 29.8. 35.4. 35.9. 38.8. 39.0 (C-13), 39.8. 42.8. 55.2 (3-OCH_3), 63.2 (C-16), 85.9 (C-17a), 111.7 (C-2), 113.5 (C-4), 126.0 (C-1), 130.2 (2C, C-3′and C-5′), 131.3 (C-10), 134.7 (C-4′), 137.4 and 137.5 (C-1′ and C-5), 157.7 (C-3), 160.4 (NCO). MS positive mode: 421 (35%, [M-CO_2-Br]$^+$), 392 (100%, [M-CO_2-C_6H_4Cl]$^+$).

9c was obtained as a white solid (method **A**: 280 mg, 90%, method **B**: 286 mg, 92%). Mp 170–173 °C; R_f = 0.70. Anal. Calcd. for $C_{33}H_{34}BrClN_2O_3$: C, 63.72; H, 5.51. Found: C, 63.58; H, 5.36%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.84 (m, 2H, 6-H_2), 3.41 (m, 1H, 16-H), 3.63–3.71 (overlapping multiplets, 2H, 16a-H_2), 5.02 (s, 2H, 3-OCH_2), 5.14 (s, 1H, 17a-H), 6.71 (s, 1H, 4-H), 6.74 (d, 1H, J = 8.4 Hz, 2-H), 7.06 (d, 1H, J = 9.0 Hz, 1-H), 7.25 (d, 2H, J = 7.8 Hz, 3′-H and 5′-H), 7.29–7.33 (overlapping multiplets, 3H, 4″-H, 2′-H and 6′-H), 7.37 (m, 2H, 3″-H and 5″-H), 7.43 (m, 2H, 2″-H and 6″-H).

^{13}C NMR (δ, ppm): 18.6 (C-18), 24.9, 26.5, 28.3, 29.8, 35.4, 35.9, 38.8, 39.0 (C-13), 39.9, 42.8, 63.2 (C-16), 69.9 (OCH_2), 85.9 (C-17a), 112.5 (C-2), 114.6 (C-4), 126.1 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 130.2 (2C: C-3′ and C-5′), 131.6 (C-10), 134.7 (C-4′), 137.1 (C-1″), 137.5 (2C: C-5 and C-1′), 157.0 (C-3), 160.4 (NCO).

MS positive mode: 621 (100%, M$^+$), 493 (55%, [M−Cl−CH_2Br]$^+$).

2.1.4. Reaction of 16-iodomethyl nitrone 5 or 6 with phenyl isocyanate 7a

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) (was reacted with NIS (113 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml) and phenyl isocyanate (0.06 ml, 0.50 mmol) were added.

10a was obtained as a white solid (method **A**: 235 mg, 84%, method **B**: 252 mg, 90%). Mp 186–188 °C; R_f = 0.73. Anal. Calcd. for $C_{27}H_{31}IN_2O_3$: C, 58.07; H, 5.60. Found: C, 57.95; H, 5.78%. ^{1}H NMR (δ, ppm): 1.17 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.05 (m, 1H, 16-H), 3.51 (m, 2H, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 5.15 (s, 1H, 17a-H), 6.61 (d, 1H, J = 2.4 Hz, 4-H), 6.65 (dd, 1H, J = 8.6 Hz, J = 2.4 Hz, 2-H), 7.02 (d, 1H, J = 8.6 Hz, 1-H), 7.29 (d, 2H, J = 7.2 Hz, 2′-H and 6′-H), 7.41 (t, 1H, J = 7.2 Hz, 4′-H), 7.47 (t, 2H, J = 7.2 Hz, 3′-H and 5′-H). ^{13}C NMR (δ, ppm): 11.1 (C-16a), 18.8 (C-18), 25.0, 26.5, 29.8, 30.3, 35.5, 38.8, 39.0 (C-13), 39.8, 42.7, 55.2 (3-OCH_3), 62.5 (C-16), 86.0 (C-17a), 111.7 (C-2), 113.5 (C-4), 126.0 and 128.8 (C-1 and C-4′), 129.9 (2C: C-3′and C-5′), 131.5 (C-10), 137.5 and 138.9 (C-5 and C-1′), 157.7 (C-3), 160.6 (NCO). MS positive mode: 515 (5%, [M−CO_2]$^+$), 440 (31%, [M-CO_2-C_6H_5]$^+$), 387 (100%, [M−CO_2−I]$^+$).

11a was obtained as a white solid (method **A**: 270 mg, 85%, method **B**: 283 mg, 89%). Mp 102–107 °C; R_f = 0.60. Anal. Calcd. for $C_{33}H_{35}IN_2O_3$: C, 62.46; H, 5.56. Found: C, 62.63; H, 5.75%. ^{1}H NMR (δ, ppm): 1.17 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.05 (m, 1H, 16-H), 3.52(m, 2H, 16a-H_2), 5.01 (s, 2H, 3-OCH_2), 5.15 (s, 1H, 17a-H), 6.70 (s, 1H, 4H), 6.73 (d, 1H, J = 8.5 Hz, 2-H), 7.03 (d, 1H, J = 8.5 Hz, 1-H), 7.29–7.33 (overlapping multiplets, 3H, 2′-H, 6′-H, 4″-H), 7.36–7.43 (overlapping multiplets, 5H, 3′-H, 4′-H, 5′-H, 3″-H and 5″-H), 7.48 (m, 2H, 2″-H and 6″-H). ^{13}C NMR (δ, ppm): 11.1 (C-16a), 18.8 (C-18), 25.0, 26.5, 29.8, 30.3, 35.5, 38.8, 39.0 (C-13), 39.8, 42.7, 62.5 (C-16), 69.9 (OCH_2), 85.9 (C-17a), 112.5 (C-2), 114.5 (C-4), 126.0 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 128.8 (C-4′), 129.9 (2C: C-3′ and C-5′), 131.8 (C-10), 137.2 (C-1″), 137.6 (C-5), 138.9 (C-1′), 156.9 (C-3), 160.6 (NCO). MS positive mode: 591 (15%, [M−CO_2]$^+$), 509 (100%, [M−I]$^+$).

2.1.5. Reaction of 16-iodomethyl nitrone 5 or 6 with 4-methoxyphenyl isocyanate 7b

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was reacted with NIS (113 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml) and 4-methoxyphenyl isocyanate (0.07 ml, 0.50 mmol) were added.

10b was obtained as a white solid (method **A**: 280 mg, 95%, method **B**: 283 mg, 96%). Mp 139–142 °C; R_f = 0.55. Anal. Calcd. for $C_{28}H_{33}IN_2O_4$: C, 57.15; H, 5.65. Found: C, 57.02; H, 5.77%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.86 (m, 2H, 6-H_2), 3.04 (m, 1H, 16-H), 3.50 (m, 2H, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 3.84 (s, 3H, 4′-OCH_3), 5.05 (s, 1H, 17a-H), 6.62 (s, 1H, 4-H), 6.66 (d, 1H, J = 8.6 Hz, 2-H), 6.96 (d, 2H, J = 8.4 Hz, 3′-H and 5′-H), 7.05 (d, 1H, J = 8.6 Hz, 1-H), 7.19 (d, 2H, J = 8.4 Hz, 2′-H and 6′-H). ^{13}C NMR δ ppm: 11.0 (C-16a), 18.8 (C-13), 25.0, 26.5, 29.8, 30.4, 35.2, 38.8, 38.9 (C-13), 39.8, 42.8; 55.2 (3-OCH_3), 55.5 (4′-OCH_3), 62.4 (C-16), 85.9 (C-17a), 111.7 (C-2), 113.5 (C-4), 115.2 (2C: C-3′and C-5′), 126.0 (C-1), 131.3 and 131.5 (C-1′ and C-10), 137.5 (C-5), 157.7 (C-3), 159.6 (C-4′), 160.8 (NCO). MS positive mode: 440 (18%, [M−CO_2−C_7H_7]$^+$), 417 (100%, [M−CO_2−I]$^+$).

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009

11b was obtained as a white solid (method **A**: 322 mg, 97%, method **B**: 325 mg, 98%). Mp 119–122 °C; R_f = 0.50. Anal. Calcd. for $C_{34}H_{37}IN_2O_4$: C, 61.45; H, 5.61. Found: C, 61.73; H, 5.43%. ^{1}H NMR (δ, ppm): 1.16 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.04 (m, 1H, 16-H), 3.51(m, 2H, 16a-H_2), 3.84 (s, 3H, 4′-OCH_3), 5.02 (s, 2H, 3-OCH_2), 5.06 (s, 1H, 17a-H), 6.70 (s, 1H, 4-H), 6.73 (d, 1H, J = 8.5 Hz, 2-H), 6.98 (m, 2H, 3′-H and 5′-H), 7.05 (d, 1H, J = 8.5 Hz, 1-H), 7.19 (m, 2H, 2′-H and 6′-H), 7.31 (m, 1H, 4″-H), 7.37 (t, 2H, J = 7.3 Hz, 3″-H and 5″-H), 7.41 (d, 2H, J = 7.3 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm): 11.1 (C-16a), 18.8 (C-18), 24.9, 26.5, 29.8, 30.4, 35.2, 38.8, 38.9 (C-13), 39.8, 42.8, 55.5 (4′-OCH_3), 62.4 (C-16), 69.9 (OCH_2), 85.9 (C-17a), 112.5 (C-2), 114.6 (C-4), 115.1 (2C: C-3′ and C-5′), 126.0 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 131.3 (C-1′), 131.8 (C-10), 137.2 (C-1″), 137.6 (C-5), 156.9 (C-3), 159.6 (C-4′), 160.6 (NCO). MS positive mode: 515 (42%, $[M-CO_2-C_7H_7O]^+$), 497 (100%).

2.1.6. Reaction of 16-iodomethyl nitrone 5 or 6 with 4-chlorophenyl isocyanate 7c

As described in Section 2.1, oxime **1** (157 mg, 0.50 mmol) or oxime **2** (195 mg, 0.50 mmol) was reacted with NIS (113 mg, 0.50 mmol). The solvent was evaporated off, and toluene (5 ml) and 4-chlorophenyl isocyanate (77 mg, 0.50 mmol) were added.

10c was obtained as a white solid (method **A**: 267 mg, 90%, method **B**: 276 mg, 93%). Mp 110–112 °C; R_f = 0.77. Anal. Calcd. for $C_{27}H_{30}ClIN_2O_3$: C, 54.70; H, 5.10. Found: C, 54.61; H, 4.98%. ^{1}H NMR (δ, ppm): 1.17 (s, 3H, 18-H_3), 2.86 (m, 2H, 6-H_2), 3.02 (m, 1H, 16-H), 3.50 (d, 2H, J = 4.2 Hz, 16a-H_2), 3.76 (s, 3H, 3-OCH_3), 5.12 (s, 1H, 17a-H), 6.62 (d, 1H, J = 2.3 Hz, 4-H), 6.67 (dd, 1H, J = 8.5 Hz, J = 2.3 Hz, 2-H), 7.06 (d, 1H, J = 8.5 Hz, 1-H), 7.23 (d, 2H, J = 7.7 Hz, 3′-H and 5′-H), 7.44 (d, 2H, J = 7.7 Hz, 2′-H and 6′-H). ^{13}C NMR (δ, ppm): 10.8 (C-16a), 18.8 (C-18), 24.9, 26.5, 29.8, 30.3, 35.8, 38.8, 39.1 (C-13), 39.9, 42.8, 55.2 (3-OCH_3), 62.5 (C-16), 85.9 (C-17a), 111.7 (C-2), 113.5 (C-4), 126.0 (C-1), 130.2 (2C: C-3′and C-5′), 131.3 (C-10), 134.7 (C-4′), 137.4 and 137.6 (C-1′ and C-5), 157.7 (C-3), 160.5 (NCO). MS positive mode: 440 (100%, $[M-CO_2-C_6H_5Cl]^+$), 421 (37%, $[M-CO_2-I]^+$).

11c was obtained as a white solid (method **A**: 305 mg, 91%, method **B**: 312 mg, 93%). Mp 151–155 °C; R_f = 0.70. Anal. Calcd. for $C_{33}H_{34}ClIN_2O_3$: C, 59.25; H, 5.12. Found: C, 59.47; H, 5.23%. ^{1}H NMR (δ, ppm): 1.17 (s, 3H, 18-H_3), 2.85 (m, 2H, 6-H_2), 3.02 (m, 1H, 16-H), 3.50 (m, 2H, 16a-H_2), 5.02 (s, 2H, 3-OCH_2), 5.12 (s, 1H, 17a-H), 6.70 (s, 1H, 4-H), 6.74 (d, 1H, J = 8.5 Hz, 2-H), 7.06 (d, 1H, J = 8.5 Hz, 1-H), 7.23 (d, 2H, J = 7.1 Hz, 3′-H and 5′-H), 7.31 (t, 1H, J = 6.9 Hz, 4′-H), 7.37 (m, 2H, 2′-H and 6′-H), 7.41 (m, 2H, 3″-H and 5″-H), 7.45 (m, 2H, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, 55 °C): 10.8 (C-16a), 18.8 (C-18), 24.9, 26.5, 29.8 30.3, 35.8, 38.7, 39.1 (C-13), 39.9, 42.8, 62.5 (C-16), 69.9 (OCH_2), 85.9 (C-17a), 112.5 (C-2), 114.6 (C-4), 126.1 (C-1), 127.4 (2C: C-2″ and C-6″), 127.9 (C-4″), 128.5 (2C: C-3″ and C-5″), 129.1 (2C: C-2′ and C-6′), 130.2 (2C: C-3′ and C-5′), 131.6 (C-10), 134.7 (C-4′), 137.1 (C-1″), 137.5 (2C: C-5 and C-1′), 156.9 (C-3), 160.5 (NCO). MS positive mode: 625 (11%, $[M-CO_2]^+$), 497 (100%, $[M-CO_2-I]^+$).

2.2. Cell cultures and antiproliferative assays

Human cancer cell lines (Hela, MCF-7 and A431, isolated from cervical adenocarcinoma, breast adenocarcinoma and skin epidermoid carcinoma, respectively) and noncancerous human foreskin fibroblasts were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino-acids and an antibiotic–antimycotic mixture (AAM). A2780 cells (isolated from ovarial cancer) were maintained in RPMI medium supplemented with 10% FBS, 1% AAM and 1% L-glutamine. All cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK). For pharmacological investigations, 10 mM stock solutions of the tested compounds were prepared with dimethyl sulfoxide (DMSO). The highest applied dimethyl sulfoxide concentration of the medium (0.3%) did not have any substantial effect on the determined cellular functions. All the chemicals, if otherwise not specified, were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary). The antiproliferative effects were determined *in vitro* on the four cell lines: Hela, A431, MCF-7 and A2780. The cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded onto 96-well plates at a density of 5000 cells/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 dimethyl sulfoxide and the absorbance was determined at 545 nm with an ELISA reader [12]. 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. Sigmoidal dose–response curves were fitted to the measured data. Calculations of IC_{50} values and statistical analyses (*t*-test and ANOVA) were performed by means of GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA).

2.3. Cell cycle analysis by flow cytometry

Flow cytometric analysis was performed in order to characterize the cellular DNA content of treated A2780 cells. After treatment for 24 or 48 h, cells (200,000/condition) were trypsinized (Gibco BRL, Paisley, UK), washed with phosphate-buffered saline (PBS) and fixed in 1.0 ml of cold 70% ethanol for 30 min on ice. After two washing steps in cold PBS, DNA was stained with PI (10 μg/ml) in the presence of RNA-ase (50 μg/ml). The samples were then analyzed with CyFlow (Partec GmbH, Münster, Germany). In each analysis, 20,000 events were recorded, and the percentages of the cells in the different cell-cycle phases (subG1, G1, S and G2/M) were calculated by using ModFit LT (Verity Software House, Topsham, ME, USA) [13].

3. Results and discussion

3.1. Synthesis and structure determination

Coskun and Parlar recently described the cycloaddition of acyclic nitrones with phenyl isocyanate [14]. The cycloadditon was carried out in refluxing acetonitrile with a 3-fold excess of phenyl isocyanate in reaction times of 1.5–24 h, with yields of 52–96%, depending on the structure of the starting nitrone. It was demonstrated that the structure of the oxime influenced the reaction rate and the yield. When the C-phenyl oxime was substituted with electron-withdrawing substituents on the phenyl ring, the yields were moderate and the reaction proceeded only after prolonged heating. When electron-donating substituents were present, the reaction time was shorter and the yield was higher. There have been other reports on the cycloaddition of acyclic [2,3] or cyclic nitrones [1,2,15–17] with phenyl isocyanate, but there is no evidence of such reactions on the steroid core.

In the present work we describe 1,3-dipolar cycloadditions of steroidal cyclic nitrone dipoles (**3**–**6**) with substituted or nonsubstituted phenyl isocyanates (**7**). Seco-oximes of estrone 3-methyl or 3-benzyl ether (**1**, **2**) used for the electrophile-induced cyclization were obtained from the D-seco-aldehydes using hydroxylamine hydrochloride and sodium acetate [18]. D-seco-aldehydes are available in several steps from estrone 3-methyl or 3-benzyl ether by employing a Grob fragmentation as the key step [19,20].

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009

The cyclization was first carried out with two different electrophile triggers: NBS or NIS in acetonitrile, as reported earlier [6,7]. When TLC monitoring revealed that the starting oxime (**1**, **2**) had been consumed the solvent was evaporated off, toluene and 1 equiv. of phenyl isocyanate (**7**) were added, and the solution was refluxed for the time indicated in Table 1. The desired condensed homosteroidal oxadiazolidinone derivatives (**8**–**11**) were formed in higher yields and shorter reaction times as compared with the literature results [14]. An electron-donating methoxy group on the phenyl ring (**7b**) promoted the reaction (Entries 3, 4, 9 and 10) and, surprisingly, the dual nature of the chloro substituent (**7c**) also accelerated the reaction (Entries 5, 6, 11 and 12). The lowest extent of reaction was observed with the unsubstituted reagent (**7a**, Entries 1, 2, 7 and 8). The reactions were totally chemoselective: no side-products were formed under the reaction conditions applied. The newly formed stereogenic centers displayed the same configurations as earlier: a 16β-substituent and a 17aβ-hydrogen at the anellation of the piperidine and oxadiazolidinone rings [7].

A further aim of the present work was to compare the reaction rates and the chemo- and stereoselectivities of the cycloadditions carried out under reflux or with microwave irradiation. The microwave-induced reactions were performed in a similar manner (100 °C and a 1-min reaction time) independently of the nature of the substituent in the phenyl isocyanates, yielding the oxadiazolidinones (**8**–**11**) chemo- and stereoselectively. This method greatly shortened the reaction time and improved yields were achieved.

In the NMR spectra of the products **8**–**11**, the proton and carbon chemical shifts were assigned through COSY, HSQC and HMBC experiments. The relative configurations of the products were deduced from the NOESY spectra, in which the cross-peaks for the protons of 18-Me, 17a-H and 16-CH_2X proved their *cis* arrangement. The overlapping signals of C-2′ and C-6′ did not appear in the ^{13}C NMR spectrum of the compounds, recorded with the *J*-MOD pulse sequence. A feasible explanation for this phenomenon is the dynamic effect, which broadened the C-2′ and C-6′ signals, which were merged therefore into the noise. To confirm this theory, the spectrum of **11c** was recorded at 55 °C in $CDCl_3$ and the signal of C-2′ and C-6′ appeared at 129.1 ppm, indicating that the temperature stepped the coalescence temperature obviously over. As a further evidence for this dynamic effect is a cross-peak between the overlapping multiplets of 2′-H and 6′-H and the signals of C-2′ and C-6′ in the HSQC spectrum of **8a**, recorded at room temperature. This cross-peak indicated the chemical shift of C-2′ and C-6′ at 128.0 ppm, the latter signal could not be seen on the ^{13}C-axis. The most stable structures the products were confirmed by molecular modeling with all possible chiral arrangements of chiral C atoms 17a and 16. The conformational protocol comprised a stochastic search via the Merck Molecular Force Field (MMFF94), and a subsequent minimization of the resulting low-energy conformations at the *ab initio* level, using the HF/6–311G** basis set. The resulting structures proved to be rigid for **8**–**11**; no minor conformation was found (Scheme. 1).

Neutral steroids are difficult to analyze by desorption/ionization methods coupled with mass spectrometry, and there have been only a few literature reports on the analysis of derivatized steroids through MALDI TOF mass spectrometry [21–24]. We recently described the synthesis and stereochemical investigation of *N*-containing heterocyclic steroids which were efficiently measured by this technique, using C70 fullerenes as matrix [25–27]. Since the N atoms are capable of protonation, no derivatization was needed. Those promising results led us to carry out MALDI TOF measurements of the oxadiazolidinones (**8**–**11**) with positive mode detection. Molecular or quasimolecular ions were not detected, because of the cleavage of carbon dioxide from the molecules. The MS spectra revealed fragment ions formed by the cleavage of carbon dioxide and/or the phenyl group (or substituted phenyl group) deriving from the isocyanate and/or the appropriate halogen atom.

3.2. Determination of the antiproliferative properties of the newly synthetized compounds 8–11

Various types of A- and D-ring-substituted estrone analogs have been synthetized and tested for their inhibitory effects on cell proliferation [28–32]. One of the major requirements for the pharmacological use of antiproliferative estrone derivatives is the lack of estrogenic activity. The introduction of a sulfamate function at position 3 or 17 or D-ring expansion drastically reduces the estrogeneity [30]. There are only a few literature descriptions of the antitumor behavior of D-homoestrone derivatives [33,34]. We recently reported the cytostatic effect of D-homoestrone on HeLa cells (IC_{50} = 5.5 μM) [34]. Hillisch et al. patented the finding that some D-homoestra-1,3,5(10)-trien-3-yl 2-substituted sulfamates are potential pharmaceuticals for the treatment of tumorous diseases [33]. To the best of our knowledge, there are no examples in the literature of the antiproliferative action of condensed D-homoestrone derivatives containing heterocyclic D and E rings. The introduction of *N* atoms into the rings of the steroidal skeleton may influence the ability of the molecules to bind proteins since the donor *N* atoms can serve as hydrogen-bond acceptor [35].

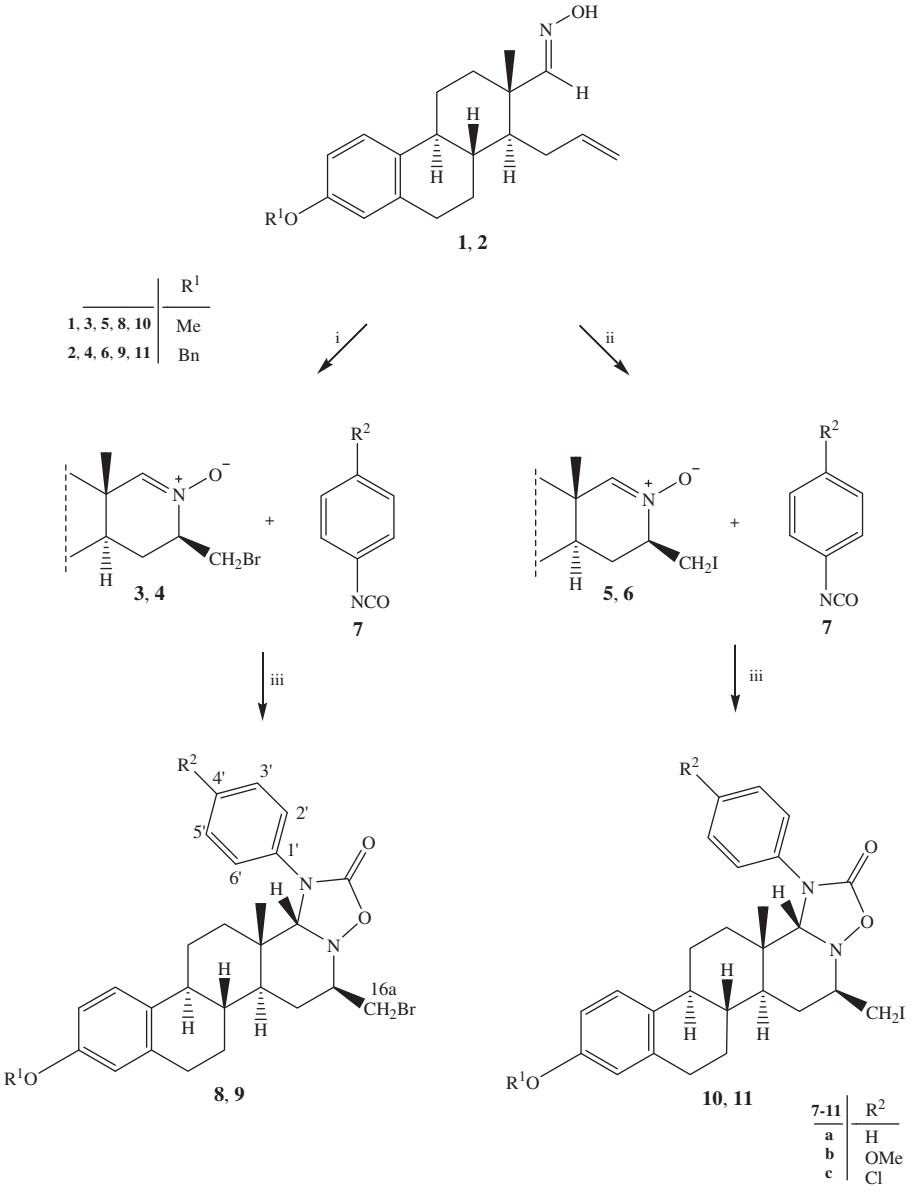
There are some literature data relating to the antimitotic properties of oxadiazoles and their partially or fully saturated derivatives.

Tahir et al. described a novel oxadiazoline derivative (A-204197) with antiproliferative properties. This new tubulin-binding agent is active against tumor cell lines which are resistant to known microtubule inhibitors [36]. The oxadiazoline analog of combrestatin A-4 containing a naphthalene ring was found to be the promising tubulin inhibitor, with potent antiproliferative activities against three cancer cells. A molecular docking study demonstrated the importance of the oxadiazoline moiety due to the hydrogen-bond acceptor ability [35]. Gopalsamy et al. Reported on a novel series of PAI-1 inhibitors (the level of PAI-1 is acutely elevated in cancer) containing an oxadiazolidinedione moiety [37]. An additional oxadiazolidinone derivative as a heterocyclic acid surrogate exhibited improved pharmacokinetic properties as a VLA-4 antagonist [38]. Ouyang et al. found that an oxadiazole derivative caused the mitotic arrest of A431 human epidermoid cells and cells from multidrug-resistant tumors (EC_{50} = 7.8 nM) [39].

The aims of our present study included the characterization of the antiproliferative properties of the newly synthetized compounds **8**–**11** on a panel of human adherent cancer cell lines. These steroidal oxadiazolidinones bearing different functional groups at positions C-3, C-16a or C-4′, displayed different growth-inhibitory effects (Table 2). They were active exclusively against gynecological cancer cell lines: none of them inhibited the proliferation of A431 human epidermoid cells. It can be concluded that the substituent on C-4′ has an impact on the anticancer properties, since compounds bearing 4′-methoxy groups (**8**–**11b**) appeared to be totally inactive (IC_{50} > 30 μM). Modification of the phenolic ether function led to minor changes in the antiproliferative results: 3-benzyloxy derivatives were slightly more efficient than their 3-methoxy counterparts. The most potent compound was the 16-iodo-*N*-phenyl-3-benzyloxy derivative **11a**, with IC_{50} = 2.19 μM for A2780 ovarian carcinoma cells, a concentration at least 5 times lower than its 50% inhibition of cell growth for the other cell lines.

Cancer selectivity is a crucial point in the design and development of an innovative anticancer agent. As a first step to describe this property of **11a**, the viability assay was repeated on noncancerous human skin fibroblast cells at 1 and 10 μM. Similarly to cisplatin, **11a** did not substantially disturb the proliferation of fibroblast cells at 1 μM, but its growth-inhibitory effect was significantly lower at higher concentration (Table 3).

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009



Scheme 1. Reagents and conditions: (i) 1 equiv. of NBS; acetonitrile; N_2 atmosphere; ice-water bath; 0.5 h; (ii) 1 equiv. of NIS; acetonitrile; N_2 atmosphere; 0–5 °C; 0.5 h; (iii) 1 equiv. of phenyl isocyanate or substituted phenyl isocyanate; toluene; reflux; 0.5–3 h or 1 equiv. of phenyl isocyanate or substituted phenyl isocyanate; toluene; microwave irradiation; 100 °C; 1 min.

In order to shed light on the mechanism of the antiproliferative action of **11a**, cell cycle analyses were performed after 24 and 48 h of exposure (Fig. 1). Treatment with 3 μM **11a** for 24 h resulted in a statistically significant decrease in the population of cells in the synthetic phase, and this became more marked at 10 μM. This higher concentration led to an increase in the ratio of cells in the G1 phase, indicating a blockade in the G1–S transition during the cell cycle. A more marked and concentration-dependent disturbance in the cell distribution was evident after 48 h of exposure.

The G1–S transition is a crucial phase in the cell cycle, tightly governed by complex machinery-regulating factors [40]. The important role of cyclin D and its interacting factors is illustrated by the fact that some component of the machinery has been found to be altered in virtually all human tumors [41]. The recently reported antiproliferative action of selected estrone-16-oxime ethers was explained by the mRNA-level induction of tumor suppressor p16 and the repression of retinoblastoma protein and cyclin-dependent kinase 4, as indicated by RT-PCR studies. A decreased

Table 2
Experimentally determined IC_{50} values of the synthetized oxadiazolidinone derivatives **8–11.**

	IC_{50} values (μM)[a]			
	HeLa	A431	A2780	MCF-7
8a	11.27	>30	13.78	9.57
10a	6.74	>30	6.28	10.5
8b	>30	>30	>30	>30
10b	>30	>30	>30	>30
8c	9.22	>30	13.93	7.64
10c	4.91	>30	4.99	7.20
9a	5.46	>30	3.24	5.68
11a	13.43	>30	2.19	12.46
9b	>30	>30	>30	>30
11b	>30	>30	>30	>30
9c	12.53	>30	5.46	8.38
11c	10.96	>30	4.63	9.16
Cisplatin	5.66	8.81	0.86	7.99

[a] Mean values from 2 independent determinations with 5 parallel wells; standard deviation <15%.

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009

Table 3
Antiproliferative effects of **11a** and cisplatin on noncancerous human foreskin fibroblast cells.

	Growth inhibition (%) ±SEM		
	11a	Cisplatin	*P* value[a]
1 μM	0.13 ± 2.60	1.92 ± 1.78	NS
10 μM	15.39 ± 0.91	26.30 ± 2.35	<0.01

[a] *P* values were calculated with the unpaired *t*-test. NS: not significantly different.

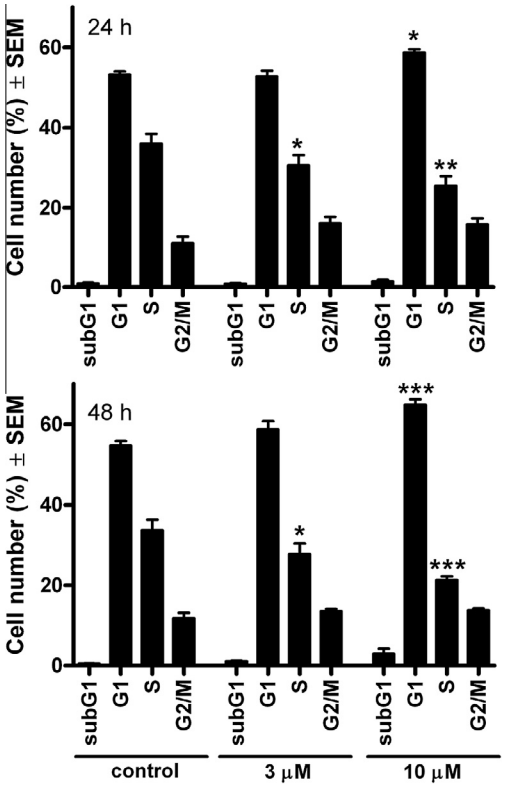


Fig. 1. Effects of **11a** on the A2780 cell cycle distribution after incubation for 24 h (upper panel) or 48 h (lower panel). *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, as compared with the control cells.

expression of phosphorylated retinoblastoma protein was additionally detected in Western blot experiments [9]. These earlier results permit the suggestion that **8**–**11** may also exert their cancer cell growth-inhibitory effects by disrupting the regulation of the cell cycle.

Acknowledgments

The authors thank the Hungarian Scientific Research Fund (OTKA K101659) and TÁMOP-4.2.2./B-10/1-2010-0012 for financial support. Special thanks should be given to Reinhard Machinek (University of Göttingen) for his useful recommendations.

References

[1] Grashey R. In: Padwa A, editor. 1,3-Dipolar Cycloaddition Chemistry, vol. 1. New York: Wiley; 1984. p. 734–814.
[2] Dondas HA, Grigg R, Hadjisoteriu M, Markandu J, Kennewell P. Thornton–Pett M X = Y–ZH systems as potential 1,3-dipoles. Part 51: halogen-induced inter- and intramolecular formation of nitrones from oximes and alkenes. Tetrahedron 2001;57:1119–28.
[3] Dondas HA, Grigg R, Hadjisoteriu M, Markandu J, Thomas WA. Kennewell P X = Y–ZH systems as potential 1,3-dipoles. Part 50: phenylselenyl halide induced formation of cyclic nitrones from alkenyl oximes. Tetrahedron 2000;56:10087–96.
[4] Pérez P, Domingo LR, Aurell MJ. Contreras R Quantitative characterization of the global electrophilicity pattern of some reagents involved in 1,3-dipolar cycloaddition reactions. Tetrahedron 2003;59:3117–25.
[5] Tiecco M, Testaferri L, Bagnoli L, Marini F, Santi C, Temperini A, et al. Enantioselective synthesis of heterocyclic compounds mediated by organoselenium reagents. Arkivoc 2006;2006(vii):186–206.
[6] Mernyák E, Benedek G, Schneider G, Wölfling J. Stereoselective synthesis of condensed aza-D-homo-estrone derivatives by 1,3-dipolar cycloaddition. Synlett 2005:637–9.
[7] Mernyák E, Bikádi Zs, Hazai E, Márk L, Schneider G, Wölfling J. Steroidal ä-alkenyl oximes as ambident nucleophiles: electrophile induced formation of oxazepane derivatives in the bis-estrone series. Lett Org Chem 2008;5:17–21.
[8] Mueck AO, Seeger H. 2-Methoxyestradiol – biology and mechanism of action. Steroids 2010;75:625–31.
[9] Berényi A, Minorics R, Iványi Z, Ocsovszki I, Ducza E, Thole H, Messinger J, Wölfling J, Mótyán G, Mernyák E, Frank E, Schneider G, Zupkó I. Synthesis and investigation of the anticancer effects of estrone-16-oxime ethers in vitro. Steroids 2013;78:69–78.
[10] Ayan D, Roy J, Maltais R, Poirier D. Impact of estradiol structural modifications (18-methyl and/or 17-hydroxy inversion of configuration) on the in vitro and in vivo estrogenic activity. J Steroid Biochem Mol Biol 2011;127:324–30.
[11] Kovács D, Kádár Z, Mótyán G, Schneider G, Wölfling J, Zupkó I, Frank É. Synthesis, characterization and biological evaluation of some novel 17-isoxazoles in the estrone series. Steroids 2012;77:1075–85.
[12] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and citotoxicity assays. J Immunol Methods 1983;65:55–63.
[13] Vermes I, Haanen C. Reutelingsperger C Flow cytometry of apoptotic cell death. J Immunol Methods 2000;243:167–90.
[14] Coskun N, Parlar A. Cycloaddition of acyclic nitrones with phenyl isocyanate: synthesis and ring-opening reactions of 1,2,4-oxadiazolidin-5-ones. Synth Commun 2006;36:997–1004.
[15] Dunn PJ, Graham AB, Grigg R, Saba IS. Thornton–Pett M X = Y–ZH systems as potential 1,3-dipoles. Part 55: cascade 1,3-azaprotio cyclotransfer-cycloaddition reactions between ketoximes and divinyl ketone. Tetrahedron 2002;58:7701–13.
[16] Buchlovic M, Hebanova S, Potacek M. 1,3-Dipolar cycloadditions of new 2,5-bifunctionalized five-membered cyclic nitrones. Tetrahedron 2012;68:3117–22.
[17] Coskun N. Regio and diastereoselective addition of imidazoline 3-oxides to aryl isocyanates. Tetrahedron Lett 1997;38:2299–302.
[18] Mernyák E, Huber J, Benedek G, Pfoh R, Rühl S, Schneider G, et al. Electrophile- and Lewis acid-induced nitrone formation and 1,3-dipolar cycloaddition reactions in the 13á- and 13â-estrone series. Arkivoc 2010;2010(xi):101–13.
[19] Wölfling J, Mernyák E, Forgó P, Schneider G. Stereoselective halogenation of the 16-hydroxymethyl-3-methoxy-13α-estra-1,3,5(10)-trien-17-ols and their solvolytic investigation. Steroids 2003;68:451–8.
[20] Wölfling J, Mernyák E, Frank É, Falkay G, Márki Á, Minorics R, Schneider G. Synthesis and receptor-binding examinations of the normal and 13-epi-D-homoestrones and their 3-methyl ethers. Steroids 2003;68:277–88.
[21] Khan MA, Wang Y, Heidelberger S, Alvelius G, Liu S, Sjövall J, Griffiths W. Analysis of derivatised steroids by matrix-assisted laser desorption/ionisation and post-source decay mass spectrometry. Steroids 2006;71:42–53.
[22] Griffiths WJ, Alvelius G, Sjovall J. Derivatisation for the characterisation of neutral oxosteroids by electrospray and matrix-assisted laser desorption/ionisation tandem mass spectrometry: The Girard P derivative. Rapid Commun Mass Spectrom 2003;17:924–35.
[23] Wang Y, Hornshaw M, Alvelius G, Bodin K, Liu S, Sjovall J, Griffiths WJ. Matrix-assisted laser desorption/ionization high-energy collision-induced dissociation of steroids: analysis of oxysterols in rat brain. Anal Chem 2006;78:164–73.
[24] Kosanam H, Prakash PKS, Yates CR, Miller DD, Ramagiri S. Rapid screening of doping agents in human urine by vacuum MALDI-linear trap mass spectrometry. Anal Chem 2007;79:6020–6.
[25] Mernyák E, Kozma E, Hetényi A, Márk L, Schneider G, Wölfling J. Stereoselective synthesis of spiro and condensed pyrazolines of steroidal α, β-unsaturated ketones and nitrilimines by 1,3-dipolar cycloaddition. Steroids 2009;74:520–5.
[26] Montsko G, Vaczy A, Maasz G, Mernyak E, Frank E, Bay Cs, et al. Analysis of nonderivatized steroids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using C70 fullerene as matrix. Anal Bioanal Chem 2009;395:869–74.
[27] Mernyák E, Márk L, Frank É, Schneider G, Wölfling J. Electrophile-induced generation of cyclic azomethine imines from steroidal δ-alkenyl hydrazones. Steroids 2009;74:474–82.
[28] Stander A, Joubert F. Joubert A Docking, synthesis and in vitro evaluation of antimitotic estrone analogs. Chem Biol Drug Des 2011;77:173–81.
[29] Cushman M, He HM, Katzenellenbogen JA, Lin CM. Hamel E Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by bonding to the colchicine binding site. J Med Chem 1995;38:2041–9.

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and antiproliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009

[30] Leese MP, Leblond B, Newman SP, Purohit A, Reed MJ. Potter BVL anti-cancer activities of novel D-ring modified 2-substituted estrogen-3-O-sulfamates. J Steroid Biochem 2005;94:239–51.
[31] Agoston GE, Shah JH, LaVallee TM, Zhan X, Pribluda VS. Treston AM Synthesis and structure-activity relationships of 16-modified analogs of 2-methoxyestradiol. Bioorg Med Chem 2007;15:7524–37.
[32] Shah JH, Agoston GE, Suwandi L, Hunsucker K, Pribluda V, Zhan XH, Swartz GM, LaVallee TM. Treston AM Synthesis of 2- and 17-substituted estrone analogs and their antiproliferative structure-activity relationships compared to 2-methoxyestradiol. Bioorg Med Chem 2009;17:7344–52.
[33] Hillisch A, Peters O, Gege C, Siemeister G, Unger E, Menzenbach B antitumoral D-homoestra-1,3,5(10)-trien-3-yl 2-substituted sulfamates, US RE42 132E; 2011.
[34] Minorics R, Bózsity N, Wölfling J, Mernyák E, Schneider G, Márki Á, et al. Antiproliferative effect of normal and 13-epi-D-homoestrone and their 3-methyl ethers on human reproductive cancer cell lines. J Steroid Biochem Mol Biol 2012;132:168–74.
[35] Hu Y, Lu X, Chen K, Yan R, Li QS. Zhu HL Design, synthesis, biological evaluation and molecular modelling of 1,3,4-oxadiazoline analogs of combrestatin-A4 as novel antitubulin agents. Bioorg Med Chem 2012;20:903–9.
[36] Tahir SK, Han EKH, Credo B, Jae HS, Pietenpol JA, Scatena CD, Wu-Wong JR, Frost D, Sham H, Rosenberg SH. Ng SC A-204197, a new tubulin-binding agent with antimitotic activity in tumor cell lines resistant to know microtubule inhibitors. Cancer Res 2001;61:5480–5.
[37] Gopalsamy A, Kincaid SL, Ellingboe JW, Groeling TM, Antrilli TM, Krishnamurthy G, Aulabaugh A, Friedrichs GS. Crandall DL Design and synthesis of oxadiazolidinediones as inhibitors of plasminogen activator inhibitor-1. Bioorg Med Chem 2004;14:3477–80.
[38] Venkatraman S, Lim J, Cramer M, Gardner MF, James J, Alves K, Lingham RB, Mumford RA. Munoz B Influence of acid surrogates toward potency of VLA-4 antagonist. Bioorg Med Chem 2005;15:4053–6.
[39] Ouyang X, Piatnitski E, Pattaropong V, Chen X, He HY, Kiselyov AS, Velankar A, Kawakami J, Labelle M, Smith L, Lohman J, Lee SP, Malikzay A, Fleming J, Gerlak J, Wang Y, Rosler RL, Zhou K, Mitelman S, Camara M, Surguladze D, Doody JF. Tuma MC Oxadiazole derivatives as a novel class of antimitotic agents: synthesis, inhibition of tubulin polimerization, and activity in tumor cell lines. Bioorg Med Chem 2006;16:1191–6.
[40] Sunley K, Butler M. Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. Biotechnol Adv 2010;28:385–94.
[41] Nevins JR. The Rb/E2F pathway and cancer. Hum Mol Genet 2001;10:699–703.

Please cite this article in press as: Mernyák E et al. Cycloaddition of steroidal cyclic nitrones to C=N dipolarophiles: Stereoselective synthesis and anti-proliferative effects of oxadiazolidinones in the estrone series. Steroids (2013), http://dx.doi.org/10.1016/j.steroids.2013.06.009