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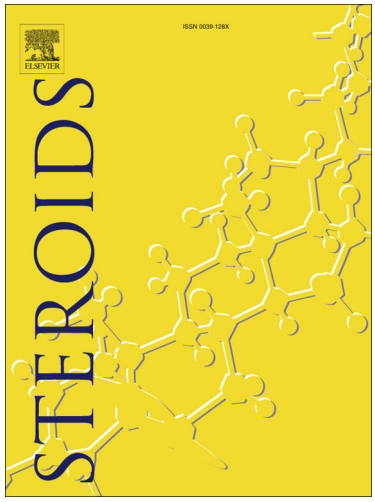

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Multicomponent synthesis of 4,4-dimethyl sterol analogues and their effect on eukaryotic cells.

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ABSTRACT

Most sterols, such as cholesterol and ergosterol, become functional only after the removal of the two methyl groups at C-4 from their biosynthetic precursors. Nevertheless, some findings suggest that 4,4-dimethyl sterols might be involved in specific physiological processes. In this paper we present the synthesis of a collection of analogues of 4,4-dimethyl sterols with a diamide side chain and a preliminary analysis of their *in vitro* activity on selected biological systems. The key step for the synthesis involves an Ugi condensation, a versatile multicomponent reaction. Some of the new compounds showed antifungal and cytotoxic activity.

KEYWORDS

antifungal; cytotoxic; 4,4-dimethyl sterols; Ugi reaction

INTRODUCTION

Sterols have an extremely widespread distribution in living organisms: protozoa, algae, fungi, higher plants and animals [1]. Although sterols have multiple roles, the most important is as structural constituents of eukaryote membranes, where they enhance the packing of the acyl chains of phospholipids in the hydrophobic phase of the bilayer, increase its mechanical strength, and reduce its permeability [2].

The outline for similarity and differences in sterol biosynthesis across kingdoms has been established [3]. Although the most abundant sterols are cholesterol, ergosterol and sitosterol [1], chemical surveys of the sterol composition of eukaryotes show that there are at least 250 different sterols, many of them being biosynthetic intermediates, present in small amounts in tissues. Taking into account that all sterols derive from the lanostane or cycloartane skeletons, many of these intermediates possess methyl groups at C-4 and/or C-14.

Most sterols become functional only after the removal of the two methyl groups at C-4 [4]. Nevertheless, some reports indicate that a variety of C-4 methylated sterols have specific physiological activity in different organisms. In animals, for example, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol and related sterols can activate the nuclear maturation of oocytes and markedly improve fertilization rates *in vitro* [5]. On the other hand, recent studies show that 4-methylsterols regulate *Schizosaccharomyces pombe* fission under low oxygen and cell stress [6].

Synthetic, non natural 4,4-dimethylsterols may have biological activities also: both 4,4-dimethylcholestrol (**1**) and 4,4-dimethylcholest-5-en-3-one (**2**) (Figure 1) are able to suppress the inflammatory response induced by a phorbol derivative on mice skin [7].

In recent papers we have shown that some sterol analogues with a diamide side chain are able to exert some interesting biological activities. Particularly, compounds **3** and **4** (Figure 2) showed inhibitory effect on the growth of fungi associated with plant pathologies, but have a low *in vitro* toxicity in mammalian cells [8,9]. Furthermore, our previous findings suggest that an aromatic fragment bound to the N-22 is important for the activity.

Taking these previous results into account, in this paper we describe the synthesis of a small library of 4,4-dimethyl sterol analogues with a general structure **5**, structurally related to **3** and **4**, and some preliminary *in vitro* studies about their effect on eukaryotic cells.

EXPERIMENTAL

SYNTHESIS

General

All the reagents were purchased from Sigma-Aldrich Chemical Co. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Melting points were determined on a Fisher Johns apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H and 125.1 MHz for ^{13}C). Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constant (J) values are in Hz. All solvents and reagents were of analytical grade. All new compounds gave satisfactory combustion analysis data (purity ≥ 98%) on an Exeter CE 440 Elemental Analyzer.

Methyl (17β)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxylate (**7**)

Methyl (17β)-3-oxoandrost-4-ene-17-carboxylate [10] (**6**, 1.6 g, 4.8 mmol, obtained from progesterone as previously described [11]) was dissolved in anhydrous THF (100 ml) under argon, and potassium *t*-butoxide (1.1 g, 9.6 mmol) was added in portions. After stirring the yellow solution for 5 min at 0 ºC, methyl iodide (13,7 g, 6.03 ml, 96 mmol) was added, after which the solution becomes cloudy yellow. The reaction was monitored by TLC, and completed within 60 minutes. Then, H_2O (20 ml) and saturated solution of ammonium chloride (20 ml) were added. The mixture was evaporated *in vacuo* in order to get rid of the THF. The residue was taken in ethyl acetate (200 ml), washed with brine, dried over Na_2SO_4 and evaporated *in vacuo* to give 1.72 g of a yellow oil crude product, which was subsequently purified by column chromatography

on silica (cyclohexane / EtOAc 95:5) to give the dimethylated product **7** (771 mg, 2.1 mmol, 63%). M.p.:123-125°C. ^{1}H NMR ($CDCl_3$): 0.67 (H18, 3H, *s*); 0.85 (H19, 3H, *s*); 1.23 (H4a and H4b, 6H, *s*); 1.51 (H8, 1H, *dq*, J = 10.7 and 5.5); 2.36 (H17, 1H, *t*, J = 9.4); 2.47 (H2β, 1H, *ddd*, J = 18.9, 8.4 and 1.7); 2.56 (H2α, 1H, *ddd*, J = 18.9, 11.1 and 8.2); 3.03 (-CO-C$\underline{H}_3$, 3H, *s*); 5.55 (H6, 1H, *dd*, J = 5.3 and 2.5). ^{13}C NMR ($CDCl_3$): 13.6 (C18); 19.6 (C19); 21.4 (C11); 23.9 (C16); 24.7 (C15); 27.5 (C4b); 30.4 (C4a); 31.6 (C8); 31.9 (C7); 32.4 (C1); 33.9 (C2); 37.4 (C10); 38.4 (C12); 44.2 (C13); 48.9 (C4); 49.1 (C9); 51.5 (-CO-$\underline{C}H_3$); 55.4 (C17); 56.4 (C14); 119.9 (C6); 150.1 (C5); 174.7 (C20); 216.8 (C3). Anal. calculated for $C_{23}H_{34}O_3$ C, 77.05; H, 9.56; found: C, 77.11; H, 9.67.

(17β)-4,4-Dimethyl-3-oxoandrost-5-ene-17-carboxylic acid. (**8**)

To a solution of ester **7** (771 mg, 2.1 mmol) in ethylene glycol (10.5 ml) an aqueous solution of KOH (1.82 ml, 40 % m/v) was added, and the resulting suspension was heated under reflux for 20 minutes until a clear solution was formed. Then H_2O (50 ml) and HCl 10% (50 ml) were added, and the precipitate extracted with EtOAc (3x20 ml). The organic fraction was dried over Na_2SO_4 and evaporated *in vacuo*. The crude product was purified by column chromatography on silica (hexane / EtOAc gradient), to yield the steroidal acid **8** (511 mg,67% yield). M.p.: 217-218°C. ^{1}H NMR ($CDCl_3$): 0.73 (H18, 3H, *s*); 0.85 (H19, 3H, *s*); 1.21 (H4a, 3H, *s*); 1.23 (H4b, 3H, *s*); 1.51 (H8, 1H, *dq*, J = 10.6 and 5.4); 2.33 (H17, 1H, *t*, J = 9.3); 2.45 (H2β, 1H, m); 2.56 (H2α, 1H, m); 5.56 (H6, 1H, *dd*, J = 5.2 and 2.5). ^{13}C NMR ($CDCl_3$): 12.6 (C18); 18.6 (C19); 20.4 (C11); 22.9 (C16); 23.7 (C15); 26.6 (C4a); 29.5 (C4b); 30.6 (C8); 30.9 (C7); 31.3 (C2); 33.0 (C1); 36.2 (C10); 37.2 (C12); 42.9 (C13); 47.7 (C4); 48.1 (C9); 54.4 (C17); 55.5 (C14); 119.0 (C6); 149.0 (C5); 175.2 (C20); 215.7 (C3). Anal. calculated for $C_{22}H_{32}O_3$ C, 76.77; H, 9.36; found: C, 76.88; H, 9.45.

(*17β)-N-((t-butylcarbamoyl)methyl)-N-(4-methoxyphenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9a**).

To a solution of the aniline (0.07 mmol, 1.1 eq.) in methanol (1 ml), 5 µL of formaldehyde (37% aq) was added, and the mixture was stirred for 30 minutes at room temperature. Then, 20 mg of the steroidal acid **8** (0.06 mmol, 1 eq.) and *t*-butyl isocyanide (6.6 µL, 0.07 mmol, 1.1 eq.) were added. The reaction was kept under the same condition until total disappearance of the acid (72 h). The solvent was evaporated under reduced pressure and the residue was taken in EtOAc and washed with NaOH (5% aq.). The crude product was purified by silica gel column chromatography (hexane / EtOAc gradient) to give compound **9a** with a 66% yield.

M.p.: 174°C. ^{1}H NMR ($CDCl_3$): 0.83 (H18, 3H, *s*); 0.84 (H19, 3H, *s*); 1.19 (H4b, 3H, *s*); 1.21 (H4a, 3H, *s*); 1.36 (-NH-C($C\underline{H}_3$)$_3$, 9H, *s*); 1.52 (H8, 1H, *m*); 2.47 (H2β, 1H, *m*); 2.50 (H17, 1H, *t*, J = 9.1); 2.56 (H2α, 1H, *m*); 3,83 (-N-Ar-O$C\underline{H}_3$, 3H, *s*); 3.86 (-N(Ar)-$C\underline{H}_aH_b$-CO-, 1H, *d*, J = 14.5); 4.38 (-N-$CH_a\underline{H}_b$-CO-, 1H, *d*, J = 14.5); 5.50 (H6, 1H, *dd*, J = 2.5 and 5.2); 6.42 (-$N\underline{H}$-C(CH_3)$_3$, 1H, *bs*); 6.89 (2H, *d*, J = 9) and 7.12 (2H, *m*).
^{13}C NMR ($CDCl_3$): 13.8 (C18); 19.3 (C19); 21.0 (C11); 24.6 (C15); 26.1 (C16); 27.2 (C4a); 28.7 (-NH-C($\underline{C}H_3$)$_3$); 30.1 (C4b); 31.1 (C8); 31.6 (C7); 32.1 (C1); 33.6 (C2); 37.1 (C10); 38.3 (C12); 38.7 (C13); 45.3 (C4); 48.6 (C9); 51.0 (-NH-$\underline{C}$(CH_3)$_3$); 51.5 (C17); 55.4 (-N-Ar-O$\underline{C}H_3$); 56.1 (-N-$\underline{C}H_2$-CO-); 56.2 (C14); 114.6, 129.9, 136.3 and 158.9 (aromatic carbons); 119.6 (C6); 149.8 (C5); 168.7 (-N(Ar)CH_2-$\underline{C}$O-); 175.1 (C20); 216.5 (C3). HRMS (ESI): calculated for [M+H$^+$] $C_{28}H_{44}NO_4$ 458.3265, found 458.3289. Anal. calculated for $C_{33}H_{48}N_2O_3$ C, 76.11; H, 9.29; N, 5.38; found: C, 76.21; H, 9.41; N, 5.21.

Further elution gave 12% of *2-(t-butylamino)-2-oxoethyl (17β)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxylate* (**10**), the Passerini condensation product. M. p.: 131-133 ºC. ^{1}H NMR ($CDCl_3$): 0.73 (H18, 3H, *s*); 0.73 (H18, 3H, *s*); 0.86 (H19, 3H, *s*); 1.24 (H4a and H4b, 6H, *s*); 1.38 (-NH-C($C\underline{H}_3$)$_3$, 9H, *s*); 1.54 (H8, 1H, *m*); 2.45 (H17, 1H, *t*, J = 9.3); 2.48 (H2β, 1H, *ddd*, J =18.9, 8.5 and 1.7); 2.57 (H2α, 1H, *ddd*, J =18.9, 8.5 and 1.7); 4.42 (-CO-$C\underline{H}_aH_b$-CO-, 1H, *d*, J = 15.1); 4.51 (-CO-$CH_a\underline{H}_b$-CO-, 1H, *d*, J = 15.1); 5.56 (H6, 1H, *dd*, J = 5.24 and 2.5); ^{13}C NMR ($CDCl_3$): 13.7 (C18); 19.5 (C19); 21.3 (C11); 23.9 (C16); 24.5 (C15); 27.4 (C4a); 28.9 (C26); 30.3 (C4b); 31.5 (C8); 31.7 (C7); 32.3 (C1); 33.8 (C2); 37.3 (C10); 38.6 (C12); 44.4 (C13); 48.8 (C4); 49.0 (C9); 51.5 (-NH-$\underline{C}$(CH_3)$_3$); 55.2 (C17); 56.4 (C14); 63.4 (-CO-$\underline{C}H_2$-CO-); 119.7 (C6); 150.1 (C5); 166.4(-CO-CH_2-$\underline{C}$O-); 172.5 (C20); 216.6 (C3). HRMS (ESI): calculated for [M+H$^+$] $C_{28}H_{44}NO_4$ 458.3265, found 458.3265. Anal. calculated for $C_{33}H_{48}N_2O_3$ C, 76.11; H, 9.29; N, 5.38; found:C, 76.21; H, 9.41; N, 5.21.

The same procedure was repeated with different amines to give compounds **9b-i**, that in all cases were obtained with a small amount (between 5% to 15%) of compound **10**

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-chlorophenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9b**).
M.p.: 158-160°C. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{34}H_{47}ClN_2NaO_3$ 589.3167, found 589.3203.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-phenyl-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9c**)

Colourless oil. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{34}H_{48}N_2NaO_3$ 555.3557, found 555.3562.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-methylphenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9d**)
M.p.: 184°C. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{35}H_{50}N_2NaO_3$ 569.3714, found 569.3734.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-chlorophenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9e**)
M.p.: 89-91°C. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{34}H_{47}ClN_2NaO_3$ 589.3167, found 589.3180.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-fluorophenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9f**)
Colourless oil. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{34}H_{47}FN_2NaO_3$ 573.3463, found 573.3467.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-ethyl-6-methylphenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9g**)
M.p.: 110ºC HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{37}H_{54}N_2NaO_3$ 597.4027, found 597.4050.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-naphthyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9h**)
Colourless oil. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{38}H_{50}N_2NaO_3$ 605.3714, found 605.3679.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-fluorophenyl)-4,4-dimethyl-3-oxoandrost-5-ene-17-carboxamide* (**9i**)
Colourless oil. HRMS (ESI$^+$): calculated for [M+Na$^+$] $C_{34}H_{47}FN_2NaO_3$ 573.3463, found 573.3481.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-methoxyphenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5a**).
Compound **9a** (13 mg, 0.02 mmol) was dissolved in 2 ml of CH_2Cl_2 / MeOH (1:1) and $NaBH_4$ (2 eq.) were added. The resulting slurry was stirred at room temperature for 15

minutes, until completion of the reaction. The solvent were removed *in vacuo*, and the crude product was purified by column chromatography on silica (hexane / EtOAc gradient) to give compound **5a** with 95% yield**.** M.p.: 248°C. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{35}H_{52}N_2NaO_4$ 587.3819, found 587.3849.

The same procedure was used for the reduction of the 3-oxo moiety in compounds **9b-i** and **10**, to give the corresponding 3β-hydroxy analogs.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-chlorophenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5b**)
M.p.: 122-124°C. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{34}H_{49}ClN_2NaO_3$ 591.3324, found 591.3304.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-phenyl-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5c**)
Colourless oil. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{34}H_{50}N_2NaO_3$ 557.3714, found 557.3753.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(4-methylphenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5d**)
M.p.: 202-204ºC. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{35}H_{52}N_2NaO_3$ 571.3870, found 571.3882.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-chlorophenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5e**)
Colourless oil. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{34}H_{49}ClN_2NaO_3$ 591.3324, found 591.3270.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-fluorophenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5f**)
Colourless oil. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{34}H_{49}FN_2NaO_3$ 575.3619, found 575.3618.

(*17β*)-*N-((t-butylcarbamoyl)methyl)-N-(2-ethyl-6-methylphenyl-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide* (**5g**)
Colourless oil. HRMS (ESI^+): calculated for $[M+H^+]$ $C_{37}H_{57}N_2O_3$ 577.4364, found 577.4414.

(*17β*)-*N*-((t-butylcarbamoyl)methyl)-*N*-(2-naphthyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide (**5h**)

M.p.: 174ºC. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{38}H_{52}N_2NaO_3$ 607.3870, found 607.3851.

(*17β*)-*N*-((t-butylcarbamoyl)methyl)-*N*-(4-fluorophenyl)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxamide (**5i**)

Colourless oil. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{34}H_{49}FN_2NaO_3$ 575.3619, found 575.3675.

2-(t-butylamino)-2-oxoethyl-(17β)-4,4-dimethyl-3-hydroxyandrost-5-ene-17-carboxylate (**11**).

M.p.: 177ºC. HRMS (ESI^+): calculated for $[M+Na^+]$ $C_{28}H_{45}NNaO_4$ 482.3241, found 482.3312.

BIOLOGICAL STUDIES

Effect on Fusarium strains.

Fungal inocula.

Fusarium virguliforme (Centro de Referencia de Micología Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario Nº CCC220.05) and *Fusarium solani* (Instituto Spegazzini, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata Nº LPSC868) were cultured in 3% (w/v) malt extract-agar médium (Oxoid Ltd, Basingstoke Hants, England) in 9 cm Petri dishes at 25ºC and in darkness. In order to obtain the spores, fungi was cultured for 7-10 days. Harvesting was carried out by suspending spores in sterilized water (with oligoelements). Colony forming unit (CFU) values was determined by plating 10 µl of 10-times serially diluted suspensions and counting germination spores/dilution concentration.

Minimum inhibitory concentration by broth microdilution.

Test compound were dissolved in methanol to a concentration of 1µg / µl. Microdilution was performed in sterile disposable microtitre plates (96 U-bottomed wells). Every well was filled with 0,2 ml of 3% (w/v) sterile malt extract broth. Aliquots of each compound were dispensed in every well ranging from 0 to

100 µM. Compounds were tested by duplicate. Stock inoculum suspension were adjusted to 10^5 CFU / ml. Ten µl were dispensed into each well, except the first row. The first row of the plate contains only sterile malt extract broth as negative control of contamination. Microtitre plates were incubated 72 hs at 25ºC in darkness and humidity control. Germ-tube inhibition was determined by direct observation. The minimum inhibitory concentration (MIC) was determined as the lowest test compound concentration that completely inhibits spore germination [12]. The maximum concentration of ethanol used did not elicit an inhibitory effect.

Effect on Trypanosoma cruzi

Epimastigotes of *Trypanosoma cruzi* CL Brener strain were grown in a culture medium containing 33 g / L brain-heart infusion; 3 g / L tryptose; 3 g / L Na_2HPO_4; 0.4 g / L KCl; 0.3 g / L glucose; pH was about 7.5, without the need for adjustment. After sterilization (10 min at 121 °C) penicillin (100 IU/mL), streptomycin (100 mg/mL) haemin (20 mg/mL, added as 1 ml of a 2 mg/mL solution in 0.1 N NaOH per litre of medium) and 20% v/v heat-inactivated (45 min at 57 °C) fetal calf serum were added. Cultures were performed at 28 °C in 600 ml cylindrical flasks with screw caps, containing 100 ml of medium. Shaking was performed manually, twice a day. All cultures were started with inocula taken from exponential growth parasite in the same medium. The susceptibilities of the epimastigote form of *T. cruzi* to synthetic compounds were tested by culturing them in cell-free medium at 28°C with the inhibitors at a 100 µM final concentration. The inhibitory effect on *T. cruzi* growth was observed by reading parasites in a Neubauer chamber every two day for seven days [13]

Effect on mammalian cells

Cells cultures

Simian Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS) and 50 µg/ml gentamicin (MEM 5%) and maintained after monolayer formation in MEM 1.5%. The human cancer cell line A549 was grown in MEM 10% and maintained after monolayer formation in MEM 5%.

Treatment solutions

Compounds were dissolved in dimethylsulfoxide and diluted with MEM 1.5% or MEM 5% for testing. The maximum concentration of DMSO tested (1%) exhibited no toxicity under *in vitro* conditions.

Cytotoxicity assay

The compounds were evaluated for cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Vero or A549 cells were seeded in 96-well plates and grown at 37°C with 5% CO_2 until monolayer was formed. The culture medium was replaced by fresh medium containing the compounds at various concentrations and cells were further incubated for 24 h or 48 h. Then, monolayers were subjected to MTS-based colorimetric assay for determining cell viability, according to the manufacturer's instructions (CellTiter 96 AQueous One Solution Reagent; Promega, Leiden, The Netherlands). The absorbance at 490 nm was measured on a BioTek microplate reader. Results were expressed as a percentage of absorbance of treated cell cultures with respect to untreated ones. Sigmoidal dose–response curves were fitted and the CC_{50} values were calculated as the concentration of the compound that caused a 50% reduction in absorbance.

Antiproliferative assay

A549 cells were seeded at a density of 10^4 cells/well and solutions containing different concentration of the tested compound were added in 96-well plates. After a 48 h incubation period, viability was determined by the addition of 20 µL of MTS solution according to the manufacturer's instructions (CellTiter 96 AQueous One Solution Reagent; Promega, Leiden, The Netherlands). The absorbance at 490 nm was measured on a BioTek microplate reader. Results were expressed as a percentage of absorbance of treated cell cultures with respect to untreated ones. Sigmoidal dose–response curves were fitted and the IC_{50} values were calculated as the concentration of the compound that caused a 50% reduction in absorbance.

RESULTS AND DISCUSSION

The synthesis of the novel compounds is shown in Scheme 1. The key step implies the construction of the diamide side chain applying an Ugi condensation, one of the most versatile multicomponent reactions (MCR). The Ugi four-component reaction (U-4CR), which is based on the exceptional reactivity of the isocyanides functional group, occurs when a carbonyl compound, an amine, a carboxylic acid and an isocyanides react together to give an α-aminoacylamide. The structure of the reaction product can be easily diversified by a systematic variation of the starting materials.

The carboxylic component for the U-4CR was obtained as depicted in Scheme 1. The known intermediate **6** [10], which was obtained from progesterone in three steps, was treated with methyl iodide and potassium *t*-butoxide to give the C-4 dimethylated ketone **7**. Further hydrolysis of the methyl ester gave the desired 17β-carboxylic acid **8** with a total yield of 12% from progesterone.

Treatment of compound **8** with formaldehyde, an aromatic amine and *t*-butyl isocyanide gave the desired Ugi adducts. The U-4CR generally took place smoothly and in good yields, and worked well with a set of structurally diverse anilines (Table 1). The reaction was completely regioselective, providing that no reaction of the 3-keto moiety was observed, probably due to the much higher reactivity of formaldehyde as the carbonylic component. Nevertheless all reactions gave a small amount of compound **10**, a by-product coming from the Passerini condensation [14].

Following this procedure a set of nine compounds was obtained (**9a-i**), which were treated with sodium borohydryde to give the corresponding 3β-hydroxy compounds **5a-i**, the target sterol analogues. Finally, reduction of compound **10** gave the analogue **11** with almost quantitative yield.

The structures of intermediates **7**, **8** and some representative members of the library were fully characterized by NMR spectroscopy. On the other hand, granted that the structures of the compounds are closely related, and in order to obtain a complete and unambiguous characterization of the library, we decided to perform a direct analysis of the purified steroids via HRESI MS. In all cases, main peaks were observed for $[M+H]^+$ and $[M+Na]^+$. Figure 3 shows a representative example spectrum for compound **9c**.

The molecular formula was shown to be $C_{34}H_{48}N_2O_3$ on the basis of its HRMS ESI (m/z 533.3739 $[M+H]^+$ and 555.3562 $[M+Na]^+$). In all cases a product ion resulting from the loss of *t*-butylamine from the side chain was observed (structure A, in this example m/z 460.2835). The structure of the side chain was revealed by ESI MS/MS. For example, in the case of **9c**, the ESI MS/MS of the A ion, used as a precursor, yielded the products ions m/z 327.2297, 106.0639 and 299.2335, which can be assigned to structures B, B´ and C respectively. Fragments B and C are common to all the compounds analyzed, whereas ions A and B´ are characteristic for each particular structure because their contain the moiety coming from each different amine. In addition, ions showing water loss were observed for the 3β-hydroxy analogs **5a-i** (Supplementary Material). The analysis described above was applied to all of the new compounds and allowed the determination of their structure unambiguously.

Compounds both having 3-keto and 3β-hydroxy moieties were tested *in vitro* for their inhibitory properties towards *Fusarium virguliforme*, the causal agent of sudden death syndrome in soy bean, and *Fusarium solani*, that produces root rots on various crops, but is also involved in human mycoses [15]. Most of the new compounds showed a measurable antifungal activity against either of the two fungi. The results, expressed as their minimum inhibitory concentration (MIC) are shown in Table 2. Values for

compounds **3** and **4**, non-methylated at C4 and previously reported, were included for comparison.

Firstly, methylation at C-4 seems to conserve, or even reduce, the antifungal activity (compare compounds **5b** and **5c** vs. their non-methylated counterparts **4** and **3** respectively). Nevertheless, some compounds show a higher activity than that reported previously by us [9].

In sight of these findings, we decided to evaluate the effect of the new analogs on the viability of mammalian cells, providing that any compound with a potential use as an antifungal agent should exert a selective toxicity. Thus, the *in vitro* cytotoxic concentration 50% (CC_{50}) was determined on Vero cells; the results are shown in Table 2. It can be seen that several of the new compounds show a selective toxicity towards fungi, with no effect on Vero cells at the maximum concentration tested.

Moreover, two of the compounds (**9h** and **5h**) having a naphthyl subtituent in the side chain, resulted toxic only against *F. solani* and not against *F. virguliforme* or Vero cells, suggesting some selectivity on their action.

On the other hand, compound **10**, the by-product from the Passerini reaction, showed a noteworthy toxic effect on Vero cells, about five-fold higher than the rest of the analogs coming from the Ugi condensations, suggesting that it might have cytotoxic properties. Thus, as a preliminary study, we tested its action on the A549 cells, an adenocarcinomic human epithelial cell line. Even though compound **10** seems not to be quite toxic on this line (CC_{50} about 45 μM), its inhibitory concentration 50% is only 1.6 μM when growing cells are treated.

The main difference between compound **10** and the other compounds is that the side chain is linked by a labile ester bond to the steroidal nucleus rather than a more stable amide bond. To rule out the possibility that the ester bond was cleavaged during the assay, we measured the cytotoxycity of the acid **7** and the methyl ester **8**, but none of them showed effect at concentrations lower than 200 μM. This might indicate that compound **10** is toxic itself.

Finally, taking into account the different behaviour of these analogs on fungal and mammalian cells, we tested the most active compounds against *Trypanosoma cruzi*, the ethiological agent of Chagas´ disease [16]. Unfortunately, none of these analogs exerted a significant inhibitory effect (at a 100 μM dose) on the epimastigote form of the parasite.

In conclusion, in this paper we have confirmed that the application of multicomponent reactions in the synthetic chemistry of steroids is a powerful tool that allows a rapid and efficient generation of collections of diverse compounds. On the other hand, we have shown that the 4,4-dimethyl substitution, not fully explored in previous research, may render novel synthetic analogs with a variety of biological effects, such as antifungal and cytotoxic activities. Encouraged by these findings, we are currently performing further studies in order to develop both structure-activity relationships and to gain insight into the mechanism of action of the new compounds in relevant biological systems.

ACKNOWLEDGEMENTS

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Table 1. Yields of Ugi reactions.

Table 2. Biological activity of the new 4,4-dimethyl sterol analogues.

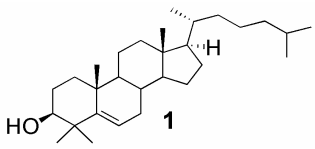
Figure 1. Known non natural 4,4-dimethylsterols with biological activity.

Figure 2. Previously synthesised sterol analogs and general structure of new 4,4-dimethyl derivatives.

Figure 3. Representative example for the structural determination by HRMS.

Scheme 1. Synthesis of new 4,4-dimethyl sterol analogues. Reactives and conditions: **a.** *t*-BuOK / CH_3I / THF / 0°C, 1 hour. **b.** KOH / water / ethylene

glycol / reflux, 20 minutes. **c.** R-NH_2 / HCHO / *t*-BuNC / MeOH / r.t., 3 days. d. $NaBH_4$ / CH_2Cl_2 / MeOH / r.t., 15 minutes.



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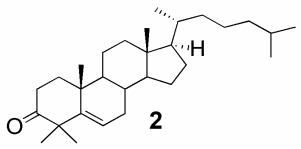

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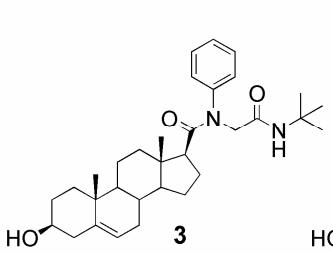

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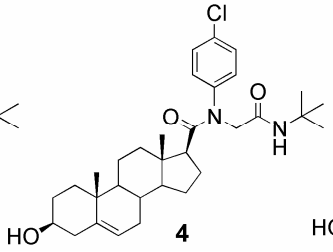

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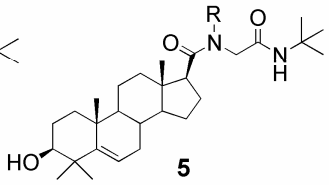

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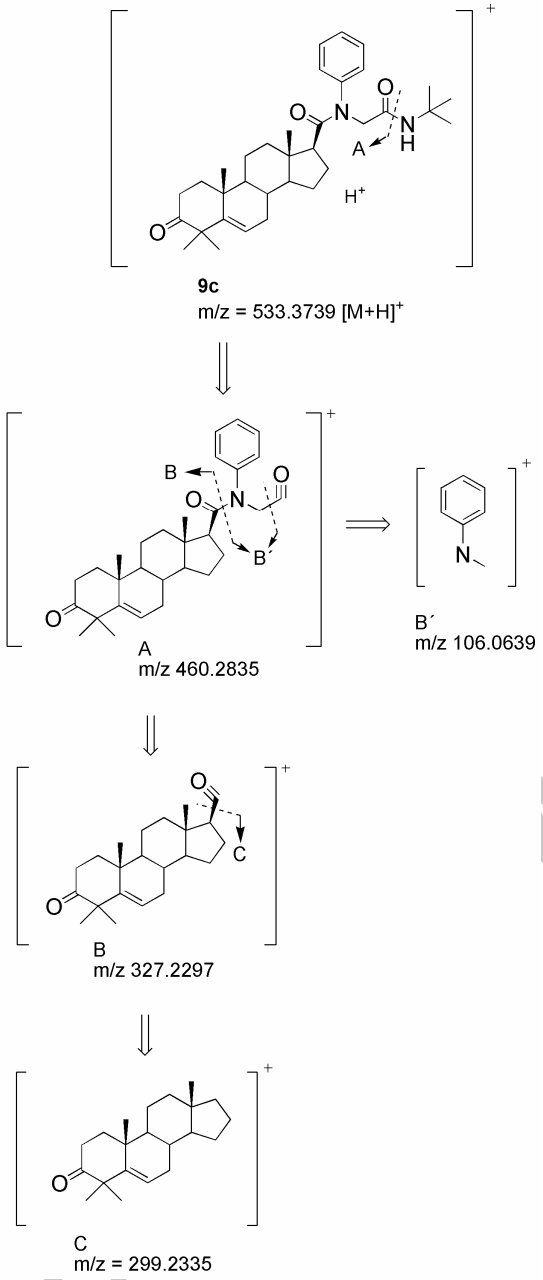

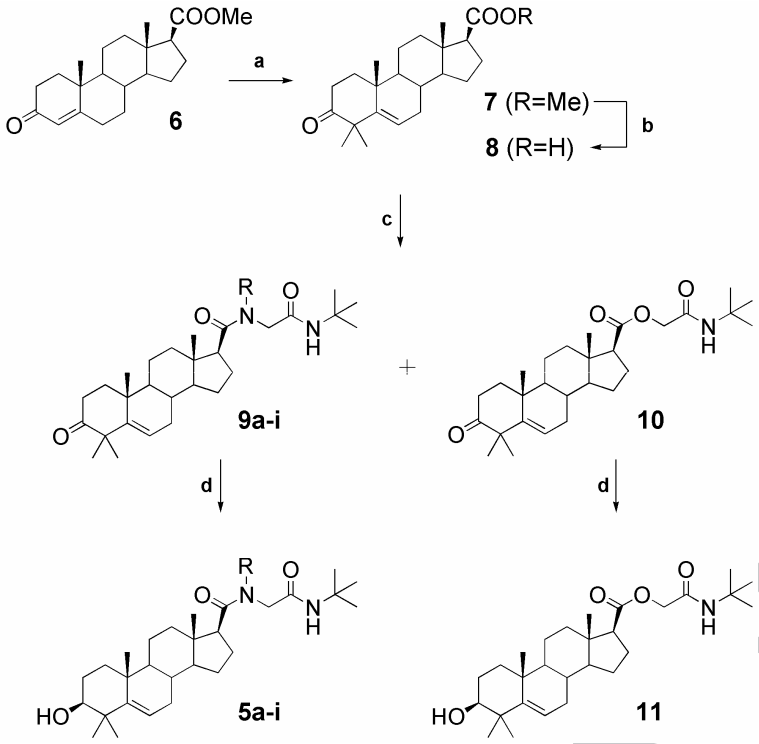

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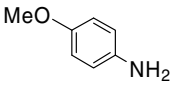
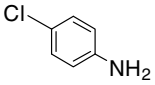
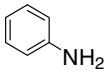
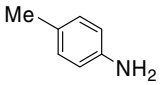
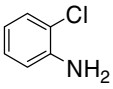
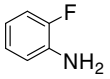
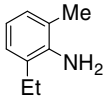
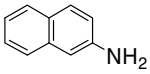
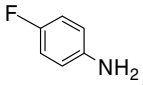
9a		63
9b		71
9c		84
9d		87
9e		61
9f		69
9g		55
9h		89
9i		83

a. Isolated yields.

	F. solani	*F. virguliforme*	*Vero cells*
	MIC[a]	*MIC*[a]	CC_{50}[b]
9a	27	>100	>200
9b	35	44	>200
9c	96	48	>200
9d	37	92	>200
9e	88	88	59
9f	46	93	65
9g	52	>100	61
9h	26	>100	>200
9i	18	>100	85
5a	18	>100	>200
5b	35	88	>200
5c	>100	96	92
5d	46	92	>200
5e	35	44	88
5f	>100	>100	65
5g	>100	>100	87
5h	17	>100	>200
5i	36	91	91
10	55	55	14
11	110	55	>200
3	46	28	>200
4	50	50	>200

a Micromolar concentration. Mean results from duplicates. The MIC of the control (Benomyl, a commercial antifungal) is 9 μM for both species.

b. Micromolar concentration. Mean results from duplicates.

HIGHLIGHTS

Analogues of 4,4-dimethyl sterols with a diamide side chain were synthetized.

The synthesis involved a multicomponent Ugi reaction.

Some compounds showed a selective activity against the fungal plant pathogen *Fusarium*.

A by-product of the synthesis resulted cytotoxic against mammalian cells.

Graphical abstract