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Synthesis and preliminary biological screening of sterol analogues as new antifungal agents against plant pathogens

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ABSTRACT

In this paper we report the synthesis of a new family of sterol analogues that have two amidic bonds on the side chain. These azasterols were obtained by a straightforward procedure including an Ugi condensation that allows the facile attachment of a polyfunctionalized side chain into the steroidal framework.

Some of the new compounds showed an interesting inhibitory effect on the growth of two pathogenic fungi involved in plant diseases.

1. Introduction

Plants are constantly exposed to a variety of pathogenic microorganisms present in their environments. Diseases caused by pathogens, including bacteria, fungi and viruses, significantly contribute to the overall loss in crop yield worldwide and constitute an emerging threat to the global food security [1]. Many of the currently available antimicrobial agents for agriculture are highly toxic and non-biodegradable and cause extended environmental pollution [2].

Several sterol analogues are known to behave as antiproliferative agents against fungi, yeast and protozoa [3,4]. An important class of antifungal steroids are azasteroids in which one or more nitrogen atoms are present in the side chain [5]. The natural alkaloid verazine **1** and the synthetic piperinidyl derivative **2** (Fig. 1) are two compounds belonging to this class.

Taking these previous results into account, our group started a research programme devoted to the synthesis of new sterol analogues with activity against plant pathogens.

We aimed at choosing a synthetic strategy based on a simple and fast generation of new compounds having a high structural diversity. Multicomponent reactions (MCRs), which are generally defined as reactions where more than two starting materials react to form a product by incorporating essentially all the atoms of the adducts, are best suited to achieve this goal. The structure of the reaction product can be easily diversified by a systematic variation of the starting materials [6].

One of the most versatile MCRs is the Ugi four-component reaction (U-4CR), which is based on the exceptional reactivity of the isocyanide functional group [7]. The U-4CR constitutes a homogeneous group of reactions in which an amino component, an acid, a carbonyl compound and an isocyanide react together to give an α-aminoacylamide [6,7]. We have recently shown that this approach can be successfully applied to the synthesis of heterocyclic steroids [8,9].

2. Experimental

2.1. Synthesis

2.1.1. 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 NMR and mass spectral/combustion analysis data. Assignments of the NMR signals of the side chain correspond to the numbering shown in Figs. 2 and 3.

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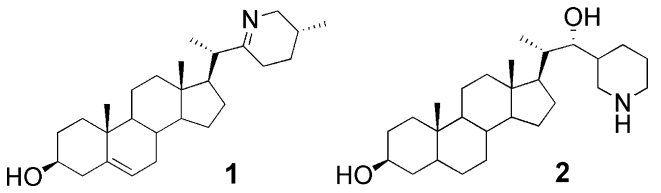


Fig. 1. Antifungal azasteroids.

2.1.2. General synthetic procedure

The steroidal acid **3** (3β-hydroxyandrost-5-en-17β-carboxylic acid, 50 mg, 0.16 mmol [10]) was suspended in 1 mL of methanol and 1.1 equivalent of the corresponding amine and 15 μL of formaldehyde (37% aq.) were added. The mixture was stirred for 15 min at room temperature and then 1.1 equivalent of the isonitrile was added. The reaction was kept under the same condition until total disappearance of the acid (usually 48 h). The solvent

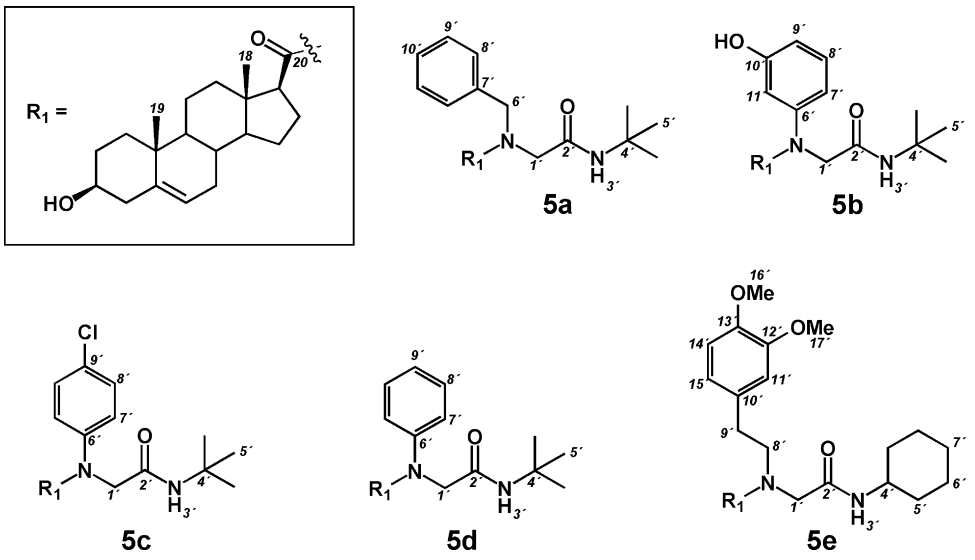


Fig. 2. Structures of the synthesized 21-azasteroids.

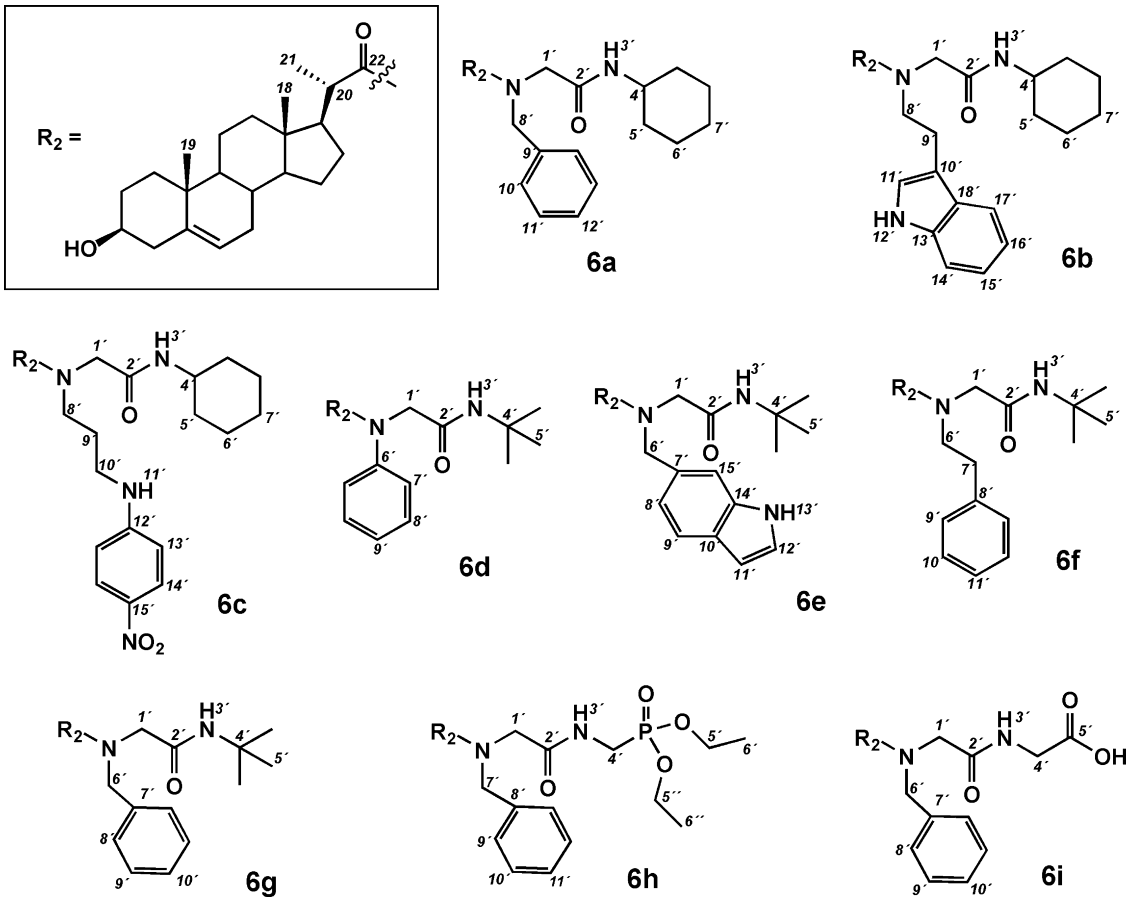


Fig. 3. Structures of the synthesized 23-azasteroids.

was evaporated under reduced pressure and the residue was taken in EtOAc and washed with NaOH (5% aq.). The compounds were purified by silica gel column chromatography (hexane/EtOAc gradient).

When starting from acetate **4** (3β-acetoxy-23,24-dinorchol-5-en-20-carboxylic acid [11]) the same procedure was followed, but after 48 h of reaction 0.2 mL of K_2CO_3 (satd.) was added to the reaction. The mixture was stirred for an additional period of 24 h and worked up as usual.

2.1.3. N-((tert-butylcarbamoyl)methyl)-N-benzyl-3β-hydroxyandrost-5-en-17β-carboxamide
(***5a***)

Colourless oil. ^{1}H NMR ($CDCl_3$): 0.82 (H18, 3H, *s*); 0.97 (H19, 3H, *s*); 1.36 (H5′, 9H, *s*); 2.51 (H17, 1H, *t*, 9.5); 3.45 (H3, 1H, *m*); 3.95 and 4.21 (H1′, 1H each signal, *d*, 14.0); 4.65 and 4.72 (H6′, 2H, *d*, 15.9); 5.28 (H6, 1H, *d*, 5.0); 6.55 (H3′, 1H, *bs*); 7.21 (H8′, 2H, *dd*, 7.1 and 1.0); 7.35 (H9′, 2H, *m*); 7.38 (H10′, 1H, *m*). ^{13}C NMR ($CDCl_3$): 13.5 (C18); 19.1 (C19); 20.6 (C11); 24.5 (C16); 25.8 (C15); 28.5 (C5′); 31.5 (C7); 31.6 (C2); 31.6 (C8); 36.3 (C10); 37.1 (C1); 38.2 (C12); 41.6 (C4); 45.3 (C13); 49.6 (C9); 51.2 (C4′); 51.7 (C17); 52.8 (C6′); 55.3 (C1′); 56.0 (C14); 71.2 (C3); 121.1(C6); 127.1 (C10′); 128.0 (C8′); 128.6 (C9′); 136.5 (C7′); 140.7 (C5); 168.8 (C2′); 175.2 (C20). HRMS (ESI): calculated for [M+H$^+$] $C_{33}H_{49}N_2O_3$ 521.3743, found 521.3754. 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.

2.1.4. N-((tert-butylcarbamoyl)methyl)-N-(3-hydroxyphenyl)-3β-hydroxyandrost-5-en-17β-carboxamide
(***5b***)

Mp: 149 °C. ^{1}H NMR ($CDCl_3$): 0.82 (H18, 3H, *s*); 0.96 (H19, 3H, *s*); 1.37 (H5′, 9H, *s*); 2.56 (H17, 1H, *t*, 9.0); 3.49 (H3, 1H, *m*); 4.03 and 4.36 (H1′, 1H each signal, *d*, 14.1); 5.28 (H6, 1H, *d*, 5.0); 6.06 (H3′, 1H, *bs*); 6.54 (H11′, 1H, *bs*); 6.68 (H9′, 1H, *d*, 7.7); 6.96 (H7′, 1H, *bs*); 7.09 (H8′, 1H, *t*, 8.7); 8.6 (H12′, 1H, *bs*). ^{13}C NMR ($CDCl_3$): 13.9 (C18); 19.4 (C19); 20.9 (C11); 24.7 (C16); 26.1 (C15); 28.7 (C5′); 31.5 (C2); 31.8 (C7); 31.8 (C8); 36.5 (C10); 37.2 (C1); 38.3 (C12); 42.1 (C4); 45.5 (C13); 49.7 (C9); 51.4 (C4′); 51.8 (C17); 55.8 (C1′); 56.1 (C14); 71.7 (C3); 115.3 (C9′); 116.3 (C11′); 118.5 (C7′); 121.4 (C6); 130.1(C8′); 140.7 (C5); 144.0 (C6′); 158.0 (C10′); 169.9 (C2′); 175.5 (C20). HRMS (ESI): calculated for [M+H$^+$] $C_{32}H_{47}N_2O_4$ 523.3536, found 523.3518. Anal. calculated for $C_{32}H_{46}N_2O_4$ C, 73.53; H, 8.87; N, 5.36; found C, 73.30; H, 8.75; N, 5.31.

2.1.5. N-((tert-butylcarbamoyl)methyl)-N-(4-chlorophenyl)-3β-hydroxyandrost-5-en-17β-carboxamide
(***5c***)

Mp: 116 °C. ^{1}H NMR ($CDCl_3$): 0.81 (H18, 3H, *s*); 0.98 (H19, 3H, *s*); 1.36 (H5′, 9H, *s*); 2.47 (H17, 1H, *t*, 9.0); 3.48 (H3, 1H, *m*); 3.84 and 4.34 (H1′, 1H each signal, *d*, 14.4); 5.29 (H6, 1H, *d*, 5.0); 6.27 (H3′, 1H, *bs*); 7.18 (H7′, 2H, *bs*); 7.36 (H8′, 2H, *d*, 8.7). ^{13}C NMR ($CDCl_3$): 13.7 (C18); 19.4 (C19); 20.8 (C11); 24.7(C16); 26.0 (C15); 28.7 (C5′); 31.6 (C2); 31.8 (C7); 31.8 (C8); 36.5 (C10); 37.2 (C1); 38.4 (C12); 42.2 (C4); 45.6 (C13); 49.8 (C9); 51.2 (C4′); 51.9 (C17); 55.8 (C1′); 56.2 (C14); 71.7 (C3); 121.3 (C6); 129.4 (C7′); 129.8 (C8′); 133.7 (C9′); 140.8 (C5); 142.2 (C6′); 168.3 (C2′); 174.7(C20). HRMS (ESI): calculated for [M+H$^+$] $C_{32}H_{46}ClN_2O_3$ 541.3197, found 541.3181. Anal. calculated for $C_{32}H_{45}ClN_2O_3$ C, 71.02; H, 8.38; N, 5.18; found C, 71.21; H, 8.41; N, 5.21.

2.1.6. N-((tert-butylcarbamoyl)methyl)-N-phenyl-3β-hydroxyandrost-5-en-17β-carboxamide
(***5d***)

Mp: 110 °C. ^{1}H NMR ($CDCl_3$): 0.82 (H18, 3H, *s*); 0.97 (H19, 3H, *s*); 1.36 (H5′, 9H, *s*); 2.51 (H17, 1H, *t*, 9.0); 3.45 (H3, 1H, *m*); 3.92 and 4.37 (H1′, 1H each signal, *d*, 14.4); 5.28 (H6, 1H, *d*, 5.0); 6.65 (H3′, 1H, *bs*); 7.28 (H9′, 1H, *t*, 7.9); 7.36 (H7′, 2H, *bs*); 7.52 (H8′, 2H, *t*, 7.5). ^{13}C NMR ($CDCl_3$): 13.5 (C18); 19.1 (C19); 20.6 (C11); 24.5 (C16); 25.8 (C15); 28.4 (C5′); 31.5 (C7); 31.6 (C2); 31.6 (C8); 36.3 (C10); 37.0 (C1); 38.2 (C12); 41.6 (C4); 45.3 (C13); 49.6 (C9); 51.1 (C4′); 51.7 (C17); 55.3 (C1′); 56.0 (C14); 71.1 (C3); 121.0 (C6); 127.7 (C7′); 127.9 (C9′); 129.4 (C8′); 140.7 (C5); 143.2 (C6′); 168.6 (C2′); 175.2 (C20). HRMS (ESI): calculated for [M+H$^+$] $C_{32}H_{47}N_2O_3$ 507.3587, found 507.3580. Anal. calculated for $C_{32}H_{46}N_2O_3$ C, 75.85; H, 9.15; N, 5.53; found C, 75.73; H, 9.21; N, 5.42.

2.1.7. N-((cyclohexylcarbamoyl)methyl)-N-(3,4-dimethoxyphenethyl)-3β-hydroxyandrost-5-en-17β-carboxamide
(***5e***)

Mp: 107 °C. ^{1}H NMR ($CDCl_3$): 0.73 (H18, 3H, *s*); 1.01 (H19, 3H, *s*); 2.48 (H17, 1H, *t*, 9.1); 2.79 (H9′, 2H, *m*); 3.52 (H3, 1H, *m*); 3.45 and 3.91 (H8′, 1H each signal, *m*); 3.61 and 4.42 (H1′, 1H each signal, *d*, 15.0); 3.86 and 3.87 (–OMe, 3H each signal, *s*); 5.35 (H6, 1H, *d*, 5.0); 6.59 (H3′, 1H, *d*, 8.0); 6.65 (H11′, 1H, *d*, 2.1); 6.68 (H15′, 1H, *dd*, 8.0 and 2.1); 6.81 (H14′, 1H, *d*, 8.0). ^{13}C NMR ($CDCl_3$): 13.9 (C18); 19.4 (C19); 21.0 (C11); 24.6 (C16); 24.7 (C6′); 25.4 (C7′); 25.9 (C15); 31.5 (C2); 31.8 (C7); 31.9 (C8); 32.9 (C5′); 34.7 (C9′); 36.5 (C10); 37.2 (C1); 38.8 (C12); 42.2 (C4); 45.4 (C13); 48.1 (C4′); 50.0 (C9); 51.1 (C17); 51.1 (C8′); 51.6 (C1′); 55.9 (C14); 55.9 (–OMe); 71.6 (C3); 111.5 (C14′); 112.0 (C11′); 120.8 (C15′); 121.3 (C6); 130.4 (C10′); 140.8 (C5); 147.9 (C13′); 149.1 (C12′); 168.9 (C2′); 174.8 (C20). HRMS (ESI): calculated for [M+H$^+$] $C_{38}H_{57}N_2O_5$ 621.4267, found 621.4240. Anal. calculated for $C_{38}H_{56}N_2O_5$ C, 73.51; H, 9.09; N, 4.51; found C, 73.47; H, 8.99; N, 4.67.

2.1.8. N-((cyclohexylcarbamoyl)methyl)-N-benzyl-3β-hydroxypregn-5-ene-20-carboxamide
(***6a***)

Mp: 114 °C. ^{1}H NMR ($CDCl_3$): 0.62 (H18, 3H, *s*); 0.99 (H19, 3H, *s*); 1.11 (H21, 3H, *d*, 6.4); 2.77 (H20, 1H, *m*); 3.50 (H3, 1H, *m*); 3.70 (H4′, 1H, *m*); 3.88 and 4.03 (H1′, 1H each signal, *d*, 14.5); 4.67 and 4.75 (H8′, 1H each signal, *d*, 16.0); 5.34 (H6, 1H, *m*); 6.60 (H3′, 1H, *d*, 7.5); 7.20 (H10′, 2H, *dd*, 7.4 and 1.2); 7.31 (H11′, 2H, *m*); 7.38 (H12′, 1H, *t*, 7.7). ^{13}C NMR ($CDCl_3$): 12.3 (C18); 17.5 (C21); 19.3 (C19); 21.0 (C11); 24.6 (C16); 24.7 (C6′); 25.4 (C15); 25.4 (C7′); 31.3 (C7); 31.7 (C2); 31.8 (C8); 32.9 (C5′); 36.4 (C10); 37.2 (C1); 37.7 (C12); 38.0 (C20); 40.9 (C13); 42.0 (C4); 48.0 (C4′); 49.4 (C9); 51.2 (C1′); 52.8 (C8′); 53.0 (C17); 56.1 (C14); 71.4(C3); 121.4 (C6); 126.7 (C10′); 127.9 (C11′); 128.9 (C12′); 136.2 (C9′); 140.8 (C5); 168.3 (C2′); 178.6 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{37}H_{55}N_2O_3$ 575.4213, found 575.4224. Anal. calculated for $C_{37}H_{54}N_2O_3$ C, 77.31; H, 9.47; N, 4.87; found C, 77.25; H, 9.62; N, 4.99.

2.1.9. N-((cyclohexylcarbamoyl)methyl)-N-(2-(1H-indol-3-yl)ethyl)-3β-hydroxypregn-5-ene-20-carboxamide
(***6b***)

Mp: 195 °C. ^{1}H NMR (CDCl3): 0.44 (H18, 3H, *s*); 0.91 (H21, 3H, *d*, 6.3); 0.94 (H19, 3H, *s*); 2.53 (H20, 1H, *m*); 3.07 (H9′, 2H, *t*, 7.0); 3.42 (H3, 1H, *m*); 3.62 (H4′, 1H, *m*); 3.71 and 3.81 (H8′, 2H, *m*); 3.86 and 4.07 (H1′, 1H each signal, *d*, 15.3); 5.27 (H6, 1H, *m*); 6.78 (H3′, 1H, *d*, 7.4); 7.01 (H11′, 1H, *s*); 7.11 (H15′, 1H, *dt*, 7.2 and 1.1); 7.18 (H16′, 1H, *dt*, 7.2 and 1.1); 7.39 (H14′, 1H, *bd*, 8.2); 7.57 (H17′, 1H, *bd*, 7.8). ^{13}C NMR ($CDCl_3$): 11.8 (C18); 17.0 (C21); 19.0 (C19); 20.8 (C11); 24.5 (C9′); 24.6 (C16); 24.7 (C6′); 25.4 (C15); 25.4 (C7′); 31.6 (C2); 31.6 (C7); 31.8 (C8); 32.9 (C5′); 36.3 (C10); 37.0 (C1); 37.5 (C12); 37.5 (C20); 40.9 (C13); 42.0 (C4); 48.0 (C4′); 49.4 (C9); 50.2 (C8′); 52.8 (C17); 52.8 (C1′); 56.0 (C14); 71.2 (C3); 110.6 (C10′); 111.3 (C14′); 117.7 (C17′); 119.2 (C15′); 121.3 (C6); 121.9 (C16′); 122.7 (C11′); 126.9 (C18′); 136.5 (C13′); 140.7 (C5); 169.1 (C2′); 178.6 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{40}H_{58}N_3O_3$ 628.4478,

found 628.4473. Anal. calculated for $C_{40}H_{57}N_3O_3$ C, 76.51; H, 9.15; N, 6.69; found C, 76.44; H, 9.27; N, 6.42.

2.1.10. N-((cyclohexylcarbamoyl)methyl)-N-(3-(4-nitrophenylamino)propyl)-3β-hydroxypregn-5-ene-20-carboxamide
(***6c***)

Mp: 124 °C. ^{1}H NMR ($CDCl_3$): 0.65 (H18, 3H, *s*); 1.00 (H19, 3H, *s*); 1.12 (H21, 3H, *d*, 6.4); 1.80 and 2.02 (H9′, 2H, *m*); 2.67 (H20, 1H, *m*); 3.27 (H10′, 2H, *m*); 3.47 and 3.55 (H8′, 2H, *m*); 3.53 (H3, 1H, *m*); 3.71 (H4′, 1H, *m*); 3.86 and 4.02 (H1′, 1H each signal, *d*, 14.1); 5.34 (H6, 1H, *m*); 5.47 (H11′, 1H, *bt*); 6.55 (H13′, 2H, *dd*, 8.9 and 1.9); 8.05 and 8.08 (H14′, 2H, *m*). ^{13}C NMR ($CDCl_3$): 11.8 (C18); 17.0 (C21); 19.0 (C19); 20.8 (C11); 24.6 (C16); 24.7 (C6′); 25.3 (C7′); 25.4 (C15); 28.4 (C9′); 31.6 (C2); 31.6 (C7); 31.8 (C8); 32.8 (C5′); 36.3 (C10); 37.0 (C1); 37.5 (C12); 37.9 (C20); 40.6 (C10′); 40.9 (C13); 42.0 (C4); 44.3 (C8′); 48.1 (C4′); 49.4 (C9); 52.6 (C1′); 52.8 (C17); 56.0 (C14); 71.7 (C3); 111.0 (C13′); 121.4 (C6); 126.5 (C14′); 137.5 (C15′); 140.7 (C5); 153.4 (C12′); 169.2 (C2′); 178.1 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{39}H_{59}N_4O_5$ 663.4485, found 663.4489. Anal. calculated for $C_{39}H_{58}N_4O_5$ C, 70.66; H, 8.82; N, 8.45; found C, 70.52; H, 8.95; N, 8.33.

2.1.11. N-((tertbutylcarbamoyl)methyl)-N-phenyl-3β-hydroxypregn-5-ene-20-carboxamide
(***6d***)

Colourless oil. ^{1}H NMR ($CDCl_3$): 0.34 (H18, 3H, *s*); 0.96 (H19, 3H, *s*); 1.09 (H21, 3H, *d*, 6.3); 2.44 (H20, 3H, *m*); 3.51 H3, 1H, *m*); 3.99 and 4.29 (H1′, 1H each signal, *d*, 14.5); 5.32 (H6, 1H, *m*); 6.49 (H3′, 1H, *bs*); 7.21 (H9′, 1H, *t*, 7.7); 7.39 (H7′, 2H, *m*); 7.42 (H8′, 2H, *m*). ^{13}C NMR ($CDCl_3$): 11.8 (C18); 17.4 (C21); 19.3 (C19); 21.0 (C11); 24.6 (C16); 25.4 (C15); 28.6 (C5′); 31.3 (C7); 31.7 (C2); 31.8 (C8); 36.3 (C10); 37.1 (C1); 37.7 (C12); 38.3 (C20); 39.4 (C13); 42.1 (C4); 49.9 (C9); 51.0 (C4′); 53.0 (C17); 53.0 (C1′), 55.9 (C14); 71.6 (C3); 121.4 (C6); 127.6 (C9′), 128.2 (C7′); 129.8 (C8′); 140.6 (C5); 142.9 (C6′); 168.2 (C2′); 177.6 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{34}H_{51}N_2O_3$ 535.3900, found 535.3911. Anal. calculated for $C_{34}H_{50}N_2O_3$ C, 76.36; H, 9.42; N, 5.24; found C, 76.25; H, 9.59; N, 5.12.

2.1.12. N-((tertbutylcarbamoyl)methyl)-N-((1H-indol-6-yl)methyl)-3β-hydroxypregn-5-ene-20-carboxamide
(***6e***)

Mp: 126 °C. ^{1}H NMR ($CDCl_3$): 0.66 (H18, 3H, *s*); 0.95 (H19, 3H, *s*); 1.17 (H21, 3H, *d*, 6.4); 1.29 (H5′, 9H, *s*); 2.94 (H20, 1H, *m*); 3.53 (H3, 1H, *m*); 3.94 and 4.17 (H1′, 1H each signal, *d*, 14.1); 4.47 and 4.93 (H6′, 1H each signal, *d*, 16.8); 5.34 (H6, 1H, *m*); 6.34 (H3′, 1H, *bs*); 6.51 (H11′, 1H, *m*); 6.51 (H12′, 1H, *m*); 7.01 (H8′, 1H, *dd*, 8.5 and 1.5); 7.37 (H9′, 1H, *d*, 8.0); 7.58 (H15′, 1H, *bs*). ^{13}C NMR ($CDCl_3$): 12.3 (C18); 17.7 (C21); 19.3 (C19); 21.0 (C11); 24.3 (C16); 28.3 (C15); 28.7 (C5′); 31.6 (C7); 31.7 (C2); 31.8 (C8); 36.5 (C10); 37.2 (C1); 37.7 (C12); 38.5 (C20); 41.8 (C13); 42.2 (C4); 50.0 (C9); 51.1 (C4′); 52.0 (C1′); 53.0 (C17); 53.1 (C6′); 56.1 (C14); 71.7 (C3); 101.4 (C11′); 111.5 (C15′); 119.3 (C8′); 121.5 (C6); 121.5 (C9′); 122.9 (C12′); 128.9 (C10′); 129.0 (C7′); 135.5 (C14′); 140.7 (C5); 168.6 (C2′); 178.1 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{37}H_{54}N_3O_3$ 588.4165, found 588.4203. Anal. calculated for $C_{37}H_{53}N_3O_3$ C, 75.60; H, 9.09; N, 7.15; found C, 75.48; H, 9.13; N, 7.25.

2.1.13. N-((tertbutylcarbamoyl)methyl)-N-phenethyl-3β-hydroxypregn-5-ene-20-carboxamide
(***6f***)

Colourless oil. ^{1}H NMR ($CDCl_3$): 0.70 (H18, 3H, *s*); 1.02 (H19, 3H, *s*); 1.06 (H21, 3H, *d*, 6.4); 1.31 (H5′, 9H, *s*); 2.64 (H20, 1H, *m*); 2.90 (H7′, 2H, *m*); 3.52 (H3, 1H, *m*); 3.67 (H6′, 2H, *m*); 3.72 and 4.03 (H1′, 1H each signal, *d*, 15.2); 5.34 (H6, 1H, *m*); 6.51 (H3′, 1H, *bs*); 7.21 (H11′, 1H, *t*, 7.7); 7.39 (H9′, 2H, *m*); 7.42 (H10′, 2H, *m*). ^{13}C NMR ($CDCl_3$): 12.4 (C18); 17.5 (C21); 19.3 (C19); 21.0 (C11); 24.6 (C16); 25.4 (C15); 28.6 (C5′); 31.6 (C7); 31.7 (C2); 31.8 (C8); 36.3 (C10); 37.2 (C1); 37.6 (C20); 37.7 (C12); 39.5 (C13); 42.2 (C4); 42.2 (C7′); 49.9 (C9); 51.3 (C4′); 52.7 (C6′); 52.9 (C1′); 53.0 (C17); 56.1 (C14); 71.7 (C3); 121.5 (C6); 126.8 (C11′); 128.6 (C10′); 128.8 (C9′); 137.7 (C8′); 140.7 (C5); 168.9 (C2′); 177.7 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{36}H_{55}N_2O_3$ 563.4213, found 563.4233. Anal. calculated for $C_{36}H_{54}N_2O_3$ C, 76.82; H, 9.67; N, 4.98; found C, 76.70; H, 9.51; N, 4.90.

2.1.14. N-((tertbutylcarbamoyl)methyl)-N-benzyl-3β-hydroxypregn-5-ene-20-carboxamide
(***6g***)

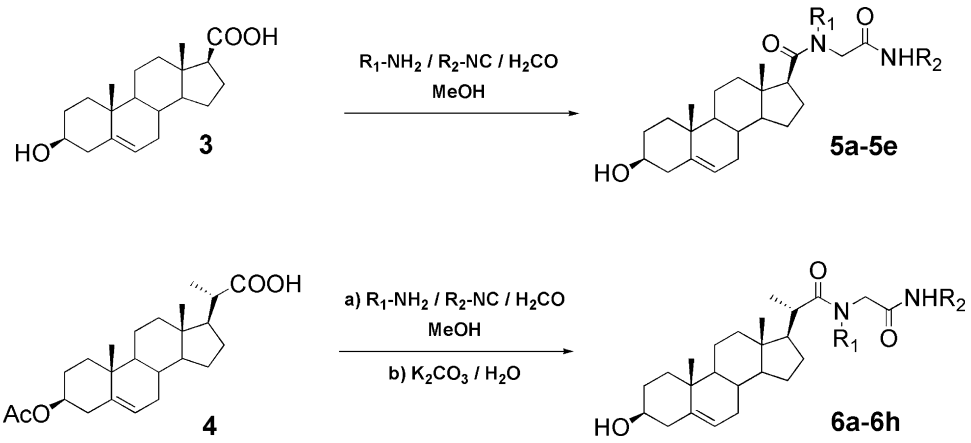
Mp: 97 °C. ^{1}H NMR ($CDCl_3$): 0.63 (H18, 3H, *s*); 1.00 (H19, 3H, *s*); 1.13 (H21, 3H, *d*, 6.4); 1.31 (H5′, 9H, *s*); 2.82 (H20, 1H, *m*); 3.52 (H3, 1H, *m*); 3.78 and 4.02 (H1′, 1H each signal, *d*, 14.1); 4.64 and 4.74 (H6′, 1H each signal, *d*, 16.5); 5.34 (H6, 1H, *m*); 6.36 (H3′, 1H, *bs*); 7.25 (H10′, 1H, *t*, 7.7); 7.32 (H8′, 2H, *m*); 7.42 (H9′, 2H, *m*). ^{13}C NMR ($CDCl_3$): 12.3 (C18); 17.6 (C21); 19.4 (C19); 21.0 (C11); 24.3 (C16); 28.3 (C15); 28.4 (C5′); 31.6 (C7); 31.8 (C2); 31.9 (C8); 36.5 (C10); 37.2 (C1); 37.2 (C11′); 37.7 (C12); 38.5 (C20); 41.8 (C13); 42.3 (C4); 50.0 (C9); 51.4 (C4′); 52.4 (C1′); 52.8 (C6′); 53.0 (C17); 56.1 (C14); 71.7 (C3); 121.5 (C6); 126.9 (C8′); 127.9 (C10′); 128.9 (C9′); 136.4 (C7′); 140.7 (C5); 168.4 (C2′); 178.2 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{35}H_{53}N_2O_3$ 549.4056, found 549.4070. Anal. calculated for $C_{35}H_{52}N_2O_3$ C, 76.60; H, 9.55; N, 5.10; found C, 76.51; H, 9.40; N, 5.32.

2.1.15. Diethyl {[N-benzyl-N-[(3β-hydroxypregn-5-ene-20-yl)-carbonyl]-glycyl]-amino}-methylphosphonate
(***6h***)

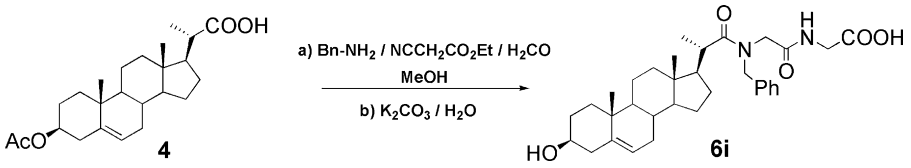
Colourless oil. ^{1}H NMR ($CDCl_3$): 0.64 (H18, 3H, *s*); 1.01 (H19, 3H, *s*); 1.18 (H21, 3H, *d*, 6.5); 1.35 (H6′, 6H, *m*); 2.79 (H20, 1H, *m*); 3.54 (H3, 1H, *m*); 3.71 (H4′, 2H, *m*); 4.04 (H1′, 2H, *m*); 4.15 (H5′, 4H, *m*); 4.67 and 4.74 (H7′, 1H each signal, *d*, 16.6); 5.36 (H6, 1H, *m*); 6.72 (H3′, 1H, *bs*); 7.21 (H9′, 2H, *dd*, 7.1 and 1.0); 7.35 (H10′, 2H, *m*); 7.38 (H11′, 1H, *m*). ^{13}C NMR ($CDCl_3$): 12.2 (C18); 16.4 (C6′, *d*, J_{C-P} = 6.1); 17.5 (C21); 19.5 (C19); 21.0 (C11); 24.4 (C16); 25.4 (C15); 31.3 (C7); 31.4 (C2); 31.8 (C8); 34.7, (C4′, *d*, J_{C-P} = 158.9); 36.8 (C10); 37.6 (C1); 37.6 (C12); 37.6 (C20); 41.3 (C13); 41.8 (C4); 49.4 (C9); 50.4 (C1′); 52.5 (C7′); 52.6 (C17); 56.1 (C14); 62.1 (C5′, *d*, J_{C-P} = 18.3); 71.7 (C3); 121.5 (C6); 126.8 (C9′); 128.0 (C10′); 129.0 (C11′); 136.2 (C8′); 140.7 (C5), 169.3 (C2′); 178.5 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{36}H_{56}N_2O_6P$ 643.3876, found 643.3876. Anal. calculated for $C_{36}H_{55}N_2O_6P$ C, 67.27; H, 8.62; N, 4.36; found C, 67.06; H, 8.79; N, 4.15.

2.1.16. N-benzyl-N-[(3β-hydroxypregn-5-ene-20-yl)-carbonyl]-glycylglycine
(***6i***)

Mp: 156 °C. ^{1}H NMR ($CDCl_3$): 0.62 (H18, 3H, *s*); 0.99 (H19, 3H, *s*); 1.11 (H21, 3H, *d*, 6.4); 1.35 (H6′, 6H, *m*); 2.82 (H20, 1H, *m*); 3.47 (H3, 1H, *m*); 3.92 and 4.09 (H4′, 1H each signal, *d*, 17.6); 4.00 and 4.11 (H1′, 1H each signal, *d*, 15.4); 4.51 and 4.82 (H6′, 1H each signal, *d*, 14.5); 5.34 (H6, 1H, *m*); 6.72 (H3′, 1H, *bs*); 7.23 (H8′, 2H, *dd*, 7.1 and 1.0); 7.31 (H9′, 2H, *m*); 7.38 (H11′, 1H, *t*, 7.7). ^{13}C NMR ($CDCl_3$–CD_3OD 9:1): 11.7 (C18); 16.8 (C21); 18.8 (C19); 20.5 (C11); 23.9 (C16); 25.4 (C15); 31.3 (C7); 31.4 (C2); 31.8 (C8); 36.8 (C10); 37.6 (C1); 37.6 (C12); 37.6 (C20); 41.3 (C13); 41.8 (C4); 49.1 (C6′); 49.2 (C4′); 49.3 (C1′); 49.4 (C9); 52.6 (C17); 55.8 (C14); 70.8 (C3); 120.9 (C6); 126.4 (C8′); 127.5 (C9′); 128.6 (C10′); 136.2 (C7′); 140.4 (C5); 169.0 (C2′); 174.1 (C5′), 178.6 (C22). HRMS (ESI): calculated for [M+H$^+$] $C_{33}H_{47}N_2O_5$ 551.3485, found 551.3497. Anal. calculated for $C_{33}H_{46}N_2O_5$ C, 71.97; H, 8.42; N, 5.09; found C, 71.79; H, 8.31; N, 5.17.



Scheme 1. Synthesis of compounds **5a–5e and 6a–6h**.



Scheme 2. Synthesis of compound **6i**.

2.1.17. Antifungal activity

Direct bioautography on TLC was used to assess the inhibitory activity. A concentration level 50 μg/spot of each compound assayed was used. Benomyl was used as a positive control. When appropriate, as large inhibitory halos were observed, minimum inhibitory concentration (MIC) was measured by the same method.

3. Results

The synthesis of the novel compounds is shown in Scheme 1. The known steroidal acids **3** and **4** were obtained as described previously [10,11]. Treatment of **3** with formaldehyde, an amine and an isonitrile led to the desired compounds of general structures **5a**–**5e**.

When the 3β-acetate **4** was used as the carboxylic component in the U-4CR, the resulting adducts were hydrolysed *in situ* to achieve the compounds **6a**–**6h**. As expected, the Ugi product obtained from ethyl isocyanoacetate gave the free acid **6i** after the hydrolysis step (Scheme 2).

The U-4CR generally took place smoothly and in good yields, and worked well with a set of structurally diverse amines, which can also include additional functional groups (Table 1).

The structure of all new compounds (Figs. 2 and 3) was unequivocally assigned by the usual methods (1 and 2D NMR experiments and mass spectrometry). In some cases, the NMR spectra showed that the compounds were present in the solution as a mixture of two conformers. These conformers are expected to originate from the *cis–trans* rotation around the N-substituted side chain amide bond [12]. Because the interchange between the *cis* and *trans* isomers of these bonds is generally slow in the NMR time scale, the NMR spectrum is the composite of the NMR spectra of the two configurational isomers (Fig. 4). For clarity, only the chemical shifts and coupling constants for the most populated conformer in each case was described in Section 2.

Bioautography offers a rapid and convenient approach to identify novel antifungal compounds and requires only microgram quantities [13]. The new compounds were tested *in vitro* using a bioautography method for their inhibitory properties towards *Fusarium virguliforme* (causal agent of sudden death syndrome in soy bean) and *Fusarium lateritium* (causal agent of sweetpotato chlorotic leaf distortion). The results are shown in Table 1.

Most of the new compounds showed a measurable antifungal activity against either of the two fungi tested. Although this is a preliminary screening, the activity might be species-specific (*F. virguliforme* seems to be more susceptible towards this family of compounds). On the other hand, it is interesting to note that whereas the homologous pair **5d** and **6d** elicited a significant activity, compound **6g** was inactive, in contrast to its homologue **5a**, which induced a small but measurable halo on the plate. This may indicate that the antifungal properties depend not only on the moieties attached to the side chain but also on the steroidal skeleton.

Finally, we determined the minimum inhibitory concentration (MIC) of two of the most active compounds. Compounds **5d** and **6i** had MIC values against *F. lateritium* of 8.6 and 10 nmol/spot respectively whereas **5d** showed a MIC value against *F. virguliforme* of 7.7 nmol/spot.

Table 1
Yields of the new compounds and their antifungal activity.

	Yield (%)[a]	*F. lateritium*[b]	*F. virguliforme*[b]
5a	76	0.3 ± 0.1	–
5b	69	–	0.5 ± 0.1
5c	75	–	0.4 ± 0.1
5d	82	1.2 ± 0.2	1.1 ± 0.1
5e	79	–	1.0 ± 0.2
6a	63	–	–
6b	62	–	0.8 ± 0.1
6c	69	–	–
6d	71	0.7 ± 0.2	0.5 ± 0.1
6e	69	–	0.4 ± 0.1
6f	78	–	0.3 ± 0.1
6g	77	–	–
6h	73	1.1 ± 0.2	–
6i	63	1.3 ± 0.1	–

[a] Isolated yields.
[b] Diameters of inhibition zone in cm. Mean values ± standard error from four independent experiments testing 50 μg of compound/spot. The inhibition halo of the control (Benomyl, a commercial antifungal) is 2.7 ± 0.1 cm at the same concentration.

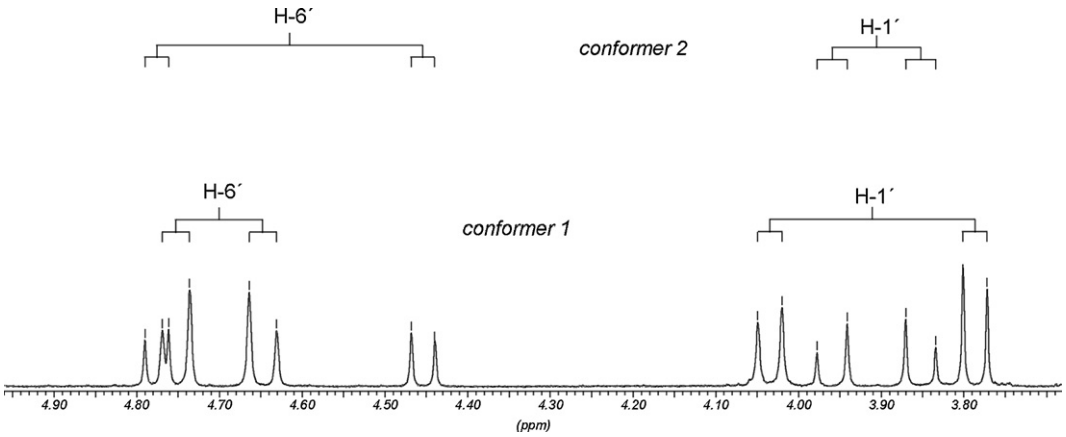


Fig. 4. Side chain methylene signals (^{1}H NMR, 500 MHz) for compound **6g** showing a 2:1 mixture of two conformers. Assignments of the signals correspond to the numbering depicted in Fig. 3.

Although examples of sterol analogues bearing amide bonds in the side chain are known [3], they were obtained using standard peptide chemistry, which imposes some limitations to the type of accessible structures. In this preliminary study, we demonstrated that the use of the U-4CR allows the facile attachment of a polyfunctionalized side chain into the steroidal framework rendering complex azasteroids by a simple procedure. In addition, our approach also leads to novel structures with remarkable antifungal activity against two pathogenic species of agricultural relevance.

Some natural and synthetic steroids with a nitrogen atom in their side chains were found to inhibit fungal growth through the inhibition of the enzyme sterol 24-methyl transferase, responsible for alkylation at C-24 during ergosterol biosynthesis [14]. Due to the structural features of our novel compounds, this mechanism of action cannot be discarded. Further studies are under way to expand this procedure to obtain a larger library of analogues in order to establish a structure–activity relationship and their possible mechanism of action on fungal growth.

Acknowledgements

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