Stereoselective Synthesis of 7α- and 7β-Aminocholesterol as Δ8-Δ7 Sterol Isomerase Inhibitors, with Fungicidal Activities towards Resistant Strains

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A mixture of epimeric 7α- and 7β-aminocholesterol was shown to be a stronger inhibitor of yeast cell growth than morpholine inhibitors. In fact, this epimeric mixture inhibits Δ8-Δ7-sterol reductase. This epimeric mixture is fungicidal and active against *Saccharomyces cerevisiae* resistant strains. Therefore, 7α- and 7β-aminocholesterol were selectively synthesized. According to in vitro bioassay studies on resistant strains such as *Candida tropicalis* [Amphotericin B resistant; minimum inhibitory concentration (MIC) of Amp B: 12.5 μg/mL], the MIC values for 7β-aminocholesterol and 7α-aminocholesterol were found to be 0.8 μg/mL and 1.5 μg/mL, respectively.

Introduction

There has been a significant rise in the incidence of fungal infections over the past two to three decades, particularly in those caused by opportunistic pathogens in immune-compromised patients as in cancer chemotherapy,[1] in the fields of organ and bone marrow transplants,[2] and in AIDS.[3] The prolonged survival of profoundly immunocompromised patients has revealed mucosal and invasive fungal infections to be the major causes of morbidity and mortality in HIV disease. Moreover, antifungal resistance has become a clinically relevant problem.[4]

Most antifungal drugs are sterol biosynthesis inhibitors (SBIs), acting as site-specific inhibitors at different steps of the ergosterol biosynthesis, the predominant sterol in most fungi.[5] Morpholine derivatives contain a nitrogen atom protonated in biological medium, and mimic the carbocationic high energy intermediates (HEI) involved in the Δ8-Δ7-sterol isomerase and Δ^{14}-sterol reductase. The Δ8-Δ7 isomerase reaction is conducted by the initial addition of a proton to the α face of C-9 giving a stabilized carbonium ion at C-8. This high energy intermediate is converted into Δ7 by removal of the 7β- (mammals and plants) or the 7α-hydrogen atom (fungi).[6] Sterol biosynthesis inhibitors (SBI), like morpholine and piperidine, widely used in agriculture against cereal powdery mildew − containing an amine function which is protonated in biological media − are mimics of the carbocationic intermediates involved in sterol isomerase and Δ14 reductase.[7,8] But the use of these drugs is limited by their only fungistatic activity. With the growing appearance of resistance towards most drugs, there is still a need for efficient new drugs.

In the frame of our work on aminosterol derivatives,[9,13] an α/β (77% α and 23% β) 7-aminocholesterol epimeric mixture was shown to inhibit Δ8-Δ7 isomerase and Δ14 reductase and to arrest cell proliferation. However, the novel feature of this compound is a strong cytotoxicity, even against SBI-resistant strains including inhibitors of sterol isomerase and sterol reductase.[13]

7-Aminocholesterol showed also strong antiproliferative properties on three cell lines: murine leukemia P388, KB, and a continuous human non small-cell bronchopulmonary carcinoma line (NSCLC-N6) with a cell blockade in Phase G1.[14] Thus, these molecules are promising for immune-compromised patients in cancer chemotherapy.

The pivotal importance of Δ8-Δ7 isomerase, added to the problematic chromatographic separation of the two epimers, have led us to develop a stereoselective synthesis of 7α- and 7β-aminocholesterol (**Iα** and **Iβ**).

Results and Discussion

Chemistry

The selective synthesis of 7α- and 7β-aminocholesterol was attempted by classical methods (Scheme 1). Treatment of cholesteryl acetate with chromium trioxide/pyridine com-

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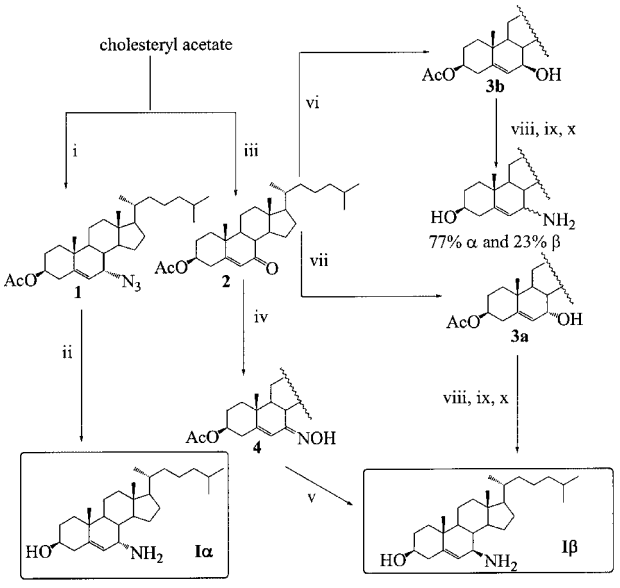
plex gave the allylic ketone[15] **2**. This ketone, reduced by sodium borohydride in the presence of cerium trichloride heptahydrate and methanol at −15 °C,[16,17] gave the corresponding 7β-hydroxy compound **3b**, and by L-selectride reduction the 7α-hydroxy epimer[18] **3a**. Conversion of the 7β-hydroxy compound **3b** into the tosylate could be accomplished in good yield with an excess of tosyl chloride (TsCl) in pyridine. On heating of the 7β-tosylate with sodium azide in dimethylformamide (DMF), an epimeric mixture of 7α-/7β-azido derivatives (77% α and 23% β, evaluated by ^{1}H NMR spectra) was reproducibly obtained [characteristic shifts of the azide β epimer: δ = 3.27 (ddd, $J_{7\alpha\text{-}8}$ = 8.5, $J_{7\alpha\text{-}6}$ = 1, $J_{7\alpha\text{-}4}$ = 1.5 Hz, 0.23 H, 7α-H), 5.30 ppm (dd, $J_{6-7\alpha}$ = 1, J_{6-4} < 1 Hz, 0.23 H, 6-H); characteristic shifts of the azide α epimer: δ = 3.49 (ddd, $J_{7\beta\text{-}8}$ = 4.5, $J_{7\beta\text{-}6}$ = 5, $J_{7\beta\text{-}4}$ = 1.5 Hz, 0.77 H, 7β-H), 5.51 ppm (dd, $J_{6\text{-}7\beta}$ = 5 and $J_{6\text{-}4}$ < 1 Hz, 0.77 H, 6-H)]. In the conversion step of the 7-α alcohol **3a** into the tosylate, the major product, 7-dehydrocholesterol, was formed by elimination (confirmed by NMR and UV spectra) and the required 7β-azido derivative was only obtained as minor product. Consequently, we developed a convenient novel synthetic route for 7α- and 7β-aminocholesterol (Scheme 1).

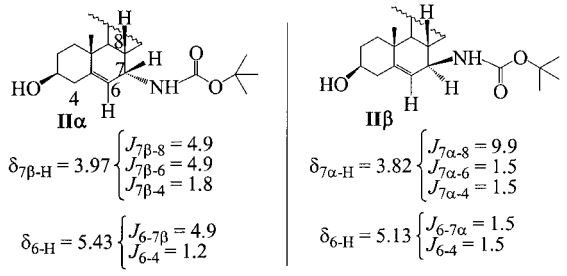


Scheme 1. Reagents and conditions: i: Me_3SiN_3, DDQ, CH_2Cl_2; ii: $LiAlH_4$, THF; iii: CrO_3/pyridine complex, CH_2Cl_2; iv: $HONH_3^+Cl^-$, pyridine; v: DIBAH (1 M in CH_2Cl_2)/CH_2Cl_2; vi: $CeCl_3{\cdot}7H_2O$, THF/MeOH; vii: L-selectride/THF, − 78 °C; viii: TsCl/pyridine; ix: NaN_3/DMF; x: $LiAlH_4$/THF

For 7α-aminocholesterol (**Iα**), the azido group was introduced directly by trimethylsilyl azide action in the presence of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in the axial allylic position C-7 of cholesteryl acetate according to the same previously described protocol for benzylic compounds.[19] The azido derivative **1** was then reduced with lithium aluminium hydride to give amine **Iα**.

For 7β-aminocholesterol (**Iβ**), the oxime derivative was obtained by treatment of ketone **2** with hydroxylamine hydrochloride in pyridine. Oxime **4** was selectively reduced by diisobutylaluminiumhydride (DIBAH) in dichloromethane to give amine **Iβ**. No oxime rearrangement to a secondary amine occurred, as is usually observed.[20]

The structure of amine **Iβ** was confirmed by a combination of ^{1}H, ^{13}C and DEPT NMR measurements. These amine structures were also confirmed as *N*-(*tert*-butoxycarbonyl)-3β-hydroxy-7α-aminocholesterol (**IIα**) and *N*-(*tert*-butoxycarbonyl)-3β-hydroxy-7β-aminocholesterol (**IIβ**). The ^{1}H NMR data revealed the differences shown in Scheme 2.



Scheme 2

Biology

Compounds **Iα** and **Iβ** were tested against five strains and compared to Amp B, Bifonazole and 5-Fluorocytosine (Table 1). Minimum inhibitory concentrations (MIC) after 48 h of incubation of **Iβ** against *Candida albicans* (1.5 μg/mL), and *Saccharomyces cerevisiae* (0.4 μg/mL) were compared to those of **Iα** (6.2 μg/mL and 3.1 μg/mL, respectively). These epimers were then tested against resistant strains such as *C. tropicalis* (Amp B resistant; MIC for Amp B: 12.5 μg/mL) with MIC(**Iβ**) = 0.8 μg/mL and MIC(**Iα**) = 1.5 μg/mL. Against *C. tropicalis* (5-FC resistant) 5-FC was inactive, contrary to **Iβ** (MIC = 1.5 μg/mL) and **Iα** (MIC = 3.1 μg/mL). Finally, the MIC value for Bifonazole (50 μg/mL) on a *C. albicans* azole-resistant strain was compared to those of **Iβ** (0.8 μg/mL) and **Iα** (3.1 μg/mL). Compounds **Iα** and **Iβ** were also fungicidal against *S. cerevisiae* (MIC = 5 μg/mL and 0.8 μg/mL, respectively) and *C. albicans* (MIC = 8 μg/mL and 2 μg/mL, respectively).

Table 1. MIC values [μg/ml]

Strain	**Iα**	**Iβ**	Amp B	Bifonazole	5-FC
S. cerevisiae ATCC 28 383	3.1	0.4	0.3	6.2	6.2
C. albicans CIP 1180-79	6.2	1.5	0.4	12.5	50
C. tropicalis CIP 1275-81 (Amp B resistant)	1.5	0.8	12.5	−	−
C. tropicalis CIP 1745-88 (5-FC resistant)	3.1	1.5	−	−	> 100
C. albicans CIP 1760-88 (azole-resistant)	3.1	0.8	−	50	−

Conclusion

An amine function at C-7β, protonated in physiological media, is spatially a better mimic than a 7α-ammonium group with respect to C-8 and C-14 carbocations. Not surprisingly, **Iβ** is more potent than **Iα** against all strains tested.

Our approach was first to compare the activity of 7α- and 7β-aminocholesterol with the three main families of antifungal drugs against *Candida* and the corresponding resistant strains.[21]

No significant variations in the cytotoxicity of the two epimers were observed against the three resistant strains. Moreover, unlike morpholine derivatives and most SBIs, these potentially lymphotropic molecules are fungicidal and are consequently promising candidates against systemic and deeply invasive infections.

Experimental Section

General: Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded with a Jeol 270 or 400 spectrometer. The chemical shift values are expressed in δ values (ppm) relative to tetramethylsilane as internal standard and coupling constant (*J*) values are expressed in Hz. High-resolution mass spectra were recorded with an HP 5889 A quadripolar instrument. The IR spectra were obtained with a Perkin−Elmer spectrometer. Elemental analyses were performed at the Institut Supérieur de la Matière et du Rayonnement, UMR CNRS 6507, Université de Caen. Melting points were measured with a Mettler FP 52 instrument and are uncorrected. All commercial reagents were provided by Aldrich.

3-acetyl-7α-azidocholesterol (1): Under argon, at room temperature, trimethylsilyl azide (10 mL, 69.97 mmol) was added to a stirred solution of cholesteryl acetate (3 g, 6.997 mmol) and DDQ (6.3 g, 13.99 mmol) in dry dichloromethane (25 mL), The mixture was stirred for 36 h, after which it was quenched with 10% aqueaous $NaHCO_3$ (2 × 10 mL), water and dried. The solution was concentrated under reduced pressure. The product was purified by column chromatography on silica gel (eluent: hexane/dichloromethane, 6:4) to give 1.67 g (51%) of a white powder, m.p. 110 °C (from ethanol). IR (KBr): $\tilde{\nu}$ = 2103 (N_3 azide) and 1727 (C=O ester) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.67 (s, 3 H, 18-H_3), 0.87 [dd, *J* = 6.6, *J* = 1.92, 6 H, $CH(CH_3)_2$], 0.92 (d, *J* = 6.6, 3 H, 21-H_3), 1.03 (s, 3 H, 19-H_3), 2.04 (s, 3 H, CH_3COO), 3.56 (ddd, $J_{7\beta\text{-}8}$ = 4.5, $J_{7\beta\text{-}6}$ = 5, $J_{7\beta\text{-}4}$ = 1.5, 1 H, 7β-H of 7-α azide), 4.66 (m, 1 H, 3-H), 5.56 (dd, $J_{6\text{-}7\beta}$ = 5.1, $J_{6\text{-}4}$ = 1.6, 1 H, 6-H of 7-α azide). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 58.2 (C-7), 73.6 (C-3), 119.1 (C-6), 147.3 (C-5), 170.3 (C=O). MS (EI): *m*/*z* (%) = 469 (5) [M$^+$], 441 (100), 351 (52).

7α-Aminocholesterol (Iα): Under nitrogen, a solution of **1** (0.4 g, 0.85 mmol) in tetrahydrofuran (5 mL) was added dropwise over 10 min to a stirred solution of lithium aluminium hydride (0.4 g, 0.85 mmol) in tetrahydrofuran (15 mL) at 0 °C. The mixture was refluxed for 4 h, after which it was cooled and quenched by careful addition of saturated aqueous sodium sulfate. The solution was filtered, dried and the solvents evaporated under reduced pressure. The crude product was purified by column chromatography on basic alumina using a dichloromethane/methanol (9:1) mixture as eluent to give 7α-aminocholesterol (0.370 g, 70%) as amorphous white solid. IR (KBr): $\tilde{\nu}$ = 3445−3000 (OH alcohol and NH_2 amine) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.69 (s, 3 H, 18-H_3), 0.86 [d, *J* = 6.5, 6 H, $CH(CH_3)_2$], 0.92 (d, *J* = 6.5, 3 H, 21-H_3), 1.05 (s, 3 H, 19-H_3), 3.58 (ddd, $J_{7\beta\text{-}8}$ = 4.8, $J_{7\beta\text{-}6}$ = 5.3, $J_{7\beta\text{-}4}$ = 1.2, 1 H, 7β-H of α amine), 4.62 (br. s, 2 H, NH_2, D_2O exchange), 5.55 (dd, $J_{6\text{-}7\beta}$ = 5.2, $J_{6\text{-}4}$ < 1, 1 H, 6-H of α amine). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 57.1 (C-7), 71.8 (C-3), 118.7 (C-5), 152 (C-6). MS (EI): *m*/*z* (%) = 401 (71), 384 (4), 351(29), 289 (100). This amine was also derivatized as *N*-(*tert*-butoxycarbonyl)-3β-hydroxy-7α-aminocholesterol. ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si): δ = 0.67 (s, 3 H, 18-H_3), 0.85 [dd, *J* = 6.6 and *J* = 1.17, 6 H, $CH(CH_3)_2$], 0.92 (d, *J* = 6.4, 3 H, 21-H_3), 1.02 (s, 3 H, 19-Me), 1.44 [s, 9 H, $C(CH_3)_3$], 3.52 (m, 1 H, 3-H), 3.97 (ddd, $J_{7\beta\text{-}8}$ = 4.9, $J_{7\beta\text{-}6}$ = 4.9, $J_{7\beta\text{-}6}$ = 1.2, 1 H, 7β-H of α epimer), 5.43 (dd, $J_{6\text{-}7\beta}$ = 4.9, $J_{6\text{-}4}$ = 1.2, 1 H, 6-H of α epimer).

3β-Acetyl-7-oxocholesterol (2): Prepared according to a literature procedure,[15] m.p. 149.5 °C (from ethanol). IR (KBr): $\tilde{\nu}$ = 2900 (CH_2), 1725 (C=O ester) and 1675 (C=O conjug. ketone) cm^{-1}. ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C): δ = 0.68 (s, 3 H, 18-H_3), 0.865 [d, *J* = 6.5, 6 H, $CH(CH_3)_2$], 0.94 (d, *J* = 6.5, 3 H, 21-H_3), 1.05 (s, 3 H, 19-H_3), 2.07 (s, 3 H, CH_3CO), 4.84 (m, 1 H, 3-H), 5.75 (d, *J* = 1.2, 1 H, 6-H).

3β-Acetyl-7-(hydroximino)cholesterol (4): Compound **2** (0.707 g, 1.59 mmol) and hydroxylamine hydrochloride (0.14 g, 2.067 mmol) was refluxed in pyridine (3 mL) under nitrogen for 24 h. The mixture was diluted with water (10 mL) and extracted with dichloromethane (3 × 7 mL). The organic layer was then treated with 0.1 N HCl (10 mL), 5% aqueous $NaHCO_3$ (10 mL) and water, after which it was dried and the solvents were evaporated under reduced pressure. Oxime **4** was crystallized from acetone (0.582 g, 80%). IR (KBr): $\tilde{\nu}$ = 3469 (N−O−H), 2900 (CH_2), 1719 (CO ester), 1646 (C=NO) cm^{-1}. ^{1}H NMR(400 MHz, $CDCl_3$, Me_4Si): δ = 0.69 (s, 3 H, 18-H_3), 0.86 [dd, *J* = 6.6, *J* = 1.6, 6 H, $CH(CH_3)_2$], 0.92 (d, *J* = 6.5, 3 H, 21-H_3), 1.13 (s, 3 H, 19-H_3), 2.04 (s, 3 H, CH_3COO), 4.69 (m, 1 H, 3-H), 5.30 (d, *J* = 1.2, 1 H, 6-H), 7.47 (br. s, 1 H, C=NOH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 73 (C-3), 113.7 (C-6), 151.3 (C-5), 157.8 (C-7), 170.3 (C=O).

7β-aminocholesterol (Iβ): DIBAH (12.4 mL of a 1 M CH_2Cl_2 solution, 1.24 mmol) was added dropwise to a solution of compound **4** (1.08 g, 2.4 mmol) in dry dichloromethane (20 mL) at 0 °C. The stirred mixture was maintained at the same temperature for 2 h and then at room temperature for 10 h. The solution was diluted with dichloromethane (20 mL), treated with sodium fluoride (0.042 g, 10 mmol) and water (1 mL). After vigorous stirring, the mixture was filtered. The crude product was purified by column chromatography on silica [ethyl acetate and ethyl acetate/methanol (95:5)] to give amine **Iβ** (0.45 g, 42%) as white solid. $C_{27}H_{47}NO$ (401.68): C 80.7, H 11.7, N 3.5; found C 80.3, H 11.3, N 3.2. IR (KBr): $\tilde{\nu}$ = 3469−3000 (OH alcohol and NH_2 amine), 2900 (CH_2) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si): δ = 0.76 (s, 3 H, 18-H_3), 0.88 [dd, *J* = 6.6, *J* = 1.6, 6 H, $CH(CH_3)_2$], 1.11 (d, *J* = 6.48, 3 H, 21-H_3), 1.14 (s, 3 H, 19-H_3), 3.46 (m, 1 H, 3-H), 3.54 (ddd, $J_{7\alpha\text{-}8}$ = 8.5, $J_{7\alpha\text{-}6}$ = 1.2, $J_{7\alpha\text{-}4}$ = 1.5, 1 H, 7α-H of amine β epimer), 4.60 (br. s, 2 H, NH_2, D_2O exchange), 5.26 (dd, $J_{6\text{-}7\alpha}$ = 1.2, J_{6-4} < 1, 1 H, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 55 (C-7), 71.6 (C-3), 119.4 (C-5), 150.3 (C-6). MS (EI): *m*/*z* (%) = 401 (42) [M$^+$], 386 (32), 384 (29), 289 (100), 248 (12), 176 (44). This amine was also derivatized to *N*-(*tert*-butoxycarbonyl)-3β-hydroxy-7β-aminocholesterol. ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si): δ = 0.66 (s, 3 H, 18-H_3), 0.84 [dd, *J* = 6.5, *J* = 1.17, 6 H, $CH(CH_3)_2$], 0.90 (d, *J* = 6.4, 3 H, 21-H_3), 1.02 (s, 3 H, 19-H_3), 1.44 [s, 9 H, $C(CH_3)_3$], 3.52 (m, 1 H, 3-H], 3.82 (dd, $J_{7\alpha\text{-}8}$ = 9.9, $J_{7\alpha\text{-}6}$ = 1.5, $J_{7\alpha\text{-}4}$ = 1.5, 1 H,

7α-H of β epimer), 5.13 (dd, $J_{6\text{-}7\alpha}$ = 1.5, $J_{6\text{-}4}$ = 1.5, 1 H, 6-H of β epimer).

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