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Bioorganic & Medicinal Chemistry 16 (2008) 209–221
Bioorganic & Medicinal Chemistry

# Novel cholesterol biosynthesis inhibitors targeting human lanosterol 14α-demethylase (CYP51)

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Received 23 April 2007; revised 15 September 2007; accepted 1 October 2007
Available online 4 October 2007

**Abstract**—Novel cholesterol biosynthesis inhibitors, a group of pyridylethanol(phenylethyl)amine derivatives, were synthesized. Sterol profiling assay in the human hepatoma HepG2 cells revealed that compounds target human lanosterol 14α-demethylase (CYP51). Structure–activity relationship study of the binding with the overexpressed human CYP51 indicates that the pyridine binds within the heme binding pocket in an analogy with the azoles.


## 1. Introduction

Cholesterol-lowering therapy with 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase inhibitors (statins) has been found as an effective method of reducing mortality and myocardial infarction among patients with coronary artery disease. However, a significant number of patients receiving statin therapy continue to have high residual risk.[1,2] Even if statins are considered as relatively safe drugs,[3] considerable attention has been paid recently to the statin-based risk of muscular adverse drug reactions, hepatotoxicity, and statin resistance. By blocking HMG–CoA reductase, statins might deplete ubiquinone (Co-Q-10) in the blood. However, there seems to be insufficient evidence from human studies to link statin therapy unequivocally to pathologically significantly decreased tissue Co-Q-10 levels.[4,5] It was observed that derangements in mevalonate kinase, but not more distal enzymes of cholesterologenesis, are associated with a skeletal myopathy, suggesting a critical role for the isoprenoids in the maintenance of muscle.[6] Consequently, the development of novel cholesterol lowering agents that are effective below the farnesyl pyrophosphate branchpoint of the mevalonate pathway remains a challenge.

The first enzyme below the farnesyl pyrophosphate branchpoint is squalene synthase. There are potent inhibitors of squalene synthase that are also being developed as cholesterol lowering agents. Takeda has one, lapaquistat (TAK-475), that is in Phase III clinical development.[7]

Oxidosqualene cyclase (lanosterol synthase) is the second enzyme bellow the farnesyl pyrophosphate branchpoint that has been identified as a target for novel anticholesterolemic drugs that could complement statins.[8] A novel series of 4-piperidinopyridines and 4-piperidinopyrimidines showed potent and selective inhibition of the rat enzyme.[9]

The subsequent enzyme of the cholesterol synthesis pathway, the lanosterol 14α-demethylase (CYP51), is another potential anticholesterolemic drug target. This enzyme, which is the most evolutionarily conserved member of the cytochrome P450 gene superfamily, is found in both prokaryotes and eukaryotes.[10] CYP51 resides in endoplasmatic reticulum in eukaryotes.[11] In animals it catalyzes the oxidative removal of the 14α-methyl group (C32) of lanosterol and 24,25-dihydrolanosterol to produce 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (FF-MAS). FF-MAS is the substrate for the consecutive enzyme in cholesterol synthesis, sterol Δ14-reductase

*Keywords*: Cholesterol biosynthesis inhibitors; Human lanosterol 14α-demethylase (CYP51) inhibitors; Cholesterol intermediates profile; Pyridylethanolamines.
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doi:10.1016/j.bmc.2007.10.001

(DHCR14), which produces 4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol (T-MAS).[12] Human lanosterol 14α-demethylase is a relatively well-characterized target at the level of gene,[13,14] regulation of expression,[15–18] tissue-specificity,[19–21] and also protein and enzymatic activity.[22–24] However, due to the membrane character of the enzyme, crystallization attempts have not been so far successful. There is also no crystal structure available for the yeast CYP51 enzymes that are approximately 40% identical to the human counterparts and represent common antifungal drug targets.[25,26] Only the crystal structure of the soluble 14α-sterol demethylase from *Mycobacterium tuberculosis* is known.[27] This bacterial enzyme is 28% identical to the human CYP51 and provides a template for analysis of eukaryotic orthologs. So far there have been many studies directed toward fungal CYP51 inhibition. Most dealt with azole-type inhibitors and characteristics of azole binding site.[28,29] Interestingly, ketoconazole and fluconazole, well-known antifungal agents, showed differing affinities for human CYP51 and experimental evidence was presented for structural difference of azole binding site in CYP51 between human and *Mycobacterium tuberculosis*.[30] A comparison of human CYP51 homology model with that of fungal analog from *Candida albicans* was based on the crystal structure.[31]

We have identified structurally diverse derivatives of pyridylethanol(phenylethyl)amines interacting with human CYP51. Taking into account the importance of the modified sterol composition after inhibition, a novel sterol profiling assay has been developed to monitor the effect of compounds on de novo synthesis of cholesterol. This allowed us to predict the targeted enzyme and also to perform a structure–activity relationship study. Binding studies on the purified, overexpressed human CYP51 enzyme confirmed predictions from the sterol profiling ex vivo assay demonstrating its predictive power. Our studies contribute to understanding of structural requirements for human CYP51 inhibition. Recently published pharmacophore model based on the three-dimensional quantitative structure–activity relationship study[32] offers a pharmacophore model consisting of hydrogen bond acceptor and hydrophobic features.

## 2. Chemistry

Different synthetic approaches have been used for the synthesis of target compounds with regard to the starting materials.

### 2.1. Procedure A

The synthesis of target compounds is shown in Scheme 1. Reactions were carried out according to the procedures described recently.[33]

### 2.2. Procedure B

The synthesis of target compounds is shown in Scheme 2. Reactions were carried out according to the procedures described recently.[33]

### 2.3. Procedure C

Amides **9a** and **9b** were prepared by coupling commercially available primary amines with the corresponding carboxylic acid using EDC and HOBT activation.[34] Amides **9a** and **9b** were further reduced by borane-dimethyl sulfide complex to provide secondary amines **10a** and **10b**.[35] By the reaction of reductive alkylation of the secondary amines using sodium triacetoxyborohydride, tertiary amines **11a** and **11b** were prepared.[36] Free bases **11a** and **11b** were converted to dihydrobromide salts **12a** and **12b**, respectively (Scheme 3).

### 2.4. Procedure D

2-Naphthaleneacetonitrile was reduced by borane-dimethyl sulfide complex to provide primary amine **13**.[35] Final compound **16** was prepared with same individual reaction steps as under procedure B (Scheme 4).

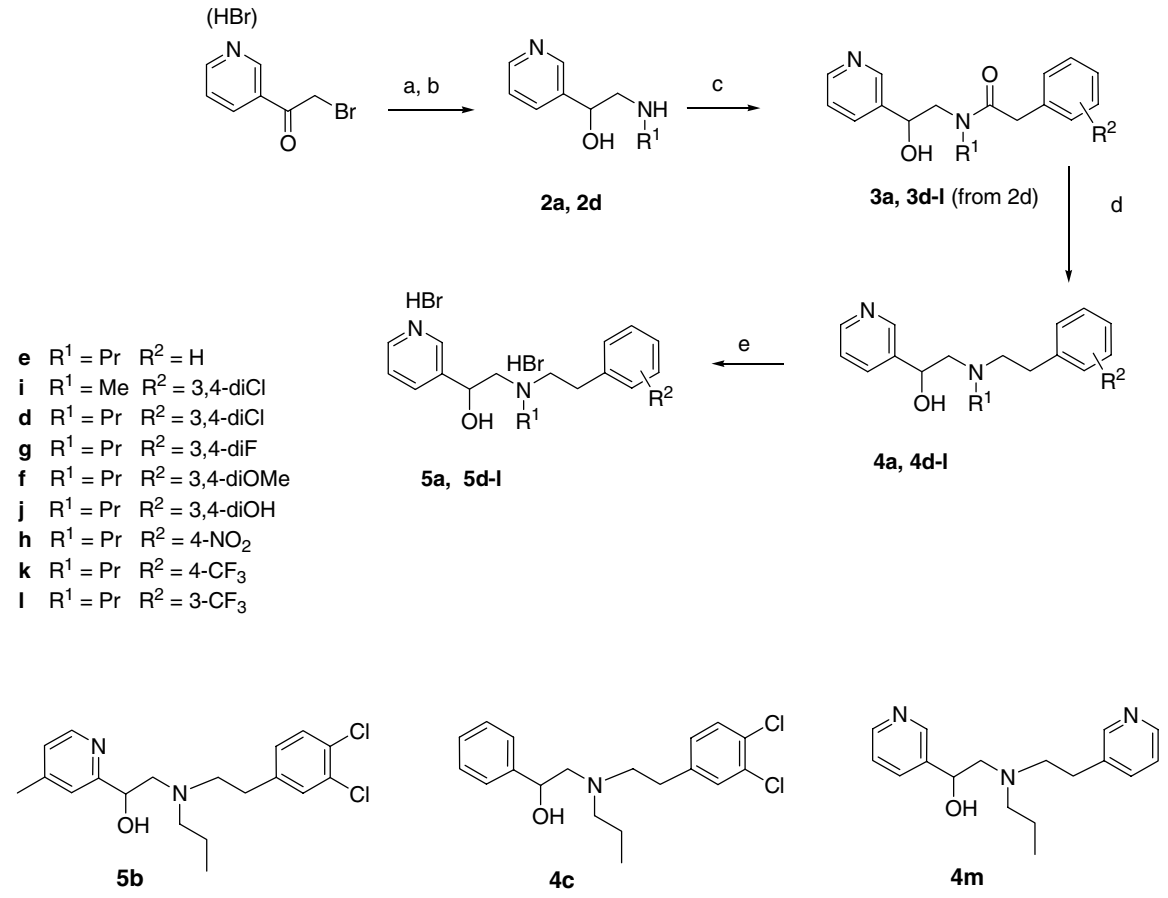
### 2.5. Procedure E

3-(Oxiran-2-ylmethoxy)pyridine **17** was prepared by the reaction of 2-(chloromethyl)oxirane with pyridin-3-ol in alkaline solution. Oxirane ring opening with phenylethanamine, $K_2CO_3$, and KI afforded secondary amine **18**. Reductive alkylation using sodium triacetoxyborohydride gave final product **19** (Scheme 5).

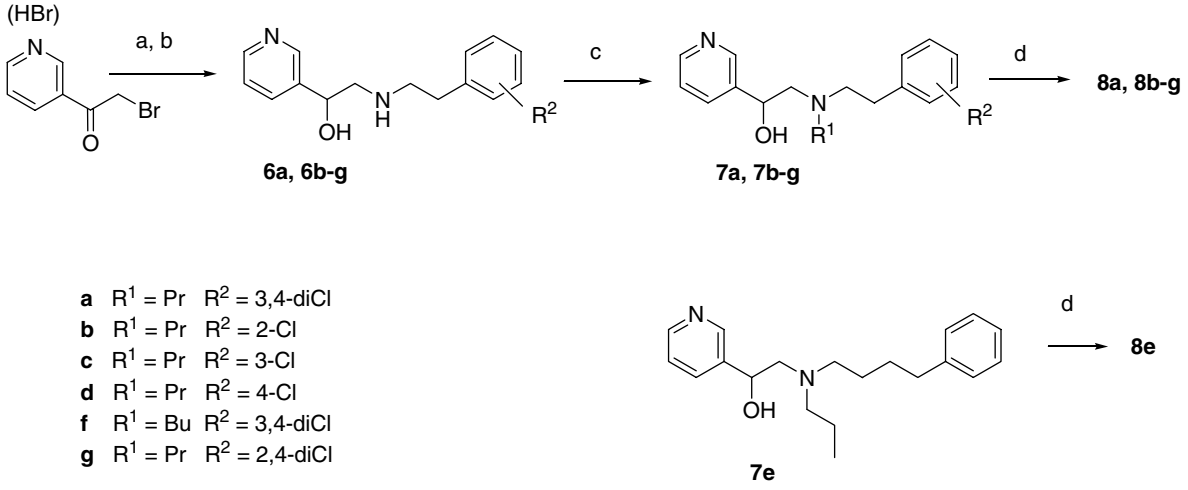
## 3. Biology

A novel ex vivo screening assay has been developed in human hepatoma HepG2 cells as described in detail in the Experimental section.[37] Figure 1a represents an outline of the post-lanosterol portion of the cholesterol biosynthesis. It indicates sterol intermediates that are measured in our assay. $^3$H-labeled de novo synthesized lanosterol, FF-MAS (follicular fluid meiosis activating sterol, 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol), T-MAS (testis meiosis activating sterol, 4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol), zymosterol, desmosterol, 7-dehydrocholesterol, and cholesterol have been separated on a reverse phase column and quantified by the radioactivity detector. Even if structurally very similar, these sterols can be separated and unequivocally identified using reference standards. Figure 1b shows the relative quantities of de novo produced sterol intermediates and cholesterol in normal, non-treated HepG2 cells. De novo produced cholesterol represents 20% of the radiolabeled sterol pool. Lanosterol (25%) and lathosterol (25%) are two bulky sterol intermediates. A mixture of 7-dehydrocholesterol and zymosterol that was not separated under our experimental conditions represents 23% of the pool. Two minor intermediates are FF-MAS (2%) and desmosterol (5%). T-MAS was not detected by the radio-HPLC assay in HepG2 cells. T-MAS is a bulky sterol intermediate in mammalian testis where it is identified easily.[38]

Figure 2 shows $^3$H labeled sterols isolated from HepG2 cells treated with 10 μM solutions of compounds, potential cholesterol biosynthesis inhibitors. Normal medium

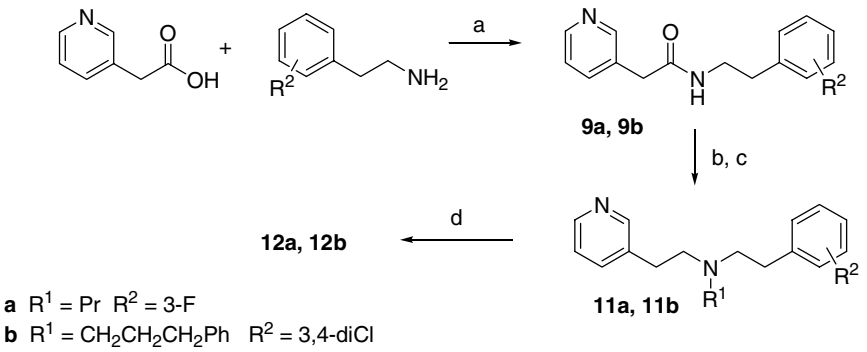



**Scheme 1.** Reagents and conditions: (a) $NaBH_4$, anhyd EtOH, rt, 2h; (b) $R^1NH_2$, anhyd EtOH, reflux, 5h; (c) $R^2$-phenylacetic acid, HOBt, EDC, NMM, DMF, rt, 12 h; (d) $BH_3{\cdot}Me_2S$, THF, reflux, 10 h; (e) HBr, EtOH, 0 °C, 2 h. (a is 4-pyridyl analog of i).

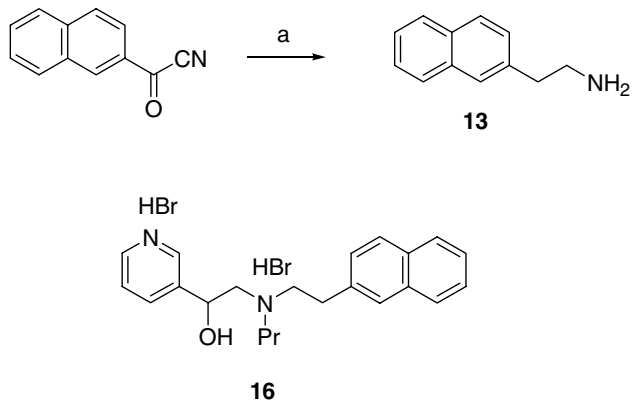



**Scheme 2.** Reagents and conditions: (a) $NaBH_4$, anhyd EtOH, rt, 2 h; (b) $R^2$-phenethylamine, anhyd EtOH, reflux, 12 h; (c) aldehyde, $NaBH(OAc)_3$, $CH_2ClCH_2Cl$, rt, Ar 2 h; (d) HBr, EtOH, 0 °C, 2 h. (a is 4-pyridyl analog of d).

and atorvastatin were used as a control (group C). As expected, atorvastatin showed a complete blockage of cholesterol biosynthesis. Due to the blockage of HMG–CoA reductase, an enzyme in the early part of cholesterol biosynthesis, no post-lanosterol intermediates have been observed. Tested compounds have been grouped according to the sterol profile. Group S inhibitors show no accumulation of cholesterol, low accumulation of post-lanosterol cholesterol biosynthesis intermediates, and high accumulation of lanosterol, a substrate for the CYP51 enzyme. Notable accumulation of lanosterol is observed for group W albeit at lower amount compared to the S group inhibitors and de novo synthesis of cholesterol is not completely blocked. With-




**Scheme 3.** Reagents and conditions: (a) HOBt, EDC, NMM, DMF, rt, 12h; (b) $BH_3$·MeS, THF, reflux, 10 h; (c) aldehyde, $NaBH(OAc)_3$, $CH_2ClCH_2Cl$, rt, Ar 12 h; (d) HBr, EtOH, 0 °C.




**Scheme 4.** Reagents and conditions: (a) $BH_3$·$Me_2S$, THF, reflux, 5 h.

in the group D compound **12a** shows high FF-MAS accumulation and is potentially a strong sterol Δ14-reductase (DHCR14) inhibitor. Group N compounds are not inhibitors of cholesterol biosynthesis. Their sterol profile shows no effect on de novo synthesis of cholesterol in HepG2 cells.

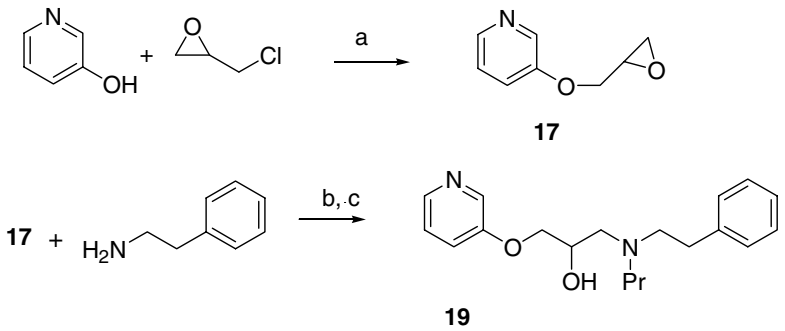
Human histidine-tagged CYP51 enzyme (lanosterol 14α-demethylase) was overexpressed in *Escherichia coli* and purified to homogeneity as described previously.[39] Affinities of novel compounds were evaluated as apparent dissociation constants determined by the spectrophotometric method.[40] Figure 3 shows type II spectral response of the purified human CYP51 to ketoconazole and **5d**. It is evident that **5d** produces a similar spectral response as ketoconazole.

In Table 1 apparent dissociation constants and maximum spectral responses are compared to lanosterol accumulation for a selected set of compounds out of that presented in Figure 2. Type II spectral response for compounds listed in Table 1, except **5b**, is very similar to that of **5d** and shows a characteristic saturation curve (Fig. 3c). This is a strong indication that novel compounds interact with the human lanosterol 14α-demethylase in a manner similar to that previously recognized for some azoles.[23,40] CYP51 inhibitors that do not have a nitrogen atom for heme iron atom coordination have spectra quite different from those of ketoconazole or fluconazole.[41]

Ketoconazole and fluconazole show strong and weak affinity toward human CYP51, respectively, in accordance with the result published previously.[30]

## 4. Discussion

Due to the massive increase of hyperlipidemias and cardiovascular diseases in the developed world and an increasing number of patients not responding to the currently available therapies searching for novel hypolipidemic drugs remains a very important task.




**Scheme 5.** Reagents and conditions: (a) NaOH, $H_2O$, 35 °C, 12 h; (b) $K_2CO_3$, KI, anhyd EtOH, 80 °C, 4 h; (c) $CH_3CH_2CHO$, $NaBH(OAc)_3$, $CH_2ClCH_2Cl$, rt, Ar 12 h.

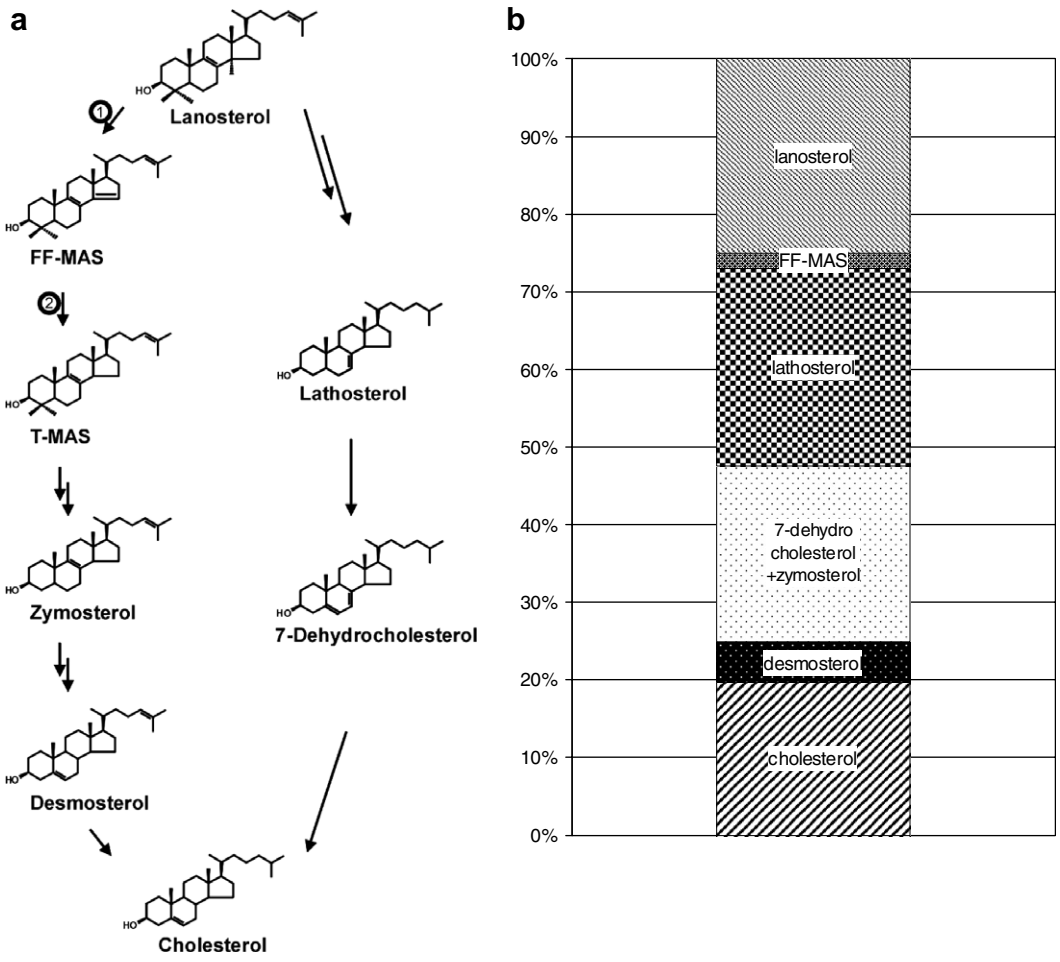



**Figure 1.** (a) An outline of the cholesterol biosynthesis pathway. Sterol intermediates are shown that have been separated and identified in this study. Enzymes CYP51 and DHCR14, targets of the novel compounds, are indicated as **1** and **2**, respectively. (b) Relative amount of radiolabeled de novo synthesized sterols in non-treated HepG2 cells. T-MAS is bellow the detection limit.

Our aim was to synthesize compounds that would inhibit cholesterol synthesis after the farnesyl pyrophosphate branchpoint leaving isoprene pathways untouched in order to avoid statin side effects such as skeletal myopathy. Several compounds structurally not related to pyridylethanol(phenylethyl)amines were already shown to inhibit the post-squalene cholesterol biosynthesis. SKF 104976, an analog of lanosterol,[42] inhibits human lanosterol 14α-demethylase (CYP51). AAY 9944 (*trans*-1,4-bis(2-chlorobenzaminomethyl) cyclohexane dihydrochloride) inhibits several human post-squalene cholesterol biosynthesis enzymes: two sterol reductases, DHCR14 and DHCR7, and the sterol Δ8-7-isomerase (EBP). SR 31747 (*N*-cyclohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride) was shown to be a selective EBP inhibitor.[43,44]

Research and development of CYP51 inhibitors for the treatment of fungals infections is now directed toward efficient and more selective drugs. It was suggested that increased azole doses to combat resistant *candidemia*s may well inhibit also the endogenous human CYP51 which may lead to severe side effects. Therefore, interest in the development of human CYP51 inhibitors lies primarily in development of potential anticholesterolemic drugs and in characterization of structural differences between human and fungal orthologues.[30,31] Spectroscopic titration of the two purified enzymes (human and *C. albicans*) for ketoconazole showed that the affinity difference was less than 10-fold.[23] Interestingly, ketoconazole has higher affinity for the human enzyme than for that from *Mycobacterium tuberculosis*.[30]

As demonstrated above in Figure 2 series of pyridylethanol(phenylethyl)amines can be divided into four groups according to the sterol profile obtained from the ex vivo assay. We demonstrated that sterol profile is indicative of compound affinity toward human CYP51. Moreover, in case of specific structural change some compounds exerted notable change in the sterol profile that implies binding affinity toward post CYP51 enzyme, most probably DHCR14.

Measurements on the purified enzyme (Table 1) confirmed the classification based on the sterol profile differences. Group S compounds showed strong CYP51 inhibition and have $K_d$ values between 0.34 and

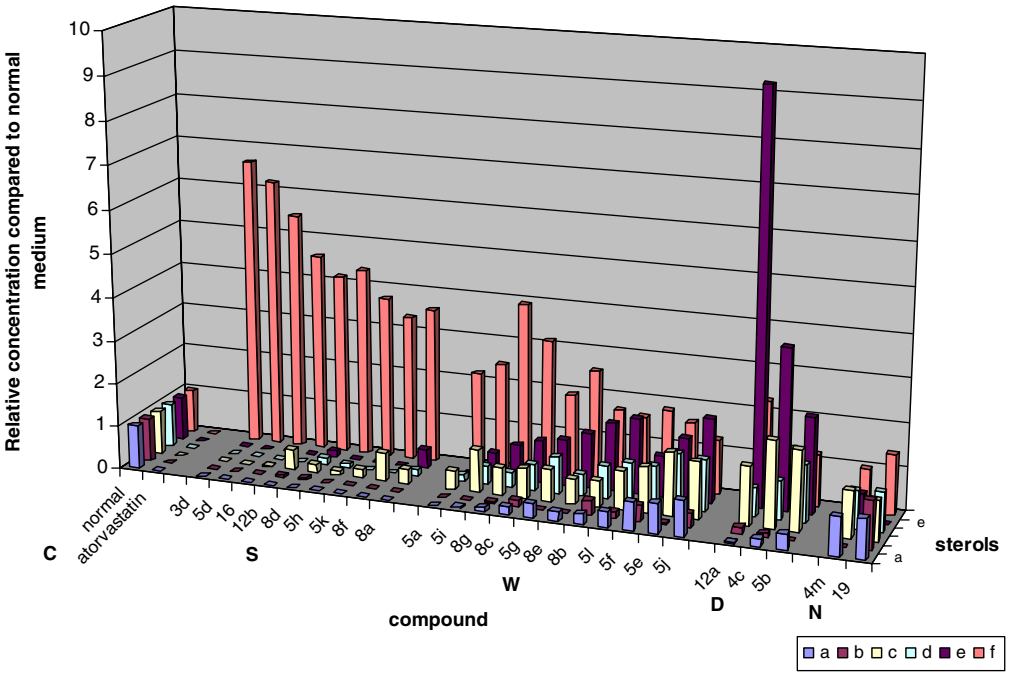



**Figure 2.** The average amount of radiolabeled sterols (a) cholesterol, (b) desmosterol, (c) zymosterol + 7-dehydrocholesterol, (d) lathosterol, (e) FF-MAS, and (f) lanosterol in cell extracts compared to the normal medium, after treatment with 10 μM solution of novel compounds. (Numerical data are available in the supplementary data).

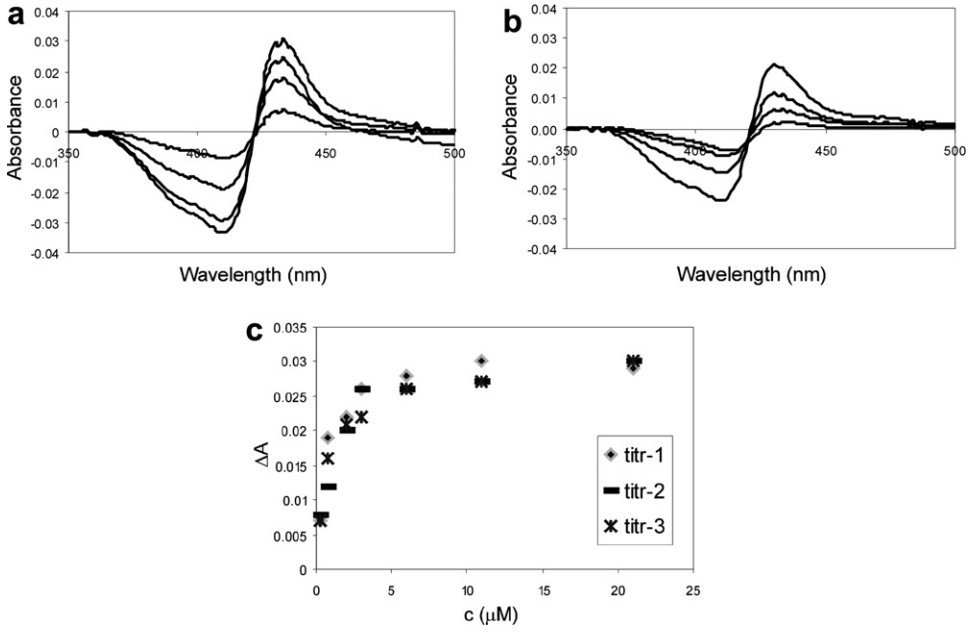



**Figure 3.** Type II cytochrome P450 spectral response of the overexpressed purified human CYP51 (2 μM) to ketoconazole (a) and **5d** (b) at concentrations of 0.5 μM, 1 μM, 2 μM, and 10 μM. (c) Spectrophotometric titration of human CYP51 (0.5 μM) with **5d**. $\Delta A(A_{432} - A_{412})$ is absorbance difference between 432 and 412 nm.

3.3 μM. Group W has one order of magnitude larger affinity constants (27–35 μM) compared to group S and therefore contains weak CYP51 inhibitors.

Compound **5d** showed to be a strong human lanosterol 14α-demethylase (CYP51) inhibitor in the ex vivo assay and this was confirmed also by measurements on the *E. coli* overexpressed purified human CYP51 enzyme. Analogs of **5d** were prepared with the aim to improve the inhibitory potential against the human CYP51 and to study structure–activity relationship.

Lead optimization was directed toward the substitution pattern on the phenyl ring (**5e**, **5f**, **5g**, **5h**, **5j**, **5k**, **5l**, **8c**, **8d**, **8e**, **8h**, **12a**), the amino group has been substituted by different functions (**5i**, **8f**, **12b**), pyridine ring has been

**Table 1.** Apparent dissociation constants ($K_d$, μM) and maximal spectral response per nanomole of purified human CYP51 ($\Delta A_{max}$/nmol) for selected compounds

| Group | Compound | $K_d$ (μM) | $\Delta A_{max}$/nmol | Lanosterol (ex vivo) |
|---|---|---|---|---|
| S | **5d** | 0.4 ± 0.1 | 0.057 ± 0.003 | 6 ± 3 |
| S | **8d** | 3.3 ± 0.5 | 0.062 ± 0.003 | 4 ± 2 |
| S | **16** | 0.34 ± 0.06 | 0.053 ± 0.001 | 5 ± 2 |
| W | **5g** | 27 ± 3 | 0.054 ± 0.002 | 1.9 ± 0.6 |
| W | **5f** | 29.2 ± 0.5 | 0.057 ± 0.005 | 1.8 ± 0.6 |
| W | **8b** | 35 ± 4 | 0.050 ± 0.003 | 1.7 ± 0.5 |
| D | **5b**[*] | ≫1000[a] | | 1.2 ± 0.3 |
| N | **4m** | 260 ± 90 | 0.06 ± 0.01 | 1.0 ± 0.1 |
| | Ketoconazole | 0.11 ± 0.01 | 0.080 ± 0.001 | |
| | Fluconazole | 40 ± 3 | 0.053 ± 0.001 | |

Lanosterol (ex vivo)—fold increase in lanosterol, that is, relative quantity of $^3$H-lanosterol from the ex vivo assay (numerical data from Fig. 2).
[a] No type II spectral response up to 300 μM.
[*] 2-Pyridyl analog of **5d**.

substituted on different positions (**5a**, **5b**, **8a**). Deshydroxy pyridylethanol analogs have been prepared (**12a**, **12b**), phenyl ring has been replaced by pyridine ring, and the opposite (**4m**, **4c**), $OCH_2$ group has been introduced between pyridine ring and the alkyl chain (**19**) and different length of the alkyl side chain between tertiary nitrogen and phenyl ring has been introduced (**8e**). The replacement of the phenyl by a naphthalene ring has also been made (**16**).

3,4-DiCl substitution of the phenyl ring is most suitable for CYP51 inhibition. Mono chloro substitution of the phenyl ring showed a marked decrease in cholesterol inhibition with regard to **5d**: 4-Cl analog (**8d)** is more potent than 3-Cl analog (**8c**), the least potent is 2-Cl analog (**8b**). **8d** and **8b** have weaker affinity for CYP51 (Table 1). 2,4-diCl analog (**8g**) is weaker inhibitor than **5d**. Removal of both Cl atoms (**5e**) additionally decreased lanosterol accumulation and cholesterol lowering effect. Compounds **5k** and **5l** with mono $CF_3$ substitution are as potent as their Cl analogs.

Replacement of 3,4-dichloro phenyl ring substitution by 3,4-difluoro substitution (**5g**) showed a marked decrease in affinity (Table 1) and reduction of lanosterol accumulation. Introduction of more polar groups, either 3,4-dimethoxy (**5f**) or 3,4-dihydroxy (**5j**) in the phenyl ring, clearly follows this trend. Replacement of 3,4-dichlorophenyl moiety with 3-pyridyl group (**4m**) resulted in the complete lack of cholesterol lowering activity and in the loss of affinity for CYP51 (Table 1). On the other side, replacement of the 3,4-dichlorophenyl moiety by a 2-naphthyl group (**16**) showed a minor increase in affinity and comparable lanosterol accumulation with regard to **5d**.

Comparison of compounds **5e** and **8e** showed that the longer alkyl chain connecting the phenyl moiety to the tertiary amine nitrogen ($(CH_2)n$, $n = 2$ and 4, respectively) increased CYP51 inhibition. To study *N*-alkyl substitution, compounds **5d** ($R^1$ = Pr), **8f** ($R^1$ = Bu), **5i** ($R^1$ = Me), and **12b** ($R^1 = CH_2CH_2CH_2Ph$) were synthesized. They all showed the CYP51 inhibition ability of the same order of magnitude. These findings confirmed that lipophilic interactions are very important for the potent binding of pyridylethanol(phenylethyl)amines to human CYP51. It seems that these features correlate well with the pharmacophore model presented by Ekins et al.[32] although our compounds have in general smaller molecular weight and direct comparison might not be trivial.

Replacement of the 3-pyridine moiety by 4-pyridine (**8a**) showed an activity of the same order of magnitude as in **5d**. This was also confirmed within pair of compounds **5i** and **5a** ($R^1$ = Me). According to the ex vivo test, 2-pyridinyl substitution (**5b**) completely changed the sterol profile (group D), possibly inhibiting DHCR14 and other enzymes from the later steps of cholesterol biosynthesis. This modification led to loss of affinity for CYP51 (Table 1). The distance between the two nitrogen atoms in the molecule seems to be essential for CYP51 affinity. Same conclusion can be drawn from the loss of CYP51 inhibition in **19**which has an $OCH_2$ group inserted in the alkyl chain. Replacement of the 3-pyridine moiety by the phenyl group (**4c**) resulted in loss of affinity for CYP51 and showed a different sterol profile. **4c** most likely inhibits DHCR14 and some other enzymes from the later steps of cholesterol biosynthesis. This clearly indicates that the pyridine nitrogen is essential for strong CYP51 inhibition. Sterol profiles of **4c** and **5b** are similar, although it seems they are weaker than **12a**, presumably, a potent DHCR14 inhibitor.

As demonstrated above we have obtained good correlation of results between Hep G2 cell assay and purified enzyme assay. Most of our compounds are hydrophobic, a property that is favorable both for cell membrane permeation and CYP51 binding. However, it has to be pointed out that Hep G2 cell assay was discriminatory between hydrophilic and hydrophobic statins, potent HMG CoA inhibitors.[45] Moreover, we also found that rosuvastatin was less potent inhibitor of cholesterol synthesis than atorvastatin in our Hep G2 cell assay.[46]

In conclusion, novel pyridylethanol(phenylethyl)amines show a post-lanosterol cholesterol biosynthesis inhibition effect. For majority of compounds biological data

suggest CYP51 as a main target of inhibition. Studies on the purified human CYP51 enzyme confirmed predictions from the sterol profiling ex vivo assay demonstrating its predictive power. Replacement of the pyridine ring with a phenyl ring resulted in a loss of CYP51 affinity indicating that the pyridine nitrogen is important for CYP51 inhibition. Moreover, spectroscopy titration provided evidence that the pyridine nitrogen corresponds to that in azoles. We demonstrated that lipophilic properties of the structure moiety apparently play a crucial role in the strength of CYP51 inhibition in accordance with the previous experimental and theoretical work.

## 5. Experimental

### 5.1. General chemical methods

Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification. Analytical TLC was performed on silica gel (60 F 254) plates (0.25 mm) and components visualized with ultraviolet light. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). Melting points were determined on a hot stage microscope and are uncorrected. $^1$H NMR and $^{13}$C NMR experiments were carried out at 300 MHz or 600 MHz in $CDCl_3$ or DMSO-$d_6$ solution with TMS as an internal standard.

#### 5.1.1. General synthesis procedure A[33]

**5.1.1.1. 2-((3,4-(Dichlorophenethyl)(methyl)amino)-1-(pyridin-4-yl)ethanol dihydrobromide (5a).** Yield: 14%. $^1$H NMR $\delta$ 2.49 (3H, s), 2.97–3.57 (6H, m, 6H), 5.44 (1H, bs), 7.35 (1H, m), 7.62–7.70 (2H, m), 8.10 (2H, d, $J$ = 6.1 Hz), 8.97 (2H, d, $J$ = 6.3 Hz); mp 185–190 °C; IR (KBr): 3210, 2964, 2706, 1632, 1595, 1498, 1395, 1264, 1135, 1079, 1029, 809 cm$^{-1}$; MS (FAB): M + H$^+$: 325; Anal. Calcd for $C_{16}H_{20}Cl_2N_2OBr_2$: C, 39.46, H, 4.14, N, 5.75. Found C, 39.46, H, 4.22, N, 5.59.

**5.1.1.2. 2-((3,4-Dichlorophenethyl)(propyl)amino)-1-(4-methylpyridin-2-yl)ethanol dihydrobromide (5b).** Yield: 9%. $^1$H NMR $\delta$ 0.92 (3H, t, $J$ = 7.3 Hz), 1.74 (2H, m), 2.43 (3H, s), 3.06–3.57 (8H, m), 5.19 (1H, bs), 7.32–7.65 (5H, m), 8.49 (1H, d, $J$ = 5.0 Hz); mp 109–112 °C; IR (KBr) 3426, 1706, 1637, 1474, 820, 780 cm$^{-1}$; MS (FAB): M + H$^+$: 367; Anal. Calcd for $C_{19}H_{26}N_2OCl_2Br_2$: C, 43.13, H, 4.95, N, 5.29. Found C, 42.65, H, 5.34, N, 4.79.

**5.1.1.3. 2-(((3,4-Dichlorophenethyl)(propyl)amino)-1-phenylethanol (4c).** Yield: 8%. $^1$H NMR $\delta$ 0.97 (3H, t, $J$ = 7.3 Hz), 1.50–1.60 (2H, m), 2.53–2.91 (8H, m), 4.67 (1H, dd, $J_1$ = 10.3 Hz in $J_2$ = 3.4 Hz), 7.08 (1H, dd, $J_1$ = 8.3 Hz, $J_2$ = 2.0 Hz), 7.31–7.43 (7H, m)); $^{13}$C NMR $\delta$ 11.72, 20, 28, 32.84, 55.29, 55.79, 62.93, 69.37, 125.74, 127.43, 128.10, 128.31, 130.33, 130.57, 140.28, 142.16; IR (NaCl) 3422, 2956, 2870, 1466, 1395, 1130, 1031, 888, 700 cm$^{-1}$; MS (FAB): M + H$^+$: 352; HRMS $m/z$ Calcd for $C_{19}H_{23}Cl_2NO$ [MH$^+$] 352.123495. Found 352.124200.

**5.1.1.4. 2-((3,4-(Dichlorophenethyl(propyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5d).** Yield: 9%. $^1$H NMR $\delta$ 094 (3H, t, $J$ = 7.5 Hz), 1.78 (2H, m), 3.10–3.54 (6H, m), 5.48 (1H, bs), 7.34–7.74 (3H, m), 8.06 (1H, dd, $J_1$ = 8.1 Hz, $J_2$ = 5.7 Hz), 8.63 (1H, d, $J$ = 8.1 Hz), 8.91 (1H, d, $J$ = 5.1 Hz), 9.04 (1H, s); $^{13}$C NMR $\delta$ 10.84, 16.41, 27.93, 52.51, 53.73 or 53.90, 55.29, 57.20, 64.32, 126.31, 129.51 or 129.61, 130.67, 131.05, 138.20, 140.25, 141.72, 142.22, 142.92; mp 170-172 °C; IR (KBr) 3411, 3168, 2954, 2685, 1626, 1598, 1536, 1473, 1348, 1210, 1129, 1028, 805, 680 cm$^{-1}$; MS (FAB): M + H$^+$: 353; Anal. Calcd for $C_{18}H_{24}Cl_2N_2OBr_2$ + 4/5 $H_2O$: C, 40.83, H, 4.87, N, 5.29. Found C, 40.86, H, 4.74, N, 5.21.

**5.1.1.5. 2-(Phenethyl(propyl)amino)-1-(pyridin-3-yl) ethanol dihydrobromide (5e).** Yield: 13%. $^1$H NMR $\delta$ 0.92 (3H, t), 1.76 (2H, m), 2.48 (2H, m), 3.05–3.51 (6H, m), 5.42 (1H, bs), 7.35 (1H, m), 7.23–7.37 (5H, m), 8.03 (1H, dd, $J_1$ = 8.0 Hz, $J_2$ = 5.5 Hz), 8.58 (1H, d, $J$ = 8.1 Hz), 8.88 (1H, d, $J$ = 4.6 Hz), 9.00 (1H, s, 1H); mp 127–130 °C; IR (KBr) 3267, 2955, 1589, 1455, 1075, 806, 704 cm$^{-1}$; MS (FAB): M + H$^+$: 285; Anal. Calcd for $C_{18}H_{26}N_2OBr_2$: C, 48.45, H, 5.87, N, 6.28. Found C, 48.38, H, 6.09, N, 6.08.

**5.1.1.6. 2-((3,4-Dimethoxyphenethyl)(propyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5f).** Yield: 25%. $^1$H NMR $\delta$ 0.93 (3H, t, $J$ = 7.2 Hz), 1.75 (2H, m), 3.00 (2H, m), 3.22–3.42 (6H, m, 6H), 3.73 (6H, m), 5.27 (1H, bs), 6.78–6.97 (3H, m), 7.66 (1H, dd, $J_1$ = 7.9 Hz in $J_2$ = 5.2 Hz), 8.16 (1H, d, $J$ = 8, 1 Hz), 8.67 (1H, dd, $J_1$ = 5.2 Hz, $J_2$ = 1.5 Hz), 8.79 (1H, s); $^{13}$C NMR $\delta$ 11.27, 16.79, 28.95, 53.51 or 53.72, 55.19, 55.52, 55.92, 58.09, 65.17, 112.37, 113.08 or 113.19, 121.18, 124.98, 129.58, 137.62, 138.54, 146.03, 147.16, 148.10, 149.16; mp 147–149 °C; IR (KBr) 3452, 3176, 2959, 1626, 1537, 1473, 1345, 1208, 1074, 1208, 1074, 1028, 895, 811. 681, 620 cm$^{-1}$; MS (EI): M + H$^+$: 345; Anal. Calcd for $C_{20}H_{29}N_2O_3Br_2$: C, 47.45, H, 5.97, N, 5.53. Found C, 47.37, H, 6.06, N, 5.51.

**5.1.1.7. 2-((3,4-Difluorophenethyl)(propyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5g).** Yield: 17%. $^1$H NMR $\delta$ 0.87 (3H, t, $J$ = 7.2 Hz), 1.66 (2H, m), 2.99 (2H, m), 3.15 (2H, m), 3.35–3.39 (4H, m), 5.20 (1H, dd, $J_1$ = 10.5 Hz in $J_2$ = 3.0 Hz), 7.09–7.33 (3H, m), 7.79 (1H, dd, $J_1$ = 7.9 Hz in $J_2$ = 5.3 Hz), 8.38 (1H, d, $J$ = 8.1 Hz), 8.61 (1H, dd, $J_1$ = 5.4 Hz in $J_2$ = 1.3 Hz), 8.70 (1H, d, $J$ = 1.5 Hz); mp 147–149 °C; IR (KBr) 3166, 2955, 1612, 1520, 1451, 1345, 1289, 1210, 1121, 1072, 943, 809, 680 cm$^{-1}$; MS (FAB): M + H$^+$: 321; Anal. Calcd for $C_{18}H_{24}N_2OF_2Br_2$: C, 44.83, H, 5.02, N, 5.81. Found C, 44.71, H, 5.10, N, 5.91.

**5.1.1.8. 2-((4-Nitrophenethyl(propyl)amino)-1-(pyridin-3-yl) ethanol dihydrobromide (5h).** Yield: 7%. $^1$H NMR $\delta$ 0.93 (3H, t, $J$ = 7.5 Hz), 1.79 (2H, m) 3.26–3.54 (8H, m), 5.46 (1H, bs), 7.65 (2H, m), 8.05 (2H, dd, $J_1$ = 8.1 Hz, $J_2$ = 5.6 Hz), 8.22 (1H, dd, $J_1$ = 8.3 Hz in $J_2$ = 4.6 Hz), 8.61 (1H, d, $J$ = 8.2 Hz), 8.88 (1H, dd, $J_1$ = 5.6 Hz, $J_2$ = 1.1 Hz), 9.02 (1H, d, $J$ = 1.7 Hz); mp

214–218 °C; IR (KBr) 3405, 3178, 2951, 1598, 1524, 1344, 1212, 1072, 857, 746, 680. $cm^{-1}$; MS (FAB): M + $H^+$: 330; Anal. Calcd for $C_{18}H_{25}ClN_2OBr_2$: C, 44.01, H, 5.13, N, 8.55. Found C, 44.15, H, 5.33, N, 8.25.

**5.1.1.9. 2-((3,4-(Dichlorophenethyl(methyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5i).** Yield: 15%. $^1H$ NMR $\delta$ 2.49 (3H, s), 2.98–3.53 (6H, m), 5.40 (1H, bs), 7.35 (1H, m), 7.60–7.70 (2H, m), 8.02 (1H, dd, $J_1$ = 7.9 Hz, $J_2$ = 5.5 Hz), 8.53 (1H, d, $J$ = 7.9 Hz), 8.87 (1H, d, $J$ = 4.4 Hz), 8.98 (1H, s); mp 157–161 °C; IR (KBr) 3228, 2932, 2852, 1588, 1470, 1131, 1029, 814 $cm^{-1}$; MS (FAB): M + $H^+$: 325; Anal. Calcd for $C_{16}H_{20}Cl_2N_2OBr_2$: C, 39.46, H, 4.14, N, 5.75. Found C, 39.52, H, 4.31, N, 5.59.

**5.1.1.10. 4-(2-((2-Hydroxy-2-(pyridin-3-yl)ethyl)(propyl)amino)ethyl)benzene-1,2-diol dihydrobromide (5j).** Yield: 4 %. $^1H$ NMR $\delta$ 0.91 (3H, t, $J$ = 7.3 Hz), 1.73 (2H, m), 2.90 (2H, m), 3.17–3.50 (6H, m), 5.37 (1H, bs), 6.50–6.70 (3H, m), 8.05 (1H, m), 8.60 (1H, d, $J$ = 8.4 Hz), 8.87 (1H, d, $J$ = 5.2 Hz), 8.97 (1H, s); IR (NaCl) 3374, 1609, 1528, 1458, 1364, 1287, 1119, 806 $cm^{-1}$; MS (FAB): M + $H^+$: 317; Anal. Calcd for $C_{18}H_{26}N_2O_3Br_2$+ 5/3 $H_20$: C, 42.54, H, 5.82, N, 5.51. Found C, 42.69, H, 6.10, N, 5.34.

**5.1.1.11. 2-(Propyl(4-(trifluoromethyl)phenethyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5k).** Yield: 1%. $^1H$ NMR $\delta$ 0.95 (3H, t, $J$ = 7.2 Hz), 1.78 (2H, m), 3.24–3.53 (8H, m), 5.44 (1H, bs), 7.60-7.76 (4H, m), 8.08 (1H, dd, $J_1$ = 8.0 Hz in $J_2$ = 5.6 Hz), 8.60 (1H, d, $J$ = 8.2 Hz), 8.90 (1H, d, $J$ = 4.6 Hz), 9.02 (1H, s); $^{13}C$ NMR $\delta$ 20.81, 26.40, 38.77, 62.60 or 63.69, 64.05 or 65.27, 67.18 or 67.28, 74.36, 132.49, 153.44, 136.33, 136.99, 137.40, 137.81, 139.85, 150.27, 151.95, 152.25, 152.91; mp 192–195 °C; IR (KBr) 1636, 1534, 1324, 1127 $cm^{-1}$; MS (FAB): M + $H^+$: 353; Anal. Calcd for $C_{19}H_{25}F_3N_2OBr_2$: C, 44.38, H, 4.90, N, 5.45. Found C, 44.15, H, 5.00, N, 5.32.

**5.1.1.12. 2-(Propyl(2-(trifluoromethyl)phenethyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (5l).** Yield: 6%. $^1H$ NMR $\delta$ 0.96 (3H, t, $J$ = 7.2 Hz), 1.78 (2H, m, 2H), 3.28–3.56 (8H, m), 5.44 (1H, bs), 7.49–7.78 (4H, m), 8.07 (1H, dd, $J_1$ = 7.8 Hz in $J_2$ = 5.9 Hz), 8.61 (1H, d, $J$ = 7.8 Hz), 8.91 (1H, d, $J$ = 5.4 Hz), 9.00 (1H, s); $^{13}C$ NMR $\delta$ 10.81, 16.19, 26.37, 52.79 or 53.57, 54.38 ali 55.00, 57.35, 64.36, 126.03, 126.48, 127.25, 127.77, 132.16, 132.95, 135.09, 140.36, 141.45, 142.56, 142.75; mp 180–183 °C; IR (KBr) 3410, 3166, 2984, 1626, 1537, 1452, 1315, 1177, 1126, 1045, 807, 775, 681 $cm^{-1}$; MS (FAB): M + $H^+$: 353; Anal. Calcd for $C_{19}H_{25}F_3N_2OBr_2$: C, 44.38, H, 4.90, N, 5.45. Found C, 44.30, H, 4.97, N, 5.42.

**5.1.1.13. 2-(Propyl-3-yl)ethyl)amino)-1-(pyridin-3-yl) ethanol (4m).** Yield: 6 %. $^1H$ NMR $\delta$ 0.92 (3H, t, $J$ = 7.3 Hz), 1.55–1.63 (2H, m), 3.20–3.30 (2H, m), 3.47–3.73 (6H, m), 5.04 (1H, dd, $J_1$ = 8.1 Hz, $J_2$ = 2.9 Hz), 7.25–7.36 (2H, m), 7.65–7.81 (2H, m), 8.42–8.62 (4H, m); IR (NaCl) 3359, 2966.1635, 1426, 1193, 1078, 714 $cm^{-1}$; MS (FAB): M + $H^+$: 286; HRMS *m/z* Calcd for $C_{17}H_{23}N_3O$ [$MH^+$] 286.191938. Found 286.192060.

### 5.1.2. General synthesis procedure B[33]

**5.1.2.1. 2-((3,4-(Dichlorophenethyl(propyl)amino)-1-(pyridin-4-yl)ethanol dihydrobromide (8a).** Yield: 20%. $^1H$ NMR $\delta$ 0.94 (3H, t, $J$ = 7.5 Hz), 1.76 (2H, m), 3.07–3.57 (8H, m), 5.47 (1H, bs), 7.32–7.73 (3H, m), 8.07 (2H, d, $J$ = 6.4 Hz), 8.94 (2H, d, $J$ = 6.4 Hz); mp 156–158 °C; IR (KBr) 3433, 3252, 2966, 1631, 1590, 1498, 1475, 1402, 1228, 1076, 807, 762 $cm^{-1}$; MS (FAB): M + $H^+$: 353; Anal. Calcd for $C_{18}H_{24}Cl_2N_2OBr_2$: C, 41.97, H, 4.70, N, 5.44. Found C, 41.71, H, 4.64, N, 5.19.

**5.1.2.2. 2-((2-Chlorophenethyl(propyl)amino)-1-(pyridin-4-yl)ethanol dihydrobromide (8b).** Yield: 20%. $^1H$ NMR $\delta$ 0.95 (3H, t, $J$ = 7.5 Hz), 1.77 (2H, m) 3.23–3.51 (8H, m), 5.40 (1H, bs), 7.32–7.53 (4H, m), 7.96 (1H, dd, $J_1$ = 8.0 Hz in $J_2$ = 5.3 Hz), 8.49 (1H, d, $J$ = 7.5 Hz), 8.84 (1H, d, $J$ = 5.3 Hz), 8.94 (1H, s); mp 184–187 °C; IR (KBr) 3414, 3172, 2930, 1626, 1537, 1476, 1348, 1209, 1094, 808, 763, 681 $cm^{-1}$; MS (FAB): M + $H^+$: 319; Anal. Calcd for $C_{18}H_{25}ClN_2OBr_2$: C, 44.98, H, 5.24, N, 5.83. Found C, 44.89, H, 5.47, N, 5.55.

**5.1.2.3. 2-((3-Chlorophenethyl(propyl)amino)-1-(pyridin-4-yl)ethanol dihydrobromide (8c).** Yield: 4%. $^1H$ NMR $\delta$ 0.91 (3H, t, $J$ = 7.5 Hz), 1.71 (2H, m) 3.04–3.40 (8H, m), 5.27 (1H, bs), 7.25–7.43 (4H, m), 7.87 (1H, dd, $J_1$ = 7.9 Hz in $J_2$ = 5.5 Hz), 8.39 (1H, d, $J$ = 8.2 Hz), 8.72 (1H, dd, $J_1$ = 5.4 Hz, $J_2$ = 1.2 Hz), 8.81 (1H, d, $J$ = 1.5 Hz); mp 133–135 °C; IR (KBr) 3465, 3168, 2963, 1616, 1525, 1448, 1288, 1209, 1071, 943, 807, 679 $cm^{-1}$; MS (FAB): M + $H^+$: 319; Anal. Calcd for $C_{18}H_{25}ClN_2OBr_2$: C, 44.98, H, 5.24, N, 5.83. Found C, 44.53, H, 5.16, N, 5.89.

**5.1.2.4. 2-((4-Chlorophenethyl(propyl)amino)-1-(pyridin-4-yl)ethanol dihydrobromide (8d).** Yield: 8%. $^1H$ NMR $\delta$ 0.92 (3H, t, $J$ = 7.5 Hz), 1.75 (2H, m, 2H) 3.04–3.49 (8H, m, 8H), 5.36 (1H, bs), 7.33 (2H, d, $J$ = 8.5 Hz), 7.43 (2H, d, $J$ = 7.8 Hz), 7.95 (1H, dd, $J_1$ = 7.8 Hz in $J_2$ = 5.6 Hz), 8.47 (1H, d, $J$ = 7.9 Hz), 8.83 (1H, d, $J$ = 4.4 Hz), 8.94 (1H, s); mp 194–196 °C; IR (KBr) 3406, 3176, 2956, 1624, 1538, 1489, 1414, 1348, 1210, 1092, 811, 680, 645 $cm^{-1}$; MS (FAB): M + $H^+$: 319; Anal. Calcd for $C_{18}H_{25}ClN_2OBr_2$: C, 44.98, H, 5.24, N, 5.83. Found C, 44.72, H, 5.41, N, 5.47.

**5.1.2.5. 2-((4-Phenylbutyl)(propyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (8e).** Yield: 8%. $^1H$ NMR $\delta$ 0.92 (3H, t, $J$ = 7.3 Hz), 1.58–1.74 (6H, m), 2.55–2.68 (2H, m), 3.11–3.47 (6H, m), 5.35 (1H, d, $J$ = 7.8 Hz), 7.17–7.34 (5H, m), 8.06 (1H, dd, $J_1$ = 8.0 Hz in $J_2$ = 5.7 Hz), 8.59 (1H, d, $J$ = 7.9 Hz), 8.87 (1H, d, $J$ = 5.7 Hz), 8.95 (1H, s); mp 129–131 °C; IR (KBr) 3449, 3170, 2363, 1609, 1545, 1472, 1379, 1322, 1258, 1076, 908, 807, 753, 702 $cm^{-1}$; MS (FAB): M + $H^+$: 313; Anal. Calcd for $C_{20}H_{30}N_2OBr_2$: C, 50.65, H, 6.38, N, 5.91. Found C, 50.29, H, 6.64, N, 5.79.

**5.1.2.6. 2-(Butyl(3,4-dichlorophenethyl)amino)-1-(pyridin-3-yl)ethanol dihydrobromide (8f).** Yield: 19%. $^1$H NMR $\delta$ 0.91 (3H, t, $J$ = 7.2 Hz), 1.33–1.40 (2H, m), 1.73 (2H, m), 3.12 (2H, m), 3.32–3.61 (6H, m), 5.47 (1H, dd, $J_1$ = 10.5 Hz in $J_2$ = 3.0 Hz), 7.23 (1H, d, $J$ = 6.8 Hz), 7.48 (2H, d, $J$ = 8.3 Hz), 8.08 (1H, dd, $J_1$ = 8.3 Hz in $J_2$ = 6.0 Hz), 8.61 (1H, d, $J$ = 8, 7 Hz), 8.77 (1H, d, $J$ = 5.6 Hz), 8.88 1H, d, $J$ = 1.7 Hz); mp 134–137 °C; IR (KBr) 3479, 3232, 2932, 1726, 1517, 1420, 1264, 1159, 1028, 811, 682 cm$^{-1}$; MS (FAB): M + H$^+$: 367; Anal. Calcd for $C_{19}H_{26}N_2OCl_2Br_2$: C, 43.13, H, 4.95, N, 5.29. Found C, 43.33, H, 5.13, N, 5.12.

**5.1.2.7. 2-(Butyl(2,4-dichlorophenethyl)amino)-1-(pyridin-3-yl)ethanol dibenzenesulfonate (8g).** Yield: 8%. $^1$H NMR $\delta$ 0.94 (3H, t, $J$ = 7.5 Hz), 1.36 (2H, m), 1.72 (2H, m), 3.48 (8H, m), 5.31 (1H, bs), 7.28–7.34 (10H, m), 7.46–7.60 (3H, m), 7.88 (1H, dd, $J_1$ = 8.0 Hz in $J_2$ = 5.3 Hz); mp 133–135 °C; IR (KBr) 3390, 2960, 1633, 1561, 1476, 1444, 1382, 1235, 1167, 1123, 1015, 820, 728, 611, 563 cm$^{-1}$; MS (FAB): M + H$^+$: 367; Anal. Calcd for $C_{31}H_{36}Cl_2N_2O_7S_2$: C, 54.46, H, 5.31, N, 4.10. Found C, 54.70, H, 5.50, N, 4.11.

### 5.1.3. Synthesis procedure C

**5.1.3.1. *N*-(3,4-Dichlorophenethyl)-3-phenyl-*N*-(2-(pyridin-3-yl)ethyl)propan-1-amine dihydrobromide (12b).** To a solution of 3,4-dichlorophenethylamine (5.00 g, 26.3 mmol) and 2-(pyridin-3-yl)acetic acid hydrochloride (4.90 g, 28.2 mmol) in DMF (20 mL) was added HOBT (5.80 g, 28.2 mmol). The pH of the solution was adjusted to 8 by adding *N*-methylmorpholine. EDC (5.63 g, 29.1 mmol) was added. After the reaction mixture was stirred at rt overnight, the solvent was evaporated under reduced pressure and the residue dissolved in EtOAc (30 mL). The organic layer was washed with aq saturated $NaHCO_3$ (30 mL) and NaCl (30 mL) solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure to give a product which was chromatographed on silica (MeOH/EtOAc, 2:10) to give dark yellow oil **9b**; yield: 6.60 g, 85%.

The solution of compound **9b** (1.38 g, 4.5 mmol) in dry THF (10 mL) was heated to reflux and 2 M solution of borane-dimethyl sulfide complex in diethyl ether (8.0 mL, 17.0 mmol) was added in drops over the period of 15 min, allowing dimethyl sulfide to distill off. The reaction mixture was refluxed for 10 h. The THF solution was then hydrolyzed during addition of 6 N HCl (3.0 mL, 18.0 mmol). After 30 min, the clear solution obtained was cooled to rt and neutralized with 6 N NaOH (4.5 mL, 25.0 mmol). The reaction mixture was stirred at rt for another 1 h. EtOAc (20 mL) was added and the organic layer was washed with aq saturated $NaHCO_3$ (20 mL) and NaCl (20 mL) solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure to give a product which was further chromatographed on silica (MeOH/EtOAc, 2:10) to give colorless oil **10b**; yield: 0.87 g, 65%.

Secondary amine **10b** (0.30 g, 1.2 mmol) and $PhCH_2CH_2CHO$ (0.27 mL, 2.5 mmol) were dissolved in 1,2-dichloroethane (10 mL) and then treated with $NaBH(OAc)_3$ (0.337 g, 1.6 mmol). The mixture was stirred at rt under an Ar atmosphere overnight. The reaction mixture was quenched by adding aq saturated $NaHCO_3$ (20 mL) solution and the product was extracted with EtOAc (20 mL). The EtOAc extract was dried ($Na_2SO_4$) and the solvent was evaporated under reduced pressure to give the crude free base, which was chromatographed on silica (MeOH/EtOAc, 2:10) to give a colorless oil **11b**; yield: 0.26 g, 62%. The product was converted to the dihydrobromide salt **12b**; yield: 0.27 g, 74% (96% pure by area% HPLC analysis).

Overall yield: 17%. $^1$H NMR $\delta$ 2.00–2.11 (2H, m), 2.64–2.69 (2H, m), 3.07–3.13 (2H, m), 3.28–3.59 (6H, m), 7.19–7.70 (8H, m), 8.05 (1H, dd, $J_1$ = 8.0 Hz, $J_2$ = 5.6 Hz), 8.59 (1H, d, $J$ = 8.1 Hz), 8.87 (1H, d, $J$ = 5.6 Hz), 9.00 (1H, d, $J$ = 1.5 Hz); mp 154–157 °C; IR (KBr) 3460, 1636, 1557, 1474, 1032, 819, 684 cm$^{-1}$; MS (FAB): M + H$^+$: 413; Anal. Calcd for $C_{24}H_{28}Cl_2N_2Br_2$: C, 50.11, H, 4.91, N, 4.87. Found C, 50.05, H, 4.43, N, 5.09.

**5.1.3.2. *N*-(3-Fluorophenethyl)-*N*-(2-(3-pyridinyl) ethyl) propan-1-amine dihydrobromide (12a).** Yield: 22%. $^1$H NMR $\delta$ 0.94 (3H, t, $J$ = 7.5 Hz), 1.76 (2H, m) 3.09–3.53 (10 H, m), 7.07–7.43 (4H, m), 8.05 (1H, dd, $J_1$ = 7.9 Hz in $J_2$ = 5.8 Hz), 8.60 (1H, dd, $J_1$ = 11.6 Hz in $J_2$ = 1.5 Hz), 8.87 (1H, d, $J$ = 5.5 Hz), 9.01 (1H, s); $^{13}$C NMR $\delta$ 10.86, 16.66, 25.87, 28.78, 51.55, 52.80, 53.67, 113.70, 115.74, 125.13, 126.66, 130.52, 136.64, 139.73, 141.05, 142.81, 146.13, 163.83; mp 142–144 °C; IR (KBr) 3424, 2938, 1612, 1561, 1473, 1442, 1243, 1146, 938, 780, 678 cm$^{-1}$; MS (FAB): M + H$^+$: 287; Anal. Calcd for $C_{18}H_{25}ClN_2OBr_2$: C, 48.23, H, 5.62, N, 6.25. Found C, 48.22, H, 5.81, N, 6.08.

### 5.1.4. Synthesis procedure D

**5.1.4.1. 2-(2-(Naphthalen-2-yl)ethyl](propyl)amino]-1-(pyridin-3-yl)ethanol dihydrobromide (16).** (2-Naphthalen-2-yl)-acetonitrile (5.0 g, 30.0 mmol) in dry THF (15 mL) was heated to reflux and 2 M solution of borane-dimethyl sulfide complex in diethyl ether (16.5 mL, 33.0 mmol) was added. The dimethyl sulfide liberated in the course of the reaction was allowed to distill off. The heating was continued for 5 h. The THF solution was then hydrolyzed during addition of 6 N HCl (14 mL, 84 mmol). After 30 min, the clear solution obtained was cooled to rt and neutralized with 6 N NaOH (21 mL, 126 mmol). The reaction mixture was stirred at rt for another 1 h. EtOAc (30 mL) was added and the organic layer was washed with aq saturated $NaHCO_3$ (30 mL) and NaCl (30 mL) solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure to give a product which was further chromatographed on silica (MeOH/EtOAc, 2:10 to gradient elution MeOH/EtOAc, 10:2) to give a pale yellow solid **13**. Final compound **16** was further prepared by reaction steps described in procedure B. Yield: 7%. $^1$H NMR $\delta$ 0.94 (3H, t, $J$ = 7.5 Hz), 1.75–1.83 (2H, m) 3.15–3.59 (8H, m), 5.43 (1H, bs), 7.45–8.03 (8H, m), 8.56 (1H, d, $J$ = 7.9 Hz), 8.87 (1H, d, $J$ = 5.3 Hz), 8.99 (s, 1H, $H_2$-Py); $^{13}$C NMR $\delta$ 10.89, 16.30 or 16.47, 26.55, 52.83,

53.52, 54.19, 5.13, 56.94 or 57.27, 64.39 or 64.48, 123.61, 125.69, 125.93, 126.46 or 126.54, 127.57, 128.75, 131.29, 133.07, 140.13, 141.99, 143.30; mp 130–132 °C; IR (KBr) 3166, 2953, 1626, 1538, 1453, 1350, 1217, 1094, 807, 755, 681, 621 $cm^{-1}$; MS (FAB): $M + H^+$: 335; Anal. Calcd for $C_{22}H_{28}N_2OBr_2$: C, 53.24, H, 5.69, N, 5.64. Found C, 52.87, H, 5.63, N, 5.15.

### 5.1.5. Synthesis procedure E

**5.1.5.1. 1-(Phenethyl(propyl)amino)-3-(pyridin-3-yloxy) propan-2-ol (19).** To a solution of NaOH (1.0 g, 25.0 mmol) in water (15 mL) was added pyridin-3-ol (2.38 g, 25.0 mmol), followed by 2-(chloromethyl)oxirane, keeping the reaction temperature below 35 °C. The mixture was then stirred at room temperature for 12 h. After cooling, the solvent was evaporated under reduced pressure to give a product which was further chromatographed on silica (MeOH) to give a brown oil **17**, yield: 1.134 g, 30%.

To a solution of compound **17** (1.5 g, 10.0 mmol) in anhyd EtOH (30 mL) were added phenethylamine (1.25 mL, 10.0 mmol), $K_2CO_3$ (1.38 g, 3.3 mmol), and KI (0.015 g, 0.1 mmol). The mixture was stirred at 80 °C for 4 h. After cooling, the solvent was evaporated under reduced pressure to give a product which was further chromatographed on silica (MeOH) to give a brown oil **18**, yield: 1.252 g, 46%.

Secondary amine **18** (0.982 g, 3.6 mmol) and $CH_3CH_2CHO$ (0.8 mL, 7.4 mmol) were dissolved in 1,2-dichloroethane (30 mL) and then treated with $NaBH(OAc)_3$ (1.33 g, 6.3 mmol). The mixture was stirred at rt under an Ar atmosphere overnight. The reaction mixture was quenched by adding aq saturated $NaHCO_3$ (30 mL) solution and the product was extracted with EtOAc (30 mL). The EtOAc extract was dried ($Na_2SO_4$) and the solvent was evaporated under reduced pressure to give the crude free base, which was chromatographed on silica (MeOH) to give a brown oil **19**; yield: 0.226 g, 20% (95% pure by area% HPLC analysis).

Overall yield: 3%. $^1H$ NMR $\delta$ 0.93 (3H, t, $J = 7.2$ Hz), 1.52 (2H, m), 1.53–1.61 (4H, m), 2.78 (4H, s), 3.91 (2H, m), 4.29 (1H, d, $J = 10.5$ Hz), 7.15–7.58 (9H, m); $^{13}C$ NMR $\delta$ 12.21, 21.20, 34.39, 57.61, 57.75, 59.14, 65.98, 70.15, 127.09, 128.01, 128.40, 130.03, 134.61, 136.03, 142.10, 169.13; IR (NaCl) 3380, 2970, 1726, 1563, 1462, 1369 $cm^{-1}$; MS (FAB): $M + H^+$: 315; HRMS *m/z* Calcd for $C_{19}H_{25}N_2O_2$ [$MH^-$] 313.191603. Found 313.192350.

**5.1.5.2. Reagents and solvents for the biological section.** [$^3H$] acetate (NEN$^{TM}$Life Science Products); trypsin, calf serum, L-glutamine, Dulbecco's modified Eagle's medium (DMEM high) (Sigma); Bio-Rad reagent (Bio-Rad Lab); ketoconazole (Sigma); commercial sterol standards (Steraloids): lathosterol, zymosterol, 7-dehydrocholesterol, desmosterol, lanosterol, ergosterol, and cholesterol; laboratory standards: FF-MAS (Laboratory of Reproductive Biology, The Juliane Marie Center for Children, Women and Reproduction, University Hospital of Copenhagen, DK-2100 Copenhagen, Denmark); atorvastatin (Lek d. d.)

**5.1.5.3. Cell culture incubation with new compounds and atorvastatin.** Human hepatoma cell line ($HepG_2$-ATCC No. HB-8065) was split in the recommended ratio (1:2-3), 75 $cm^2$cell flasks, using four flasks per experimental condition. Cells were incubated at 37°C with 5% $CO_2$ in DMEM high containing 5% calf serum and 1% L-glutamine. After 24 h culturing the medium was replaced with the one supplemented with 10 μM concentration of a selected compound. 10 μM solution of atorvastatin served as a positive control.

After 24 h the growth medium was replaced and [$^3H$] acetate added in a concentration of 40 μCi per ml of the medium (400 μCi per flask). After 4 h cells were harvested using 2 mL of trypsin and the cell pellet resuspended in 1 mL of distilled water. Cells were homogenized using the freeze and thaw method. From cell homogenate sterols were extracted and protein concentration determined using the Bio-Rad protein assay.

**5.1.5.4. Sterol extraction.** Homogenates were transferred into 4 mL glass vials. Internal standard ergosterol (0.25 mg/mL) was added prior to extraction process. Sterols were extracted in 3 mL of extraction solution (75% *n*-heptane: 25% isopropanol (vol./vol.)), with addition of 100 μL of 0.3 M $NaH_2PO_4$ (pH 1.0). Closed vials were vigorously shaken (1800 rpm) for 2 h. After extraction procedure vials were centrifuged (2000*g*, 10 min) and organic phase transferred to fresh conical glass tubes. Extraction procedure was repeated using 1 ml of extraction solution for 15 min. Organic phases were pooled.

Primary extracts were dried in vacuum centrifuge, redissolved in 2 ml of *n*-heptane, and incubated for 10 min at the room temperature with mild shaking. After centrifugation (10 min, 2000*g*) extracts were transferred into fresh glass tubes and stored in dark and cold.

**5.1.5.5. HPLC analysis.** The organic phase was dried, reconstituted in mobile phase for HPLC reversed phase separation, and loaded onto a Prism-RPN, 5 μm, 250 × 4.6 mm HPLC column running in 100% acetonitrile at 1.00 mL/min at 40 °C temperature. Scintillation liquid was added after UV detection at 30 mL/h, at the room temperature, to evaluate tritium labeled sterols on the radiodetector.

Sterol's retention times were determined by comparing eluted peaks with runs of the following standards: lathosterol, zymosterol, 7-dehydrocholesterol, desmosterol, lanosterol, cholesterol, and FF-MAS. The non-availability of $^3H$-labeled sterol standards disables the exact quantification of $^3H$-labeled sterols. Thus, radiodetected sample peak areas were compared to normal medium peak areas resulting in the relative levels of sterols.

Results were also normalized on ergosterol quantity and protein concentration. Ergosterol served as an internal

standard for the extraction procedure. Bradford assay has been used to determine the protein concentration. Protein concentration is not a direct measure of cell viability, however, it correlates to the cell number.

Two separate experiments with two replicas were done. Each replicon of compound was calculated with each non-treated value resulting in four calculations of one experiment. Results are presented as a mean of 8 calculated ratios and a corresponding standard deviation.

**5.1.5.6. Determination of the apparent dissociation constant on purified human CYP51.** CYP51 was expressed and purified by the methods described previously[39] (see SDS page in the supplementary data). Binding of nine novel compounds to the enzyme was determined spectrophotometrically. Human CYP51 was titrated with an inhibitor and the resulting absorbance difference spectra were plotted as a function of the inhibitor concentration. Spectra were taken at the room temperature in buffer containing 50 mM phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, and 10% glycerol (buffer A) on dual-beam UV spectrophotometer. Ketoconazole and **4m** were dissolved in DMSO. Other compounds were dissolved in water. Inhibitor induced spectral changes were monitored as type II spectral difference response.[21,36] CYP51 solution in buffer A was first blanked with reference (buffer A) and than titrated with inhibitor until maximal spectral response was reached. Spectra were recorded from 350-500 nm and absorbance difference between 412 nm and 432 nm calculated. For determination of the apparent dissociation constant binding assays were repeated three times for each novel compound. Concentration range for the titration was determined from the preliminary screen and was for **16** and **5d**: 0.25–21 μM, for **8d**: 1–33 μM, for **5g**, **5f**, and **8b**: 5–140 μM, for **5a**: 20–200 μM, and for **4m**: 80–400 μM. Total volume added during titration did not exceed 1.5% of CYP51 solution. Concentration of CYP51 protein for the titration with **16** and **5d** was 0.5 μM, for the titration with other tested compounds concentration of CYP51 was 2 μM. Measured data were fitted to the hyperbola with the equation: $\Delta A = A_{max}[I]_{free}/(K_d + [I]_{free})$ by using nonlinear regression in the SPSS software. The concentration of free inhibitor was expressed as: $[I]_{free} = [I]_{total} - [CYP51]\Delta A/A_{max}$, where $\Delta A$ is absorbance difference between 412 nm and 432 nm from type II difference spectra and $A_{max}$ maximal spectral response.

## Acknowledgments

This work was supported by the funds from Lek Pharmaceuticals d. d., Slovenian Research Agency Grants L-6707, J1-6713, and P1-0527, the ESSR supported activity Center of Excellence—Biotechnology with Pharmacy, and by European Community (STEROLTALK Project No. LSHG-CT-2005-512096). Matej Seliškar and Klementina Fon Tacer were supported by fellowships from Slovenian Research Agency. We thank Mogens Baltsen and Dr. A.G. Byskov (Laboratory of Reproductive Biology, University Hospital of Copenhagen) for FF-MAS and T-MAS and Prof. Dr. M.R. Waterman (Institute of Biochemistry, Medical Faculty, Vanderbilt University, Nashville, TN) for the human CYP51 expression plasmid.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.10.001.

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