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PYRIDYL SUBSTITUTED BENZOCYCLOALKENES: NEW INHIBITORS OF 17α-HYDROXYLASE/ 17,20-LYASE (P450 17α)

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Compounds capable of inhibiting 17α-hydroxylase / 17,20-lyase (P450 17α) are of great interest for the therapy of prostatic cancer since they block androgen biosynthesis. In order to evaluate the inhibitory activity of a series of benzocycloalkenes developed in our group, an *in vitro* assay was established using rat testicular microsomes as source of the enzyme, non labelled progesterone as substrate and a HPLC procedure for separation of the steroids. The inhibitory activities of 33 test compounds were compared to ketoconazole (IC_{50} 67 μM), a known inhibitor of P450 17α, which recently has been successfully used in prostate cancer patients. Several compounds of the present study were stronger inhibitors of P450 17α than ketoconazole. The most active compounds were compound **12**(5-methoxy-2-(4-pyridylmethyl)-1-tetralone: IC_{50} 13 μM) and compound **13**(5-methoxy-2-(4-pyridyl)-1-tetralone: IC_{50} 13 μM).

KEY WORDS: inhibitors of 17α-hydroxylase / 17,20-lyase (P450 17α), androgen dependent prostate carcinoma, pyridyl substituted benzocycloalkenes, *in vitro* assay, HPLC

INTRODUCTION

The microsomal cytochrome P450 dependent monooygenase 17α-hydroxylase / 17,20-lyase (EC 1.14.99.9 / EC 4.1.2.30; P450 17α) is a key enzyme for the formation of androgens. It catalyzes the 17α-hydroxylation of progesterone (especially in rats) and pregnenolone (especially in humans). These hydroxylated steroids undergo scission of the C-17,20 carbon bond to yield androstenedione or dehydroepiandrosterone and acetic acid (Figure 1). Both reactions are catalyzed by the same, gene product[1] and occur at the same active site[2]. In humans P450 17α is located in the gonads as well as in the adrenal cortex whereas in rats it is only located in the gonads.

Inhibitors of P450 17α are of increasing interest for the treatment of prostatic carcinoma, as nearly 80% of these malignancies are androgen dependent. The antifungal drug ketoconazole (cis-1-acetyl-4-(4-((2-(2,4-dichlorphenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl)methoxy)phenyl)piperazine) blocks ergosterol biosynthesis in the yeast cell by inhibition of the cytochrome P450 dependent lanosterol

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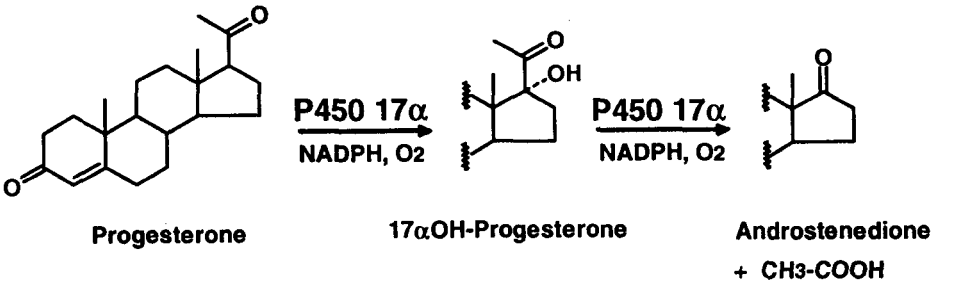


FIGURE 1 The conversion of progesterone to androstenedione catalyzed by P450 17α

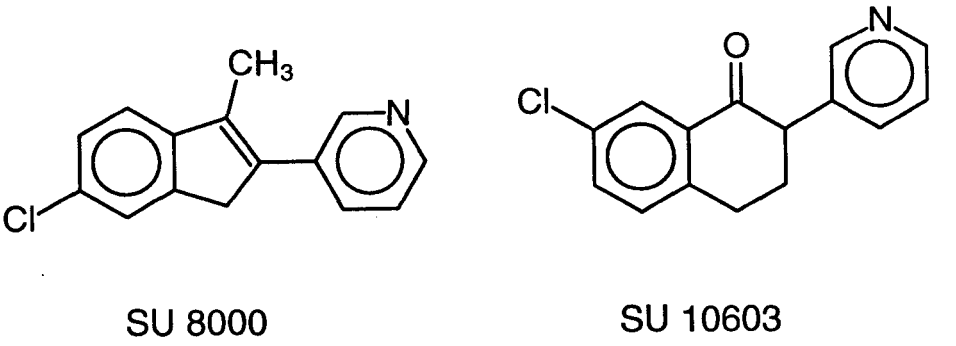


FIGURE 2 Structures of SU 8000 and SU 10603

14α-demethylase[3]. It also blocks testosterone biosynthesis by inhibition of P450 17α[4] and has already been successfully used in patients with prostatic cancer[5]. Ketoconazole, however, is far from being an optimal drug: it rather is a universal inhibitor of cytochrome P450 dependent enzymes[6].

The indene SU 8000 and the tetralone SU 10603 (Figure 2) are well known to inhibit P450 17α (K_i-values 0.04 μM and 0.3 μM, respectively[1]). Structurally related to these compounds are aromatase (P450 arom) inhibitors developed in our group[7,8,9]. The aromatase inhibitory activity of compounds **1-33** differed greatly by a factor of more than 100[7,8,9]. Since inhibition of aromatase as a side effect of P450 17α inhibitors should not be disadvantageous in male subjects we have determined the inhibitory activities of compounds **1-33** toward P450 17α in search of new leads for anti-prostate cancer drugs.

MATERIALS AND METHODS

Animals and Chemicals

Rats were purchased from Charles River (Sulzfeld, FRG). All inhibitors were synthesized in our laboratories[7,8,9]. Ketoconazole was kindly provided by H. Vanden Bossche from Janssen Pharmaceutica (Beerse, Belgium). Biochemials were obtained from Sigma (Deisenhofen, FRG), Serva (Heidelberg, FRG) and Fluka (Neu-Ulm, FRG).

Enzyme Preparation

Adult male Sprague-Dawley rats were sacrificed by CO_2 and cervical dislocation. The testes were removed, washed twice with ice-cold NaCl (0.9%), decapsulated, weighed and homogenized in a buffer containing 0.25 M sucrose, 0.01 M Tris and 0.001 M EDTA (pH 7.4; 3 ml per g tissue). The homogenate was centrifuged at 12,000 × g for 30 min in order to remove cell debris and mitochondria. The resulting supernatant was subsequently centrifuged at 105,000 × g for 1 h. The pelleted microsomes were washed once with 0.1 M sodium phosphate buffer (pH 7.4) and resuspended in a few ml (about 0.25 ml per testis) of 0.1 M sodium phosphate buffer containing 20% glycerol. All steps were carried out at 4°C. Protein content was determined by the method of Lowry *et al.* [10] using bovine serum albumin (fraction V) as standard. Aliquots of this preparation, which generally had a content of about 30 mg protein per ml, were stored at -70°C until used.

Enzyme Assay

The assay was performed in 1.5 ml micro test tubes (Eppendorf). Microsomes (about 250 μg protein) were incubated with 6.25 nmol progesterone (in 5 μl methanol), 125 nmol NADPH and inhibitor (in 5 μl methanol) in a total volume of 250 μl. The incubation medium was 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM dithiothreitol as described by Barrie *et al.* [11]. The temperature was 32°C. After a preincubation time of 5 min the reaction was started by addition of enzyme. After 20 min it was terminated by addition of 50 μl 1 N HCl.

Extraction of Steroids

Extraction of steroids was accomplished by the addition of 1.0 ml ethyl acetate by vortexing for 10 min. The tubes were then centrifuged for 5 min at 2,500 × g. The organic phase was removed, vortexed twice with 250 μl of incubation buffer and 50 μl of 1 N HCl and then dried. Aliquots of 25 μl methanol containing 250 pmol of fluorocortisol acetate as internal standard were added to the extracts. 20 μl were submitted to HPLC.

HPLC Procedure

The HPLC system consisted of a high pressure pump (Jasco 880 PU, Groß Zimmern, FRG), an injector (Latek model 7125, Eppelhein, FRG) with a 50 μl loop and a diode array detector (Perkin Elmer LC-480 Auto Scan, Überlingen, FRG) with a 4.5 μl cell (optical path 1 cm). A 125 × 4 mm Nucleosil® 120 3 μm RP-8 column with an analog guard column of 8 mm length (Macherey und Nagel, Düren, FRG),

operated at ambient temperature, was used to separate steroids which were eluted with methanol/water (50:50) at a flow rate of 1 ml/min. UV absorbance was monitored at 240 nm. Peak areas were determined using a data evaluation software (Perkin Elmer LC-DES plus). Enzymatic activity was measured as absorbance of all products, i.e. 17α-hydroxyprogesterone, androstenedione and testosterone. The lower limit for detection of all steroids measured was affuscimately 5 pmol.

RESULTS

The enzyme assay described herein differs from other procedures by the fact that there is no radiolabelled substrate used: The absorbance of the 4-ene-3-oxo-steroids at 240 nm is sufficient for their quantification. The rate of reaction was linear with time and protein concentration during the incubation period. The enzyme activity was about 22 nmol per mg protein per hour. In a first run all compounds were tested at 125 μM. Figure 3 shows a HPLC profile of an incubation in absence and in presence of ketoconazole. For compounds causing an inhibition over 80%, IC_{50} values were also determined.

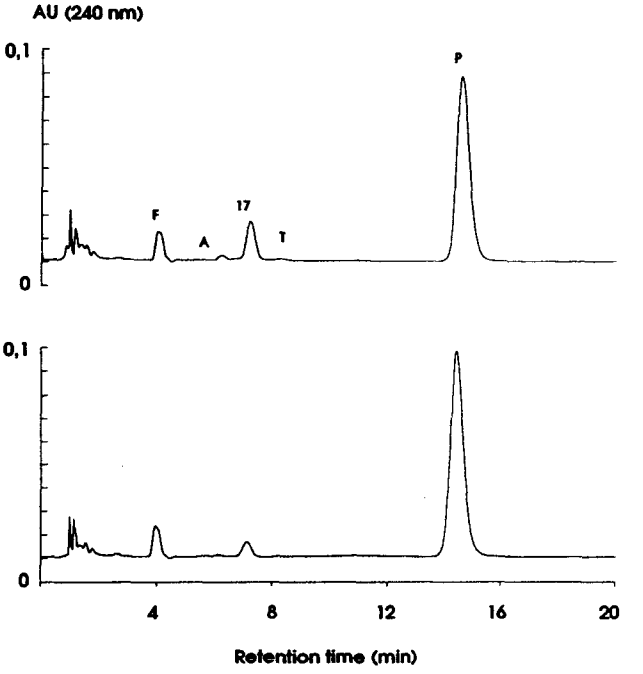
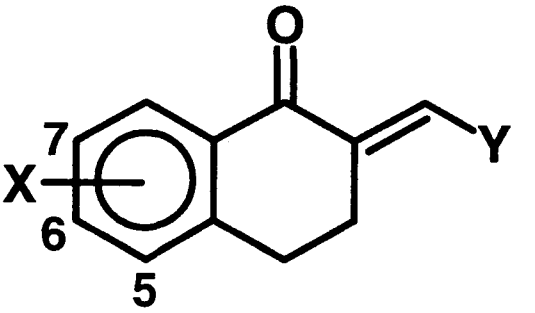


FIGURE 3 HPLC analysis of an incubation without (above) and with ketoconazole (below). A = androstenedione; 17 = 17α-hydroxyprogesterone (main product); T = testosterone; P = progesterone (substrate); F = fluorocortisol acetate (internal standard).

TABLE 1
Inhibition of rat testicular P450 17α by substituted 2-(pyridylmethylene)- and 2-(phenylmethylene)-1-tetralones



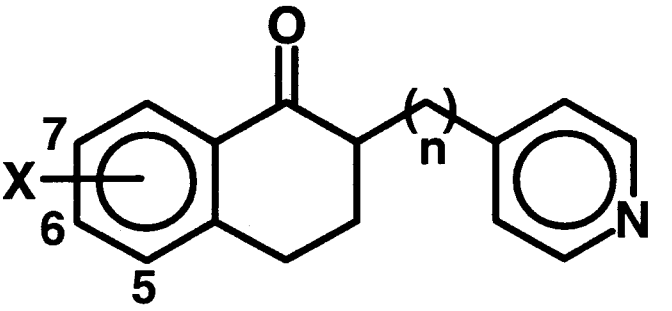
Compound	X	Y	E/Z	% Inhibition[a]
1	H	4-pyridyl	E	37
2	5-OCH_3	4-pyridyl	E	53
3	5-OH	4-pyridyl	E	23
4	6-OH	4-pyridyl	E	8
5	7-OH	4-pyridyl	E	10
6	H	4-pyridyl	Z	70
7	H	3-pyridyl	E	48
8	H	2-pyridyl	E	4
9	H	phenyl	E	0

[a]Concentration of inhibitor: 125 μM. Concentration of substrate: 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 62%. All values are the mean of at least 2 determinations.

The inhibiting activities of the test compounds toward 17α-hydroxylase/17,20-lyase are given in Tables 1-5. The unsaturated tetralones **1-9** (Table 1) caused inhibitions ranging from 0 to 70%. The 2-pyridyl and phenyl substituted compounds **8** and **9** did not show inhibiting activity while the 4- and 3-pyridyl substituted compounds **1** and **7** were active. The 3-pyridyl substituted compound **7** exceeded the activity of the 4-pyridyl derivative **1**. The Z-isomer (compd **6**), was more active than the corresponding E-isomer (compd **1**). A methoxy substituent in position-5 (compd **2**) increased inhibitory activity whereas hydroxy substituents in position-5,-6 and-7 decreased it (compds **3**, **4** and **5**).

The hydrogenated tetralones **10**, **12**, **16**, **17** and **18** (Table 2) were stronger inhibitors than the corresponding unsaturated analogues (compds **1** to **5**). The 5- and 6-methoxy as well as the 5- and 7-hydroxy derivatives (compds **12**, **15**, **16** and **18** were more active, whereas the 6-hydroxy compound **17** was less active than the unsubstituted compound **10**. Removal of the CH_2-spacer between the tetralone and 4-pyridine moiety did not influence enzyme inhibition significantly (compd **10** vs. **11** and **12** vs. **13**). An additional CH_2-spacer decreased activity slightly (compd **12** vs. **14**).

TABLE 2
Inhibition of rat testicular P450 17α by substituted 2-(4-pyridylalkyl)-1-tetralones



Compound	X	n	% Inhibition[a]
10	H	1	67
11	H	0	71
12	5-OCH_3	1	84
13	5-OCH_3	0	86
14	5-OCH_3	2	70
15	6-OCH_3	1	83
16	5-OH	1	83
17	6-OH	1	34
18	7-OH	1	79

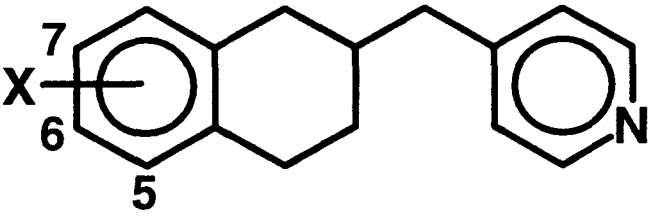
[a]Concentration of inhibitor: 125 μM. Concentration of substrate: 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 62%. All values are the mean of at least 2 determinations.

The tetralines **19-22** (Table 3) were stronger inhibitors than the corresponding unsaturated tetralones **1, 3, 4** and **5**. The unsubstituted compound **19** turned out to be more active than the corresponding saturated tetralone **10**. Substitution of compound **19** with hydroxy substituents in position-5, -6 and -7 led to a loss of activity (compds **20** to **22**).

In case of the unsaturated indanones **24** to **28** (Table 4) the unsubstituted compound **24** showed less inhibiting properties than the corresponding tetralone **1**. The 5-methoxy and 4-hydroxy derivatives **26** and **27**, however, turned out to be more active than **1**. The 4-methoxy substituted compound **25** was as potent as the unsubstituted compound **24** whereas the 5-hydroxy derivative **28** was nearly inactive.

The hydrogenated indanone **29** (Table 5) was more active than the corresponding unsaturated indanone **24** and as active as the corresponding tetralone **10**. 5-Methoxy substitution did not influence activity (compd **31**) whereas 4-hydroxy substitution decreased enzyme inhibitory properties (compd **30**). The 4-hydroxy substituted indan **32** was more active than the corresponding indanone **30**. The 5-hydroxy indan was the most potent compound of this series.

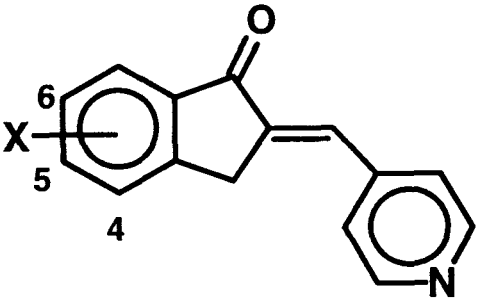
TABLE 3
Inhibition of rat testicular P450 17α by substituted 2-(4-pyridylmethyl)-tetralines



Compound	X	% Inhibition
19	H	85
20	5-OH	55
21	6-OH	70
22	7-OH	67

[a]Concentration of inhibitor: 125 μM. Concentration of substrate: 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 62%. All values are the mean of at least 2 determinations.

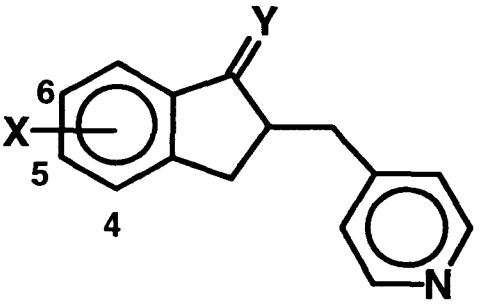
TABLE 4
Inhibition of rat testicular P450 17α by substituted 2-(4-pyridylmethylene)-1-indanones



Compound	X	% Inhibition
24	H	24
25	4-OCH_3	27
26	5-OCH_3	40
27	4-OH	51
28	5-OH	7

[a]Concentration of inhibitor: 125 μM. Concentration of substrate: 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 62%. All values are the mean of at least 2 determinations.

TABLE 5
Inhibition of rat testicular P450 17α by substituted 2-(4-pyridylmethyl)-1-indanones and 2-(4-pyridylmethyl)-indans



Compound	X	Y	% Inhibition[a]
29	H	=O	66
30	4-OH	=O	33
31	5-OCH_3	=O	67
32	4-OH	-H, -H	43
33	5-OH	-H, -H	75

[a]Concentration of inhibitor: 125 μM. Concentration of substrate: 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 62%. All values are the mean of at least 2 determinations.

TABLE 6
IC_{50}-values of the most active compounds

Compound	IC_{50} μM[a]	Compound	IC_{50} μM[a]
12	13	**16**	25
13	13	**18**	39
15	19	**19**	22

[a]IC_{50} is the concentration of inhibitor required to give 50% inhibition. Concentration of substrate: 25 μM. The IC_{50} of ketoconazole under identical experimental conditions was 67 μM. All values are the mean of at least 2 determinations.

Comparing the IC_{50} values of the most active compounds (Table 6) it becomes apparent that they are clearly superior to ketoconazole. Maximum activity was shown by compound **12** (IC_{50} 13 μM) and compound **13** (IC_{50} 13 μM).

DISCUSSION

The present work describes a series of new P450 17α inhibitors. They probably are competitive inhibitors like ketoconazole[6] as shown by a parallel course of the corresponding dose response curves. Comparison of the tetralones **1**, **7**, **8** and **9** demonstrates the indispensableness of a nitrogenous ligand which is capable of interacting

with the heme iron of the cytochrome P450 component of the enzyme. In case of the pyridylmethylene tetralones position-3 is most favourable for the nitrogen atom. The addition of a further CH_2-spacer lowers the activity (compd **12** vs. **14**). Thus, it can be concluded that a spacer with 3 or 4 carbon atoms is necessary between C-2 of the tetralone moiety and the nitrogen. On the other hand in SU 10603 the spacer consists of only 2 carbon atoms. The lack of activity of the 2-pyridyl derivative **8** can be explained by limited accessibility of the nitrogen atom for the interaction with the heme iron.

The finding that the E-configurated α, β-unsaturated ketones are less active than the pyridylmethyl derivatives shows that the fixation of the pyridine nitrogen into the plane of the tetralone structure is not suitable for maximum inhibitory activity. The more flexible hydrogenated compounds, however, are able to attach to the enzyme in a way more favourable to strong inhibition: they possibly mimick the Z-isomer (compd **10** vs. **6**).

The oxo group in position-1 of the tetralones and indanones is not essential for P450 17α inhibitory activity.

Ring diminution of the tetrahydronaphthalenes to the indans does not lead to an increase in inhibitory activity.

The potency of the tetralones and indanones is increased by insertion of a methoxy group in position-5. This might be indicative of a hydrophilic interaction between the inhibitor and the enzyme at this position. Methoxy groups in other positions do not influence activity strongly. On the other hand, an impairment of inhibitory activity is observed by insertion of more polar substituents like hydroxy groups. This is also true for the tetralines. Thus, not the polarity but the property to act as hydrogen bond acceptor might be the crucial criterion. This is in accordance with the conception that the 5-methoxy group mimicks the 3-oxo group of progesterone, the natural substrate of rat P450 17α. Superposition of the 5-methoxy tetralone and progesterone by overlapping the 5-methoxy group and the 3-oxo group places the nitrogen atom in the region of C-17 and C-20, the site of hydroxylation. A definite distance between the hydrogen bond acceptor (OCH_3-oxygen) and the pyridyl nitrogen seems to be a prerequisite for strong inhibition (in compd **2** the distance[9] is about 9.7 Å). This seems to be a reasonable explanation for the superiority of the 5-methoxy indanone over the 4-methoxy derivative (compds **26** and **25**).

The goal of further studies must be the development of more potent P450 17α inhibitors. The selectivity profile of these compounds has to be looked into more closely since they could be potential inhibitors of other P450 enzymes than P450 17α and P450 arom. The enzymes of the adrenal cortex involved in the biosynthesis of gluco- and mineralocorticoids (cholesterol side chain cleavage enzyme, 21-hydroxylase, 11β-hydroxylase and 18-hydroxylase) are of special interest.

Acknowledgements

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