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European Journal of Medicinal Chemistry 43 (2008) 872–879
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Antimycobacterial activity of *diphenylpyraline* derivatives

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Received 6 December 2006; received in revised form 20 June 2007; accepted 21 June 2007
Available online 10 July 2007

Abstract

2-Substituted derivatives of *diphenylpyraline* and their 1-phenyl and 1-phenethyl analogues have been prepared in several steps from dihydropyridine-2(1*H*)-thiones. The structures of all new compounds have been confirmed by NMR spectroscopy. Their activity against *Mycobacterium tuberculosis* $H_{37}Rv$ as well as their cytotoxicity against human cells (HEK-293) have been determined via in vitro assays. The antimycobacterial potency was in general increased by substitution in ring position 2. The most promising modifications were a 2-isopropyl derivative and a 1,2-diphenyl analogue.

Keywords: 4-Alkoxypiperidine; Antimycobacterial activity; In vitro assay; Piperidin-4-ol; Synthesis

1. Introduction

Tuberculosis is the leading infectious cause of death today afflicting more than 14 million and killing 1.7 million people per year [1]. Due to the spreading of multi-drug resistant strains new antimycobacterial and non-toxic drugs are urgently needed [2]. The H_1-antihistaminic agent *diphenylpyraline* (**1**) has been proved to be far more active against *Mycobacterium tuberculosis* than against *Escherichia coli* and *Staphylococcus aureus* [3]. As outlined in Ref. [4] many derivatives with different substitution on nitrogen or at the aromatic ring system have been prepared, but their antimycobacterial properties have not yet been reported. Recently we published the synthesis of a number of 2-substituted derivatives of **1** [4]. This paper deals with the preparation of some new analogues and with the investigation of the antimycobacterial activities of the so far prepared 2-substituted *diphenylpyraline* derivatives. By comparison with the activity of **1** structure–activity relationships will be discussed.

2. Chemistry

Considering that enhanced lipophilicity was argued to increase the antimycobacterial activity of H_1-antihistamines [3], we prepared 2-alkyl and 2-aryl derivatives of **1** as well as the corresponding 1-phenyl analogues. Furthermore, some 1-phenethyl analogues of **1** were prepared. In contrast to the syntheses of 2-unsubstituted *diphenylpyraline* derivatives which have been summarized in Ref. [4] our method is based on the conversion of easily available 4-amino-5,6-dihydropyridine-2(1*H*)-thiones **2** and **3**. The synthesis of 2-isopropyl and 2-phenyl derivatives using this method has already been reported [4]. Their 2,2-dimethyl analogues were prepared from the pyridinethiones **2** and **13** [5] using slightly modified procedures.

Abbreviations: ATCC, American type culture collection; CC, Column chromatography; CH_3I, Iodomethane; CH_2Cl_2, Dichloromethane; DMAP, 4-Dimethylaminopyridine; DMEM, Dulbecco's modified eagle medium; Ether, Diethylether; EtOH, Ethanol; FCS, Fetal calf serum; HCl, Hydrochloride or hydrogen chloride; HEK-293 cells, Human embryonic kidney 293 cells; MABA, Microplate alamar blue assay; MeOH, Methanol; MIC, Minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaOH, Sodium hydroxide; NMR, Nuclear magnetic resonance; PBS, Phosphate-buffered saline; SDS, Sodium dodecylsulfate; TAACF, Tuberculosis antimicrobial acquisition and coordinating facility.

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doi:10.1016/j.ejmech.2007.06.012

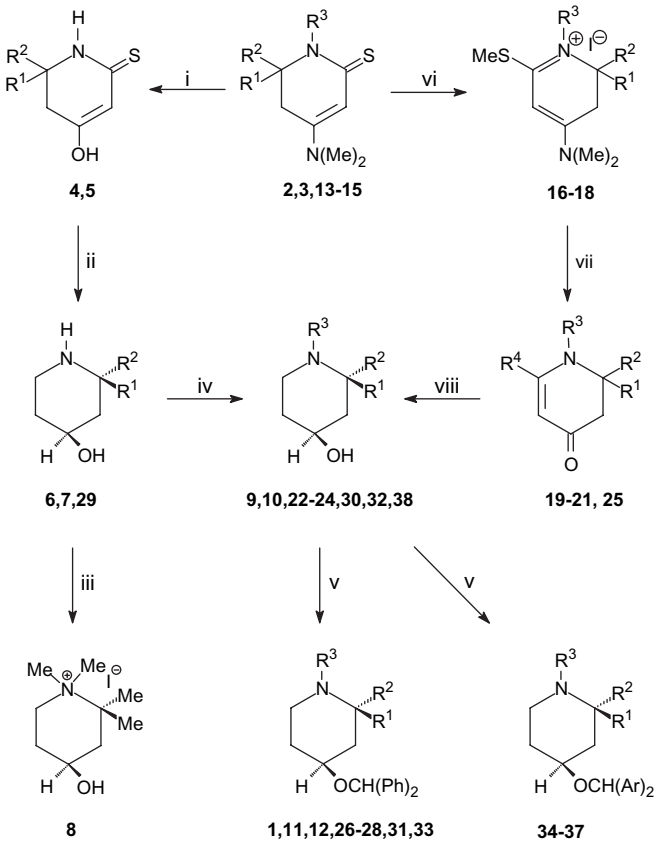
Alkaline hydrolysis of **2** and **3** yielded the 4-hydroxy derivatives **4** and **5**. The catalytic hydrogenation of **4** with Raney nickel W-7 [6] afforded a mixture of the piperidinols *cis*-**6** and *trans*-**6**, which were separated by column chromatography [4]. By a similar procedure the piperidinol **7** was obtained from **5**. Since the methylation of **7** with iodomethane gave quantitatively the quaternary salt **8**, we converted *cis*-**6**, *trans*-**6** and **7** to their 1-methyl derivatives *cis*-**9**, *trans*-**9** [4] and **10** via a Leuckart reaction. The *diphenylpyraline* derivatives *cis*-**11**, *trans*-**11** [4] and **12** were obtained in good yields by etherification with alkyl bromides (method A) in alkaline medium (Scheme 1).

The corresponding 1-phenyl substituted analogues were prepared from 1-phenyl substituted 4-dimethylamino-pyridinethiones **13**–**15**. The latter were obtained in a Dimroth rearrangement from 2-phenylimino-thiopyrane derivatives [7,8]. In contrast to their 1-unsubstituted analogues **2** and **3**, compounds **13**–**15** were stable against caustic soda. The replacement of the 4-dimethylamino group by a hydroxy group failed. However, their methoiodides **16**–**18** were successfully hydrolyzed to the dihydropyridin-4(1*H*)-ones **19**–**21**. Hydrogenation of **19** and **20** with Raney nickel W-2 [9] afforded the cis and trans configurated piperidin-4-ols **22** and **23**, which were separated by column chromatography [4]. When compound **21** was treated that way, a large excess of catalyst was required. Better results were obtained by the use of the more active Raney nickel W-7. When the 1,2-diphenyl compound **20** was treated with that catalyst, the dihydropyridine ring was cleaved and high amounts of a pentanol derivative were formed [4]. But when its 2,2-dimethyl analogue **21** was stirred at 70 °C in an inert-gas atmosphere with Raney nickel W-7 the methylthio group was selectively removed giving **25**. Under hydrogen pressure the same reaction afforded the piperidinol **24** in good yields. The latter was readily converted to ether **28** with bromodiphenylmethane (method A). However, the cis and trans forms of compounds **22** and **23** were – in order to circumvent *N*-substitution – rather etherified with diphenylmethanol (method B) to give the cis and trans forms of **26** and **27** [4].

The 1-phenethyl derivative **31** was prepared in two steps from commercially available 4-piperidinol (**29**), whereas its 2-isopropyl analogue *cis*-**33** was obtained from *cis*-**6**. The piperidinols **29** and *cis*-**6** were treated with phenethyl bromide in aprotic solvent giving the corresponding 1-alkyl derivatives **30** and *cis*-**32** in good yields. The formation of a quaternary by-product was not observed in the ^{1}H NMR spectrum of the reaction mixture. Compounds **31** and *cis*-**33** were afforded by etherification of **30** and *cis*-**32** with diphenylmethanol (method B).

Since higher lipophilicity has been argued to increase the antimycobacterial activity of *diphenylpyraline* derivatives [3], selected bis-(4-chlorophenyl) analogues **34**, *cis*-**35**, **36** and *cis*-**37** have been prepared by reaction of the corresponding piperidinols **38**, *cis*-**9**, **30**, and *cis*-**32** with bis-(4-chlorophenyl)-methanol (method B).

The assignment of most of the signals in the NMR spectra and the structural elucidation of the new compounds were achieved by the method which is outlined in Ref. [4]. Through-space interactions were observed from protons in ring position 4 or 6 to axial protons or protons of axial methyl groups in ring position 2. For all newly synthesized 2-substituted



Compound	R^1	R^2	R^3	R^4
1,34,38	H	H	Me	--
2,4,cis-6	iPr	H	H	--
3,5,7	Me	Me	H	--
trans-6	H	iPr	H	--
cis-9,cis-11,cis-35	iPr	H	Me	--
trans-9,trans-11	H	iPr	Me	--
10,12	Me	Me	Me	--
13,16,19,cis-22,cis-26	iPr	H	Ph	--
14,17,20,cis-23,cis-27	Ph	H	Ph	--
15,18,21,24,28	Me	Me	Ph	--
19	iPr	H	Ph	MeS
20	Ph	H	Ph	MeS
21	Me	Me	Ph	MeS
trans-22,trans-26	H	iPr	Ph	--
trans-23,trans-27	H	Ph	Ph	--
25	Me	Me	Ph	H
29	H	H	--	--
30,31,36	H	H	$Ph(CH_2)_2$	--
cis-32,cis-33,cis-37	iPr	H	$Ph(CH_2)_2$	--

Scheme 1. Reagents and reaction conditions: (i) NaOH, 60–110 °C, 24–72 h; (ii) Raney nickel W-7, ethanol, 45 psi (H_2), 20–50 °C, 48–96 h; (iii) CH_3I, $CHCl_3$, rt, 1 h; (iv) HCOOH, CH_2O, 100 °C or $Ph(CH_2)_2Br$, K_2CO_3, 110 °C, 16 h; (v) method A: $(Ph)_2CHBr$, K_2CO_3, 140 °C, 3–16 h; method B: $(Ph)_2CHOH$ or $(Ar)_2CHOH$, *p*-toluene sulfonic acid, PhMe, 130–140 °C, 6–18 h; (vi) CH_3I, $CHCl_3$, rt, 16–18 h; (vii) NaOH, 100 °C, 2 h; (viii) Raney nickel W-2 or W-7, ethanol, 30–45 psi (H_2), rt, 6–16 h.

compounds chair conformation was deduced from those NOEs and w-couplings in their NMR spectra.

3. Pharmacology

3.1. Antimycobacterial activity

The antimycobacterial activities were determined as microplate assays using *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) within the tuberculosis antimicrobial acquisition and coordinating facility (TAACF) screening program by means of the microplate alamar blue assay (MABA) [13].

3.2. Cytotoxicity

Cytotoxicity was determined indirectly in a microplate assay using HEK-293 cells (human embryonic kidney 293 cells).

4. Results and discussion

For *diphenylpyraline* (**1**) the MIC (minimum inhibitory concentration) of 64 μg/ml against *M. tuberculosis H37Ra* has been published [3]. In our screening the antimycobacterial activity was determined at a concentration of 6.25 μg/ml against *M. tuberculosis* $H_{37}Rv$. At this concentration *diphenylpyraline* caused only 5% inhibition. Dimethylation or the insertion of an isopropyl group trans to the benzhydryloxy group in ring position 2 only slightly improved the activity (**12**, *trans*-**11**: ≤18% inhib.), whereas the activity of the 2,4-cis isomer *cis*-**11** was remarkable (*cis*-**11**: 68% inhib.).

Likewise, in the 1-phenyl series the 2,4-cis analogue *cis*-**26** (*cis*-**26**: 36% inhib.) was more active than its 2,4-trans isomer *trans*-**26** and the 2,2-dimethyl analogue **28** (*trans*-**26**, **28**: ≤7% inhib.). Both 1,2-diphenyl substituted *diphenylpyraline* derivatives *cis*-**27** and *trans*-**27** exhibited good antimycobacterial activity, and the 2,4-trans isomer *trans*-**27** (*trans*-**27**: 75% inhib.) was even the most active of the screened compounds.

Replacement of the 1-methyl groups of compounds **1** and *cis*-**11** by a 1-phenethyl group led to scarcely active analogues (**31**, *cis*-**33**: ≤8% inhib.).

The 2-unsubstituted bis-(4-chlorophenyl) derivatives **34** and **36** (**34**, **36**: ≥56% inhib.) were far more active than their analogues **1** and **31** indicating the remarkable positive influence of the chloro substitution. However, this observation was not transferable to the 2-substituted bis-(4-chlorophenyl) derivatives *cis*-**35** and *cis*-**37** (Table 1).

For *diphenylpyraline* (**1**) a maximum dose of 75 mg daily for oral application has been documented [10]. In our cytotoxicity assay we determined a LC_{50} of 107.59 μg/ml for **1** (Table 1). Of the more active compounds the 2-isopropyl derivatives *cis*-**11** and *trans*-**27** showed comparable cytotoxicity (*cis*-**11**: LC_{50} of 98.06 μg/ml, *trans*-**27**: LC_{50} of 111.67 μg/ml), whereas all of the more potent bis-(4-chlorophenyl) analogues **34**–**36** were distinctly more toxic (**34**–**36**: $LC_{50} \leq 25.46$ μg/ml).

Table 1
Antimycobacterial activity (alamar blue assay) and cytotoxicity of 4-alkoxypiperidines **1**–**37**

Compound	%Inhibition 6.25 μg/ml	Cytotoxicity (LC_{50}) (μg/ml)
Diphenylpyraline HCl (**1**)	5	107.59
cis-**11**	68	98.06
trans-**11**	12	69.44
12	18	104.67
cis-**26**	36	69.38
trans-**26**	7	97.06
cis-**27**	39	77.82
trans-**27**	75	111.67
28	3	65.08
31	8	14.93
cis-**33**	0	62.80
34	65	25.46
cis-**35**	74	17.92
36	56	17.26
cis-**37**	0	76.45
Isoniazid (INH)	>99	>576

5. Conclusion

This paper reports the synthesis and the screening of the antimycobacterial activity of new analogues of *diphenylpyraline*. The majority of its 2-substituted derivatives and its bis-(4-chlorophenyl) analogues showed improved activity against *M. tuberculosis* $H_{37}Rv$. The most active chloro compounds displayed high cytotoxicity. However, the 2-isopropyl derivative of *diphenylpyraline* and its 1,2-diphenyl analogue exhibited markedly increased antimycobacterial activity and similar cytotoxicity compared to *diphenylpyraline*. These compounds have potential as leads for a future study.

6. Experimental protocols

6.1. Instrumentation and chemicals

Melting points were measured using melting point apparatus Dr. Tottoli (Büchi 510) or on a digital melting point apparatus Electrothermal IA 9200 and are uncorrected. The refractive indices were measured on a Abbe refractometer (Carl Zeiss). IR spectra were measured by infrared spectrometer system 2000 FT (Perkin-Elmer) in KBr discs; frequencies are reported in cm^{-1}. NMR spectra were measured on a Varian Gemini 200 or a Varian Inova 400 (298 K) using 5 mm tubes; TMS resonance was used as internal standard. ^1H NMR (200 MHz or 400 MHz) and ^{13}C NMR (50 MHz or 100 MHz) spectra are reported in ppm, ^1H and ^{13}C resonances were assigned using $^1H,^1H$- and $^1H,^{13}C$-correlation spectra. Microanalyses were done at the Microanalytical Laboratory at the Institute of Physical Chemistry, Vienna using EA 1108 CHNS-O apparatus (Carlo Erba). Hydrogenations were performed in an Erlenmeyer flask at atmospheric pressure or in a Parr hydrogenation apparatus shaker type 3911. Column chromatography (CC): silica gel 60 (Merck), 0.063–0.200 mm, pore–diameter 60 Å, column diameter

30–40 mm, layer thickness 300–700 mm, rate of flow: 2 ml/min. TLC: plates (Merck) silica gel 60 F_{254}. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

6.2. Syntheses

6.2.1. Dihydropyridin-4-ones *19*, *20*, *21*, *25*

The preparation of compounds **19** and **20** has already been reported [8].

*6.2.1.1. 2,2-Dimethyl-6-methylthio-1-phenyl-2,3-dihydropyridin-4(1H)-one (**21**).* Compound **18** (8.04 g (20 mmol)) [7] was refluxed in 100 ml 2 M NaOH for 2 h. The aqueous phase was extracted with toluene repeatedly. The extract was washed twice with H_2O, dried with Na_2SO_4, evaporated in vacuo, and the residue was recrystallized. Yield: 4.44 g (89%); m.p. (EtOH) = 158 °C; IR (KBr) 2975, 1618, 1505, 1390, 1289, 1142, 1104, 768, 698 cm^{-1}; NMR: ^1H NMR (200 MHz, $CDCl_3$) δ 1.25 (s, 6H, $(CH_3)_2$), 2.22 (s, 3H, SCH_3), 2.58 (s, 2H, 3-H), 5.20 (s, 1H, 5-H), 7.21–7.45 (m, 5H, aromatic H) ppm; ^{13}C NMR: (50 MHz, $CDCl_3$) δ 15.95 (SCH_3), 25.51 ($(CH_3)_2$), 50.46 (C-3), 60.89 (C-2), 95.30 (C-5), 128.88, 128.97, 130.81, 139.19 (aromatic C), 167.12 (C-6), 188.50 (C-4) ppm; Anal. $C_{14}H_{17}NOS$ (C, H, N, S).

*6.2.1.2. 2,2-Dimethyl-1-phenyl-2,3-dihydropyridin-4(1H)-one (**25**).* Raney nickel W-7 (15 g) was added to a stirred solution of 3.71 g (15 mmol) of **21** in 50 ml of EtOH. The mixture was refluxed at 70 °C on an oil-bath for 72 h. The catalyst was filtered off and the filtrate was evaporated. The residue was triturated with $CHCl_3$ and filtered. The solvent was evaporated and the residue was recrystallized. Yield: 2.62 g (86%); m.p. (heptane) = 133 °C; IR (KBr) 3050, 2967, 1637, 1572, 1488, 1429, 1365, 1296, 781, 770, 709 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 1.20 (s, 6H, $(CH_3)_2$), 2.44 (s, 2H, 3-H), 4.90 (d, J = 7.7 Hz, 1H, 5-H), 7.17 (d, J = 7.7 Hz, 1H, 6-H), 7.22–7.44 (m, 5H, aromatic H) ppm; ^{13}C NMR: (50 MHz, DMSO-d_6) δ 24.51 ($(CH_3)_2$), 50.22 (C-3), 59.04 (C-2), 98.38 (C-5), 127.40, 128.05, 129.09, 142.68 (aromatic C), 152.20 (C-6), 190.70 (C-4) ppm; Anal. $C_{13}H_{15}NO$ (C, H, N).

6.2.2. Piperidin-4-ols *cis*-**6**, *trans*-**6**, **7**, **8**, *cis*-**9**, *trans*-**9**, **10**, *cis*-**22**, *trans*-**22**, *cis*-**23**, *trans*-**23**, **24**, **29**, **30**, **32**, **38**

The preparation of compounds *cis*-**6**, *trans*-**6**, *cis*-**9**, *trans*-**9**, *cis*-**22**, *trans*-**22**, *cis*-**23**, *trans*-**23** has already been reported [4]. Compounds **29** and **38** have been purchased from Aldrich.

*6.2.2.1. (RS)-(±)-2,2-Dimethylpiperidin-4-ol (**7**).* A solution of 3.15 g (20 mmol) of **5** [11] in 200 ml of EtOH was shaken at 50 °C at 45 psi for 48 h with 15 g of freshly prepared Raney nickel W-7. After filtration the solvent was evaporated. The residue was dissolved in $CHCl_3$, filtered, washed with H_2O and dried. The solvent was removed and the piperidinol distilled in vacuo. The distillate was recrystallized. Yield: 2.24 g (87%); m.p. (EtOH/H_2O) = 78 °C; bp: 66 °C/0.2 mbar; IR (KBr) 3254, 2930, 2843, 1452, 1364, 1260, 1146, 1117, 1072 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.10 (s, 3H, CH_{3ax}), 1.13 (t, J = 12.3 Hz, 1H, 3-H_{ax}), 1.16 (s, 3H, CH_{3eq}), 1.23 (dddd, J = 13.0, 12.3, 10.0, 4.7 Hz, 1H, 5-H_{ax}), 1.84 (ddd, J = 12.3, 4.3, 2.0 Hz, 1H, 3-H_{eq}), 1.90–1.98 (m, 1H, 5-H_{eq}), 2.81 (ddd, J = 13.4, 12.3, 2.9 Hz, 1H, 6-H_{ax}), 2.96 (ddd, J = 13.4, 4.7, 2.7 Hz, 1H, 6-H_{eq}), 3.80–3.88 (m, 1H, 4-H_{ax}) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.47 (CH_{3ax}), 32.64 (CH_{3eq}), 36.53 (C-5), 40.01 (C-6), 47.70 (C-3), 51.17 (C-2), 66.22 (C-4) ppm; Anal. $C_7H_{15}NO$ (C, H, N).

*6.2.2.2. (RS)-(±)-4-Hydroxy-1,1,2,2-tetramethylpiperidinium iodide (**8**).* To a solution of 1.29 g (10 mmol) of **7** in 50 ml of $CHCl_3$, 1.42 g (10 mmol) of CH_3I were added dropwise. The reaction mixture was stirred for 6 h at room temperature, then the solvent was removed in vacuo. The residue was triturated with ethyl acetate, filtered with suction and recrystallized. Yield: 1.40 g (98%); m.p. (EtOH) = 295 °C; IR (KBr) 3363, 2941, 1473, 1416, 1160, 1067, 1055 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.36 (s, 3H, CH_{3eq}), 1.39 (s, 3H, CH_{3ax}), 1.63–1.73 (m, 1H, 5-H_{ax}), 1.80 (dd, J = 14.5, 9.5 Hz, 1H, 3-H_{ax}), 1.84–1.97 (m, 2H, 3-H_{eq}, 5-H_{eq}), 2.94 (s, 3H, NCH_{3eq}), 3.02 (s, 3H, NCH_{3ax}), 3.43 (dt, J = 13.4, 4.6 Hz, 1H, 6-H_{eq}), 3.49–3.67 (m, 1H, 6-H_{ax}), 3.79–3.88 (m, 1H, 4-H_{ax}), 4.99 (d, J = 4.0 Hz, 1H, OH) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.31 (CH_{3ax}), 23.86 (CH_{3eq}), 29.13 (C-5), 41.47 (C-3), 46.67 (NCH_{3ax}), 47.75 (NCH_{3eq}), 58.61 (C-6), 61.33 (C-4), 69.61 (C-2) ppm; Anal. $C_9H_{20}INO$ (C, H, I, N).

*6.2.2.3. (RS)-(±)-1,2,2-Trimethylpiperidin-4-ol (**10**).* Formic acid (85%) (4.0 g (75 mmol)) were added to 1.92 g (15 mmol) of **7** at 0 °C. The mixture was slowly warmed until solution was obtained. Then 1.5 g (18 mmol) of a 37% aqueous solution of CH_2O was added at room temperature. The mixture was heated on a steam bath until the generation of CO_2 ceased. The solution was cooled, acidified with concentrated HCl and evaporated. The oily residue was dissolved in 2 ml H_2O, alkalified with a 25% solution of NaOH and extracted three times with ether. The combined organic layers were dried over Na_2SO_4. The solvent was removed and the piperidinol distilled in vacuo. For characterization purposes a part of the pure colourless oil was treated with an equivalent amount of HCl in EtOH. The solvent was evaporated and the residue recrystallized giving the hydrochloride of **10** as white crystals. Yield: 1.81 g (84%); m.p. (HCl, EtOH/ethyl acetate) = 208 °C; refractive index n_D^{20} (base): 1.4835 (Ref. [12]: 1.4804); IR (HCl, KBr) 3264, 2938, 2680, 1467, 1396, 1382, 1348, 1204, 1162, 1065, 1024 cm^{-1}; NMR (base): ^1H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 3H, CH_{3ax}), 1.16 (s, 3H, CH_{3eq}), 1.39 (t, J = 11.9 Hz, 1H, 3-H_{ax}), 1.53 (qd, J = 12.2, 4.6 Hz, 1H, 5-H_{ax}), 1.75 (ddd, J = 11.9, 4.2,

2.4 Hz, 1H, 3-H_{eq}), 1.91–1.98 (m, 1H, 5-H_{eq}), 2.23 (s, 3H, NCH_3), 2.45 (td, $J = 12.2$, 3.1 Hz, 1H, 6-H_{ax}), 2.66 (ddd, $J = 12.2$, 4.6, 3.4 Hz, 1H, 6-H_{eq}), 3.76–3.85 (m, 1H, 4-H_{ax}) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.00 (CH_{3ax}), 29.40 (CH_{3eq}), 35.43 (C-5), 37.07 (NCH_3), 48.41 (C-3), 49.14 (C-6), 54.13 (C-2), 66.15 (C-4) ppm; Anal. $C_8H_{18}ClNO$ (C, H, Cl, N).

*6.2.2.4. (RS)-(±)-2,2-Dimethyl-1-phenylpiperidin-4-ol (**24**).* Freshly prepared Raney nickel W-7 (15 g) was added to a solution of 2.47 g (10 mmol) of **21** in 50 ml of EtOH. The mixture was shaken at room temperature at 45 psi for 16 h and sucked off through a sintered-glass filter. The residue was thoroughly washed with EtOH and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and H_2O. The organic layer was washed once with H_2O and dried with Na_2SO_4. The solvent was evaporated and the residue was treated with an equivalent amount of HCl in EtOH. The solvent was evaporated and the residue recrystallized. Yield: 2.03 g (83%); m.p. (HCl, EtOH/ethyl acetate) = 236 °C; IR (KBr) 3341, 2925, 1490, 1377, 1138, 1072, 1028, 778, 709 cm^{-1}; NMR (base): ^1H NMR: (400 MHz, $CDCl_3$) δ 1.01 (s, 3H, CH_{3ax}), 1.08 (s, 3H, CH_{3eq}), 1.46 (d, $J = 5.1$ Hz, 1H, OH), 1.58 (dd, $J = 12.3$, 11.6 Hz, 1H, 3-H_{ax}), 1.62 (tdd, $J = 12.0$, 11.2, 4.1 Hz, 1H, 5-H_{ax}), 1.88 (ddd, $J = 12.3$, 4.4, 2.0 Hz, 1H, 3-H_{eq}), 2.01–2.08 (m, 1H, 5-H_{eq}), 2.95 (dt, $J = 12.0$, 4.1 Hz, 1H, 6-H_{eq}), 3.34 (td, $J = 12.0$, 2.7 Hz, 1H, 6-H_{ax}), 3.92–4.01 (m, 1H, 4-H_{ax}), 7.08–7.27 (m, 5H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 18.48 (CH_{3ax}), 31.56 (CH_{3eq}), 36.42 (C-5), 46.59 (C-6), 49.43 (C-3), 55.29 (C-2), 66.73 (C-4), 124.45, 127.90, 149.61 (aromatic C) ppm; Anal. $C_{13}H_{20}ClNO$ (C, H, Cl, N).

*6.2.2.5. (RS)-(±)-1-Phenethylpiperidin-4-ol (**30**).* Piperidin-4-ol (**29**). (1.0 g (10 mmol)), 3.7 g (20 mmol) of 2-phenethyl bromide (deacidified with basic aluminium oxide) and 0.7 g (5 mmol) of K_2CO_3 were refluxed over night in 50 ml of dry toluene. The solvent was evaporated and the residue acidified. Excess alkyating agent was removed with ether (30 ml). The aqueous solution was made alkaline and extracted with ether. The organic layer was dried and evaporated giving pure **30**. Yield: 1.95 g (95%); m.p.: 95–97 °C (Ref. [14] 95.5–98.5 °C); IR (KBr) 3158, 2949, 2812, 1497, 1472, 1454, 1373, 1345, 1248, 1120, 1068, 1016, 773, 748, 698 cm^{-1}; NMR: ^1H NMR: (400 MHz, $CDCl_3$) δ 1.58–1.67 (m, 2H, 3-H_{ax}, 5-H_{ax}), 1.87–1.95 (m, 2H, 3-H_{eq}, 5-H_{eq}), 2.20 (br t, $J = 9.9$ Hz, 2H, 2-H_{ax}, 6-H_{ax}), 2.55–2.60 (m, 2H, NCH_2), 2.77–2.82 (m, 2H, $PhCH_2$), 2.82–2.89 (m, 2H, 2-H_{eq}, 6-H_{eq}), 2.97 (br s, 1H, OH), 3.64–3.71 (m, 1H, 4-H_{ax}), 7.18–7.27 (m, 5H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 33.88 ($PhCH_2$), 34.53 (C-3, C-5), 51.08 (C-2, C-6), 60.46 (NCH_2), 67.96 (C-4), 125.99, 128.35, 128.66, 140.39 (aromatic C) ppm; Anal. $C_{13}H_{19}NO$ (C, H, N).

*6.2.2.6. (2RS,4SR)-(±)-2-Isopropyl-1-phenethylpiperidin-4-ol (cis-**32**).* Compound *cis*-**6** (2.28 g (16 mmol)), 5.88 g (32 mmol) of 2-phenethyl bromide (deacidified with basic aluminium oxide) and 1.10 g (8 mmol) of K_2CO_3 were heated in 80 ml bromobenzene at 180 °C on an oil-bath for 18 h. The solvent was evaporated and the residue acidified. Excess alkyating agent was removed with ether (50 ml). The aqueous solution was made alkaline and extracted with CH_2Cl_2. The organic layer was dried and evaporated. The residue was purified by column chromatography (toluene/MeOH = 4:1) over silica gel giving pure *cis*-**32**. Yield: 3.19 g (69%); m.p.: 160–162 °C; IR (KBr) 2966, 2937, 2631, 1456, 1418, 1399, 1068, 1050, 753, 703 cm^{-1}; NMR: ^1H NMR: (400 MHz, $CDCl_3$) δ 0.86, 0.91 (2d, $J = 6.7$ Hz, 6H, $2CH_3$), 1.18 (dt, $J = 11.7$, 11.2 Hz, 1H, 3-H_{ax}), 1.53 (dtd, $J = 12.2$, 11.0, 4.2 Hz, 1H, 5-H_{ax}), 1.85 (dddd, $J = 11.7$, 4.3, 2.8, 2.3 Hz, 1H, 3-H_{eq}), 1.88–1.94 (m, 1H, 5-H_{eq}), 2.01–2.19 (m, 3H, 2-H_{ax}, OH, $CH(CH_3)_2$), 2.42 (ddd, $J = 12.4$, 12.0, 2.5 Hz, 1H, 6-H_{ax}), 2.65–2.78 (m, 3H, $PhCH_2$, NCH), 2.82–2.91 (m, 1H, NCH), 3.06 (ddd, $J = 12.0$, 3.8, 3.0 Hz, 1H, 6-H_{eq}), 3.53–3.62 (m, 1H, 4-H_{ax}), 7.16 (d, $J = 7.4$ Hz, 2H, *o*-Ph-H), 7.18 (t, $J = 7.4$ Hz, 1H, *p*-Ph-H), 7.28 (t, $J = 7.4$ Hz, 2H, *m*-Ph-H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 15.41, 20.09 ($2CH_3$), 27.32 ($CH(CH_3)_2$), 31.01 ($PhCH_2$), 33.38 (C-3), 34.63 (C-5), 50.86 (C-6), 53.53 (NCH_2), 63.07 (C-2), 69.78 (C-4), 125.87, 128.34, 128.58, 140.69 (aromatic C) ppm; Anal. $C_{16}H_{26}ClNO \cdot 0.3H_2O$ (C, H, Cl, N).

*6.2.3. Analogues of diphenylpyraline **11**, **12**, **26–28**, **31**, **33–37***

The preparation of compounds *cis*-**11**, *trans*-**11**, *cis*-**26**, *trans*-**26**, *cis*-**27** and *trans*-**27** has already been reported [4].

*6.2.3.1. General procedure for the synthesis of **12** and **28**.* The piperidinols **10**, **24** (10 mmol), benzhydrylbromide (10 mmol) and K_2CO_3 (5 mmol) were stirred on an oil-bath at 140 °C. The mixture was cooled and benzene (50 ml) was added. The inorganic salts were dissolved in H_2O (10 ml). The layers were separated in a separatory funnel. The aqueous layer was extracted once with benzene (10 ml). The combined organic layers were washed twice with H_2O, dried and evaporated. The residue was purified by column chromatography (CH_2Cl_2/toluene = 3:1) over silica gel. Their hydrochlorides were afforded by treatment with equivalent amounts of a 1 M solution of HCl in ether. Crystallizing products were sucked off. Alternatively the solvent was evaporated and the residue recrystallized.

*6.2.3.1.1. (RS)-(±)-4-Benzhydryloxy-1,2,2-trimethylpiperidine (**12**).* Compound **10** (1.43 g (10 mmol)), 2.53 g (10 mmol) of benzhydrylbromide and 0.71 g (5 mmol) K_2CO_3 gave after 3 h a resinous residue. Upon treatment with ethereal HCl the hydrochloride of **12** was obtained. Yield: 1.75 g (56%); m.p. (HCl, EtOH/ether) = 160 °C; IR (HCl, KBr) 3029, 2953, 1494, 1473, 1454, 1093, 741, 698 cm^{-1}; NMR (base): ^1H NMR (400 MHz, DMSO-d_6) δ 0.75 (s, 3H, CH_{3ax}), 1.05 (s, 3H, CH_{3eq}), 1.38 (dd, $J = 12.4$, 10.4 Hz, 1H, 3-H_{ax}), 1.47 (dtd, $J = 11.7$, 11.0, 4.4 Hz, 1H, 5-H_{ax}), 1.75 (ddd, $J = 12.4$, 4.0, 1.8 Hz, 1H, 3-H_{eq}), 1.82–1.86 (m, 1H, 5-H_{eq}), 2.07 (s, 3H, NCH_3), 2.23 (td, $J = 11.8$, 2.7 Hz, 1H,

6-H_{ax}), 2.52 (dt, $J = 11.8$, 4.4 Hz, 1H, 6-H_{eq}), 3.43–3.50 (m, 1H, 4-H_{ax}), 5.61 (s, 1H, OCH), 7.18–7.39 (m, 10H, aromatic H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): 16.33 (CH_{3ax}), 28.28 (CH_{3eq}), 32.18 (C-5), 36.81 (NCH_3), 45.06 (C-3), 48.25 (C-6), 53.30 (C-2), 70.96 (C-4), 79.07 (OCH), 126.45, 126.49, 126.93, 126.94, 128.07, 128.09, 143.10, 143.25 (aromatic C) ppm; Anal. $C_{21}H_{27}NO \cdot 0.2H_2O$ (C, H, N).

*6.2.3.1.2. (RS)-(±)-4-Benzhydryloxy-2,2-dimethyl-1-phenylpiperidine (**28**).* Compound **24** (2.05 g (10 mmol)), 2.53 g (10 mmol) of benzhydrylbromide and 0.71 g (5 mmol) K_2CO_3 gave after 12 h a resinous residue. Upon treatment with ethereal HCl the hydrochloride of **28** was obtained. Yield: 1.83 g (49%); m.p. (HCl, EtOH/ether) 214 °C; IR (base, KBr) 2968, 2941, 1596, 1492, 1453, 1070, 740, 701 cm^{-1}; NMR (base): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (s, 3H, CH_{3ax}), 1.08 (s, 3H, CH_{3eq}), 1.75 (dd, $J = 12.5$, 10.2 Hz, 1H, 3-H_{ax}), 1.71–1.80 (m, 1H, 5-H_{ax}), 1.87–1.94 (m, 1H, 3-H_{eq}), 2.00–2.07 (m, 1H, 5-H_{eq}), 2.97–3.04 (m, 1H, 6-H_{eq}), 3.14 (td, $J = 11.4$, 2.8 Hz, 1H, 6-H_{ax}), 3.66–3.73 (m, 1H, 4-H_{ax}), 5.55 (s, 1H, OCH), 7.03–7.37 (m, 15H, aromatic H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.05 (CH_{3ax}), 30.24 (CH_{3eq}), 33.01 (C-5), 46.06 (C-3), 46.36 (C-6), 55.23 (C-2), 71.60 (C-4), 80.24 (OCH), 124.31, 126.97, 127.00, 127.22, 127.77, 127.80, 128.24, 142.78, 142.99, 149.66 (aromatic C) ppm; Anal. $C_{26}H_{29}NO$ (C, H, N).

*6.2.3.2. General procedure for the synthesis of **31**, **33–37**.* To a solution of the corresponding piperidinol in toluene, toluene-4-sulfonic acid monohydrate and diphenylmethanol or bis-4-(chlorophenyl)methanol were added. The flask was fitted with a Dean-Stark trap and the mixture was refluxed with stirring over night at 140 °C. The water formed during the reaction was removed by introducing dry molecular sieves (0.4 nm) in the trap. The mixture was cooled and washed with 1 M NaOH and once with water. The organic layer was dried with Na_2SO_4 and the solvent removed in vacuo. Excess diarylmethanol was removed by CC over silica gel. Fractions containing the *diphenylpyraline* analogue were collected and the solvent removed in vacuo. The residue was redissolved in acetone and a 2 M solution of hydrogen chloride in ether was added. The precipitate was sucked off and washed with a mixture of acetone and ether.

*6.2.3.2.1. (RS)-(±)-4-Benzhydryloxy-1-phenethylpiperidine (**31**).* Compound **30** (0.82 g (4 mmol)), 1.84 g (10 mmol) of diphenylmethanol and 1.90 g (10 mmol) toluene-4-sulfonic acid monohydrate in 200 ml of toluene gave a residue from which **31** was obtained upon purification with CC over silica gel (toluene/MeOH = 49:1). Yield: 1.45 g (89%); m.p. (HCl): 254–258 °C; IR (HCl, KBr) 3424, 2942, 2487, 1599, 1493, 1455, 1086, 1058, 755, 743, 700 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 1.72–1.81 (m, 2H, 3-H_{ax}, 5-H_{ax}), 1.87–1.95 (m, 2H, 3-H_{eq}, 5-H_{eq}), 2.17–2.26 (m, 2H, 2-H_{ax}, 6-H_{ax}), 2.54–2.59 (m, 2H, NCH_2), 2.76–2.86 (m, 4H, 2-H_{eq}, 6-H_{eq}, $PhCH_2$), 3.43–3.49 (m, 1H, 4-H_{ax}), 5.53 (s, 1H, OCH), 7.18–7.36 (m, 15H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 31.17 (C-3, C-5), 33.67 ($PhCH_2$), 50.91 (C-2, C-6), 60.46 (NCH_2), 72.12 (C-4), 80.11 (OCH), 126.03, 127.06, 127.31, 128.30, 128.37, 128.68, 140.23, 142.80 (aromatic C) ppm; Anal. $C_{26}H_{30}ClNO$ (C, H, N).

*6.2.3.2.2. (2RS,4SR)-(±)-4-Benzhydryloxy-2-isopropyl-1-phenethylpiperidine (cis-**33**).* Compound *cis*-**32** (0.50 g (2 mmol)), 1.11 g (6 mmol) of benzhydrol and 0.48 g (2.5 mmol) toluene-4-sulfonic acid monohydrate in 100 ml of toluene gave a residue, from which *cis*-**33** was obtained upon purification with CC over silica gel (toluene; toluene/MeOH = 49:1). Yield: 0.89 g (98%); m.p. (HCl): 147 °C; IR (HCl, KBr) 2967, 2334, 1494, 1455, 1421, 1399, 1100, 1076, 752, 702 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 0.84, 0.89 (2d, $J = 6.5$ Hz, 6H, $2CH_3$), 1.31 (q, $J = 11.4$ Hz, 1H, 3-H_{ax}), 1.61 (dtd, $J = 12.5$, 12.2, 4.1 Hz, 1H, 5-H_{ax}), 1.88–1.94 (m, 1H, 3-H_{eq}), 1.95–2.02 (m, 1H, 5-H_{eq}), 2.04–2.12 (m, 2H, 2-H_{ax}, C*H*$(CH_3)_2$), 2.31 (ddd, $J = 12.5$, 12.3, 12.0 Hz, 1H, 6-H_{ax}), 2.61–274 (m, 3H, NCH, $PhCH_2$), 2.80–2.88 (m, 1H, NCH), 3.04 (ddd, $J = 12.0$, 4.1, 3.5 Hz, 1H, 6-H_{eq}), 3.31–3.39 (m, 1H, 4-H_{ax}), 5.58 (s, 1H, OCH), 7.13–7.38 (m, 15H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 15.55, 20.12 ($2CH_3$), 27.54 (*C*H$(CH_3)_2$), 30.46 (C-3), 31.09 ($PhCH_2$), 31.50 (C-5), 50.84 (C-6), 53.47 (NCH_2), 63.16 (C-2), 74.92 (C-4), 80.03 (OCH), 125.88, 127.08, 127.10, 127.27, 127.32, 128.30, 128.33, 128.37, 128.63, 140.80, 142.73, 142.95 (aromatic C) ppm; Anal. $C_{29}H_{36}ClNO \cdot 0.2H_2O$ (C, H, Cl, N).

*6.2.3.2.3. (RS)-(±)-4-(Bis-(4-chlorophenyl)methoxy)-1-methylpiperidine (**34**).* 1-Methylpiperidin-4-ol (**38**) (0.46 g (4 mmol)), 3.04 g (12 mmol) of bis-(4-chlorophenyl)methanol and 0.96 g (5 mmol) toluene-4-sulfonic acid monohydrate in 200 ml of toluene gave a residue, from which **34** was obtained upon purification with CC over silica gel (toluene/MeOH = 39:1). Yield: 1.12 g (72%); m.p. (HCl): 116–118 °C; IR (HCl, KBr) 2957, 2418, 1489, 1089, 1069, 1014, 818, 796 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 1.66–1.76 (m, 2H, 3-H_{ax}, 5-H_{ax}), 1.81–1.89 (m, 2H, 3-H_{eq}, 5-H_{eq}), 2.05–2.15 (m, 2H, 2-H_{ax}, 6-H_{ax}), 2.24 (s, 3H, NCH_3), 2.63–2.71 (m, 2H, 2-H_{eq}, 6-H_{eq}), 3.34–3.42 (m, 1H, 4-H_{ax}), 5.44 (s, 1H, OCH), 7.22–7.30 (m, 8H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 31.42 (C-3, C-5), 46.20 (NCH_3), 53.17 (C-2, C-6), 72.28 (C-4), 78.81 (OCH), 128.29, 128.58, 133.29, 140.96 (aromatic C) ppm; Anal. $C_{19}H_{22}Cl_3NO$ (C, H, Cl, N).

*6.2.3.2.4. (2RS,4SR)-(±)-4-(Bis-(4-chlorophenyl)methoxy)-2-isopropyl-1-methylpiperidine (cis-**35**).* Compound *cis*-**9** (0.63 g (4 mmol)), 1.27 g (5 mmol) of bis-(4-chlorophenyl)-methanol and 0.95 g (5 mmol) toluene-4-sulfonic acid monohydrate in 200 ml of toluene gave a residue, from which *cis*-**35** was obtained upon purification with CC over silica gel (toluene/MeOH = 9:1). Yield: 1.65 g (96%); m.p. (HCl): 239–240 °C; IR (HCl, KBr) 2969, 2598, 2515, 1490, 1411, 1089, 1052, 1014, 823, 797 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 0.84, 0.86 (2d, $J = 7.3$ Hz, 6H, $2CH_3$), 1.29 (q, $J = 11.7$ Hz, 1H, 3-H_{ax}), 1.60 (qd, $J = 12.4$, 3.9 Hz, 1H, 5-H_{ax}), 1.60–1.66 (m, 1H, 2-H_{ax}), 1.80–1.86 (m, 1H, 3-H_{eq}), 1.87–1.94 (m, 1H, 5-H_{eq}), 1.95–2.07 (m, 2H, 6-H_{ax},

C*H*(CH_3)$_2$), 2.17 (s, 3H, NCH_3), 2.88 (ddd, $J = 11.7$, 4.0, 3.2 Hz, 1H, 6-H_{eq}), 3.23–3.32 (m, 1H, 4-H_{ax}), 5.49 (s, 1H, OCH), 7.22–7.32 (m, 8H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 14.88, 19.97 (2CH_3), 27.77 (*C*H(CH_3)$_2$), 30.20 (C-3), 31.94 (C-5), 41.68 (NCH_3), 55.63 (C-6), 66.99 (C-2), 75.18 (C-4), 78.76 (OCH), 128.31, 128.34, 128.58, 128.63, 133.28, 133.32, 140.87, 141.08 (aromatic C) ppm; Anal. $C_{22}H_{28}Cl_3NO$ (C, H, Cl, N).

*6.2.3.2.5. (RS)-(±)-4-(Bis-(4-chlorophenyl)methoxy)-1-phenethylpiperidine (**36**).* Compound **30** (0.82 g (4 mmol)), 2.53 g (10 mmol) of bis-(4-chlorophenyl)methanol and 0.95 g (5 mmol) toluene-4-sulfonic acid monohydrate in 200 ml of toluene gave a residue, from which **36** was obtained upon purification with CC over silica gel (toluene/MeOH = 9:1). Yield: 1.83 g (96%); m.p. (HCl): 207–208 °C; IR (HCl, KBr) 2940, 2404, 1489, 1090, 1066, 1015, 819, 795, 753, 698 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 1.70–1.81 (m, 2H, 3-H_{ax}, 5-H_{ax}), 1.88–1.97 (m, 2H, 3-H_{eq}, 5-H_{eq}), 2.23–2.33 (m, 2H, 2-H_{ax}, 6-H_{ax}), 2.60 (t, $J = 8.0$ Hz, 2H, NCH_2), 2.82 (t, $J = 8.0$ Hz, 2H, $PhCH_2$), 2.79–2.87 (m, 2H, 2-H_{eq}, 6-H_{eq}), 3.41–3.48 (m, 1H, 4-H_{ax}), 5.45 (s, 1H, OCH), 7.18–7.31 (m, 13H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 30.80 (C-3, C-5), 33.34 ($PhCH_2$), 50.60 (C-6), 60.21 (NCH_2), 71.96 (C-4), 78.91 (OCH), 126.14, 128.23, 128.40, 128.60, 128.63, 133.34, 139.78, 140.81 (aromatic C) ppm; Anal. $C_{26}H_{28}Cl_3NO$ (C, H, Cl, N).

*6.2.3.2.6. (2RS,4SR)-(±)-4-(Bis-(4-chlorophenyl)methoxy)-2-isopropyl-1-phenethylpiperidine (cis-**37**).* Compound *cis*-**32** (0.50 g (2 mmol)), 1.52 g (6 mmol) of bis-(4-chlorophenyl)-methanol and 0.48 g (2.5 mmol) toluene-4-sulfonic acid monohydrate in 100 ml of toluene gave a residue, from which *cis*-**37** was obtained upon purification with CC over silica gel (toluene/MeOH = 49:1). Yield: 0.98 g (94%); m.p. (HCl): 223–224 °C; IR (HCl, KBr) 2967, 2508, 1490, 1399, 1089, 1071, 1014, 822, 799, 751, 701 cm^{-1}; NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ 0.84, 0.89 (2d, $J = 6.4$ Hz, 6H, 2CH_3), 1.28 (td, $J = 11.5$, 11.2 Hz, 1H, 3-H_{ax}), 1.58 (dddd, $J = 12.3$, 12.1, 11.2, 4.2 Hz, 1H, 5-H_{ax}), 1.83–1.89 (m, 1H, 3-H_{eq}), 1.89–1.96 (m, 1H, 5-H_{eq}), 2.04–2.13 (m, 2H, 2-H_{ax}, C*H*(CH_3)$_2$), 2.30 (td, $J = 12.3$, 2.2 Hz, 1H, 6-H_{ax}), 2.61–2.74 (m, 3H, NCH, $PhCH_2$), 2.79–2.90 (m, 1H, NCH), 3.01–3.08 (m, 1H, 6-H_{eq}), 3.25–3.34 (m, 1H, 4-H_{ax}), 5.50 (s, 1H, OCH), 7.12–7.33 (m, 13H, aromatic H) ppm; ^{13}C NMR: (100 MHz, $CDCl_3$) δ 15.52, 20.12 (2CH_3), 27.51 (*C*H(CH_3)$_2$), 30.43 (C-3), 31.15 ($PhCH_2$), 31.49 (C-5), 50.76 (C-6), 53.47 (NCH_2), 63.13 (C-2), 75.20 (C-4), 78.70 (OCH), 125.93, 128.32, 128.35, 128.40, 128.59, 128.63, 133.29, 133.33, 140.74, 140.87, 141.09 (aromatic C) ppm; Anal. $C_{29}H_{34}Cl_3NO \cdot 0.2H_2O$ (C, H, Cl, N).

6.3. Biological tests

6.3.1. Antimycobacterial activity

The screening of the antimycobacterial activity of the hydrochlorides of all synthesized analogues of *diphenylpyraline* was conducted in vitro at a concentration of 6.25 μg/ml against *M. tuberculosis* H_{37}Rv (ATCC 27294) in BACTEC 12B medium using a broth microdilution assay, the microplate alamar blue assay (MABA) [13]. Isoniazid (INH) was used as standard.

6.3.2. Cytotoxicity

The cytotoxic properties of all *diphenylpyraline* analogues were detected indirectly via cell proliferation of a HEK-293 cell line using a colorimetric assay based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a coloured formazan product by proliferating cells [15]. HEK-293 cells were seeded in 96-well plates at a density of 12 000 cells/well in 180 μl DMEM (Dulbecco's modified eagle medium) supplemented with 10% FCS (fetal calf serum) and incubated at 37 °C and 5% CO_2. After 48 h 100 μl of the medium were carefully removed from each well and substituted by an equal volume of fresh medium. Then various concentrations of the hydrochlorides of the test compounds in 20 μl of an aseptically filtered mixture of 17% glycerine, 33% DMSO and 50% water were added (as negative controls, the corresponding solutions free of compounds were used). After incubation for 72 h 100 μl of the medium were carefully removed from each well and 20 μl of DMEM and 13 μl MTT reagent (5 mg MTT/ml PBS) were added to each well. The plates were shaken for 30 s at 120 rpm and incubated for 3 h. The resulting formazan dye was solubilised with 100 μl of a solution of 10% SDS (sodium dodecylsulfate) in 0.01 M aqueous hydrochloric acid. The plates were shaken at 170 rpm for 30 min, sealed with lab film and stored until complete solubilization of the purple formazan crystals. Finally the plates were shaken at 180 rpm for 30 min. The absorbance was measured spectrophotometrically on a Fluostar Galaxy (BMG LABTECH) at a sample wavelength of 544 nm and a reference wavelength of 640 nm. To obtain the LC_{50} values of the compounds, their concentrations were plotted against the absorbance and fitted to a sigmoidal curve with the Boltzmann equation using the program Microcal Origin. INH was used as standard.

Acknowledgements

Antimycobacterial data were provided by the TAACF through a research and development contract with the U.S. National Institute of Allergy and Infectious Diseases.

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