Exploring the Importance of Piperazine N-Atoms for σ2 Receptor Affinity and Activity in a Series of Analogs of 1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydroanaphthalen-1-yl)-propyl]piperazine (PB28)†

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σ2-Agonist 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydroanaphthalen-1-yl)propyl]piperazine (7, PB 28), which proved to revert doxorubicin resistance in breast cancer cells, was taken as a template to prepare new analogs. One of the two basic N-atoms was alternatively replaced by a methine or converted into an amide or ammonium function, with the aim of finding out which of them was essential for σ2 receptor affinity and activity. Some simply 4-substituted 1-cyclohexylpiperazines were also investigated. None of the compounds was as high-affinity as 7 (σ2Kᵢ = 0.68, σ1Kᵢ = 0.38 nM), proving that both basic N-atoms ensure better σ2 receptor binding. Amide 36 emerged as high-affinity (Kᵢ = 0.11 nM) and noteworthy selective (1627-fold) σ1 ligand. Small N-cyclohexylpiperazine 59 displayed the highest σ2 affinity (Kᵢ = 4.70 nM). The σ2/σ1 selectivities were generally low. Antiproliferative assay in SK-N-SH cells revealed piperidines 24 and 15 as putative σ2 agonists (EC50 1.40 and 3.64 μM respectively) more potent than 7.

Introduction

Initially considered as belonging to the opioid receptor family,1 at present, sigma (σ) receptors are known as a distinct class consisting of two subtypes, σ1 and σ2 receptors.2 They are present with high density in the central nervous system (CNS) and in peripheral organs.3,4 There are several pieces of evidence that show both σ receptors to be overexpressed in many tumor cell lines, where σ2 subtype overexpression is prevalent.5 These findings prompted the development of σ radioligands as potential tumor imaging agents by single photon emission computed tomography (SPECT) and positron emission tomography (PET) techniques.6,7 However, only σ1 receptor has been identified and cloned, showing a sequence different from any other mammalian protein.8 Recently, the σ2 receptor has been likely isolated and characterized, and it seems to belong to the histone protein family.9 The biochemical mechanism through which both the subtypes act is still under investigation. Different experiments suggest that σ receptors are endocellular proteins and play a role in signal transduction through the mobilization of Ca2+ from intracellular stores.10 σ2 Receptor agonists proved to induce cell death by both caspase-independent and caspase-dependent apoptoses.11,12 Several selective σ1 receptor ligands have been proposed to develop new classes of CNS agents for neuroprotection, anxiety, depression, psychosis, and learning and memory improvement.13 Conversely, only few σ2 agents are now under development: putative agonists have been proposed as anticancer drugs, whereas antagonists have been suggested to be useful in inhibiting extrapyramidal symptoms of neuroleptics.10 Most of the σ2 receptor ligands show a low selectivity relative to the σ1 subtype. Poorly selective 1,3-di-2-tolyguanidine (1, DTG) is the most used radioligand in binding affinity assays (Chart 1). Excellent σ2 receptor ligands were found within the series of tetrahydroisoquinoline derivatives, where the highest selectivity was displayed by N-[4-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)butyl]-2-(2-flouro-ethoxy)-3-methoxy-5-iodo-benzamide (2). The corresponding radiolabeled [18F]-derivative has been developed to be used in PET analysis.14 The σ2 receptor agonist (+)-(1R,5R)-(E)-8-3,4-dichlorobenzylidene-5-(3-hydroxyphenyl)-2-methyl-2-azabicyclo[3.3.1]nonan-7-one (3, CB 184) widely used to characterize σ2 receptors, also binds the μ opioid receptor.15 A promising agent for tumor therapy is the σ2 receptor ligand 1’-[4-[4-(fluorophenyl)-1H-indol-3-yl]-1-butyl]pirro-[isobenzofuran-1(3H),4’-piperidene] (4, siramine)16 that causes lysosome leakage leading the tumor cells to death.17 Very few are the compounds claimed to be σ2 receptor antagonists such as the moderate-affinity 1-(2-phenylethyl)-4-(2-pyridyl)piperazine (5, UMB 24),18 which displays only a poor σ2 over σ1 selectivity, (±)-3α-Tropanyl 2-(4-chlorophe-noxy)butyrate [6, (±)-SM 21] is a σ2 selective but hydrolyzable antagonist.19

In our previous works, the high-affinity σ2 receptor ligand 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydroanaphthalen-1-yl)propyl]piperazine (7),20 later named PB 28, emerged
Chart 1. Structures of the Most Known \( \sigma_2 \) Ligands

![Chart 1](image)

from a class of \( N \)-cyclohexylpiperazine derivatives.\(^{21}\) On the basis of earlier binding studies, it proved to be selective relative to \( \sigma_1 \) receptor (40-fold) and a number of other CNS receptors.\(^{22,23}\) A summary of structure–affinity relationship (SAfIR) studies on compound 7 and its analogs is reported in a recent review.\(^{24}\) Compound 7 was widely studied in functional assays and demonstrated an agonist \( \sigma_2 \) activity because it inhibited electrically evoked twitch in guinea pig ileum,\(^ {23}\) and proliferation of SK-N-SH,\(^{25}\) MCF7,\(^{26}\) and PC3 tumor cell lines.\(^ {27}\) Compound 7 also proved to down regulate P-gp pump expression and to revert multidrug resistance (MDR) in breast cancer tumor cells treated with doxorubicin,\(^ {28}\) two effects consistent with agonist \( \sigma_2 \) receptor activity. Contradictory binding results for compound 7 and its enantiomers,\(^ {21}\) prompted us to conduct a reinvestigation with an updated protocol. Although selectivity was quite lost from new assays in animal tissues,\(^ {28}\) the \( \sigma_2 \) over \( \sigma_1 \) receptor selectivity was confirmed in MCF7 and MCF7 ADR cell lines (46-fold and 59-fold respectively) for racemic compound 7.\(^ {28}\) Since piperidine derivatives provided a good pharmacophore for \( \sigma_1 \) affinity,\(^ {28,26}\) we assumed that the poor \( \sigma_2 \) over \( \sigma_1 \) selectivity found in the \( N \)-cyclohexylpiperazines series was possibly caused by the piperazine overlapping the piperidine ring. Moreover, the two \( N \)-atoms in the piperazine ring should allow reverse binding modes to both the \( \sigma \) receptor subtypes, as already suggested.\(^ {30,31}\) To find out which \( N \)-atom is essential for \( \sigma_2 \) binding, we followed an approach similar to the one conducted for investigating the \( \sigma_1 \) pharmacophore in a series of phenylalkyl-piperidine and \(-\)piperazine analogs.\(^ {30}\)

In the present work, we synthesized a series of analogs of compound 7 to explore the effect of the lack of one of the two basic piperazine \( N \)-atoms in \( \sigma \) receptor binding. Alternatively, proximal and distal \( N \)-atoms were replaced by an isosteric methine group or were transformed in amidic functions. Two piperidine derivatives were prepared with an \( N \)-atom or an \( O \)-atom in the intermediate chain to balance the increase in hydrophobicity. Moreover, one or both the piperazine \( N \)-atoms were quaternized to evaluate the effect of positive permanent charges on the ligand binding. Derivatives bearing an opened piperazinone ring or a symmetric (5-methoxy)-tetralinpropyl substituent were prepared, as well as some analogs devoid of \( N \)-cyclohexyl substituent, and with an isosteric ring replacing the piperazine ring. A series of known and rather more simply substituted \( N \)-cyclohexylpiperazines were also investigated. To further define their selectivity, most of these compounds also underwent explorative binding assays at emopamil binding protein (EBP), a sterol isomerase thought to belong to the \( \sigma \) receptor family.\(^ {27,32}\) The most significant ligands, among those herein reported, were selected to be assayed for their ability in inhibiting cell proliferation. Thus, a limited structure–activity relationship (SAR) was generated to define the structural features for \( \sigma_2 \) agonist or antagonist activity.

Chemistry

The synthetic pathway for final compounds 11, 15, 16 and 24 is depicted in the Scheme 1. PtO\(_2\)-catalyzed hydrogenation of 4-(3-cyclohexen-1-yl)pyridine (8) yielded 4-cyclohexylpiperidine (9). Alkylation of intermediate 9 with (5-chloropen-tyl)tetralin\(^ {10}\) afforded final compound 11. 4-Cyclohexyl-4-hydroxy-piperidine (13) was given by Grignard’s reaction between 1-benzyl-4-piperidine (12) and cyclohexylmagnesium bromide, followed by debenzylization with H\(_2\) and 10% palladium on activated carbon. Compounds 9 and 13 were alkylated by (3-bromopropyl)tetralin\(^ {14,15}\) affording final compounds 15 and 16. The synthesis of the final piperidine 24 was achieved in several steps. Bromopropyl intermediate 14 was reacted with diethyl malonate in the presence of NaH to afford diethyl ester derivative 17. Reduction of this latter compound by LiAlH\(_4\) yielded the diol 18, which was subsequently mesylated to the compound 19 with methanesulfonyl chloride. Nucleophilic substitution of compound 19 with NaCN provided dinitrile 20, which was subsequently hydrolyzed to the corresponding dicarboxylic acid monoamide 21 in alkaline medium (NaOH). Cyclization of such amide to the imide 22 was provided in the presence of carbonyl diimide. Imide 22 was reduced by LiAlH\(_4\) to 4-substituted piperidine derivative 23, which was condensed to the corresponding enamine with cyclohexanone in the presence of trifluoroacetic acid. Reduction in situ with NaBH\(_4\) gave the final compound 24.

The synthesis of final piperidines 29 and 33 is depicted in the Scheme 2. Carboxylic acid intermediate 26 was obtained by alkylation of 5-methoxy-1,2,3,4-tetrahydro-1-naphthalen (25)\(^ {12}\) with chloroacetic acid, through previous activation with NaH,\(^ {33}\) and then was reduced with LiAlH\(_4\) to the corresponding alcohol 27, that generated compound 28 upon mesylation. This last compound was reacted with 4-cyclohexylpiperidine (9) to achieve final compound 29. Acylation of 5-methoxy-1,2,3,4-tetrahydro-1-naphthalenamine (30) with bromoacetyl chloride gave bromoacetamide 31 according to a previously reported procedure.\(^ {34}\) The subsequent substitution with 4-cyclohexylpiperidine (9) led to the amide derivative 32, which was then reduced with borane dimethyl sulfide complex to the final diamine compound 33.

The final compound 36 was prepared by hydrolysis of carboxylic acid ethyl ester 34\(^ {35}\) in alkaline medium and
subsequent condensation of the corresponding carboxylic acid with commercial 1-cyclohexylpiperazine (35) by activation with dicyclohexylcarbodiimide (DCC) (Scheme 3).

The synthesis of final compounds 40, 41, 43, 44, 49, and 50 is reported in the Scheme 4. All these compounds were prepared starting from the common key intermediate 14. The synthesis of final compound 40 required the monomethylammonium intermediate 39. Commercially available 1-acetyl-4-cyclohexylpiperazine (37) was prepared by acetylation of 1-cyclohexylpiperazine (35) and then underwent methylation affording the corresponding methylpiperazinium iodide salt 38. Subsequent deacetylation with HCl afforded the intermediate methylpiperazinium iodide salt 39 that was subjected to alkylation with the key compound 14 to give the final compound 40. Final bis-1,4-dimethylpiperazinium iodide 41 was obtained by methylation of known compound 7 with an excess of CH3I. Intermediate 42 was synthesized as already reported with an excess of CH3I. Intermediate 42 was subjected to deacetylation with HCl to afford the intermediate methylpiperazinium iodide salts 39 and 42. Alkylation of this last compound with bromocyclohexane afforded the final piperazinium salt 49. Symmetrical compound 50 was afforded by reaction of an excess of intermediate 14 with piperazine.

Furthermore, compound 14 was used as starting material for the synthesis of final compound 54 (Scheme 5). Through standard reaction between 14 and potassium phthalimide, derivative 51 was synthesized. Then, the latter compound underwent hydrazinolysis to intermediate amine 52. Final amine 54 was obtained by derivatization of amine 52 with amide 53 which is also commercially available. Small piperazine derivatives 37 and 55–59 (Table 3) were prepared through standard procedures starting from commercial N-acetylpiperazine or N-cyclohexylpiperazine (35). Compounds 37 and 56–58 were also commercially available.

To be assayed, all target compounds obtained as free bases were converted into hydrochloride salts with gaseous HCl in

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**Scheme 1.** Synthesis of 7-Related Cyclohexylpiperidine Derivatives 11, 15, 16, and 44

![Scheme 1](image_url)

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Reagents: (a) H2, PtO2; (b) cyclohexylmagnesium bromide; (c) H2, Pd–C; (d) CH2(COOC2H5)2, NaH; (e) LiAlH4; (f) CH3SO2Cl; (g) NaCN; (h) NaOH; (i) 1,1'-carbonyldiimidazole; (j) cyclohexanone, CF3COOH; (k) NaBH4.
Scheme 2. Synthesis of 7-Related 4-Cyclohexylpiperidines with Hetero Spacer 29 and 33a

Scheme 3. Synthesis of Amide Compound 36a

Results and Discussion
Radioligand Binding and σ2 SAfIR. Several N-cyclohexylpiperazines which are analogs of σ agent 7 were found to be high-affinity σ2 ligands in our previous works21,24 and this suggested an important role for piperazine ring N-atoms in optimal σ2 receptor binding. Are both N-atoms important for σ2 receptor affinity? Is the proximal N-atom (the one linked to the intermediate chain) to be important? Or the distal one (the one bearing the cyclohexyl group)? To answer these questions, compounds 15 and 24 were first prepared. In these derivatives, the distal N-atom and the proximal N-atom, respectively, were replaced by an isosteric methine group in the piperazine ring (Table 1). The binding data at σ2 receptor for both these piperidine derivatives revealed quite similar affinities (Ki,σ < 30 nM) and different binding profile. Compounds 15 and 40 were σ2 receptor preferring ligands; 16, 24, 49, and small piperazines 57 and 59 were both σ receptors almost equally high-affinity ligands; 41 was a poor σ ligand. Furthermore, we prepared the σ3 agonist 7 and σ3 antagonist 5, and included them in the assay as positive and negative reference compounds, respectively. All selected compounds were tested for evaluating their possible σ2-mediated antiproliferative effect at 48 h in SK-N-SH cell line. The EC50 values were obtained from nonlinear iterative curve fitting by Prism, version 3.0, GraphPad software.41

Antiproliferative Assays. The functional biochemical assays were performed on human SK-N-SH neuroblastoma cell line, where the expression of σ2 receptor had been previously reported.25 Among the newly synthesized σ2 receptor ligands, strategic compounds 15, 16, 24, 40, 49, 57, and 59 were selected on the basis of their high σ2 receptor affinity (Ki,σ < 30 nM) and different binding profile. Compounds 15 and 40 were σ2 receptor preferring ligands; 16, 24, 49, and small piperazines 57 and 59 were both σ receptors almost equally high-affinity ligands; 41 was a poor σ ligand. Furthermore, we prepared the σ3 agonist 7 and σ3 antagonist 5, and included them in the assay as positive and negative reference compounds, respectively. All selected compounds were tested for evaluating their possible σ2-mediated antiproliferative effect at 48 h in SK-N-SH cell line. The EC50 values were obtained from nonlinear iterative curve fitting by Prism, version 3.0, GraphPad software.41

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N-atom resulted in an increase of hydrophobic parameter ClogD, therefore compound 16 was prepared as a 4-hydroxy-piperidine derivative of 15. This modification not only led to a small improvement of $\sigma_2$ affinity but also to a significant enhancement of $\sigma_1$ affinity, so that 16 turned out to be a nonselective ligand. Preparation of 1-cyclohexyl-4-hydroxy-piperidine derivative, isomer of 16, failed because of synthetic difficulties. As an alternative way, the hydrophobicity was lowered by introduction of an O-atom or a NH-group in the intermediate chain adjacently to the tetralin nucleus. In the case of compound 29 both $\sigma_1$ and $\sigma_2$ affinities were moderate resulting in an absence of selectivity; in the case of compound 33 a high $\sigma_1$ affinity appeared, whereas a moderate $\sigma_2$ affinity was retained, comparable to the affinity of longer-chain compound 11. One can deduce that the presence of a further basic N-atom could increase the possibility of more binding modes at the $\sigma_1$ receptor.

Another way to demonstrate the importance of N-atom basicity was to render unavailable one electron lone pair at a time for each N-atom of the piperazine ring. Thus, amide derivatives 36, 44, and 54 were prepared. Compound 36 showed lower $\sigma_2$ affinity ($K_i = 179$ nM), suggesting a certain role of proximal N-atom basicity in $\sigma_2$ receptor binding. Moreover, its $\sigma_1$ affinity reached the subnanomolar range ($K_i < 0.11$ nM) revealing the compound 36 as the highest-affinity and selective $\sigma_1$ receptor ligand among this class. The insertion of a carbonyl group in the $\alpha$-position of the distal piperazine N-atom led to the amide 44 (Table 2). This compound displayed low $\sigma_2$ affinity ($K_i = 304$ nM) and very low $\sigma_1$ affinity ($K_i > 5000$ nM). However, it should be noted that in compound 44 the cyclohexyl substituent was spaced out by a carbon atom from the piperazine N-atom. These results taken together with those for 36 confirmed the importance of the basicity of distal piperazine N-atom for $\sigma_1$ receptor binding and of both N-atoms for excellent $\sigma_2$ receptor binding within this series. Similar results were obtained when the amide 54 was assayed as opened piperazinone ring derivative ($K_i = 798$ nM); neither $\sigma_2$ nor $\sigma_1$ receptor affinity was significant (Table 2). This same behavior was evident in simpler 1,4-disubstituted piperazines.

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Scheme 4. Synthesis of Methylpiperazinium Derivatives 40, 41, and 49 and Compounds 43, 44, and 50

Reagents: (a) CH$_3$I; (b) HCl; (c) N-acetylpiperazine; (d) cyclohexanecarbonyl chloride; (e) bromocyclohexane; (f) piperazine.
when the affinities of compounds 58 and 57 were compared (Table 3). The importance of a hydrophobic group such as the cyclohexyl substituent was better stressed in the high-affinity mixed $\sigma_1/\sigma_2$ ligand 59, where a three-methylene spacer supplies the appropriate distance from N-atoms to fit the known putative $\sigma_1$ receptor model.42

To investigate if N-atoms bind the receptor in the protonated form, one or two positively charged N-atoms were inserted, by quaternization to ammonium salts 40, 41, and 49. The results for these three compounds were surprisingly interesting (Table 1). The presence of two charged methylenammonium functions in compound 41 was quite detrimental for both $\sigma_1$ and $\sigma_2$ binding, lowering the $\sigma_2$ affinity at a micromolar range ($K_i=1710$ nM). This could be either due to the low hydrophobicity provided by the doubly charged piperazine or to its conformation inferred by the two methyl groups. When only a methylenammonium function was present, the $\sigma_2$ binding was not hampered, the $\sigma_2$ affinities being rather comparable for 40 and 49 ($K_i$ values 13.0 and 6.78 nM, respectively). A greater difference was observed

### Table 1. ClogD Values and Binding Data for 7-Related $\sigma$ Ligands with Cyclohexylpiperazine or Cyclohexylpiperidine Moiety

<table>
<thead>
<tr>
<th>compound</th>
<th>A</th>
<th>B</th>
<th>X</th>
<th>Y</th>
<th>Clog D$^a$</th>
<th>$K_i$ ± SEM (nM)</th>
<th>$K_i$ ratio</th>
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<tbody>
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<td>7$^b$</td>
<td>CH$_2$</td>
<td>CH$_2$</td>
<td>N</td>
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<td>11</td>
<td>(CH$_2$)$_3$</td>
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<td>CH</td>
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<td>236 ± 85</td>
<td>72.3 ± 7.3</td>
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<td>338 ± 66</td>
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<td>$^+NCH_3$</td>
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<td>&gt;5000</td>
<td>1710 ± 400</td>
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<tr>
<td>49</td>
<td>CH$_2$</td>
<td>CH$_2$</td>
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<tr>
<td>(+)-pentazocine</td>
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<td>1860 ± 40$^d$</td>
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<tr>
<td>(±)-ifenprodil</td>
<td>88.5 ± 9.0$^d$</td>
<td>32.0 ± 1.7</td>
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$^a$ Referred to pH 7.4. $^b$ $\sigma_1$ And $\sigma_2$ data are from ref 24. $^c$ Not tested. $^d$ From ref 25.

### Table 2. ClogD Values and Binding Data for 7-Related $\sigma$ Ligands with Modified Cyclohexylpiperazine Moiety

<table>
<thead>
<tr>
<th>compound</th>
<th>A</th>
<th>X</th>
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<th>$K_i$ ± SEM (nM)</th>
<th>$K_i$ ratio</th>
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<tbody>
<tr>
<td>43$^b$</td>
<td>R$_1$</td>
<td>NH</td>
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<td>218 ± 74</td>
<td>590 ± 86</td>
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<td>R$_3$</td>
<td>3.86</td>
<td>3910 ± 610</td>
<td>798 ± 186</td>
<td>1230</td>
</tr>
</tbody>
</table>

$^a$ Referred to pH 7.4. $^b$ Previously assayed in $\sigma$ total binding: $K_i > 877$ nM, [3H]-I, guinea-pig brain (ref 20). $^c$-Hex stands for cyclohexyl. $^d$ Previous $\sigma_1$ binding: $K_i = 18.2$ nM, (+)-[3H]-pentazocine, whole rat brain (ref 37). $^e$ Not tested.
The synthesis of opened piperazinone analog 54 was achieved through the following steps:

1. Reaction of compound 14 with Br followed by addition of A (Potassium phthalimide) and B (NH₂NH₂, HCl) to give compound 51.
2. Reaction of compound 52 with reagents to give compound 53.
3. Reaction of compound 54 with OCH₃ to give compound 55.

Table 3. ClogD Values and Binding Data for Simple N-Cyclohexylpiperazine-Related σ Ligands

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>B</th>
<th>ClogD*</th>
<th>Kᵢ ± SEM (nM)</th>
<th>Kᵢ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>H</td>
<td>c-Hex</td>
<td>-0.38</td>
<td>&gt;5000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>37</td>
<td>CH₃CO</td>
<td>c-Hex</td>
<td>1.28</td>
<td>&gt;5000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>55</td>
<td>CH₃CH₂</td>
<td>c-Hex</td>
<td>0.95</td>
<td>62.4±11.3</td>
<td>5.6</td>
</tr>
<tr>
<td>56</td>
<td>CH₃CO</td>
<td>CH₃</td>
<td>-0.58</td>
<td>&gt;5000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>57</td>
<td>c-Hex</td>
<td>c-Hex</td>
<td>2.27</td>
<td>2.05±0.63</td>
<td>5.6</td>
</tr>
<tr>
<td>58</td>
<td>c-HexCO</td>
<td>c-Hex</td>
<td>3.36</td>
<td>3.59±0.98</td>
<td>56</td>
</tr>
<tr>
<td>59</td>
<td>c-Hex(CH₃)</td>
<td>c-Hex</td>
<td>4.09</td>
<td>0.25±0.07</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Referred to pH 7.4.

Scheme 5. Synthesis of Opened Piperazinone Analog 54.

between the σ₁ affinity values of the same compounds (Kᵢ values 338 and 63.7 nM, respectively). Again, a more pronounced decrease in σ₁ affinity was caused by distal N-atom quaternization (compound 40), suggesting the need of an available electron lone pair of the piperazine distal N-atom for optimal σ₁ receptor binding. On the other hand, when this N-atom was secondary (compound 43) by removal of the cyclohexyl group, both σ₁ and σ₂ affinities became moderate (Table 2). These results could be explained by either a lower hydrophobicity of 43 or a piperazine ring boat conformational arrangement, which weakens N-atoms basicity through an intramolecular H-bond between them. As a confirmation of this, piperidine 45 and morpholine 46, which are isosteric derivatives of 43, regained high σ₁ affinity (Kᵢ values 3.38 and 9.10 nM, respectively), whereas σ₂ affinity increased only for 45. Likely, a reduced interaction with the corresponding hydrophobic pocket in the σ₂ receptor might be hypothesized for the morpholine moiety of 46, because of the loss of the cyclohexyl group together with the presence of a hydrophilic O-atom. Furthermore, from these results it derives that a certain additional role is played by the 5-methoxytetralin nucleus. Still, testing the symmetrically substituted piperazine 50, σ₂ receptor affinity was poor, suggesting the need of a relatively small substituent on one of the piperazine N-atoms.

To investigate more deeply these aspects, a molecular simplification was conducted. Some simpler-substituted piperazines were tested, where the N-cyclohexyl was retained and the 5-methoxytetralin nucleus removed and replaced by a small group (Table 3). The sole removal of the proximal N-atom substituent led to the 1-cyclohexylpiperazine (35), with loss of any affinity (Kᵢ > 5000). In this case as well, a high hydrophilicity and/or an intramolecular H-bond can be claimed. The 4-ethyl substitution on the 1-cyclohexylpiperazine (compound 55) was sufficient to raise a certain affinity, which was more pronounced toward σ₁ receptor (Kᵢ = 62.4 nM). When both piperazine N-atoms were substituted with a cyclohexyl group (compound 57), better affinities were reached either for σ₂ (Kᵢ = 11.5 nM) or σ₁ receptor (Kᵢ = 2.05 nM). These affinities became even higher (σ₂, Kᵢ = 4.70; σ₁, Kᵢ = 0.25) when one of the two cyclohexyl was spaced from the N-atom by a three-methylene chain (compound 59). As already said, the inclusion of the N-atoms into an amide function results in a worse σ₂ (amide 58, Kᵢ = 201 nM) but not σ₁ affinity (Kᵢ = 3.59 nM). These results suggest that both basic N-atoms of piperazine ring are needed for an optimal σ₂ binding, whereas an amide function still allows a good interaction with σ₁ receptor likely through a H-bond interaction. Nevertheless, the hydrophobic contribution in the amide function (compounds 36, 44, 58) was beneficial since the acetamide 37 displayed no affinity at both σ receptors. The importance of more hydrophobic substituents was evidenced by the moderate affinities of ethyl-derivative 55, which were higher than 37, but lower than 57 affinities. When no cyclohexyl group was present and only one N-atom was basic (acetylderivative 56), both σ affinities were lost, as for 37.

As for affinities toward EBP site (i.e., mammalian Δ₉-Δ₇ sterol isomerase), we already demonstrated that the presence of a tetralinalkyl moiety brought EBP affinity. All the EBP binding results were rather comparable to those at σ₁ receptor (Kᵢ values 3.75). Linear relationships were evidenced for small piperazines 56, 55, 57, and 59 series and piperidines 16, 15, 24, and 11 series where the rise
1.40 
57 
15.9 ± 0.6
57 
(4%)^f
24 
1.40 ± 0.38
59 
52.1 ± 0.8

<table>
<thead>
<tr>
<th>compound</th>
<th>EC_50 ± SEM^g (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>(38%)^f</td>
</tr>
<tr>
<td>7</td>
<td>9.97 ± 0.55</td>
</tr>
<tr>
<td>15</td>
<td>3.64 ± 0.68</td>
</tr>
<tr>
<td>16</td>
<td>15.9 ± 0.6</td>
</tr>
<tr>
<td>24</td>
<td>1.40 ± 0.38</td>
</tr>
</tbody>
</table>

*Human neuroblastoma cell line. *Mean of n ≥ 2 separate experiments. *Percent inhibition at 100 μM.

Table 4. Antiproliferative Effect Measured as Inhibition of SK-N-SH Cell Proliferation

Antiproliferative effect of compounds tested in human SK-N-SH neuroblastoma cell line. Incubation was carried out for 48 h in the presence (1−100 μM) and absence of tested compound (ref 25 and 26).

Functional Assays and SAR. The results expressed as EC_50 values are reported in Table 4. As previously reported, SK-N-SH human neuroblastoma cell line expressed α_1 receptor in a low affinity state. Investigation by Scatchard analysis proved the absence of EBP binding sites in the same cell line. Thus, we speculated that the antiproliferative effect was mediated by α_2 receptor rather than the other two investigated binding sites. When tested in SK-N-SH human neuroblastoma cell line, compound 5 displayed only 38% of antiproliferative activity at 100 μM, confirming its α_2 antagonist activity, which had been previously claimed by in vivo assay. The compounds selected to be tested in the antiproliferative assay as the highest-affinity α_2 ligands were also representative of the different structural changes made. The ammonium salts 40, 41, and 49 showed a comparable low percentage of inhibition of the proliferation, despite their different α_2 affinities. This may be ascribed to the permanent charges that likely prevent the compounds to penetrate the cell and exert the effect. The symmetric ligand 57 may be claimed as α_2 receptor antagonist because it showed an antiproliferative activity as low as 4% at 100 μM. The other five tested compounds displayed antiproliferative activity in the micromolar range (EC_50 ranging from 1.40 μM to 52.1 μM), and thus they can be claimed as α_2 receptor agonists (Figure 1). The piperidine compounds 15 and 24 displayed antiproliferative activity (EC_50 3.64 and 1.40 μM respectively) higher than 7, although their α_2 receptor affinity was moderate. Furthermore, compounds 15 and 24 presented the highest ClogD values among the compounds tested (4.81 and 5.43 respectively). In the 1.40−15.9 μM range, a linear relationship was observed between the antiproliferative activity and the ClogD rise within the sequence of the most active compounds 16, 7, 15, and 24. Despite the unambiguous action of α_2 receptors in mediating the antiproliferative effect in SK-N-SH cells, a linear correlation between the α_2 receptor affinity and the activity could not be found, so that other receptor systems should be taken into account.

Conclusions

None of the newly tested compounds herein presented displayed α_2 receptor affinity comparable to that of compound 7. Therefore, one can deduce that both N-atoms in the piperazine ring are important for a subnanomolar α_2 receptor binding in this class. The loss of only one of the basic N-atoms did not lead to a dramatic drop in affinity, indicating just a weakened binding. The same conclusions were in part achieved in a recent molecular modeling work on some series of our cyclohexylpiperazines studied with a comparative molecular field analysis (CoMFA) method. Among the changes made to repress N-atom basicity, introduction of the amide function was the most detrimental for α_2 receptor binding (compounds 36 and 44). On the basis of this finding, compound 36, which was found to possess the highest α_1 affinity (K_i = 0.11 nM), reached a noteworthy selectivity (1627-fold). Small N-cyclohexylpiperazine 59 emerged as the highest-affinity α_2 ligand (K_i = 4.70 nM). Activity results from antiproliferative assay did not allow to define a clear SAR consistently with binding results. Indeed, isomeric piperidines 24 and 15 elicited putative α_2 agonist activity higher than reference compound 7, although their α_2 receptor affinities were lower. Conversely, compound 57 was found to be a possible α_2 antagonist.

Experimental Section

Chemistry. Both column chromatography and flash column chromatography were performed with 60 Å pore size silica gel as the stationary phase (1:30 w/w, 63–200 μm particle size, from ICN and 1:15 w/w, 15–40 μm particle size, from Merck respectively). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Purity of tested compounds was established by combustion analysis, confirming a purity ≥ 95%. Elemental analyses (C, H, N) were performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ± 0.4% of the theoretical values, unless otherwise indicated. HPLC analyses were performed on a 1525 Micro Binary pump instrument (Waters) equipped with a 996 Photo Diode Array (PDA) detector and on a Perkin-Elmer series 200 LC instrument equipped with a Perkin-Elmer 785A UV/vis detector. ^1^H NMR spectra were recorded on a Mercury Varian 300 MHz using CDCl_3 as solvent, unless otherwise reported. The following data were reported: chemical shift (δ) in ppm, multiplicity (s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet), integration and coupling constants in hertz. Recording of mass spectra was done on an Agilent 6890–5973 MSD gas chromatograph/mass spectrometer and on an Agilent 1100 series LC-MSD trap system VL mass spectrometer; only significant m/z peaks, with their percentage of relative intensity in parentheses, are reported. Chemicals were from Aldrich and Acros and were used without any further purification.

General Procedure to Obtain Final Amine Derivatives 11, 15, 16, and 16. In a typical reaction, the haloalkyl intermediate, 1-(5-chloropentyl)-24 or 1-(3-bromopropyl)-5-methoxy-1,2,3,4-tetrahydronaphthalene (14) (1.0 mmol), was stirred and refluxed overnight with the appropriate amine (9 or 13 or morpholine) (1.2 mmol) and Na_2CO_3 (1.0 mmol, 0.11 g) in CH_3CN. The work up for final amine compounds was carried out as previously reported for similar derivatives. Purification of the crude residue was achieved by column chromatography with
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CH$_2$Cl$_2$/MeOH (9:1) as eluent affording the title amine derivatives (11, 15, 16, and 46) as colorless oils in 70% yield, unless otherwise stated.

4-Cyclohexyl-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)-pentyl]piperidine (11). Compound 11 was obtained as a colorless oil in 40% yield (0.16 g) with CH$_2$Cl$_2$/MeOH (95:5) as eluent: $^1$H NMR δ 0.85–1.98 [m, 28H, cyclohexyl CH and 5 CH$_2$, piperidine CH(CH$_2$)$_2$, and (CH$_2$)$_3$CH(CH$_2$)$_2$], 2.28–2.35 [m, 6H, CH$_2$N(CH$_2$)$_2$], 2.55–3.05 [m, 3H, benzyl CH and CH$_2$], 3.80 (s, 3H, OCH$_3$), 6.62–7.05 (m, 3H, aromatic); signal attribution was supplementary assisted by NOESY-NMR; GC-MS m/z 398 (M$^+$ + 1, 6), 397 (M$^+$, 22), 236 (43), 180 (100); Anal. (C$_{25}$H$_{39}$NO$^+$) calcd 370 (M$^+$), 322 (23), 236 (43); LC-MS-MS 457: 361, 243.

3-[3-(5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]-1,5-dinitroline (20). A solution of 19 (3.06 mmol, 1.33 g) and NaCN (12.25 mmol, 0.600 g) in CH$_3$CN (15 mL) in the presence of 1 µL of 15-Crown-5 ether was refluxed under stirring overnight. After the mixture was cooled to room temperature, the solvent was evaporated under reduced pressure, and the residue was taken up with H$_2$O and extracted with CH$_2$Cl$_2$ (3 × 15 mL). The organic layers were collected, dried (Na$_2$SO$_4$), and concentrated under reduced pressure, giving a crude mixture which was purified by column chromatography with CH$_2$Cl$_2$ affording a yellow oil (0.635 g) in a 70% yield: $^1$H NMR δ 1.35–1.85 [m, 8H, (CH$_2$)$_3$CO, benzyl CH and CH$_2$], 2.15–2.20 [m, 2H, (CH$_2$)$_2$CH$_2$], 2.45–2.58 [m, 2H, (CH$_2$)$_2$CN], 2.60–2.82 [m, 3H, benzyl CH and CH$_2$], 3.80 (s, 3H, OCH$_3$), 6.62–7.15 (m, 3H, aromatic); LC-MS (ESI$^+$) m/z 457 [M + Na$^+$]; LC-MS-MS 370: 361, 243.

3-(Carbamoylmethyl)-6-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)hexanoic Acid (21). A solution of intermediate 20 (2.18 mmol, 0.97 g), NaOH (6.05 mmol, 0.24 g) and 15-Crown-5 ether (1 µL) in EtOH (20 mL, 90%) was refluxed for 40 h under stirring. The solvent was then removed under reduced pressure, and the residue was taken up with H$_2$O and added with 6M HCl until the solution became acidic. The acidic solution was extracted with AcOEt (3 × 15 mL), the organic layers were dried (Na$_2$SO$_4$) and concentrated under reduced pressure to give the title compound (3.17 g) as a white solid in 90% yield: $^1$H NMR δ 1.35–1.90 [m, 11H, (CH$_2$)$_3$CO, benzyl CH and CH$_2$], 2.10–2.30 [m, 2H, (CH$_2$)$_3$CO], 2.45–2.80 [m, 5H, (CH$_2$)$_2$CO, benzyl CH and CH$_2$], 3.80 (s, 3H, OCH$_3$), 6.60–7.15 (m, 3H, aromatic); LC-MS (ESI$^+$) m/z 356 [M + Na$^+$].

2-[3-(5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]propane-1,3-diol (18). A solution of 17 (5.52 mmol, 2.00 g) in anhydrous Et$_2$O (10 mL) was added in a dropwise manner to a suspension of LiAlH$_4$ (11.4 mmol, 0.422 g) in the same solvent kept under N$_2$ and at 0 °C. The mixture was then refluxed for 4 h, and after it was cooled to room temperature, few drops of H$_2$O were carefully added into the reaction pot. The obtained mixture was extracted with Et$_2$O (3 × 15 mL) and the collected organic phases were dried (Na$_2$SO$_4$) and concentrated under reduced pressure to give a crude mixture. This latter was purified by column chromatography with CH$_2$Cl$_2$/ethanol acetate (8:2) as eluent affording 18 (1.17 g) as a colorless oil in 76% yield: $^1$H NMR δ 1.32–1.85 [m, 11H, (CH$_2$)$_3$CO, benzyl CH and CH$_2$] 2.55–2.80 (m, 5H, benzyl CH and CH$_2$ and 2 OH, D$_2$O exchanged), 3.58–3.72 [m, 2H, (CH$_2$O)$_2$], 3.78–3.85 (s, 5H, OCH$_3$ and (CH$_2$O)$_2$), 6.60–7.10 (m, 3H, aromatic); GC-MS m/z 278 (M$^+$, 15), 161 (100).

2-[3-(5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]-1,3-propanediol Dimethanesulfonate (19). Methylchloride (15.5 mmol, 1.20 mL) was added to a solution of 18 (4.14 mmol, 1.15 g) and Et$_3$N (16.1 mmol, 2.26 mL) in anhydrous CH$_2$Cl$_2$ at 0 °C. The mixture was kept under stirring at the same temperature for 1 h. Then it was poured into H$_2$O and extracted with CH$_2$Cl$_2$ (3 × 15 mL). The organic layers were collected, dried (Na$_2$SO$_4$), and concentrated under reduced pressure to give a crude mixture which was purified by column chromatography with CH$_2$Cl$_2$/ethyl acetate (8:2) as eluent. The brown oil obtained underwent further purification by column chromatography with CH$_2$Cl$_2$/ethyl acetate (7:3) as eluent affording pure 19 (1.53 g) as a colorless oil in 85% yield: $^1$H NMR δ 1.40–2.25 [m, 11H, (CH$_2$)$_3$CO, benzyl CH and CH$_2$], 2.55–2.80 (m, 3H, benzyl CH and CH$_2$), 3.05 (s, 6H, 2 SO$_2$CH$_2$), 3.80 (s, 3H, OCH$_3$), 4.18–4.35 (m, 4H, 2 CH$_2$O), 6.60–7.15 (m, 3H, aromatic); LC-MS (ESI$^+$) m/z 457 [M + Na$^+$]; LC-MS-MS 370: 361, 243.
of H₂O were carefully added. The resulting mixture was extracted with Et₂O (3 × 10 mL), and the organic layers were collected and dried (Na₂SO₄). Concentration under reduced pressure gave a crude residue, which was purified by column chromatography with CH₂Cl₂/MeOH (9:1) as eluent to afford 23 (0.306 g) as a yellow oil in 70% yield; ¹H NMR δ 1.02–1.82 [m, 16H, (CH₂)₃CH(CH₃)₂, piperidin CH(CH₂)₂], 2.45–2.80 [m, 5H, (CH₂)₂NH, benzyl CH and CH₃], 3.02–3.18 [m, 2H, (C/H)₂N], 3.80 (s, 3H, OCH₃), 6.60–7.15 (m, 3H, aromatic); GC-MS m/z: 288 (M⁺ + 1, 8), 287 (M⁺, 11), 126 (100).

1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperidine (24). A solution of 23 (0.32 mmol, 0.150 g), cyclohexanone (0.96 mmol, 0.10 mL), and CF₃COOH (2 drops) in toluene (20 mL) was refluxed overnight with azeotropic removal of H₂O. After cooling, the reaction mixture was added to a solution of NaN₃ (0.16 g, 0.24 mmol) in CH₃CN (2 mL) and refluxed for 1 h. The mixture was cooled to room temperature; then it was added with excess of reagent was quenched with MeOH. Then 2 N HCl (0.17 mL) was added and the mixture was refluxed for 30 min. The mixture was cooled, the solvent was evaporated under reduced pressure to afford a white solid, which was passed over silica gel (CH₃Cl₂/MeOH 8:2 as eluent). The resulting gumy solid was recrystallized from EtOH to give the final compound 24 (0.17 g) as white crystals in 35% yield: ¹H NMR δ 1.18 [m, 2H, cyclohexyl CH(CH₂)₂], 1.85 [m, 2H, cyclohexyl CH(C/H)], 2.05–2.10 [m, 2H, cyclohexyl CH(CH₃)₂], 2.38–2.95 [m, 10H, CHN(CH₂)₂], 3.80 (s, 3H, OCH₃), 6.60–7.05 (m, 3H, aromatic); GC-MS m/z: 385 (M⁺ + 1, 13), 384 (M⁺, 50), 138 (78), 125 (100); Anal. (C₂₄H₃₈NO₂-HCl·H₂O) C, H, N.

1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]1-methylpiperazinium iodide (40). A mixture of 1-(3-bromopropyl)-5-methoxy-1,2,3,4-tetrahydronaphthalene (14) (3.2 mmol, 0.91 g) with intermediate 39 (3.2 mmol, 1.00 g) and Na₂CO₃ (3.7 mmol, 0.39 g) in CH₃CN (25 mL) and EtOH (2 mL) was refluxed and stirred overnight. After the mixture was cooled, the solvent was removed under reduced pressure, and the solid residue was dissolved in MeOH and warmed under stirring for 20 min. The methanolic solution, while still warm, was filtered and the filtrates were evaporated under reduced pressure to afford a yellow oil (160 mg), which was triturated overnight (CH₃Cl₂/MeOH 8:2 as eluent). The resulting gumy solid was recrystallized from EtOH to give the final compound 40 (1.15 g) as pale yellow crystals in 70% yield: mp: 173–175 °C; ¹H NMR (CDCl₃/OD) δ 1.18–1.70 [m, 14H, cyclohexyl (CH₂)₃, and (CH₂)₂-CH(CH₂)], 1.78–1.85 [m, 2H, cyclohexyl CH(CH₃)], 2.05–2.10 [m, 2H, cyclohexyl CH(CH₂)₂], 2.15–2.62 [m, 9H, benzyl CH and CH₃, and CH₂N(CH₂)₂], 2.77 (s, 3H, NCH₃), 3.20–3.40 [m, 5H, (CH₂)₂NCH₃, 3.80 (s, 3H, OCH₃), 6.45–6.95 (m, 3H, aromatic); LC-MS (ESI⁺) m/z: 385 (M⁺ + I); Anal. (C₂₄H₃₈NO₂·HCl·H₂O) C, H, N.

1-Cyclohexyl-1,4-dimethyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazinium dihydrochloride (41). To a solution of 33 (0.705 mmol, 0.30 g) in CH₃CN (5 mL), a solution of 3M MF in CH₃CN (0.5 mL), CH₃J (3.0 mmol, 0.2 mL) was added, and the mixture was stirred under reflux overnight. After it was cooled to room temperature, more CH₃I (6.0 mmol, 0.40 mL) was added to the mixture, which was stirred under reflux for further 80 h. After the mixture was allowed to cool to room temperature, the solvent was evaporated under reduced pressure. The residue was taken up with MeOH, and the mixture was refluxed for 30 min. The warm methanolic suspension was filtered, affording the target compound 41 (0.17 g) as white crystals in 35% yield: mp 240–241 °C; ¹H NMR (D₂O) δ 0.95–1.98 [m, 18H, cyclohexyl 5 CH₂, and (CH₂)₂CH(CH₂)₂], 2.38–2.55 (m, 2H, benzyl CH₂), 2.70–2.80 (m, 1H, benzyl CH), 2.98 (s, 3H, NCH₃), 3.10 (s, 3H, NCH₂), 3.35–3.58 (m, 2H, NCH and CH₂N), 3.65 (s, 3H, OCH₃), 3.75–3.85 (m, 8H, piperazinum ring).
4-Chloro-4-cyclohexyl-5-CH

OCH3), 6.62 (s, 3H, OCH3), 3.35 (s, 3H, NCH3), 3.52 (m, 4H, (CH2)2NCO), 3.80 (s, 3H, OCH3), 6.64 (s, 1H, NCH3). Anal. (C22H34N2O2·2H2O) C, H, N.

5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)piperazine (44). Cyclohexanecarboxylic chloride (2.57 mmol, 0.34 mL) was added in a dropwise manner to a solution of 43 (2.14 mmol, 0.615 g) and triethylamine (0.4 mL in toluene (8 mL) kept at 0 °C. The mixture was then heated at reflux for 6 h. The reaction mixture was allowed to cool to room temperature, and the solvent was evaporated under reduced pressure. The residue was taken up with H2O (10 mL) and extracted with CH2Cl2 (3 x 10 mL). The combined organic layers were dried (Na2SO4) and concentrated under reduced pressure. The crude residue was eluted by column chromatography with CH2Cl2/MeOH (95:5) affording the title compound (0.60 g) as a pale yellow crystals in 65% yield: mp 196 °C. The formazan crystals were solubilized by 100 μL of DMSO and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Supporting Information Available: Formulas, appearances, and melting points of hydrochloride salts of the novel final compounds 11, 15, 16, 24, 29, 33, 36, 44, 46, 50, and 54, elemental analyses of the novel end products, description of the preparation and spectroscopy data for the intermediate compounds 9, 13, N-benzyl-13, 26, 27, 32, 38, 39, 47, 48, 51 and 52, and purity HPLC analysis for compound 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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