1-[1-(2-Benz[b]thiophen-1-yl)cyclohexyl]piperidine Hydrochloride (BTCP) Yields Two Active Primary Metabolites in Vitro: Synthesis, Identification from Rat Liver Microsome Extracts, and Affinity for the Neuronal Dopamine Transporter

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1-[1-(2-Benz[b]thiophen-1-yl)cyclohexyl]piperidine hydrochloride (BTCP, 1) and cocaine bind to the neuronal dopamine transporter to inhibit dopamine (DA) reuptake. However, on chronic administration, cocaine produces sensitization, but 1 produces tolerance. Because metabolites of 1 might be responsible for some of its pharmacological properties, we have identified the primary metabolites of 1 produced by rat liver microsomes and determined their affinities for the DA transporter. Five monohydroxylated derivatives (3, 5, 9, 10, 14) and two degradation compounds (15, 16) were identified as metabolites through comparison with synthetic standards in HPLC and GC systems. Standards were obtained utilizing synthetic schemes previously used for the synthesis of phenylcyclohexyl metabolites. In vitro, two compounds (3, 5) showed a high affinity for the DA transporter. These active metabolites might be important in the pharmacology of 1.

Introduction

1-[1-(2-Benz[b]thiophen-1-yl)cyclohexyl]piperidine HCl (BTCP, 1) is a phenylcyclohexyl (PCP) analogue evoking behavior related to the stimulation of dopaminergic transmission by a different route to that of dextroamphetamine.1 In a drug discrimination paradigm, 1 generalizes in rats trained to discriminate cocaine from saline.2 It, like cocaine, binds strongly to the neuronal dopamine (DA) transporter to inhibit neuronal DA reuptake.3-5 1 is highly selective for the DA reuptake complex when compared to PCP as it binds very poorly to the PCP receptor inside the N-methyl-d-aspartate (NMDA)-gated Ca2+ channel. Therefore, cocaine and 1 are both potent indirect dopaminergic agonists.

DA uptake inhibition appears to be important in mediating the rewarding2 and reinforcing6,7 effects of cocaine, as evidenced by the highly significant correlation between the inhibition of DA uptake and self-administration.10,11 It has been recognized that the DA neuronal transporter can bind dopamine and reuptake blockers on different binding sites which are not completely overlapping.12-14 This observation led to the hypothesis that selective ligands could interfere with the mechanisms which make cocaine a drug of abuse15 and might help to block cocaine dependency in pharmacotherapeutic interventions.16 In rodents, 1 and cocaine have similar dose-dependent behavioral and neurochemical effects after acute administration. Chronically, however, in mice and rats cocaine induces a behavioral sensitization while 1 produces tolerance.17,18 These behavioral differences may be accounted for by the different binding sites for both drugs on the DA uptake complex.12-14,19 Alternatively, the metabolites of 1 may also be involved in its pharmacology. The enzymes responsible for the metabolism of 1 may be induced during chronic treatment, thus leading to an apparent tolerance. On the other hand, 1 may be very slowly degraded in mice and rats, leading to a relatively high circulating concentration. This would be similar to the situation with cocaine where continued infusion induces tolerance.20,21 The role for active metabolites in chronic administration studies is not known. We therefore decided to study the biotransformations of 1 initially in vitro. This study was designed to identify the primary metabolites of 1 obtained from rat liver microsomes and to measure their affinity for the rat striatal DA transporter labeled with [3H]BTCP. Putative hydroxylated metabolites of 1 were synthesized according to those already known in the PCP series and used for comparative identification (GC and HPLC) of micromolar metabolites of 1.

Chemistry

On the basis of structural similarities, the in vitro metabolism of 1 should be similar to that of PCP. Accordingly, structures of the putative metabolites to be synthesized as probes for identification were chosen according to known primary PCP metabolites.22,23 Thus, we prepared monohydroxylated derivatives of 1 bearing the hydroxyl substitution at either the β and γ position from the nitrogen atom in the piperidine ring and at either the β and γ position from the quaternary carbon atom in the cyclohexyl ring (Figure 1). In this last case cis- and trans-diastereomers are possible. Their structures were defined by the relative position of the hydroxyl group and the piperidine ring in reference to the plane of the cyclohexyl ring. (In some previous papers isomers were inaccurately defined relative to the aromatic moiety, thus cis and trans were reversed.) Finally, during identification we had to synthesize three more compounds to fully identify the primary metabolites produced: the isomeric alcohols 15 and 17 and the corresponding unsaturated compound 16 (Figure 1). In our previous synthesis in the arylcyclohexylamines series, we showed that two general synthetic paths could be used depending on the desired final structures: (i) structures unsubstituted at the cyclohexyl ring or...
obtained in the form of one diastereomer only and (ii) structures substituted at the cyclohexyl ring obtained as cis/trans-diastereomeric pairs. The first path is Bruylant’s reaction from suitable R-amino nitriles and Grignard reagents (Scheme 1). The stereospecificity of this reaction is well-known: it yields only one diastereomer whose configuration corresponds to an equatorial attack of the iminium intermediate by the Grignard reagent. For example this path would yield only the cis compound 9 (Figure 1). The second path stems from the Schmidt and Curtius reactions adapted to yield diastereomeric mixtures when the cyclohexyl moiety is substituted (Scheme 2). Moreover, depending on conditions (time and/or temperature), this azide synthesis may yield enriched mixtures of either of the anticipated diastereomers. Final chromatography is always needed to separate the isomers. The common 2-benzo[b]thiophenyl Grignard reagent necessary for both pathways was obtained by reacting MgBr2 with 2-lithiobenzo[b]thiophene. As anticipated, the piperidinol compounds 3 and 5 were easily obtained via the first path. However cyclohexanol derivatives were very difficult to obtain in a pure isomeric form. Two major problems were encountered: very low yields in the azide pathway and a great difficulty in resolving isomeric mixtures. Finally, the simplest method to obtain alcohols 9 and 10 was to prepare the ketal 7 via the first path (Scheme 1), regenerate the corresponding ketone, and reduce it by diborane or by potassium tri-sec-butylborohydride. This was done to isolate pure samples of cis-9 or trans-10 alcohols respectively after chromatography. The trans-alcohol 14 could have been obtained by either of the two paths. We used the azides route synthesis to obtain a mixture of cis- and trans-alcohols, but we were unable to achieve a complete separation of this mixture: only pure samples of the trans-alcohol 14 were isolated. Structures of isomers were determined by NMR measurements by comparison with those previously obtained for phenyl analogues in the PCP series.

**Results**

**Identification of in Vitro Metabolites.** The biotransformation of 1 by rat liver microsomes was carried out at physiological pH in the presence of a NADPH-regenerating system. Figure 1 shows the molecules extracted from the microsomal system and identified by comparison with synthesized samples. Metabolites were identified both by means of a HPLC separation in heptane/2-propanol coupled to a UV or a MS detector and by means of a GC fitted with a capillary column (OV 17) coupled to a flame ionization detector (FID) or a MS detector. Each analysis contained an internal standard (18: 4-(2-benzo[b]thiophenyl)-4-piperidinotetrahydropyran) prepared for this purpose and added to the microsomal medium before metabolite extraction. A HPLC analysis with a UV detection at 230 nm separated almost all the derivatives with the exception of 1 and 16 which were eluted at the same retention time. However, at a 289 nm wavelength where 1 absorbs less than at 230 nm, we were able to detect, but not quantify, compound 16. A GC analysis did not separate all compounds and in particular did not allow for the quantification of 1 and 16 because 16 is also a thermolysis product of 1. Nevertheless, it helped to confirm the presence of compounds difficult to detect by HPLC analysis. Metabolite identification was confirmed by HPLC and GC analysis coupled to a MS detector. The retention times measured by HPLC and GC analysis are reported in Table 1. They represent all peaks detected in the time window used. According to our hypothesis, most of the identified metabolites (3, 5, 9, 10) were similar to those found for the PCP in vitro metabolism. As for PCP, no peak corresponding to a biotransformation of the aromatic ring was detected. The cis diastereomer of 14 was
not detected although it is the sole diastereomer detected in the biotransformation of PCP.\textsuperscript{22,23,34} Alcohol 15 apparently originates in the biotransformation of the unsaturated compound 16 since we detected 15 after incubation of 16 with rat liver microsomes under the same conditions as for 1. It is interesting to note that the isomeric alcohol of 15, compound 17, was at no time detected under the conditions we used. This might be due to a regiospecific hydration of the double bond of 16 during incubation. The corresponding unsaturated compound has also been found in PCP-treated mouse liver microsomes and its production explained either by a thermolysis during treatment (GC analysis particularly) or by a Cope elimination following a N-oxidation.\textsuperscript{35,36} In our case, 1 incubated without microsomes and treated as usual did not yield such an unsaturated structure (HPLC analysis). As for PCP\textsuperscript{35} in vitro metabolization, at no time did we detect a peak which could be attributed to 1 N-oxide.

Quantification of metabolites obtained in Vitro.
To identify all of the putative metabolites and to obtain good quantification, the dependency of the biotransformation of 1 on the pH, time, protein content, and NADPH concentrations was studied in preliminary experiments. Optimal conditions, i.e., a maximum number of detectable and measurable metabolites without inhibition of the microsomal system, were found to be 1 mg/mL microsomal protein, pH = 7.4, 500 µM 1, and a NADPH-regenerating system constituted with 10 mM glucose 6-phosphate (G6P), 0.5 mM NADP, 1 unit of glucose 6-phosphate dehydrogenase (G6PDH). A 500 µM concentration of 1 probably does not comply with physiological conditions, but at lower concentrations detection and/or quantification failed and thus linearity could not be verified. Interestingly, the concentration we used here is the same as that previously used to analyze PCP metabolites (0.25 µmol in 0.5 mL).\textsuperscript{23} Evolution of the biotransformation was checked after various incubation times (5, 15, 30, 60, 90 min). The consumption of 1 was not quantifiable because it could not be completely distinguished from 16 after HPLC separation; in GC it yields in part compound 16 by thermolysis. Metabolite production is represented by nonlinear monophasic curves which reach a plateau after about 90 min (Figure 2). This nonlinear process probably reflects the formation of secondary metabolites which may possibly be dihydroxylated derivatives of 1. These were observable at much longer retention times and have not yet been identified (not shown). A degradation of metabolizing enzymes, however, cannot be excluded. After a 90 min incubation time, two major metabolites, 10 and 3, were produced, three metabolites, 5, 9, and 15, were present but were at least 4 times less abundant, and a very low amount of 14 was detected (Table 2).

Biological Activity of the Hydroxylated Derivatives of 1. The affinities of the alcoholic compounds identified as in vitro metabolites to bind for the DA transporter were measured by inhibition of the specific binding of [3H]BTCP to rat striatal membranes. Due to their insolubility in an aqueous medium, the affinities of compounds 15, 16, and 17 for the dopamine transporter were not measured. Nevertheless, such molecules are very different from 1. Particularly the lack of a nitrogen atom in their structure may be assumed to considerably reduce their affinities for [3H]BTCP binding sites. Measured affinities are reported in Table 3. Relatively large differences were observed in comparison with 1: 3, 9, and 10 displayed affinities respectively 2, 6, and 13 times lower, but 5 displayed a 4 times higher affinity.

Discussion
Eight compounds were prepared, seven of which were identified among the primary metabolites yielded by incubation of 1 with rat liver microsome preparations. We were able to quantify five compounds whose structures correspond to the monooxidation of cyclohexyl or piperidine moieties of 1. Another compound, 1-(2-benzo[b]thiopheneyl)cyclohexanol 15, was quantified which might be formed by regioslective hydration of 1-(2-benzo[b]thiopheneyl)cyclohexene (16). Compound 16 yielded 15, but not 17 after incubation of 500 µM 16 with microsomes. It is unlikely that the unsaturated or the hydroxylated compound would result from a...
degradation of 1 during incubation, extraction, or HPLC separation since when 1 was incubated in the usual fashion but without microsomes, 15 and 16 were not detected by the HPLC analysis of extracts. Given the experimental results, the hypothesis that hydration is a metabolic step is an attractive concept that requires additional study.

An analogue to compound 14 trans-(pip/OH) 1-(1-(2-benzo[b]thiophenyl)-3-hydroxy cyclohexyl) piperidine was not detected at any time in PCP-treated rat liver microsomes whereas mouse liver microsomes yielded the corresponding cis structure.22,23,34

Hydroxylation at the 3- or 4-position of the piperidine ring of 1 yields high-affinity ligands for the DA transporter. Conversely, hydroxylation at the 3- or 4-position of the cyclohexyl ring decreases the affinity. Indeed, 3 and 5 are high-affinity ligands for the neuronal dopamine transporter, which is different from the situation with PCP metabolites: all monohydroxylated compounds are low- or very low-affinity ligands for the PCP receptor, and consequently none of them has been considered as an active metabolite.33 Experiments in vivo are needed to evaluate the potential contribution of 3 and 5 to the pharmacology of 1.

Conclusion

The identification of two high-affinity ligands for the DA transporter after metabolism of 1 by rat liver microsomes leads to the hypothesis that in vivo metabolism could produce the same metabolites. Thus, considering the very important role which could be played by highly active metabolites during chronic administrations, in vivo biotransformations of 1 are currently being studied in our laboratories.

Experimental Section

Melting points were determined with a Büchi-Tottoli apparatus and remain uncorrected. Elemental analyses were performed at the CNRS Microanalytical Section in Montpellier on the hydrochloric salts and were within ±0.4% of theoretical values. 13C NMR spectra were obtained on a Brucker AC 200 spectrometer at 50.323 MHz (Brucker Spectrospin, Wissembourg, France) in 5 mm sample tubes in the FT mode. A spin–echo sequence (I mod) was used for signal assignments. HPLC/MS data given for synthetic compounds was obtained from a Shimadzu (Touzart et Matignon, Les Ulis, France) LC10AT HPLC coupled to a Fisons Trio-1000 mass spectrometer (Fisons Instruments, Les Ulis, France) coupled to a Fisons Particle Beam Interface LINC; data was treated by Fisons Masslab system software. For in vitro experiments, compounds were isolated in their hydrosoluble hydrochloride salt form. The salts were precipitated by bubbling a dry stream of HCl in an ethereal solution of bases. After filtration, the solvents collected were dried in vacuum.

1-(4-Hydroxypiperidino)cyclohexanecarbonitrile (2)

Acetone cyanohydrin (10.71 g, 0.126 mol) was added to a stirred mixture of cyclohexanone (12.35 g, 0.126 mol), anhydrous magnesium sulfate (75 g, 0.63 mol), dimethylacetamide (16.63 g, 0.189 mol), and 4-hydroxypiperidine (18.9 g, 0.189 mol). The pastelike mixture obtained was heated at 45 °C for 48 h, cooled to room temperature, poured onto ice, and vigorously stirred for 30 min. The aqueous mixture was extracted with ether (3 × 200 mL), and the organic phases were washed with water until neutrality was achieved. The dried (Na2SO4) organic layer was evaporated to dryness under reduced pressure to yield a white solid (20.2 g, 63%) used without further purification.

1-(1-(2-Benzo[b]thiophenyl)cyclohexyl)piperidin-3-ol (3)

A MgBr2 solution was prepared by the gradual addition of 1.2-dibromomethane (43 mL, 0.33 mol) to Mg turnings (8.4 g, 0.35 mol) covered with 100 mL of anhydrous ether. Simultaneously, a solution of 2-lithiobenzo[b]thiophene was prepared at −20 °C in a nitrogen atmosphere, by the careful addition of a n-butyl lithium solution (1.6 M in hexane) (200 mL, 0.32 mol) to a solution of benzo[b]thiophene (43.2 g, 0.32 mol) in ether. The resulting solution was refluxed for 2 h, cooled to room temperature and added under nitrogen to the MgBr2 solution. This, after stirring at room temperature for 30 min, yielded 2-MgBr-benzo[b]thiophene. 2 (22.8 g, 0.106 mol) in ether was added dropwise to the Grignard reagent solution, and the resulting mixture was stirred and refluxed for 16 h. After being cooled to room temperature, the yielded solution was poured onto an ice-cold saturated solution of NH4Cl in water, stirred for 30 min, and treated as follows: decantation, extraction with ether (2 × 200 mL) and CH2Cl2 (200 mL), washing of the pooled organic phases with 10% HCl (3 × 200 mL), neutralization of the aqueous phase with 20% NH4OH, extraction with ether (2 × 200 mL) and CH2Cl2 (50 mL), and washing of the pooled organic phases with water until neutrality was achieved. The dried (Na2SO4) organic layer was evaporated to dryness under reduced pressure to yield a solid material purified by crystallization (CH2Cl2) in the form of a white solid (15.3 g, 46%): mp (HCl) 185–6 °C; 13C NMR (CDCl3) δ 25.35, 26.31, 32.13, 33.82, 35.77, 48.28, 67.55, 72.68, 124.93, 127.23, 127.67, 128.65, 131.27, 138.79, 141.67, 142.48, 148.85, 315 (M+ , 16), 272 (20), 214 (52), 185 (42), 173 (25), 147 (10), 134 (25), 115 (32). Anal. (C19H26NOSCl) C, H, N.

1-(3-Hydroxypiperidino)cyclohexanecarbonitrile (4)

A mixture of cyclohexanone (4.9 g, 0.05 mol), anhydrous magnesium sulfate (42 g, 0.35 mol), dimethylacetamide (7 g, 0.08 mol), piperidin-3-ol (8 g, 0.082 mol), and acetone cyanohydrin (4.7 g, 0.055 mol), treated as described for 2, yielded a yellow oil (6.75 g, 54%) used without further purification.

1-(1-(2-Benzo[b]thiophenyl)cyclohexyl)piperidin-3-ol (5)

A Grignard reagent obtained as described for 3 from Mg turnings (4.8 g, 0.2 mol), 1,2-dibromomethane (26 mL, 0.2 mol), and a 1.6 M solution of n-butyl lithium in hexane (125 mL, 0.2 mol) and benzo[b]thiophene (27.2 g, 0.2 mol) was made to react with the α-amino nitrile (12.5, 0.06 mol) to yield an oily residue. Purification on a column (alumoxine Merck II-Ill) yielded (CH2Cl2) a white solid (3.8 g, 37%): mp (HCl) 165 °C; 13C NMR (CDCl3) δ 20.55, 23.12, 23.91, 31.37, 32.95, 33.38, 46.63, 51.77, 63.85, 70.28, 122.17, 124.50, 125.03, 125.86, 127.75, 136.25, 138.99, 139.97; MS 315 (M+ , 18), 272 (21), 215 (54), 214 (43), 185 (30), 173 (21), 147 (100), 134 (18), 115 (24). (C9H11NOSCl) C, H, N.

1-(1-Piperidino)-4,4-(ethylenedioxy)cyclohexanecarbonitrile (6)

A mixture of 1,4-hexanedione-monoethylene ketal (20 g, 0.138 mol), anhydrous magnesium sulfate (42 g, 0.35 mol), dimethylacetamide (11.35 g, 0.129 mol), piperidine (11 g, 0.129 mol), and acetone cyanohydrin (10.71 g, 0.126 mol), treated as described for 2, gave a white solid (20.2 g, 63%) which was used without further purification.

1-(1-(2-Benzo[b]thiophenyl)-4,4-(ethylenedioxy)cyclohexyl)piperidine (7)

A Grignard reagent obtained as described for 3 from Mg turnings (8.4 g, 0.35 mol), 1,2-dibromomethane (43 mL, 0.33 mol), a 1.6 M solution of n-butyl lithium in hexane (200 mL, 0.32 mol), and benzo[b]thiophene (43.2 g, 0.32 mol) was made to react with the α-amino nitrile 6 (22.2 g, 0.06 mol) to yield purified by precipitation in petroleum ether (21.2 g, 53%).

1-(1-(2-Benzo[b]thiophenyl)-4-oxo-cyclohexyl)piperidine (8)

Compound 7 (10 g, 0.028 mol) was dissolved in 5% H2SO4 (100 mL) and stirred for 16 h at room temperature. The reaction was halted by neutralization with 20% NH4OH, and the mixture was treated as follows: extraction with CH2Cl2 (3 × 50 mL), washing of the pooled organic phases with 10% HCl (3 × 50 mL), neutralization of the aqueous phase with 20%NH4OH, extraction with CH2Cl2 (3 × 50 mL), and washing of the pooled organic phases with water until neutrality was achieved. The dried (Na2SO4) organic layer was evaporated under reduced pressure to give an oily residue. This residue dissolved in a minimum of CH2Cl2 was precipitated by petroleum ether at 4 °C to produce 8 in the form of a solid (5.5 g, 58%).
cis- and trans- (pip/OH) 4-(2-Benz(b)thiophenyl)-4'-piperidinocyclohexanols (9 and 10). A 1 M diborane solution in THF (18.2 mL, 0.018 mol) was added to 8 (2.6 g, 0.0083 mol) dissolved in anhydrous THF (20 mL) in a nitrogen atmosphere. The solution was subsequently stirred at room temperature for 1 h, then diluted with ethanol and distilled in CH₂Cl₂, and treated as described for 8 to produce a 60/40% mixture of diastereomeric primary amines. Chromatography on a column of silicium dioxide, SDS, 70:30 mixture yielded 1.5 g of the isomeric mixture and 900 mg of the pure cis isomer of 9: mp (HCl) 176 °C; ¹³C NMR (CDCl₃) δ 173.33, 22.32, 22.97, 31.16, 32.27, 38.42, 47.21, 47.52, 65.95, 68.36, 122.12, 124.45, 125.22, 126.07, 126.78, 136.14, 139.01, 140.07; MS 315 (M⁺, 21), 256 (100), 230 (32), 213 (66), 185 (31), 173 (25), 147 (70), 134 (23), 115 (34). Anal. (C₁₉H₂₆NOSCl) C, H, N.

A 0.5 M solution of potassium tri-sec-butyl borohydride in THF (165 mL), diluted with anhydrous THF (66 mL), was added dropwise at −78 °C to 8 (1.3 g, 0.0035 mol) dissolved in anhydrous THF (16.5 mL) in a nitrogen atmosphere. The solution was stirred for 5 h at −78 °C and then diluted with water (11 mL) and ethanol (33 mL), and 6 N Na₂CO₃ (22 mL) and 30% H₂O₂ (22 mL) added. The resulting aqueous solution was extracted with CH₂Cl₂ (2 × 50 mL), and the pooled organic phases were treated as described for 8 to give a 20/80 mixture of cis/trans isomers (1:90). In the same conditions as above a chromatography on a silica gel column (silicium dioxide, SDS, 70:30 mixture, and 900 mg of the pure cis isomer) was maintained at 3°C and Na₂CO₃ was added until gas evolution ceased. The solution was reduced pressure to give 9 g of crude isomeric alcohols in the form of an oily residue.

Grignard reagent was obtained as described for 9. A 0.5 M solution of potassium tri-sec-butyl borohydride in THF (13 mL, 0.01 mol) was added dropwise at −20 °C and well stirred until the medium resembled a jelly; 11 (16.25 g, 0.065 mol) was then dissolved in CH₂Cl₂ (10 mL) and added dropwise, after which the medium was maintained at −20 °C. It was then vigorously stirred for 3 h and Na₂CO₃ was added until gas evolution ceased. The mixture was filtered, and the solvent was evaporated under reduced pressure to give 9 g of crude isomeric alcohols in the form of an oily residue.

3-Amino-3-(2-benz(b)thiophenyl)-1,3-cyclohexanediols (11). A Grignard reagent was obtained as described for 3 from Mg turnings (6.21 g, 0.25 mol), 1,2-dibromoethane (31.8 mL, 0.26 mol), a 20/80 mixture of THF (165 mL), diluted with anhydrous THF (66 mL), was added dropwise at room temperature, and then a 5% NaOH (5 mL) solution was added. The resulting mixture was stirred for 2 h and the reaction stopped by the addition of 15% H₂O₂ (2 mL). Separation of the two isomeric alcohols was achieved on a chromatography column (silicium dioxide, SDS, 70–200 µm) in a petroleum ether/ether mixture (70:30) to yield pure 11 (0.92 g, 40%), mp 93 °C; ¹³C NMR (CDCl₃) δ 171.33, 24.73, 25.77, 33.79, 34.13, 49.04, 74.79, 121.79, 122.29, 122.99, 123.77, 124.25, 124.98, 130.97, 135.15; MS 232 (M⁺, 100), 199 (23), 186 (53), 185 (73), 174 (21), 173 (3), 164 (114), 115 (37). Anal. (C₁₉H₂₆NOSCl) C, H, N.

A mixture of 2-(2-benz(b)thiophenyl)cyclohexene (16). A 1.6 M solution of diborane in THF (13 mL, 0.01 mol) was added dropwise under a nitrogen atmosphere to 16 (2.14 g, 0.001 mol) dissolved in anhydrous THF. The solution was then stirred at room temperature for 2 h and the reaction stopped by the addition of 15% H₂O₂ (2 mL). Separation of the two isomeric alcohols was achieved on a chromatography column (silicium dioxide, SDS, 70–200 µm) in a petroleum ether/ether mixture (70:30) to yield pure 16 (1.25 g, 40%), mp 107 °C; ¹³C NMR (CDCl₃) δ 171.61, 24.73, 24.77, 25.77, 33.79, 34.13, 49.04, 74.79, 121.79, 122.29, 122.99, 123.77, 124.25, 124.98, 130.97, 135.15, 147.21, 146.73; MS 214 (M⁺, 100), 199 (100), 186 (53), 185 (73), 174 (21), 173 (3), 167 (144), 115 (37). Anal. (C₁₉H₂₆NOSCl) C, H, N.

Microsomal Preparation. Rats deprived from food for 24 h were killed by decapitation. Their livers were perfused by a 10 mM Na₂HPO₄, 9% NaCl, 1 mM EDTA (pH 7.4) buffer, removed, washed, and cut up finely with scissors. The liver tissue was homogenized with a Elvejhem potter (10 strokes, 700 rpm at 4 °C) in three volumes of a 0.25 M sucrose, 10 mM EDTA, 0.1 mM DTT, 50 mM Na₂HPO₄/NaH₂PO₄ (pH 7.4) buffer. The homogenate was centrifuged at 105000 g, 10000 for 20 min, and the resulting supernatant was centrifuged at 105000 g for 60 min. All centrifugation stages were performed at 4 °C. The pellet was suspended in three volumes of a 100 mM K₂HPO₄/KH₂PO₄, 1 mM DTT, 1 mM EDTA, and 20% glycerol (pH 7.4) buffer and centrifuged at 105000 g for 60 min. The final pellet was suspended in one volume of the same buffer, the protein concentration was determined with the Pierce’s reagent ²¹ using BSA as standard, and the microsomes were stored at −80 °C until used.

Incubation and Extraction. A typical incubation medium contained 1 mg of microsomal protein, 100 mM potassium phosphate (pH 7.4) buffer, 10 mM MgCl₂, and a NADPH-generating system (10 mM G6P, 0.5 mM NADP and 1 unit G6PDH) in a final volume of 1 mL. Incubation mixtures were preincubated in the presence of a NADPH-generating system at 37 °C for 15 min. The reaction was initiated by the addition of 500 µM of 1. Incubations were performed in air at 37 °C in a shaking bath. Reactions were stopped by adding 1 mL of 0.2 N NaOH and calibrated by adding 100 nmol of 18 as internal standard in each reaction tube. Extraction of 1, 18, and metabolites was performed as follows and repeated three times: addition of 2 mL of ethyl acetate, vortexing for 1 min, centrifugation at 4000 g for 10 min at 4 °C. The organic layers were pooled, filtered, and evaporated under nitrogen for 12 h.
at 60 °C. The dried residue was solubilized in heptane before analysis (50 µL for GC analysis, 400 µL for HPLC analysis).

Identification, Quantification, and Extraction yield of 1, Metabolites, Synthetic Compounds, and Internal Standard. Compounds from rat liver microsomal preparations were recovered by extraction with ethyl acetate. 1, internal standard 18, and metabolites were generally recovered with a very good yield with the exception of 3. 3 Standard calibration curves were determined from peak area analysis of standards solutions in heptane of increasing amounts of metabolites (0.3 nmol to 300 nmol) and a fixed amount of 18 (100 nmol). Linear regression lines were obtained with correlation coefficients varying from 0.991 to 0.996. The metabolites were identified and quantified through reference to calibration standards (retention time and peak area). To determine the extraction yield, 500 nmol of 1 and 100 nmol of each compound were added to the microsomal preparation previously stopped by 1 mL of 0.2 N NaOH and then extracted as described above and analyzed by HPLC separation. The areas were computed by the area under the retention time and peak area. The extraction yield did not vary regardless of the amount of 1 used or regardless of the relative proportion of 1 and synthetic compounds. Finally, the recovery yields (% ± SEM) of synthetic compounds after extraction with ethyl acetate from microsomal medium were as follows: 1, 100 (0.4); 3, 87 (1); 5, 94 (1.1); 9, 92 (1.2); 10, 98 (1.6); 14, 91 (1.4); 15, 98 (1.6); 16, 99 (1.5); 17, 96 (1.7); 18, 100 (1.4).

Analysis Conditions. HPLC system: Shimadzu LC-10AD pump (Touzet et Matignon, Les Ulis, France); Lichrospher 100 NH2 column (5 µm, 250 × 4 mm, 40 °C) (Merck-Clevenot, Nogent/Marne, France); Shimadzu SPD-6A UV spectrophotometric detector (at 230 or 289 nm); heptane/2-propanol (98/2, v/v) as mobile phase (1 mL/min); 5 µL injection volumes.

GC system: Shimadzu gas chromatograph/FID GC-14B; Quadrex 007-OV-1701 fused silica capillary column (25 m × 0.25 mm × 0.1 µm) with a methyl 7% phenyl 7% cyanopropylsilicone bonded phase (Touzet et Matignon, Les Ulis, France); injector and detector temperatures: 240 °C and 270 °C, respectively; oven temperature: 60 °C for 0.5 min increasing linearly by 40 °C/min to 130 °C, 10 °C/min to 205 °C, 1 °C/min to 220 °C, then 10 °C/min to 250 °C for 6 min to purge the column; carrier gas (H2), 115 KPa, split vent 1 mL/min. For HPLC and GC data acquisition and treatment: Shimadzu Class-LC 10 system. Comparative mass spectra between standards and metabolites were obtained from Hewlett-Packard equipments (Les Ulis, France). A 1050 HPLC with a particle beam interface HP 5989B, a HP 5890 gas chromatograph, and an ECD mass spectrometer HP 5989A. Column and elution programs were as described above.

In Vitro Binding Experiments. [3H]-BTCP binding to the DA uptake complex: the method used was the same as described by Vignon et al. 3 Rat striata were dissected on ice and homogenized with an ultrarotor in a 320 mM sucrose, 10 mM Tris/HCl (pH 7.4) buffer and centrifuged at 1000g for 10 min. The supernatant was then centrifuged at 49000g for 20 min. The resulting pellet (synaptosomal homogenate) was resuspended in the same buffer (1 mL per striatum). The homogenate (0.05–0.1 mg of protein) was incubated with [3H](1-2-(benzothiophenyl)cylohexyl) piperidine (BTCP) (0.2–0.5 nM) (CEA, Service des Molecules Marquées, Saclay, France, 55 Ci/mmol) in the absence or in the presence of the competing drug in a 50 mM NaHPO4 (pH 7.4) buffer in a volume of 2 mL for 90 min at 4 °C. The incubation was terminated by filtration over GF/B Whatman glass fiber filters (Labovier, Montpellier, France) presoaked in 0.5% polyethyleneimine (Aldrich, Saint-Quentin Fallavier, France) with a MR24 Brandel cell harvester (Beckman, Gagny, France). The filters were rinsed three times with 5 mL of a mixture of 0.5 M NaCl, Tris/HCl 10 mM (pH 7.7) buffer, and the radioactivity retained was counted in 3.5 mL of ACS (Amersham) with an Excel 1410 (LK Bank, Orosy, France) liquid scintillation spectrophotometer. The nonspecific binding was determined in parallel incubations in the presence of 10 µM unlabeled 1.

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