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Synthesis and in vitro Activity of Heterocyclic Inhibitors of CYP2A6 and CYP2A13, Two Cytochrome P450 Enzymes Present in the Respiratory Tract

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The synthesis of several heterocyclic compounds (1- or 2-substituted *1H*-imidazoles and 2-substituted oxazoles, oxazolines and pyrazines) has been achieved. These compounds were tested as inhibitors of CYP2A6 and CYP2A13—two cytochrome P450 enzymes present in the respiratory tract—with a view to preventing the formation of carcinogenic metabolites of nicotine and inhibiting the metabolism of fragrances. 1-Substituted imidazoles bearing short alkyl chains displayed IC_{50} values of around 2 μM for both enzymes, together with high vapour pressures.

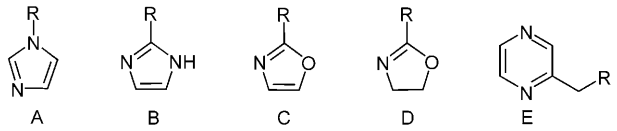
Introduction

Tissues of the respiratory tract that are exposed to inhaled xenobiotic compounds are important targets with regard to environmental toxicity. Several studies have been devoted to the expression of cytochrome P450 (CYP) genes in human lung and nasal mucosa. Most of the liver microsomal CYPs are also expressed in the respiratory tract, usually in lower amounts, but with the notable exception of CYP2A13, which is predominantly expressed in the human respiratory tract.[1] The two functional members of the human CYP2A gene family—CYP2A6 and -2A13—are involved in the metabolism of the nicotine-derived procarcinogen 4-(methylnitrosamino)-1-(pyridin-3-yl)butan-1-one (NNK),[2,3] as well as of aflatoxin B_1.[4] Inhibition of the CYP2A family members in the respiratory tract is of obvious interest because both NNK and aflatoxin require activation to generate the active intermediates that form DNA adducts and result in carcinogenicity. Further, it is reasonable to assume that these P450s, which are also present in the olfactory mucosa, metabolize fragrances, and that inhibition studies might hence be significant for the design of the compositions of new perfumes.

Many studies have already been devoted to the development of inhibitors of CYP2A6,[5] the role of that enzyme in drug and nicotine metabolism having been known for many years.[6] CYP2A13, however, has been investigated much less[7] and only limited information on inhibition studies has so far been reported on isothiocyanates[8] and on the mechanism-based inhibitor 8-methoxypsoralen.[9] X-ray structures of human liver CYP2A6[9] and human lung CYP2A13[10] have been published. According to these, the enzymes are very similar in terms of their global structures and differ only slightly with regard to the volumes of their narrow, very hydrophobic substrate-binding sites. The CYP2A13 active site is about 15–20% larger than that of CYP2A6.[10] Both enzymes are well adapted for the oxidation of small, planar substrates such as coumarins.

The design of new inhibitors that can be inhaled, therefore, requires: 1) a flat heteroaromatic structure that coordinates well with the cytochrome P450 heme iron, 2) flexible substituents that will fit into the narrow binding site, and 3) that the compounds display sufficient volatility.

We have designed and synthesized a series of potential inhibitors of the CYP2A6/2A13 enzymes. These compounds consist of heterocyclic subunits such as 1- and 2-substituted 1*H*-imidazoles (Scheme 1, A and B) and 2-substituted oxazoles (C), oxazolines (D) and pyrazines (E), which are known to bind to cytochrome P450 heme iron with high affinity. These heterocycles are substituted with short side chains, resulting in relatively high vapour pressures,[11] such that compounds could be administered directly through the respiratory tract and interact with the target enzymes.[12]



Scheme 1. Potential inhibitors of CYP2A6 and -2A13.

Results and Discussion

Synthesis of the inhibitors

Synthesis of the 1-substituted 1*H*-imidazoles of type A by direct substitution, from imidazole and various R–Br compounds, has been performed. Several inhibitors containing

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simple alkyl, alkenyl and alkynyl side chains of different lengths, as well as side chains carrying functional groups, were prepared (Table 1).

Table 1. CYP2A6 and -2A13 inhibition by 1-substituted 1*H*-imidazoles A.

	R	$IC_{50} \pm SD$ [μM] CYP2A6	CYP2A13
1	$-(CH_2)_2-CH_3$	110 ± 3	$>300^{[a]}$
2	$-(CH_2)_3-CH_3$	8.6 ± 1.3	44 ± 6
3	$-(CH_2)_4-CH_3$	2.1 ± 0.5	4.3 ± 0.4
4	$-(CH_2)_5-CH_3$	2.7 ± 0.2	2.1 ± 0.1
5	$-(CH_2)_6-CH_3$	11.0 ± 1.9	3.1 ± 0.4
6	$-CH_2-$cyclopropyl	54 ± 2	148 ± 51
7	$-CH_2-CH_2-CH(CH_3)_2$	3.1 ± 0.2	5.6 ± 0.5
8	$-CH=CH_2$	$>300^{[a]}$	>300
9	$-CH_2-CH=C(CH_3)_2$	7.7 ± 0.4	5.7 ± 0.3
10	$-CH_2-CH=CH-$Et (*cis*)	6.7 ± 0.7	11.4 ± 2.8
11	$-(CH_2)_2-CH=CH-$Et (*cis*)	7.1 ± 0.5	5.5 ± 1.7
12	$-(CH_2)_2-CH=CH-$Et (*trans*)	11.5 ± 0.7	6.8 ± 0.7
13	$-(CH_2)_4-CH=CH_2$	1.4 ± 0.3	2.4 ± 0.6
14	(*Z*)-$(CH_2)-CH=C(CH_3)-(CH_2)_2-CH=C(CH_3)_2$	5.7 ± 0.3	6.5 ± 1.9
15	$-CH_2-C\equiv C-CH_3$	64 ± 6	235 ± 21
16	$-CH_2-C_5H_4N$	136 ± 31	266 ± 73
17	$-(CH_2)_2-$COOEt	>300	238 ± 46
18	$-(CH_2)_3-$COOEt	>300	123 ± 25
19	$-(CH_2)_4-$OH	>300	>300
20	$-(CH_2)_5-$OH	122 ± 14	>300
21	$-(CH_2)_4-$COOH	178 ± 14	82 ± 25
22	$-(CH_2)_4-C\equiv N$	22.4 ± 1.1	19.1 ± 1.9

[a] A value of >300 μM indicates that no meaningful concentration-dependent inhibition curve could be recorded for the molecule, and it is not considered to be an inhibitor of the CYP enzyme.

The 2-substituted 1*H*-imidazoles B (Table 2) were prepared from the corresponding aldehydes in the presence of glyoxal and ammonia.[13] The 2-substituted oxazoles C (Table 3) and the

Table 2. CYP2A6 and -2A13 inhibition by 2-substituted 1*H*-imidazoles B.

	R	$IC_{50} \pm SD$ [μM] CYP2A6	CYP2A13
23	(*Z*)-$(CH_2)-CH=C(CH_3)-(CH_2)_2-CH=C(CH_3)_2$	$>300^{[a]}$	10.7 ± 1.2
24	*exo*-bicyclo[2.2.1]hept-3-ene	>300	238 ± 46
25	*endo*-bicyclo[2.2.1]hept-3-ene	>300	189 ± 16
26	$-CH_2-CH(CH_3-(CH_2)_2-CH=C(CH_3)_2$	>300	247 ± 18
27	$-(CH_2)_8-CH_3$	>300	277 ± 24
28	$-CH_2-CH=CH-$Et (*trans*)	>300	143 ± 27

[a] A value of >300 μM indicates that no meaningful concentration-dependent inhibition curve could be recorded for the molecule, and it is not considered to be an inhibitor of the CYP enzyme.

2-substituted oxazolines D (Table 4) were synthesized from the corresponding carboxylic acids by using published procedures.[14,15] The 2-substituted pyrazines E were also prepared as described in the literature.[16] These general methods are summarized in Scheme 2.

The compounds were first tested as inhibitors for CYP2A6[17,18] by use of commercially available microsomes from

Table 3. CYP2A6 and -2A13 inhibition by 2-substituted oxazoles C.

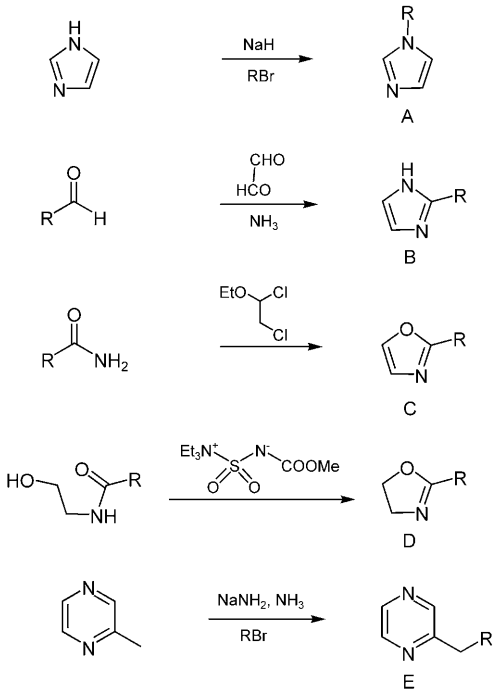
	R	$IC_{50} \pm SD$ [μM] CYP2A6	CYP2A13
29	$-C_6H_4-O-CH_3$ (*para*)	302 ± 37	49 ± 10
30	$-(CH_2)_8-CH_3$	$>300^{[a]}$	$>300^{[a]}$

[a] A value of >300 μM indicates that no meaningful concentration-dependent inhibition curve could be recorded for the molecule, and it is not considered to be an inhibitor of the CYP enzyme.

Table 4. CYP2A6 and -2A13 inhibition by 2-substituted oxazolines D.

	R	$IC_{50} \pm SD$ [μM] CYP2A6	CYP2A13
31	$-C_6H_4-O-CH_3$ (*para*)	$>300^{[a]}$	$>300^{[a]}$
32	*endo*-bicyclo[2.2.1]hept-3-ene	30.1 ± 7.5	2.3 ± 0.2

[a] A value of >300 μM indicates that no meaningful concentration-dependent inhibition curve could be recorded for the molecule, and it is not considered to be an inhibitor of the CYP enzyme.



Scheme 2. General procedures for the synthesis of inhibitors.

baculovirus-infected insect cells containing CYP2A6 + P450 reductase. The CYP2A13 enzyme was obtained from microsomal preparations derived from Sf9 insect cells coinfected with recombinant baculoviruses encoding CYP2A13 and a cytochrome P450 reductase. An apparent K_m values of 5.9 ± 0.3 μM was determined for the CYP2A13-containing microsomes. This is higher than values reported by others using reconstituted CYP2A13 and reductase (0.48 ± 0.07 μM,[19] 2.21 ± 0.63 μM[20]); the only higher K_m value was reported for CYP2A13 expressed in *Escherichia coli* as a truncated, His-tagged form (7.5 ± 1.6 μM[21]). It is possible that the variation in affinity is the

result of differences in lipid compositions, because additional lipids (dilauroyl-L-α-phosphatidylcholine) are required in the reconstituted assay and it has been shown previously that phosphatidylcholine can produce higher affinity in reconstituted cytochrome P450 systems.[22] The IC_{50} values were determined on the basis of the efficacies of the compounds in interfering with the formation of umbelliferone in a dose-dependent manner as described in the Experimental Section.

The results are given in Tables 1–5. Obviously the 2-substituted imidazoles are poor inhibitors of both isoenzymes. The reason for insufficient binding of these compounds is probably that the ligand's nitrogen cannot bind well to the heme iron. Steric interaction between the adjacent substituents and the porphyrin plane prevent optimal orbital overlap between nitrogen and iron, in particular in a rather small binding site. As a consequence, almost no effect on the activity of CYP2A6 and relatively high IC_{50} values for CYP2A13 were observed (Table 2). The only exception is the 2-neryl-substituted imidazole (Table 2, entry 23); this compound is not an inhibitor of CYP2A6 but displays an IC_{50} value for CYP2A13 that is comparable with the value for the corresponding 1-substituted imidazole (Table 1, entry 14). The selectivity for CYP2A13 is most likely to be due to the larger active site of CYP2A13.[10]

Table 5. CYP2A6 and 2A13 Inhibition by 2-substituted pyrazines E.

	R	$IC_{50} \pm SD$ [μM] CYP2A6	CYP2A13
33	$-(CH_2)_2-CH(CH_3)_2$	11.2 ± 0.2	21.6 ± 8.3
34	$-(CH_2)_4-CH=CH_2$	23.8 ± 2.2	9.8 ± 0.6
35	$-(CH_2)-$cyclopropyl	10.3 ± 0.1	39 ± 3
36	$-(CH_2)_6-CH_3$	115 ± 36	33 ± 4

[a] A value of >300 μM indicates that no meaningful concentration-dependent inhibition curve could be recorded for the molecule, and it is not considered as being an inhibitor of the CYP enzyme.

Except for one *endo*-bicyclo[2.2.1]hept-3-ene-substituted oxazoline (Table 4, entry 32), both oxazoles and oxazolidines are poor inhibitors of both enzymes; it appears that they also suffer from the substituent adjacent to the nitrogen that is expected to bind to the heme iron. The 2-substituted pyrazines are more potent because they each have one extra uncongested nitrogen for binding to iron. There is no clear difference between inhibitor efficacies towards the two enzymes, although it appears that the smaller inhibitors (Table 5, entries 33 and 35) show better efficacy towards CYP2A6 and that the larger ones have lower IC_{50} values for CYP2A13 (entries 34 and 36).

The most potent compounds belong to the 1-substituted imidazole series, especially when R is a linear alkyl or alkenyl chain.

The most potent compound for inhibition of CYP2A6 is 1-(hex-5-en-1-yl)imidazole (IC_{50} 1.4 μM, entry 13, Table 1), and it is also one of the most potent inhibitors of CYP2A13 (IC_{50} 2.4 μM). The compounds with pentyl, hexyl or isopentyl side chains (entries 3, 4 and 7) are also very potent. However, the inhibitory effect decreases significantly when R is longer than six carbons (entry 5) or shorter than five (Table 1, entries 1, 2 and 6).

Other than entry 13, the other imidazoles substituted with alkenyl residues are also potent inhibitors of both enzymes—although less active than their saturated counterparts—provided that their chains also contain five or six carbons (Table 1, entries 9, 10, 11 and 12). A smaller chain (entry 8) translates into a drop in activity. The stereochemistry of the double bond is not important (entries 11 and 12), but its presence in the terminal position in the chain results in the most potent inhibitor (entry 13). A longer chain (entry 14) with two nonconjugated double bonds also provides a relatively potent inhibitor.

An alkyne chain is not favourable (entry 15). The presence of polar functional groups decreases the potencies of the inhibitors, especially in the case of CYP2A6 (entry 18). A short-chain nitrile substituent give a reasonable inhibitory effect for both enzymes (Table 1, entry 22).

In general these inhibitors are nonselective with regard to CYP2A13 and CYP2A6, which is not surprising because the active sites of the two proteins are very similar. Further, in order to enhance volatility, most of our compounds do not carry functional groups in their side chains; these might otherwise generate selectivity through interactions with polar amino acids that could be active-site-specific for CYP2A13 and CYP2A6.[10]

The calculated vapour pressure data available for all the compounds described show values between 1–0.001 Pa. The most potent inhibitors have values of about 1–0.1 Pa, which is well in the range of many odorous materials used in the fragrance industry. This is a good indication that these molecules may be useful in applications in which they need to be delivered to the respiratory tract in order temporarily to reduce the activities of CYP2A13 and CYP2A6.

Conclusions

A potential inhibitor of CYP2A6 and CYP2A13, two cytochrome P450 enzymes present in the respiratory tract, must have two important features: 1) it should possess a subunit that binds with high affinity in the binding site of cytochrome P450—that is, to the heme-thiolate cofactor—and 2) it should display sufficient volatility for uptake into the respiratory tract. Both aspects are significant for inhibition of the activation of tobacco-specific nitrosamines, which generates the reactive intermediates that form DNA adducts and ultimately result in carcinogenicity.[2–3] CYP2A13 is expressed at the highest levels in the respiratory tract and is highly efficient for the metabolic activation of NNK;[3] new inhibitors, however, are also of interest with respect to nicotine metabolism[23–25] with CYP2A6 being the major nicotine C-oxidase.

To create inhibitors featuring these properties we have synthesized a series of substituted 1*H*-imidazoles (A/B), oxazoles (C), oxazolines (D) and pyrazines (E). Whereas structures C, D and E turned out to be rather weak inhibitors for both CYP2A6 and -2A13, the 1-substituted 1*H*-imidazoles (A) gave interesting results. The affinities of imidazoles towards the cofactor of P450 enzymes are well known and have been used in, for ex-

ample, the design of antifungal drugs, such as ketoconazole.[26] Of the 28 compounds investigated, the 1-substituted 1*H*-imidazoles are the best inhibitors, displaying sufficient vapour pressure and IC_{50} values down to 2 μM for both CYP2A6 and CYP2A13.

In general, these inhibitors are nonselective with regard to the closely related enzymes CYP2A6 and CYP2A13. Since both enzymes catalyse the activation of tobacco-specific nitrosamine promutagens, it is of advantage for an inhibitor to affect the functional activities of both enzymes. The reported inhibitor results are also of interest with respect to hepatic metabolism of nicotine by CYP2A6, in which the aim is to reach a relatively high blood nicotine level while smoking less.[18] In this case, one would have to demonstrate that inhibitors are selective for CYP2A enzymes and do not interfere with the activity of other hepatic CYPs. Furthermore, the volatile inhibitors are useful for better understanding of the metabolism of fragrances (i.e., to determine whether the smell of an odorant is related to the original fragrance molecule or to its metabolite). In vivo sensory studies demonstrating the influence of inhibitors on the olfactive profiles of odorants are underway and will be reported in due course.

Experimental Section

Chemicals were purchased from Aldrich, Sigma or Fluka and were used without further purification, if not otherwise stated. Solvents were purified by standard procedures. TLC: Merck precoated silica gel 60 F254 glass plates (0.25 mm layer); column chromatography (CC): Merck silica gel 60 (0.04–0.063 mm); visualization by use of UV light (254 nm), Schlittler reagent and/or a phosphomolybdic acid/cerium sulfate mixture; FC = flash chromatography. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra: in $CDCl_3$, Bruker DPX 400 instrument; at 298 K if not otherwise stated; δ in ppm, coupling constants (*J*) in Hz. GC-MS was performed with a Hewlett–Packard 5890 gas-chromatograph equipped with a mass selective detector (5970A) and a Macherey–Nagel column (Phe Me Si, 5%, "optima 5"); flow 1 mL min^{-1}. Temperature program: 70 °C for 5 min then increase 10 °C min^{-1}, end temperature 270 °C for 5 min.

Microsomes containing CYP2A6 and NADPH-cytochrome P450 reductase (Supersomes) were purchased from BD Biosciences Gentest (Woburn, MA, USA).

In order to clone the coding region of the CYP2A13 gene, total RNA was isolated from human olfactory epithelium cells, which were obtained from a local hospital (informed, signed consent was obtained for the use of the material), by using RNeasy midi-kit (Qiagen). The isolated RNA was reverse transcribed by using Superscript II (MMLV) Reverse Transcriptase (Gibco) to produce the corresponding cDNA product. Four consecutive PCR reactions were required to amplify the full-length sequence of the CYP2A13 gene and to introduce appropriate restriction enzyme sites. The following primer pairs were used for the four reactions: PCR1: 5′-ATA TCC TTA GGC GAC TGA GG-3′ and 5′-CAG GGC TGC TTC TGG TGA-3′; PCR2: 5′-ATA TCC TTA GGC GAC TGA GG-3′ and 5′-GTC TTG ATG TCA GTC TGG CG-3′; PCR3: 5′-TCT GGT GAC CTT GCT GGC CTG CCT GAC TGT GAT GGT CTT GAT GTC TGT TTG G-3′ and 5′-GGG ATC GTG GCA AAG CCC ACG TGT TTG GGG GAC ACG TCA ATG TCC TTA GGC GAC TGA GGA-3′; PCR4: 5′-TAT GAA TTC TAT GCT GGC CTC AGG GCT GCT TCT GGT GAC CTT GCT GGC CT-3′ and 5′-AGA AGC TTA TCA GCG GGG CAG GAA GCT CAT GGT GTA GTT TCG TGG GAT CGT GGC AAA GCC CA-3′. The product of the fourth PCR reaction spans the entire open reading frame of the CYP2A13 gene, which is flanked by the restriction enzyme recognition sites for EcoRI and HindIII. The EcoRI and HindIII DNA fragment was subcloned and sequenced for comparison to the known cDNA sequence for CYP2A13 (GenBank accession No.: AF209774) before being cloned into the expression vector pBlueBac4.5 (Invitrogen) to give a recombinant baculovirus expression vector.

Recombinant CYP2A13-encoding baculoviruses were produced by using the manufacturer's protocol (Invitrogen). Transfection of the recombinant pBlueBac4.5 and Bac-N-Blue DNA resulted in the formation of a full-length β-galactosidase gene, and recombinant viruses containing the CYP2A13 cDNA were identified as blue plaques in the presence of the chromogenic substrate X-gal. Recombinant viruses from single plaques were isolated, propagated and analysed by use of the primer pair of the first PCR reaction (PCR1). Propagation of the recombinant virus was performed by several consecutive cell infections until a high-titre (2×10^8 PFU mL^{-1}) large-volume virus stock was obtained.

Recombinant P450 reductase-encoding baculoviruses were produced by starting from the rat NADPH-dependent P450 oxidoreductase gene contained in the *Escherichia coli* expression plasmid pOR263.[27] A BamHI–HindIII fragment from pOR263 containing the coding region of the oxidoreductase gene was cloned into the expression vector pBlueBac4.5 (Invitrogen). Recombinant oxidoreductase-encoding baculoviruses were produced as described for CYP2A13, by using the manufacturer's protocol (Invitrogen).

Microsomes from baculovirus-infected Sf9 insect cells coexpressing CYP2A13 and the P450 oxidoreductase genes were prepared as follows. Insect cells were cultured in spinners at 27 °C up to a cell density of 2×10^6 cells per mL. Cells were coinfected with the two recombinant baculoviruses (multiplicity of infection of four for CYP2A13 and three for recombinant P450 reductase baculoviruses). At the time of infection, vitamin B2 was added at a concentration of 5 μg L^{-1}. After 24 h, hemin was added to the infected culture at a concentration of 10 μg L^{-1}. Cells were harvested 72 h postinfection. Microsomes containing CYP enzyme were prepared and purified as described by Zhang et al.[28] Resuspended microsomes were used to determine the P450 carbon monoxide (CO) spectrum to calculate the concentration of the P450 enzyme contained per mL of preparation, as described by Omura and Sato.[29]

1-Substituted 1*H*-imidazoles: The preparation of the 1-substituted 1*H*-imidazoles was performed by the procedure exemplified by compound **11**. Most of them have already been described[30,31] and their identities were checked by ^{1}H and ^{13}C NMR and by GC-MS.

(*Z*)-1-(Hex-3-enyl)-1*H*-imidazole (entry 11): The bromoalkenes were prepared from the corresponding alcohols:[32] (*Z*)-hex-3-en-1-ol (5 mmol, 500 mg) in dry diethyl ether (15 mL) was treated at −78 °C under Ar with a solution of PBr_3 in ether (1:10 *v/v*, 1.69 mL) for 1 h and at 0 °C for 5 h. The mixture was then poured into ice-water, extracted with hexane and washed with a saturated sodium bicarbonate solution and water. The crude bromo compound was mixed with imidazole (1.3 g, 19 mmol) in dry THF (10 mL) containing NaI (a few mg) and heated at reflux for 18 h. The solvent was evaporated under reduced pressure, the residue was redissolved in methylene chloride and extracted into water with HCl (1 N), and the solution was brought to pH 9 with K_2CO_3, extracted with ethyl acetate, washed with water and purified by FC (CH_2Cl_2/MeOH 93:7): 210 mg (28%); oil; R_f = 0.52 (CH_2Cl_2/MeOH 10:1); ^{1}H NMR ($CDCl_3$): δ = 7.50 (s, 1H), 7.04 (s, 1H), 6.91 (s, 1H), 5.50 (m, 1H), 5.28

(m, 1H), 3.95 (t, 2H), 2.49 (m, 2H), 1.92 (m, 2H), 0.89 ppm (t, 3H); ^{13}C NMR ($CDCl_3$): δ = 137.4, 135.9, 129.4, 123.8, 119.2, 47.37, 29.43, 20.90, 14.40 ppm; GC-MS: 16 min; *m/z*: 150.

(*E*)-1-(Hex-3-enyl)-1*H*-imidazole (12): 156 mg (20%); oil; R_f = 0.47 (CH_2Cl_2/MeOH 10:1); ^{1}H NMR ($CDCl_3$): δ = 7.47 (s, 1H), 7.04 (s, 1H), 6.89 (s, 1H), 5.50 (m, 1H), 5.30 (m, 1H), 3.95 (t, 2H), 2.44 (m, 2H), 1.97 (m, 2H), 0.93 ppm (t, *J* = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ = 137.39, 136.47, 129.48, 124.1, 119.23, 47.59, 34.71, 25.94, 13.97 ppm; GC-MS: 15.78 min; *m/z*: 150.

1-(Hex-5-enyl)-1*H*-imidazole (13): 647 mg (86%); oil; R_f = 0.28; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43 (s, 1H), 7.02 (s, 1H), 6.87 (s, 1H), 5.72 (m, 1H), 4.95 (m, 2H), 3.90 (t, *J* = 7 Hz, 2H), 2.05 (m, 2H), 1.76 (m, 2H), 1.36 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 138.22, 137.41, 129.71,119.16, 115.62, 47.27, 33.44, 30.80, 26.09 ppm; GC-MS: 16.13 min; *m/z*: 150.

2-Substituted 1*H*-imidazoles: The preparation of the 2-substituted 1*H*-imidazoles **23**–**28** was achieved by the general procedure described below for compound **23**. Compounds **24**–**27** have already been described[13] and were identified by their NMR spectra and GC-MS analysis.

2-((*Z*)-2,6-Dimethylhepta-1,5-dienyl)-1*H*-imidazole (entry 23): Glyoxal trimer hydrate (504 mg, 2.4 mmol) was added at 0 °C to neral (365 mg, 2.4 mmoles) in MeOH (10 mL), together with solution of ammonia in MeOH (7 N, 1.5 mL). The reaction mixture was stirred at room temperature overnight, the solvent was evaporated, and the residue was dissolved in $CHCl_3$, extracted three times with HCl (1 N), adjusted to pH 7 with a K_2CO_3 solution, extracted with AcOEt, washed with water, and dried over sodium sulfate. The solvent was evaporated and the residue was purified by FC (AcOEt) to afford brown crystals (82 mg). M.p. 71–73 °C; R_f = 0.21 (AcOEt); ^{1}H NMR ($CDCl_3$): δ = 6.9 (s, 2H), 6.12 (s, 1H), 5.34 (m, 1H), 2.57 (t, 2H), 2.55 (m, 2H), 1.9 (d, 3H), 1.69 (d, 3H), 1.64 ppm (s, 3H); ^{13}C NMR: δ = 145.7, 144.2, 133.2, 124.4, 122.1, 122.0, 114.8, 36.8, 26.4, 26.1, 24.7, 18.2 ppm; GC-MS: 16.3 min; *m/z*: 190.

2-((*E*)-Pent-2-enyl)-1*H*-imidazole (entry 28): This was obtained as a brown oil; 86 mg; R_f = 0.26 (AcOEt/MeOH 95:5); ^{1}H NMR ($CDCl_3$): δ = 6.9 (s, 2H), 5.9 (m, 2H), 3.47 (m, 2H), 2.01 (m, 2H), 0.97 ppm (t, 3H); ^{13}C NMR: 147.5, 136.5, 123.9, 121.9, 32.3, 25.8, 14.6 ppm; GC-MS: 15.4 min; *m/z*: 136.

2-Substituted oxazoles and oxazolines: The 2-substituted oxazoles and oxazolines have already been described in the literature and their identities were checked by ^{1}H and ^{13}C NMR and by GC-MS.

2-Substituted pyrazines: The 2-substituted pyrazines were prepared from 2-methylpyrazine by the procedure given[16] for compound **33**. Compounds **34** and **35** have already been described in the literature and their identities were checked by ^{1}H and ^{13}C NMR and by GC-MS.

2-(Hept-6-enyl)pyrazine (entry 33): 2-Methylpyrazine (940 mg, 912 mL,10 mmol) was added at −65 °C to sodium amide (490 mg, 12.5 mmol) in liquid NH_3 (10 mL) and the red mixture was stirred for 30 min. A solution of 1-bromohex-5-ene (7.5 mmol) in dry ether was added dropwise and the mixture was stirred for another hour. The reaction was quenched by addition of ammonium chloride (626 mg, 11.7 mmol) and the ammonia was eliminated by heating at reflux. The mixture was extracted with ether, washed with water, dried with sodium sulfate, concentrated under vacuum and purified by FC (hexane/ethyl acetate 1:1). 1.03 g (78%); oil; R_f = 0.52 (hexane/ethyl acetate 1:1); ^{1}H NMR ($CDCl_3$): δ = 8.48 (s, 1H), 8.45 (s, 1H), 8.39 (s,1H), 5.78 (m, 1H), 5.00–4.94 (m, 2H), 2.81 (t, *J* = 7 Hz 2H), 2.04 (m, 2H), 1.75 (m, 2H), 1.41 ppm (m, 4H); ^{13}C NMR ($CDCl_3$): δ = 158.29, 144.96 144.34, 142.46, 139.22, 114.82, 35.81, 33.99, 29.65, 29.12, 29.03 ppm; GC-MS: 16.23 min; *m/z*: 176.

2-(4-Methylpentyl)pyrazine (entry 32): This was obtained as an oil; 430 mg (26%); R_f 0.44 (hexane/AcOEt); ^{1}H NMR ($CDCl_3$): δ = 8.49 (d, 1H), 8.46 (s, 1H), 8.40 (d, 1H), 2.79 (t, 2H), 1.74 (m, 2H), 1.56 (h, 1H), 1.24 (m, 2H), 0.88 ppm (d, 6H); ^{13}C NMR ($CDCl_3$): δ = 158.4, 144.9, 144.2, 142.4, 38.9, 36.1, 28.2, 27.7, 22.9 ppm; GC-MS: 14.16 min; *m/z*: 164.

CYP2A6 inhibition tests: Microsomes containing CYP2A6 (1.0 pmol) were used. Tris buffer (1 M, pH 7.6) and water were added to give a buffer concentration of 0.1 M. The test compound was prepared as a stock solution in acetonitrile (50 mM). The concentration of the standard substrate coumarin was 3 μM, and the K_m value for coumarin hydroxylation was determined under the conditions described here. Initially, the test compound was used in concentrations between 0 and 0.2 mM, and the range was adjusted depending on the first results. The mixture was incubated for 10 min at 37 °C prior to the initiation of the enzymatic reaction by the addition of a solution of NADPH in water (50 mM, 5 μL). The final total volume was 0.2 mL, which is suitable for microtitre plate measurements. The samples were incubated for 60 min at 37 °C. After 60 min, the enzymatic reaction was stopped by the addition of cold trichloroacetic acid (50%, 20 μL) and the mixture was incubated at 4 °C for 15 min. A solution of NADPH in water (50 mM, 5 μL) was added to the control reaction, which corresponds to the reaction without test compound and without NADPH, and as a consequence no umbelliferone was formed. Denatured proteins and other insoluble parts were separated by centrifugation (10 min, 560*g*, room temperature). The samples were analysed spectrofluorometrically, which allows detection of the formation of umbelliferone as the enzymatic product of coumarin at an excitation wavelength of 340 nm and an emission wavelength of 480 nm. A decrease in the fluorescent signal at 480 nm with respect to the control shows that the test compound is influencing enzymatic activity and confirms the nature of an inhibitor, or alternatively a competing substrate. Graphical analysis of the data allowed calculation of the concentration at which the test compound inhibits the enzyme to the level of 50% of its maximum activity (IC_{50} value).

CYP2A13 inhibition tests: Microsomes containing CYP2A13 (1.1 pmol) were used. Tris buffer (1 M, pH 7.6) and water were added to give a buffer concentration of 0.1 M. The test compound was prepared as a stock solution in acetonitrile (50 mM). The concentration of the standard substrate, coumarin, was 6 μM, and the K_m value for coumarin hydroxylation was determined under the conditions described here. Initially, the test compound was used in concentrations between 0 and 0.2 mM, and the range was adjusted depending on the first results. The mixture was incubated for 10 min at 37 °C prior to the initiation of the enzymatic reaction by the addition of a solution of NADPH in water (50 mM, 5 μL). The final total volume was 0.2 mL, which is suitable for microtitre plate measurements. The samples were incubated for 60 min at 37 °C. After 60 min, the enzymatic reaction was stopped by the addition of cold trichloroacetic acid (50%, 20 μL) and the reaction mixture was incubated at 4 °C for 15 min. A solution of NADPH in water (50 mM, 5 μL) was added to the control reaction, which corresponds to the reaction without test compound and without NADPH, and as a consequence no umbelliferone was formed. Denatured proteins and other insoluble components were separated by centrifugation (10 min, 560*g*, room temperature). The samples

were analysed spectrofluorometrically, which allows the detection of the formation of umbelliferone as the enzymatic product of coumarin at an excitation wavelength of 340 nm and an emission wavelength of 480 nm. A decrease in the fluorescent signal at 480 nm with respect to the control shows that the test compound is influencing enzymatic activity and confirms the nature of an inhibitor, or alternatively a competing substrate. Graphical analysis of the data allowed calculation of the concentration at which the test compound inhibits the enzyme to the level of 50% of its maximum activity (IC_{50} value).

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