Metabolic instability of cyanothiazolidine-based prolyl oligopeptidase inhibitors: a structural assignment challenge and potential medicinal chemistry implications

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Abstract

As part of the development of cyanothiazolidine-based prolyl oligopeptidase inhibitors, initial metabolism studies suggested multiple sites of oxidations by P450 enzymes. Surprisingly, in-depth investigations revealed that epimerization at multiple stereogenic centers was responsible for the conversion of the single primary metabolite into a panel of secondary metabolites. The rapid isomerization of all seven detected molecules precluded the use of NMR or X-ray crystallography for complete structural determination, presenting an interesting structure elucidation challenge. Through a combination of LC-MS analysis, synthetic work, deuterium exchange studies and computational predictions we were able to characterize all metabolites and to elucidate their dynamic behavior in solution. In the context of drug development, this study reveals that cyanothiazolidine moieties are problematic due to their quick P450-mediated oxidation and the unpredictable stability of the corresponding metabolites.

Graphical abstract

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Supporting information

Calibration curves, supporting schemes and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Solving a complex puzzle: a mixture of rapidly equilibrating diastereoisomers is formed after sulfur oxidation of cyanothiazolidine-based compounds by P450 enzymes.

Keywords

Metabolism; sulfur oxidation; epimerization; cyanothiazolidine; cytochrome P450

INTRODUCTION

Prolyl oligopeptidase (POP) is a post-proline serine endopeptidase that has become a promising drug target for the treatment of neurodegenerative states such as Alzheimer's disease [1–3] and psychiatric disorders (e.g., bipolar disorder) [4]. Over the last several years, we have been interested in developing POP covalent inhibitors. In 2009 we reported a series of constrained bicyclic molecules designed as pseudopeptidic inhibitors. [5] Within this series, some cyanothiazolidine derivatives showed promising activity in cells. [6] Although clearance rates were measured, no structural characterization of the metabolites has been reported so far.

The importance of thorough drug metabolism studies is well recognized in the pharmaceutical field. [7] In the early stages of drug development, major metabolites are tested for activity and toxicity, and the resulting findings are carefully considered before promoting a drug candidate for expensive and time-consuming preclinical trials. Liquid chromatography coupled to mass spectrometry (LC-MS) has shown tremendous capabilities for the identification of drug metabolites, however further studies are often warranted, [8] for example in cases where the stereochemistry has to be assessed. Nuclear magnetic resonance (NMR), with its potential for the characterization of very complex molecules, can offer advantages over LC-MS, but it is also not free of limitations. [9] In fact, the necessary acquisition times of NMR can become a problem with low quantities and/or highly unstable compounds, even with some more expensive tandem systems, such as LC-NMR or LC-

NMR-MS. [10] The preparation of synthetic standards for comparison remains one of the most reliable strategies but is contingent on an accurate prediction as well as synthetic accessibility. In the end, a combination of different techniques is often necessary.

Herein, we present our strategy to characterize the mixture of interconverting P450 metabolites derived from our first generation of POP inhibitors. In addition to providing another example of how different techniques can be combined, this study reveals some of the issues associated with the use of cyanothiazolidine moieties as drug candidates. We hope that these findings will not only be helpful in the development of better POP inhibitors, but will also contribute to the current knowledge in the field of drug discovery by alerting medicinal chemists to the potential pitfalls of thiazolidine-based scaffolds.

RESULTS AND DISCUSSION

Metabolic profile

In previous work by Lawandi *et al.*, [5] compounds **S1** and **S2** (Figure 1A) were synthesized and tested as POP inhibitors. **S1**, in particular, showed promising activity in living cells. Prior to beginning a lead optimization study, **S1** and **S2** were incubated with human liver microsomes (HLM) and their metabolites examined by LC-MS. The chromatographic profiles (for all traces see S.I., Figures S1–6), revealed the formation of three mono-oxygenated products for **S1** (Figure 1B), and four for **S2** (Figure 1C). No other metabolites were detected (Figures S1–2). Analysis of the retention times and co-elution controls (Figures 1D and S8) confirmed the different chemical nature of these seven compounds.

Chemical intuition and a vast body of literature suggested that the sulfur atom of **S1** and **S2** would be the most likely site of oxidation by P450s, [11] the main metabolic enzymes found in HLM, although this would lead to only two diastereomeric metabolites for each substrate molecule. Alternatively, aromatic oxidation of the benzyl ring could have produced three regioisomers each.

Investigating sulfur oxidation

To identify the correct site of metabolism, we first focused our efforts on preparing synthetic standards for comparison of HPLC retention times with those of the observed metabolites. Ammonium persulfate was used to perform the sulfoxidation of **S1** and **S2** while avoiding formation of the corresponding sulfones. [12] Interestingly, oxidation of **S1** led to the formation of **2**, **4**, **7** (Figure 2A), whereas **S2** led to the formation of a mixture of **1**, **2**, **3**, **4**, **5** and **6** (Figure 2B). In summary, the two separate reactions provided the same seven compounds previously observed in the metabolism of **S1** and **S2**. Isolation of compounds **1** through **7** for NMR characterization or X-ray crystallography turned out to be unsuccessful. In fact, whenever isolation of each of these compounds was attempted through semi-preparative HPLC, a new mixture was regenerated upon solvent removal. This observation was key in revealing the scenario of a quick equilibration among the molecules under study.

Structural determination of 1–7

In order to fully characterize all seven observed compounds, we envisioned a strategy where different techniques could yield complementary pieces of structural data. Figure 3 summarizes the overall path followed and all the tools used to arrive at the final solution. First, we clarified what type of isomers (regio- or stereo-) we were dealing with: LC-MS/MS fragmentation studies revealed that **1–7** all contained a sulfoxide moiety and that they differed only by their stereochemistry. At this point, we assumed that no information on the configuration of any stereocenter was available, and we considered all possible sets of stereoisomers that could be assigned to the seven HPLC peaks. In the presence of four stereogenic centers, eight diastereomers are possible (although we observed only seven). From a mathematical perspective, 8 different numbers can be ordered in $40320 (=8!)$ unique arrangements (i.e. 1,2,3,4,5,6,7,8 or 1,8,2,7,3,6,4,5 etc.) that we will call henceforth ‘combinations’. A series of experiments was then designed to negate certain combinations and, by a process of elimination, reduce the number of possibilities to one and only one. This one solution would contain not only the structures of **1–7**, but also that of the unobserved eighth diastereomer.

In order to keep track of all of the experimental observations and evaluate their consequences on the structure assignments, we relied on a logic-testing protocol written in MatLab (v. 2012b) for which experimental results were encoded as “exclusion rules”.

LC-MS/MS studies: the seven observed isomers are diastereomeric sulfoxides

When LC separation was coupled to ESI-MS (Electrospray Ionization Mass Spectrometry), an almost identical fragmentation pattern was observed for compounds **1** through **7** (Figure 4 shows one representative piece of data), which was taken as evidence that the seven compounds were diastereomers. By taking advantage of high resolution MS (time-of-flight), we assigned structures to the main fragments, which allowed a partial structural characterization. The presence of a sulfoxide moiety was confirmed by the formation of fragment F272 which corresponded to a loss of H_2O from fragment F290 in all compounds analyzed. Loss of the oxygen atom of the sulfoxide had been reported in the literature for similar structures analyzed with the same fragmentation technique. [13] The complete LC-MS/MS data, proposed fragments and mechanisms of fragmentation are detailed in Figure S11.

Equilibration studies

Each of compounds **1–7** was rapidly isolated by analytical HPLC and without prior removal of the eluent it was allowed to equilibrate under the conditions used for the biochemical assays: potassium phosphate buffer (KPB) 0.1 M, pH = 7.4 at 37°C, under gentle shaking (250 rpm). Isomerization was monitored by LC-MS after 1 min, 10 min, 1 hour, 6 hour, 24 hours and 3 weeks of incubation time, and as a result a complex pattern of interconversion phenomena was discovered (Figure 5).

In addition to the presence of clear kinetic trends (for example, the rate of interconversion between **1** and **6** parallels the one between **2** and **7**, in both directions), it is remarkable that each of the seven compounds generates exactly the same mixture after 3 weeks, an

observation that confirmed that all seven molecules existed in equilibrium with each other under the experimental conditions.

To rationalize the interconversion pattern, we analyzed the likelihood of epimerization at each of the four stereogenic centers of **1–7**. The C-1 α to the CN group (Figure 6A) was deemed fairly prone to epimerization due to the slightly acidic character of the α -proton. Inversions of stereochemistry at the sulfoxide moiety and at position C-3 (ring junction) were also considered realistic. Indeed, previous studies on thiazolidine sulfoxides have shown that these systems can undergo ring opening, allowing the neighboring C-S bond to freely rotate and the sulfur to re-attack the C-3 on both sides of the cycle (Figure 6B). [14] Finally, inversion of the stereochemistry at C-5, for the same class of compounds, has been previously reported by Hanessian *et al.* [15] (a proposed mechanism of epimerization is illustrated in Figure S12).

We envisaged that our quest for structural assignment could benefit from looking at the chronological order of appearance of the HPLC peaks in the equilibration studies. In fact, when decrease of a peak is concomitant with appearance of a new one, one can assume that the corresponding compounds have structures differing at C-1 only, C-5 only, C-3 only, S-O only, or both C-3 and S-O. In particular, if we compare the equilibration profiles in the timeframe 1–10 min (Figure 5), the following pairs of compounds are assumed to have such correlation: (**1–6**); (**2–7**); (**4–7**); (**5–6**); (**5–3**). This assumption was taken as the first exclusion rule for the MatLab protocol, and allowed us to decrease the number of combinations from 40320 to 2304 (Figure 3).

In a control experiment, substrates **S1** and **S2** were subjected to the same conditions that triggered epimerization of **1–7** (KPB, 37°C, 1 h shaking). Surprisingly, LC-MS analysis revealed significantly higher stability of **S1** and **S2** (Figure S9) compared to the respective sulfoxide derivatives. Although no definitive conclusions can be made, one can speculate on the role of the additional oxygen in increasing the isomerization rate. First of all, the H α to the CN group is thought to acquire a stronger acidic character in the presence of the sulfoxide moiety (sulfur has a partial positive charge). Second, the hypothesized intramolecular ring opening would not be as favorable in the absence of the S-O oxygen that is able to delocalize the lone pair of the nitrogen at the ring junction. On the contrary, we believe that epimerization at C-5, further away from the sulfur, is only slightly affected by the presence of the sulfoxide. As Figure 5 illustrates, < 1 % of **2** is generated from **1** after 6 h incubation in KPB.

Time-based metabolism studies

In a following experiment, the HLM assay for **S1** and **S2** was repeated with the exception that product formation by the P450s was monitored over time (Figure 7). For both **S1** and **S2** mainly one compound each (**4** and **5**, respectively) was rapidly produced in the first 10 minutes of incubation, whereas secondary peaks appeared only at a later stage, which highly resembled what had been previously observed during equilibration studies (Figure 5). We thus concluded that compounds **4** and **5** were the major metabolites of **S1** and **S2**, respectively, and that the mixtures of products originally observed (Figure 1B–C) were the

result of the isomerization of the primary metabolites in the medium used for the HLM assay. These findings were translated into the following statement: **4** and **5** must have unchanged stereochemistry at position C1, C3 and C-5 with respect to corresponding P450 substrates **S1** and **S2**. By adding these constraints as exclusion rules to the MatLab protocol, the number of possible combinations was further decreased to 168.

A closer look at the synthetic mixtures

Attempts to use NMR to characterize the synthetic standards **1–7** (obtained, as mixtures, from the chemical oxidation of **S1** and **S2**) were unsuccessful. In fact, the simple process of removing the solvent after semi-preparative HPLC purification was sufficient to observe 30% isomerization. However, valuable information was retrieved by carefully analyzing the profiles of the synthetic products mixtures and by comparing them with previously collected data. In particular, if we look at the equilibration profile of **7** after 1 hour of incubation in KPB (presented again in Figure 8A), we notice its high resemblance to the mixture profile from chemical oxidation of **S1** (Figure 8B). This suggests that the reaction of **S1** with ammonium sulfate initially produces **7**, which later undergoes isomerization to yield **2** and **4**. In other words, **7** is a sulfoxide derivative of **S1** but with no change in the initial stereochemistry at C-1, C3 or C-5 (Figure 8C). This information can be easily translated in additional exclusion rules for the MatLab protocol. Similarly, comparison between the equilibration profile of **6** after 1 hour incubation in KPB (Figure 8D), and the mixture profile from chemical oxidation of **S2** (Figure 8E), suggests that **6** is a sulfoxide derivative of **S2** but with no change in the initial stereochemistry (Figure 8F). These last conclusions about the nature of **6** and **7** led to a decrease in the number of combinations from 168 to 16.

At this stage of the study we knew that **4** (the product of HLM microsomal oxidation of **S1**) and **7** (the product of chemical oxidation of **S1**) differed only by the stereochemistry of the sulfur atom. Interestingly, this means that P450s are able to oxidize **S1** with a stereoselectivity opposite to that of standard chemical methodology. The same can be concluded for the oxidation of **S2** which produces **5** with P450 catalysis, and **6** when ammonium persulfate is used.

Deuterium studies

When compounds **1–7** are incubated separately in KPB prepared with D₂O (d-KPB) instead of H₂O, replacement of hydrogen with deuterium atoms within the compounds can be monitored by mass spectrometry. Since initially the sole source of deuterium atom is the deuterated solvent, it is possible to use LC-MS to determine whether conversion of one compound into another involves intermolecular exchanges between metabolites and the solvent, or if the equilibration happens intramolecularly.

One of the hurdles that we encountered in the development of this assay was elucidating the behavior of the carbamate N-H. We were able to demonstrate that hydrogen-deuterium exchange at this position does happen during the experiments, but that it does not significantly affect the results of the assay when HPLC separation is performed using an acetonitrile/H₂O mixture which reverts the exchange back to hydrogen. Full discussion in regard to this can be found in Figure S13. Thus, when the four stereogenic centers are

considered, only epimerization at C-1 or C-5 is expected to proceed via incorporation of a D atom from D₂O. These are the only positions where inversion of stereochemistry involves H exchange with the solvent, whereas a lack of incorporation of deuterium is only consistent with epimerization at either C-3 or S-O.

As shown in Figure 9, upon incubation in d-KPB for 1 hour, isomer **6** equilibrated to **1** with the incorporation of one D atom, suggesting that the two compounds differ only by the stereochemistry at C-1 or C-5. The interconversion between **2** and **7** paralleled that of **1** and **6**, with one D atom incorporated. The deduced structural relationship between **1** and **6**, **2** and **7**, further narrowed down the number of combinations from 16 to 6. The graphic representation of these six combinations is reported in Figure S22.

Computational studies and resolution of the problem

To narrow it down to one possible combination we took advantage of our recently reported software IMPACTS (In-silico Metabolism Prediction by Activated Cytochromes and Transition States). [16] This program is designed to predict the site of metabolism (SoM) of xenobiotics by P450s based on a combination of docking, transition state modeling and rule-based substrate reactivity prediction, and it has been validated on a wide variety of drugs and small molecules including a diverse set of functional groups. IMPACTS was applied to substrate **S1** and **S2** with P450s 3A4, 2C19, 2C9, 2D6 and 1A2, separately. For all five P450s, the sulfur atom was predicted to be the most likely SoM (Figure 10), in agreement with the experiments, while other possible chemical transformations were ranked as significantly less favorable.

In the case of prochiral centers, IMPACTS also provides the most favorable stereoisomer formed. Figure 10 shows the predicted metabolites of **S1** and **S2** (**4** and **5**, respectively). Relying on this program for the assignment of the stereochemistry at the sulfur atom in the structures of the primary metabolites allowed us to arrive at a final solution: only one diastereomeric structure for each of the seven HPLC peaks.

To further validate our conclusions based on the predictions by IMPACTS, additional computations were carried out. The equilibration studies revealed that each compound generated the same mixture after 3 weeks of incubation (Figure 5). We rationalized that this carried information about the concentrations at the thermodynamic equilibrium, and that the relative concentrations could be explained by ground state energy differences. A number of actions from our in-house computational platform FORECASTER [17] were then used to carry out a conformational search on the 8 possible diastereomers (and the 8 enantiomers) followed by energy minimization to obtain a ranking from most to least energetically favorable (details can be found in the experimental section). The results are summarized in Figure 11. In general, the predictions are in good agreement with our structural assignments. This is particularly true for the lowest-in-energy compound, **7**, which is experimentally the major one (> 50%) at the thermodynamic equilibrium, and for compounds **3** and **5** which have very high potential energies and are difficult to detect experimentally when the thermodynamic equilibrium is reached. The difference between the experimental and calculated data is larger for the other compounds, probably because of the intrinsic errors associated with the computational method itself. Another explanation for the slight

mismatch between experiments and computations could be related to **8**, the unobserved diastereomer. In fact, it is possible that the peak for **8** was hiding beneath one of the other seven, under the experimental conditions. Different HPLC separation methods were attempted to investigate this hypothesis (data not shown), but never more than seven peaks were observed.

Encouragingly, out of the six combinations left after the deuterium studies, the best match between experimental concentrations and calculated energies overlaps with the predictions by IMPACTS. The agreement between the two very different computational methods used here (docking-based method IMPACTS and DFT) provides a high-confidence in the computational study and in the final structural assignment.

CONCLUSIONS

In the study described herein, we have shed light on a complex oxidation/epimerization process leading to several metabolites. Although this study was centered on a small number of inhibitors specifically designed to target POP, our discoveries may have wider implications. For example, the bicyclic scaffold of **S1** and **S2** has been patented as part of a series of compounds [18] targeting DPP-IV, a prolyl peptidase associated with type-2 diabetes. [19] Here too, one might expect sulfur oxidation followed by a sequence of isomerizations similar to the ones that we have discovered. Such a large number of metabolites is an important liability in drug development. Not only would toxicological studies on every isomer be challenging, but the process of ring opening could also trigger subsequent harmful covalent modifications in biological systems. In fact, the hypothesized iminium ion intermediate could exist in solution long enough to react with surrounding biomolecules. It is also reasonable to think that cyanothiazolidine moieties alone, even when not contained within a bicyclic scaffold, could be prone to the same instability issues following sulfur oxidation. These moieties are found in other previously reported DPP-IV inhibitors for example. [20, 21]

The results presented here have provided guidance in designing a second generation of POP inhibitors lacking the liabilities associated with bicyclic cyanothiazolidine moieties, while maintaining high potencies. For example a series of oxoisindoline derivatives is looking very promising. [6]

EXPERIMENTAL SECTION

Chemicals and enzymes

Human Liver Microsomes (Ultra Pool™ 150) were purchased from BD Gentest. Compounds **S1** and **S2** were previously synthesized. [5]

Instruments

HPLC-MS analyses were performed on an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array UV detector, a quadrupole MS detector, and a ChemStation (for LC 3D A.09.03) data system. LC-MS/MS studies were performed using a Dionex Ultimate 3000 UHPLC coupled to a Bruker Maxis Impact

Quadrupole-Time of Flight mass spectrometer in positive ESI mode. The data was processed using Bruker DataAnalysis software version 4.1.

Metabolism of **S1**, **S2** by HLM

Each reaction mixture (200 μ l) contained HLM (1 mg/ml) substrate **S1** or **S2** (100 μ M) in 0.1 M potassium phosphate buffer at pH 7.4. The reaction was initiated by addition of NADPH (500 μ M) and the mixture was incubated at 37°C for 1 hour. At the end of the reaction, substrates and metabolites were extracted in DCM (0.3 ml $\times$ 3). The combined organic extracts were evaporated and the residue was redissolved in acetonitrile (30 μ l) before HPLC analysis. Separation and detection of metabolites were achieved by HPLC-MS. The column used was a 150 $\times$ 4.6 mm, 5 μ M, Eclipse XDB-C8 (Agilent). Elution consisted of a gradient step from 95% mobile phase A (water + 0.05% formic acid) and 5 % mobile phase B (acetonitrile + 0.05% formic acid), to 95% phase B over 20 min, at a flow rate of 1 ml/min. The absorption was recorded at 220 nm.

Equilibration studies

Compounds **1–7** were isolated by collecting the respective peaks separated by HPLC. Upon solvent evaporation, compounds **1–7**, separately, were incubated in 0.1 M potassium phosphate buffer at pH 7.4 and 37°C for 1 min, 10 min, 1 hour, 24 hours, or 3 weeks. At the end of the incubation period, compounds **1–7** were extracted in DCM (0.3 ml $\times$ 3). The combined organic extracts were evaporated and the residue was redissolved in acetonitrile (30 μ l) before HPLC-MS analysis. Column and elution methods were the same as those used to study the metabolism of **S1** and **S2** by HLM and P450 supersomes. All the LC-MS traces are reported as SIM (Single Ion Monitoring).

Deuterium studies

Compounds **6** and **7** were isolated by collecting the respective peaks separated by HPLC. Once isolated, the compounds were incubated in 0.1 M phosphate buffer, which was prepared with deuterium oxide and adjusted to pH 7.4. After 1 hour incubation, the compounds were isolated and analyzed following the same procedure as used for the equilibration studies. All the LC-MS traces are reported as TIC (Total Ion Current).

Synthesis of the sulfoxide derivatives of **S1** and **S2**

The starting material, **S1** or **S2** (25 mg, 0.079 mol), was dissolved in a mixture of 70:30 THF: water (2 ml) before addition of ammonium persulfate (3 equiv, 0.24 mol). The reaction mixture was stirred at room temperature for 16 hours before evaporation of the solvent. Water (5 ml) and DCM (5 ml) were added to the residue with mixing. The organic layer was collected, dried over sodium sulfate before evaporation of the solvent *in vacuo*. The crude product was purified through semi-preparative HPLC on a 25 cm $\times$ 9.4 mm ZORBAX Rx-C18 column (Agilent). Elution consisted of a gradient step from 95% mobile phase A (water) in 5% mobile phase B (acetonitrile), to 95% phase B over 45 min, at a flow rate of 2 ml/min. The absorption was recorded at 220 nm.

Ground state energy calculations

All of the possible diastereomers that could exist in this mixture were considered. Since there are 4 stereogenic centers, there are thus 16 (2^4) combinations possible (8 diastereomers, each having its enantiomer). A number of actions from our in-house computational platform FORECASTER were used. First ISOMER (Inverting Stereochemical Orientations Mitigating Exhaustive Rearrangement) was used to generate the 16 stereoisomers structures from one 2D structure, and these 16 configurations were then converted to 3D by our CONVERT (Conformational Optimization of Necessary Virtual Enantiomers, Rotamers and Tautomers) program. However, at this stage, the conformations produced are derived from molecular mechanics and may not be the true minimum energy conformation. Therefore we applied our new software ALTER (Assigning Labile Torsions and Executing Rotations) to explore further potential 3D conformations. The program is a conformational search tool designed to iterate rotatable bonds by a given resolution (60 degrees in this example) and flip rings in order to systematically generate exhaustive coarse-grained representation of the potential energy surface of the given molecule. The software also takes into account possible symmetrical rotations thus limiting the redundancy of the generation. The systematic nature of the protocol results in some nonsensical conformations and these are discarded upon attempting to optimize the structure. The other plausible conformations are energy-minimized and optimized using semi-empirical techniques (RM1 is said to incorporate improvements for hydrogen bonding considerations).

The software then discarded any conformations with an energy 10 kcal/mol above that of the minimum energy conformation as it is assumed that any conformation in this range would not be observed in any ensemble, nor would the energy be improved sufficiently even if calculated using a higher level of theory such as Density Functional Theory (DFT) or *ab initio* methods. Once the eliminations are complete, the remaining structures are grouped together based on similarity of their torsion angles as multiple starting conformations may optimize to the same geometry. A cut-off of 60 degrees (meaning set angles ± 30) was defined to decide whether structures were deemed equivalent to one another. It is important to note that the energies were computed using a polarizable continuum model accounting for water solvation energies to more accurately portray the conditions used in the actual experiment. In order to reduce the required time for this computational study without significantly affecting the expected accuracy, we removed the phenyl ring of the Cbz group, formally replacing Cbz by Moc (methyloxycarbonyl). Through this substitution, eleven atoms and a rotatable bond were removed while keeping a very similar functional group (the carbamate moiety is retained). With each diastereomer, there was a clear preferred conformation noted by its lower energy and a metric used to consider its entropic favorability. In order to obtain a more confident energy value for these structures, the 16 conformations were then further optimized using DFT (M06/6-31G*+) followed by a single point energy calculation using MP2. It was noted that the enantiomeric pairs of each of the 8 isomers had similar energy and geometry with one exception where the chosen conformation was different (based on a preferential selection from the semi-empirical optimization), demonstrating the reasonable convergence of the method in its alpha version.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING SOURCES

This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) via a grant to K.A., an NSERC-AstraZeneca grant (CRD program) to N.M. and by the Center in Green Chemistry and Catalysis. P.S. and J.P. were supported by a scholarship from McGill CIHR Drug Discovery Training Program.

We thank Dr. Alexander Wahba for technical support with the HPLC-MS/MS fragmentation experiments.

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Figure 1.

(A) Structure of **S1** and **S2** with respective inhibitory potencies against human POP. [5] (B) Red: monitoring of the molecular ion $[M+H]^+$ ($m/z = 318$) during LC-MS analysis of **S1**. Blue: monitoring of $[M+O+H]^+$ ions ($m/z = 334$) after incubation of **S1** with HLM (1 mg/ml) in potassium phosphate buffer (KPB, 0.1 mM, pH 7.4), for 1 hour at 37°C. (C) Pink: monitoring of $[M+H]^+$ ($m/z = 318$) during LC-MS analysis of **S2**. Green: monitoring of $[M+O+H]^+$ ions ($m/z = 334$) after incubation of **S2** with HLM (same conditions used for **S1**). (D) Overlay of the LC-MS traces of products of mono-oxidation of **S1** and **S2**. Detailed elution conditions can be found in the experimental. LC-MS traces looking at other possible products are shown in Figures S1–S6.

Figure 2.

(A) Synthesis of **2, 4, 7** by chemical oxidation of **S1** and LC-MS trace overlap of the products of chemical (solid line) and HLM microsomal (dashed line) oxidation of **S1**. (B) Synthesis of **1, 2, 3, 4, 5, 6** from chemical oxidation of **S2** and LC-MS trace overlap of the products of chemical (solid line) and HLM microsomal (dashed line) oxidation of **S2**.

Figure 3.

(A) Flowchart summarizing all the techniques used and the information collected from each experiment. The goal of the study was to assign a structure to all seven HPLC peaks. (B) Graphical representation of the partial structural information collected through each of the experiments and *in silico* calculations. This strategy allowed us to assign a structure (**1–7**) to each of the 7 peaks on the HPLC trace of metabolites and identify the only unobserved diastereomer.

Figure 4.

Representative fragmentation pattern shared by compounds **1–7**. The proposed structures for the main fragments strongly suggest that **1–7** are diastereomeric sulfoxides.

Figure 5.

Equilibration of each of compounds **1–7** separately when incubated in KPb at 37°C over time. The arrows in the highlighted graphs show pairs of compounds that are assumed to differ at only one stereocenter.

Figure 6.

(A) Carbon labeling for the structural scaffold of **1–7**. (B) Proposed mechanism of epimerization at S-O and C-3 for **1–7**. (C) Additional stereocenters considered prone to epimerization.

Figure 7.

Monitoring of $[M+O+H]^+$ ions ($m/z = 334$) after incubation of **S1** (A) and **S2** (C) with HLM (1 mg/ml) in KPB (0.1 mM, pH 7.4), for different periods of time, at 37°C. The background from spontaneous oxidation was subtracted, but the original spectra can be found in Figure S10. (B, D) Schematic representation of the sequence of chemical transformations happening during incubation of **S1** and **S2** with HLM.

Figure 8.

Equilibration profiles of **7** (A) and **6** (D) when isolated and incubated in KPB for 1h; mixture of products from chemical oxidation of **S1** (B) and **S2** (E). Partial assignment of the stereochemistry of **7** (C) and **6** (F) based on observations illustrated in panels A–D.

Figure 9.

LC-MS traces revealing incorporation of deuterium atoms when compounds **6** or **7** are incubated in d-KPB for 1 hour, at 37°C. The HPLC separation was performed using H₂O and acetonitrile as eluents. Original spectra can be found in Figures S14–21.

Figure 10.

(A) Predicted transition state for the reaction of **S1** with P450 3A4 (orange: enzyme heme group shown as the iron-oxo reaction intermediate; green: compound **S1**; light blue: protein residues surrounding the ligand; grey: protein binding site surface); predicted metabolites of **S1** (B) and **S2** (C) for the five major P450s.

Figure 11.

Calculated energies for **1–8** (kcal), reported as relative to the predicted most stable isomer, **7**. The experimental order of stability can be deduced from the LC-MS peak areas after 3 weeks of incubation in KPb, when thermodynamic equilibrium is reached.