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Enrichment of Relevant Oxidative Degradation Products in Pharmaceuticals With Targeted Chemoselective Oxidation

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ABSTRACT

The ability to produce and isolate relatively pure amounts of relevant degradation products is key to several aspects of drug product development: (a) aid in the unambiguous structural identification of such degradation products, fulfilling regulatory requirements to develop safe formulations (International Conference on Harmonization Q3B and M7); (b) pursue as appropriate safety evaluations with such material, such as chronic toxicology or Ames testing; (c) for a specified degradation product in a late-stage regulatory filing, use pure and well-characterized material as the analytical standard. Producing such materials is often a resource- and time-intensive activity, either relying on the isolation of slowly formed degradation products from stressed drug product or by re-purposing the drug substance synthetic route. This problem is exacerbated if the material of interest is an oxidative degradation product, because typical oxidative stressing (H_2O_2 and radical initiators) tends to produce a myriad of irrelevant species beyond a certain stress threshold, greatly complicating attempts for isolating the relevant degradation product. In this article, we present reagents and methods that may allow the rapid and selective enrichment of active pharmaceutical ingredient with the desired oxidative degradation product, which can then be isolated and used for purposes described above.

Introduction

One challenging aspect of the drug product (DP) development is the degradation propensity of the active pharmaceutical ingredient (API), which may directly impact the safety of the drug. The essential requirement to developing safe and efficacious drug products dictates a thorough understanding of the degradation pathways operating on the API in the DP, which often requires the identification of the degradation product(s). This activity is guided by ICH Q3B and ICH M7 regulatory guidance calling for identifying degradation products that meet an established threshold, which is based on the total daily intake of the drug of interest.

Definitive structural identification of degradation products (which are also referred as degradates) ideally requires isolation of the authentic degradate in the DP in enough quantity for nuclear magnetic resonance (NMR) and mass spectrometric studies. Once structurally identified, an integral part of the identification of degradation products is to determine a relative response factor (RRF), that is, the relative absorbance at a given wavelength, compared to the API. The RRF determination enables the pharmaceutical scientists to calculate the weight percent formation of the degradation products accurately. The process of determining RRF often requires tens of milligrams of pure material. Aside from the structure identification and RRF determination, isolated pure degradation products, if pure enough and well characterized, could also be used as sources of material for safety assessment qualification studies, and even as analytical standards for the specific impurities supporting world market applications for new drugs, and the eventual commercial manufacturing activities for the product.

Two most common degradation pathways observed in DP are hydrolytic and oxidative. The focus of this article is oxidative degradation because it presents more complex reaction profile under simulated oxidative conditions. Oxidative degradation in DP during storage is commonly caused by the endogenously present peroxide impurities, which may react directly as a neutral peroxide species or via catalytically driven radical processes. As reported in the

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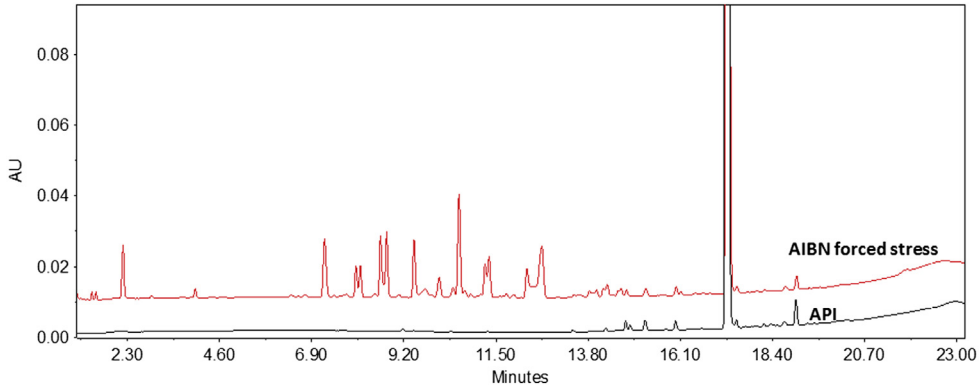


Figure 1. Oxidative forced stress experiment with AIBN for an MSD development candidate.

literature,[1] for the purpose of understanding a molecule's oxidative sensitivity as well as degradate isolation, the API is enriched with oxidative degradation products by the reaction of azonitriles, such as 2,2'-azobisisobutyronitrile (AIBN) or H_2O_2 with API. But these processes, when performed at higher temperature or under extended time to yield larger amount of degradates for isolation, may produce a mixture of irrelevant degradates, exemplified in Figure 1 by a typical forced stress experiment with AIBN for one of our development candidates. It is also possible, and often practiced, to enrich DP with desired oxidative degradation products under accelerated stress (i.e., high temperature and humidity). But this takes long lead times and gives low conversion to degradates (e.g., see Hartauer et al. [2]). Stressing at higher temperatures can help the formation of higher levels of the degradates of interest, but it is often accompanied by other unintended reactions, creating less tractable preparatory chromatographic isolations. Given the timelines under which pharmaceutical scientists operate in the development area, faster and practical ways of enriching API with relevant oxidative degradates and their isolation in pure form is highly desirable.

In this article, we present methods using oxidants used in organic synthesis to enrich a given API with commonly observed oxidative degradation products. This collection of methods and reagents can be viewed as an *Oxidation Toolkit* for the pharmaceutical scientists, and we will use this terminology in the rest of the discussion. We also present high yield conversion with common reagents used in oxidative forced stress studies (an AIBN-like reagent and H_2O_2 in gas phase). Hence, this is not a collection of reagents to replace those typically used to assess oxidative sensitivity for APIs as described above but rather to use broader chemistry, not related to the DP excipients, to produce large amounts of degradates (rather than trace amounts). Here we list reagents and methods for oxidation reactions relevant to different functional groups, and the pharmaceutical scientist will prioritize screening based on the information available, which generally comes from forced stress and accelerated stress experiments performed on the API and on the DP. Although application of this idea could be found in isolated cases in the literature,[2-4] and was introduced earlier as a "tool box" by Baertschi et al.,[1] this concept has further been developed in this article to include a much broader array of reagents and methods. Principles behind this toolkit will be elaborated, along with a few examples of enriching API with known degradation products. The driver in choosing the reagents in the toolkit is their effectiveness and ease of use.

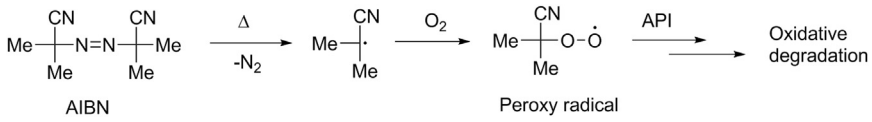
Discussion

The main reagent for the commonly observed oxidative degradation of the API in DP is oxygen. But the abundant atmospheric molecular oxygen cannot react directly with most organic molecules because of the difference in the electronic ground states—triplet ground state for molecular oxygen and singlet ground state for the majority of organic molecules. The selection rules derived from quantum mechanics prohibit such reactions because these are "spin forbidden." For the reactions to occur, the molecular oxygen has to be excited to the singlet state ($^1O_2$), or the organic molecules have to react with other "reactive oxygen species," such as hydroxyl radical (·OH) or hydrogen peroxide (H_2O_2).

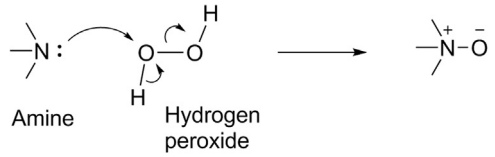
Oxidation of the API by the singlet oxygen ($^1O_2$) is often encountered in photo degradation processes. Although photo degradation is a valid degradation mechanism in DP, it is controlled effectively, most of the time, by the use of proper protective packaging. On the other hand, oxidative degradation with hydroperoxides (ROOH) or with hydroxy (·OH)/alkoxy (·OR)/peroxy (OOR) radicals cannot be controlled in most instances, which leads to the presence of degradates above ICH Q3B identification thresholds. The oxidation toolkit presented in this article is geared toward accessing these latter types of degradates.

Oxidative forced stress studies[1] are commonly performed on the API in solution phase using azonitriles such as AIBN, and H_2O_2. Forced stress of API with AIBN emulates peroxy radical−mediated (1-electron) oxidation (Scheme 1). On the other hand, oxidation with H_2O_2 follows a 2-electron pathway, which is generally observed for *N*- or *S*-oxidation (Scheme 2).

Analysis of experimental data (chromatographic, spectroscopic, mass spectrometric) obtained from accelerated stress experiments on DP and from forced stress studies of API, along with the analysis of structure reactivity of the API molecule often indicate the plausible structures of degradates. Sometimes, it is possible, using compelling chemical reactivity argument, to establish a proposed structure in the absence of isolated pure material with comprehensive



Scheme 1. Formation of peroxy radicals from AIBN and subsequent oxidative degradation of API.



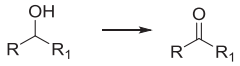
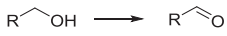
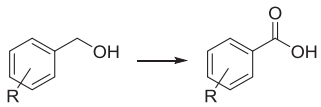
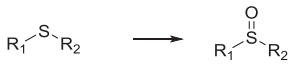
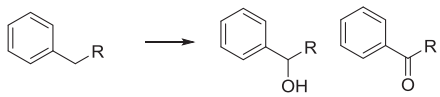
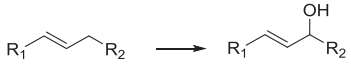
Scheme 2. Two-electron oxidation of amines with hydrogen peroxide.

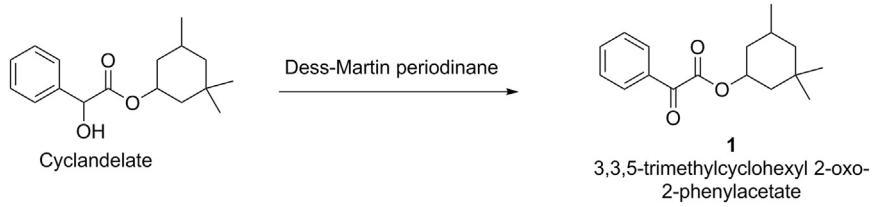
spectroscopic data. But, for the definitive structure ID, the degradation products need to be isolated in pure form. Even when it is possible to isolate the pure degradation products from the stressed DP or from appropriate forced stress experiments on API, the process may be time-consuming and often the amounts of isolated degradates are quite small to be used in other activities, such as RRF determination. Ideally, pharmaceutical scientists need to have other means (than stressing DP or relying upon limited opportunities offered by established forced stress experimental conditions) to enrich the API with degradation product(s), preferably in a clean manner. Here we present examples of enrichment of API with degradation products (and their isolation in selected cases) using a set of reactions which are referred to as oxidation toolkit.

Table 1 lists some of the most common transformations observed in the oxidative degradation of the API in DP as evidenced by literature references[1,5,6] and by in-house examples, along with recommended reagents for the enrichment of the API with desired degradation products. Although process chemists will well know these reactions to enable preparation of these degradates, it is useful for the pharmaceutical scientist to be aware and have access as needed. It should be noted that the reagents listed for the enrichment of the API with desired degradation products are only a subset of the reagents reported in the chemistry literature. The choice of reagents, in Table 1, is based on the ease of their use and their general effectiveness to enrich API with key degradation products. So, it is expected that the practicing pharmaceutical scientists will eventually add new tools (reagents and methods) to this toolkit as the situation warrants.

Entry 1 (Table 1) shows oxidation of a secondary alcohol to ketone and entry 2 shows the oxidation of a primary alcohol to aldehyde. For both oxidations, Dess-Martin periodinane is an

Table 1
Specific Oxidative Transformations and Recommended Reagents to Enrich API With Oxidative Degradates

Entry	Transformation	Reagents
1		(i) Dess-Martin periodinane (see preparation of 1, Scheme 3) (ii) Pyridinium chlorochromate (Corey and Suggs[7]) (iii) Pyridinium dichromate (Corey and Schmidt[8]) (iv) $RuO_2.xH2O/NaIO_4$ (Doromy and Castro[9]; Baldwin et al.[10])
2		(i) Dess-Martin periodinane (see preparation of 1, Scheme 3) (ii) Pyridinium chlorochromate (Corey and Suggs[7]) (iii) Pyridinium dichromate (Corey and Schmidt[8])
3		(i) TPAP/NMO (Houri et al.[12])
4		(i) $KMnO_4$ (Jursic[13]; Shaabani et al.[14])
5		(i) MTO/H_2O_2 (Coperet et al.[15]) (ii) *m*-CPBA (Ray et al.[16])
6		(i) H_2O_2 (see preparation of 2, Scheme 5) (ii) Peracetic acid (Hartauer et al.[2]) (iii) $O_2/RuCl_3$ (Jain and Sain[17])
7		(i) Oxone (Trost and Curran[18]) (ii) $H_2O_2/[VO_2F(dmpz)_2]$ (Hussain et al.[19])
8		(i) Oxone (Trost and Curran[18])
9		(i) $NaNO_2/HCl$ (see preparation of 3, Scheme 6 and Owens[20])
10		(i) *m*-CPBA (March[23])
11		(i) $FeCl_3$ (see enrichment of dextromethorphan with 7 and 8, Scheme 7 and Nanda et al.[24]) (ii) AAPH (see enrichment of ibuprofen with 5 and 6, Scheme 8 and Betigeri et al.[25])
12		(i) H_2O_2 (g) (see enrichment of rosuvastatin calcium with 4, Scheme 9 and Zhu et al.[29]) (ii) Dess-Martin periodinane (see preparation of 1, Scheme 3)
13		(i) SeO_2 (Suksamram et al.[30]; Khurana et al.[31])



Scheme 3. Oxidation of cyclandelate with Dess-Martin periodinane.

effective reagent and provides easy reaction setup and postreaction workup. This has been demonstrated (Scheme 3) in our lab for the enrichment of cyclandelate with its degradation product, 1, which was isolated in 86% yield. It should be noted that the whole process of enrichment, workup, and isolation has easily been performed in a single day. Two other reagents, pyridinium chlorochromate and pyridinium dichromate, can also affect the similar transformations.[7,8] Combination of $RuO_2.xH_2O$ (catalytic) and $NaIO_4$ (oxidant) has been used successfully for selective oxidation of secondary alcohol to ketone.[9,10] Although the reagents listed produce the desired degradation products most of the time, there will be times when these might not work. Such an example is the oxidation of lovastatin to its oxolactone, 9 (Scheme 4). All 4 reagents, listed under entry 1 (Table 1), failed to do the oxidation of lovastatin in a *chemoselective* fashion. But, a slight variation of the dichromate oxidant, namely, cetyltrimethylammonium dichromate, has been reported[11] to accomplish the desired chemoselective oxidation to 9.

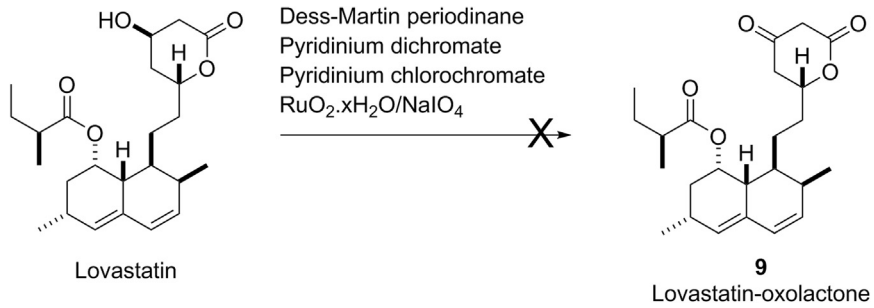
Exhaustive oxidation of primary alcohols yields carboxylic acids (entries 3 and 4, Table 1). A very useful catalyst for the transformation of primary aliphatic alcohol to the corresponding acid is tetrapropylammonium perruthenate, *n*-Pr_4NRuO_4 (TPAP), sometimes called Ley-Griffith reagent. The co-oxidant in this reaction is *N*-methylmorpholine *N*-oxide (NMO). An example of the reaction of TPAP/NMO with a primary alcohol to yield the corresponding carboxylic acid could be found in the work reported by Houri et al.[12] The oxidation was complete in 3 h and isolated yield of the carboxylic acid was 65%. Potassium permanganate, $KMnO_4$, is also an efficient reagent for the exhaustive oxidation of alcohols to carboxylic acids, particularly benzylic alcohols to benzoic acids. Oxidation with permanganate can be performed efficiently in aqueous medium in the presence of surfactants,[13] or if nonaqueous reaction medium is preferred, the oxidation could be performed in the presence of an ion-exchange resin.[14]

A common oxidative degradation is the formation of *N*-oxides of pyridines (entry 5, Table 1) or of *N*-containing heterocycles. Such oxidation can be carried out very effectively by the method of Coperet et al.[15] using methyltrioxo rhenium (MTO) as catalyst in the presence of H_2O_2. The transformation is carried out in a biphasic reaction medium, where the substrate and the reaction product remain soluble in dichloromethane layer, whereas the oxidant stays in the aqueous phase. This enrichment method is fast and very easy to execute in the laboratory. Although MTO/H_2O_2 should be the method of choice, the same transformation could also be affected by H_2O_2 or *m*-chloroperoxybenzoic acid.[16]

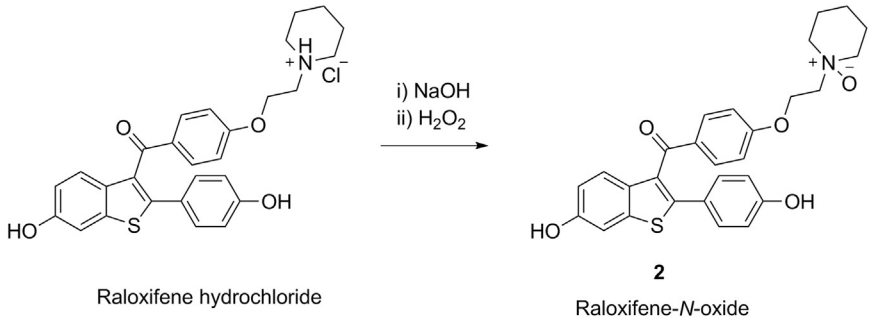
Oxidation of tertiary amines to their corresponding *N*-oxides with H_2O_2 is a 2-electron process, where the lone pair of electrons on the *N*-atom is involved (Scheme 2). So, it is important to maintain the pH of the reaction medium at or above the p*K*a of the oxidizable amine. At pH below the p*K*a, the amine-*N* will remain protonated rendering the lone pair of electrons inaccessible toward desired reaction. This principle has been demonstrated in the synthesis of the *N*-oxide of raloxifene (2, Scheme 5) from raloxifene hydrochloride. Raloxifene hydrochloride was neutralized *in situ*, before reaction with H_2O_2, by 1 equivalent of NaOH. Recrystallization (trituration) provided the desired degradation product 2 in 75% yield. Application of the principles of oxidation toolkit enabled us to enrich the API with 2 and made its subsequent isolation an efficient process. It should be noted here that the enrichment of raloxifene hydrochloride—containing tablets with only 1% of 2 was accomplished after 8 weeks of stressing the tablets at 105°C.[2] The synthesis of 2 has also been reported[2] by Hartauer et al., in multi-gram scale, from the *free base* of raloxifene hydrochloride using peracetic acid. The *N*-oxidation of tertiary amines can also be conducted under oxygen atmosphere with $RuCl_3$ as catalyst.[17]

Sulfoxides (entry 7, Table 1) and sulfones (entry 8, Table 1) are common oxidative degradation products of sulfides, which are formed by the reaction of hydroperoxides with API in DP. It should be noted that the oxidation of sulfoxide group in the API will also produce sulfone as the degradation product. An efficient way of forming sulfone from sulfide or sulfoxide is to use oxone as oxidant in H_2O-MeOH reaction medium.[18] By controlling the amount of oxone used and reaction time, it is possible to get sulfoxide as the major product (instead of sulfone) in some instances. A more chemoselective way of making sulfoxides is to use a vanadium catalyst [$VO_2F(dmpz)_2$] in the presence of H_2O_2.[19]

An aromatic sulfide could be oxidized to sulfoxide using HNO_2,[20] produced from the reaction of $NaNO_2$ and HCl (entry 9, Table 1). The operating mechanism, in this case, is via a cation radical intermediate,[21] *not* a standard 2-electron pathway, which is in play with



Scheme 4. Ineffective reagents for the chemoselective oxidation of lovastatin to lovastatin-oxolactone.



Scheme 5. Oxidation of the piperidine-N in raloxifene hydrochloride to the corresponding N-oxide.

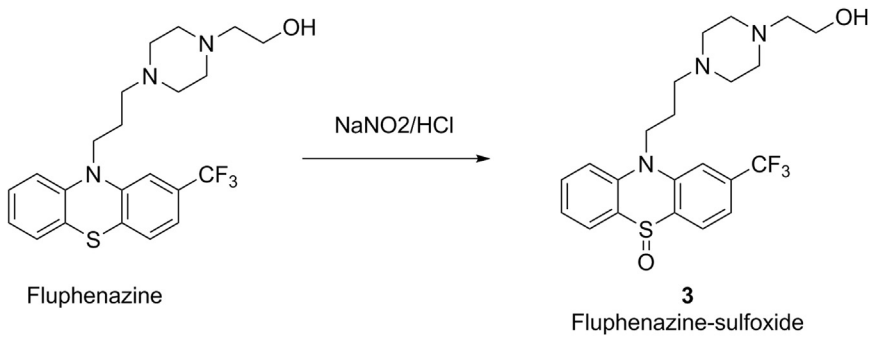
peroxide- or oxone-mediated oxidation. This unique mechanism provides an advantage to oxidize aromatic sulfides to sulfoxide selectively, in the presence of amines or pyridines which are also susceptible to oxidation to corresponding *N*-oxides. This methodology has successfully been applied to make the sulfoxide degradation product (3) of fluphenazine (Scheme 6) in 70% isolated yield, without oxidizing the piperidine-nitrogen to corresponding *N*-oxide. Isolation of 3 was accomplished by preparative thin layer chromatography (TLC), an easily executable and efficient method.

Epoxides, which are mutagenic alerting structures,[22] are readily formed from olefinic moieties in the API, by the action of hydroperoxides. There are many established ways to make epoxides from olefins. The most widely used method,[23] also known as Prilezhaev reaction, employs the oxidant *m*-chloroperoxybenzoic acid (entry 10, Table 1). This method of enriching API with its corresponding epoxide is fast, efficient, and easy to execute.

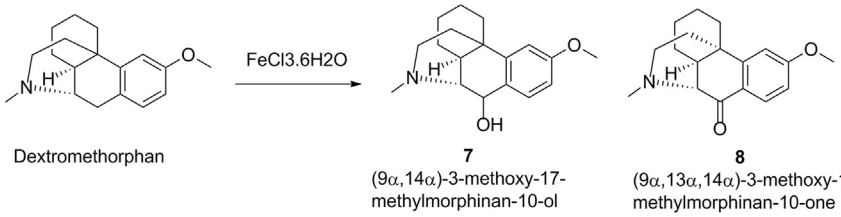
Oxidative degradation products arising from the oxidation of benzylic position are commonly observed in DP. Usually, this kind of oxidation is affected by alkoxy (·OR) or peroxy (·OOR) radicals,[1,5] or by transition metal ions.[24] Hence, for the enrichment of the API with such oxidative degradation products, any of these 2 mechanisms could be exploited (entry 11, Table 1), depending on the substrate. For example, oxidative degradation products (7 and 8) of dextromethorphan were obtained by using $FeCl_3$ (Scheme 7). In this instance, the oxidative mechanism goes through an aromatic cation radical intermediate.[24] On the other hand, a radical-initiated mechanism was exploited to enrich ibuprofen with its degradation products (5 and 6, Scheme 8), and 2,2'-azobis(2-amidinopropano) dihydrochloride (AAPH) was used to generate the reactive peroxy radical species.[25] AAPH is a variant of AIBN. In this case, AAPH provided a cleaner and faster enrichment profile than that obtained from the use of AIBN.

Oxidation of allylic carbon to its corresponding alcohol (entry 13, Table 1) and oxidation of allylic alcohol to ketone (entry 12, Table 1) are also affected by the action of alkoxy or peroxy radicals, because of the low bond dissociation energy of the allylic C-H bond.[5] In solution, alkoxy radical can be generated by the homolysis of 2,2'-azobisisobutyronitrile (AIBN) in acetonitrile-water in the absence of any alcoholic solvent,[26] whereas hydroxy radical, which is more reactive than alkoxy radicals because of the difference in bond dissociation energy,[27] can be generated in solution using Fenton chemistry.[28] Reaction of hydrogen peroxide in gas phase with solid API is shown[29] to be effective in generating oxidative degradation products. Dess-Martin periodinane, as described earlier, can also act as an oxidant for the oxidation of allylic alcohol to ketone. All the above methods can be used for the oxidation of allylic or benzylic carbons, depending on the reactivity of individual API molecule. Here we present an example of the oxidation of an allylic alcohol to the corresponding ketone (Scheme 9), using H_2O_2 in gas phase. It was found that hydrogen peroxide, in the gas phase, can react with solid API, rosuvastatin calcium, to generate the oxidative degradation product 5-keto-rosuvastatin (4). The degradate, 4, was generated cleanly (24% by peak area) in 1 week from the oxidation of the allylic alcohol in rosuvastatin. It should be noted that the degradate 4 cannot be generated using H_2O_2 in solution, under typical forced stress condtions[1] (2-electron oxidation). Allylic oxidation to allylic alcohol (entry 13, Table 1) can be accomplished by the use of selenium dioxide (SeO_2).[30,31]

Qualification of degradation products is required, as per ICH Q3B, when they reach a certain threshold. The qualification process involves synthesis of pure degradate which is then doped into the API to be used in the animal toxicological studies. The independent synthesis of the degradate is a time-consuming and expensive process in the drug development area. Application of the oxidation toolkit may expedite this process considerably, saving time and resources. Using the principles of the oxidation toolkit, an API can be enriched with its desired oxidative degradation product in a clean chemoselective fashion. This material can then be used



Scheme 6. Oxidation of fluphenazine to fluphenazine-sulfoxide.



Scheme 7. Iron(III)-mediated oxidation of dextromethorphan.

directly in the animal toxicological studies for the qualification of the degradate. We were able to use this principle to enrich rosuvastatin calcium with 1% of 5-keto-rosuvastatin (4), in a clean chemoselective way. The enriched API was then successfully used to qualify 4 in animal toxicological studies.

Materials

All chemicals were used as received. Dess-Martin periodinane, hydrogen peroxide (H_2O_2) solution (35%), pyridinium dichromate, pyridinium chlorochromate, sodium nitrite, ibuprofen, 1-hydroxyibuprofen, 2-(4-isobutyrylphenyl)-propanoic acid, raloxifene hydrochloride, and titanium(III) chloride were purchased from Sigma-Aldrich (St. Louis, MO). Cyclandelate and fluphenazine hydrochloride were obtained from Chem-Impex International (Wood Dale, IL) and Matrix Scientific (Columbia, SC), respectively. Lovastatin was obtained from TCI America (Portland, OR). Rosuvastatin calcium was obtained from Hanmi Fine Chemical Co. (Kyonggi-Do, Korea). Trifluroacetic acid (TFA), hydrochloric acid, and HPLC-grade solvents were obtained from Fisher Scientific (Fair Lawn, NJ).

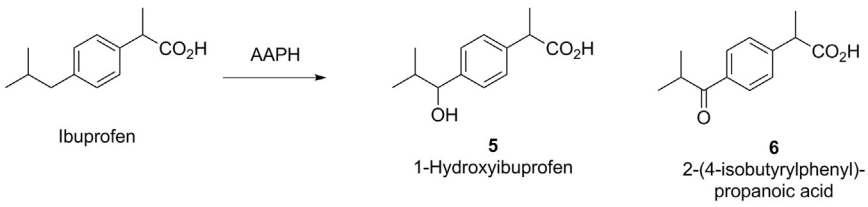
Experiment and Analysis

Reversed-phase chromatography for the analysis of the oxidation experiments involving rosuvastatin was carried out on an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a quaternary pump, heated column compartment, diode array detector, and autosampler. The chromatographic conditions included a Waters Symmetry Shield RP18 column of dimensions 4.6 mm × 150 μm and 3.5 μm particle size held at 45°C; flow rate was maintained at 1.5 mL/min; injection volume was 60 μL; tray temperature was 20°C; detector wavelength was 242 nm; mobile phase A consisted of a mixture of 0.01 M potassium dihydrogen phosphate solution pH 2.5 and acetonitrile (95:5); mobile phase B consisted of acetonitrile. The mobile phase gradient is listed in the Supporting Information (Table S1).

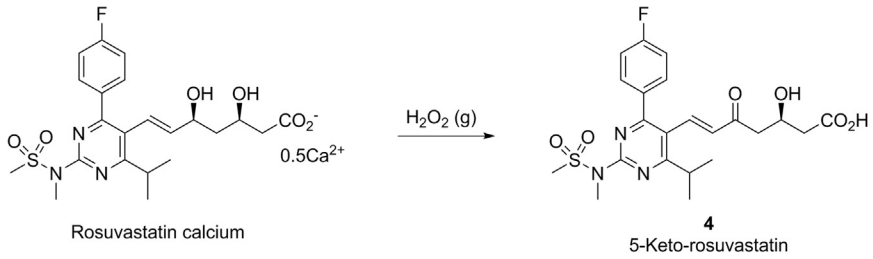
For the liquid chromatography-mass spectrometry (LC-MS) analysis of reactions and isolated products, samples were injected (0.5 μL) onto a Waters Acquity UPLC BEH C18 column (1 cm × 500 mm, 1.7 μm) from Waters Corporation (Milford, MA) and eluted with a linear gradient delivered at a flow rate of 300 μL/min by an Acquity ultra-performance liquid chromatography system (Waters Corporation). Mobile phases consisted of water/acetonitrile/TFA at a ratio of 99.95%, 0%, and 0.05% (v:v:v) for solvent A and a ratio of 0%, 99.95%, and 0.05% (v:v:v) for solvent B. The following elution conditions were set: 5% of solvent B for 0.1 min, followed by a linear increase of B from 5% to 99% within 2 min. Column temperature was maintained at 50°C. An SQD detector (Waters Corporation) was used coupled to the UPLC system. The mass spectrometer was equipped with an electrospray ion source. The capillary was set to 3.0 kV. The cone and extraction voltages were 20.0 eV and 3.0 eV, respectively. Source and desolvation temperatures were 120°C and 400°C, respectively.

For the LC-MS analysis of raloxifene hydrochloride, raloxifene-*N*-oxide (2), fluphenazine, and fluphenazine-sulfoxide (3), samples were injected (3 μL) onto a BEH C18 RP Shield column (5 cm × 2.1 mm, 1.7 μm) from Waters Corporation and eluted with a linear gradient delivered at a flow rate of 300 μL/min by an Acquity ultra-performance liquid chromatography system (Waters Corporation). Mobile phases consisted of water/acetonitrile/TFA at a ratio of 99%, 1%, and 0.08% (v:v:v) for solvent A and a ratio of 1%, 99%, and 0.08% (v:v:v) for solvent B. The following elution conditions were set: 2% of solvent B for 0.5 min, followed by a linear increase in B from 2% to 98% within 5 min. Column temperature was maintained at 26°C. A Synapt-G1 (Waters Corporation) hybrid mass spectrometer operated in the MSe mode and acquiring data with the time-of-flight analyzer was used coupled to the UPLC system. The instrument was operated for maximum resolution with all lenses optimized on the $[M + H]^+$ ion from the Leu-enkephalin. The capillary was set to 3.0 kV. The cone and extraction voltages were 40.0 eV and 4.0 eV, respectively, and Ar was admitted to the collision cell. Spectra were acquired within a mass range 100-2000 amu and accumulating data for 1.5 s per cycle. The low collision energy in the trap was set to 12.0 V. The high collision energies were ramped from 18.0 V to 32.0 V during each scan. Source and desolvation temperatures were 100°C and 150°C, respectively. Time to mass calibration is made with CsI cluster ions acquired under the same conditions.

High-resolution solution NMR data were collected on either a Bruker Avance HD III 700 (Bruker BioSpin Corporation, Billerica, MA) NMR spectrometer equipped with a 1.7-mm HCN TCI MicroCryoprobe or a Varian VNMRS 600 MHz NMR spectrometer equipped with a 5-mm One NMR probe. Compounds 1-3 were each dissolved in about 150 μL of DMSO-d6 (CIL, 99.96+% D) and placed into a 3-mm Wilmad NMR tube and capped. The sample was used for solution NMR experiments using standard pulse sequences (proton, zTOCSY, ME-gHSQC, gHMBC, and ROESY experiments) at 25°C. Water suppression was used (as needed) for various experiments to aid in the structure elucidation of the oxidative



Scheme 8. Benzylic oxidation of ibuprofen with AAPH.



Scheme 9. Oxidation of rosuvastatin calcium to 5-keto-rosuvastatin.

degradates. All data were analyzed and archived using Mnova 12.0 (Mestrelabs).

Analytical TLC was performed on EM Reagent 0.25-mm silica gel 60-F plates.

Authentic commercial compounds were used to identify degradation products of ibuprofen by comparing retention time in HPLC and UV-vis spectra. Oxidation product of rosuvastatin calcium was identified by comparing HPLC retention time and UV-vis spectrum (in-house method) from previous in-house research. Commercially available compounds corresponding to the degradation products of cyclandelate, raloxifene, and fluphenazine were not available, prompting isolation and structural characterization by NMR and mass spectrometry. Isolation of degradation products was accomplished either by recrystallization or by normal-phase preparative chromatography using ISCO Combiflash Sg 100c instrument with Biotage Flash 40 cartridge/column. When appropriate, isolation was performed with preparative TLC using silica gel preparative TLC plates (1000 μm, 20 × 20 cm) from Sorbent Technologies Inc.

Enrichment of API With Degradation Products, Isolation and Structural Characterization

Warning

Reagents for oxidation should be handled with care as indicated in corresponding Material Safety Data Sheet.

Enrichment of Cyclandelate With Oxidative Degradation Product, 3,3,5-Trimethylcyclohexyl 2-oxo-2-phenylacetate (1), and Isolation and Structural Characterization

To a solution of cyclandelate (300 mg, 1.1 mmol) in dichloromethane (6 mL) was added solid Dess-Martin periodinane (700 mg, 1.7 mmol). The resulting mixture was stirred at room temperature for 2 h and filtered through 0.45-micron polytetrafluoroethylene syringe filter. The filtrate was concentrated on a rotary evaporator and purified by preparative normal-phase chromatography (40 g silica gel column, 0% to 30% ethyl acetate in hexanes over 20 min) to yield the desired product 1 as a colorless viscous liquid (260 mg, 86%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.99 (dd, J = 8.4, 1.3 Hz, 2H, Ar*H*), 7.65 (m, 1H, Ar*H*), 7.51 (dd, J = 8.3, 7.4 Hz, 2H, Ar*H*), 7.31 (s, 1H, Ar*H*), 5.22 (m, 1H, C*H*), 2.14, 1.03 (m, 2H, CH_2), 1.84, 1.27 (m, 2H, CH_2), 1.38, 0.84 (m, 2H, CH_2), 1.00 (s, 3H CH_3), 0.99 (s, 3H, CH_3), 0.95 (d, J = 6.5 Hz, 3H, CH_3); ^{13}C (150 MHz, DMSO-d6): δ 187.0, 163.6, 134.7, 132.8, 130.0, 128.9, 74.0, 47.4, 43.8, 40.1, 27.2, 25.4, 33.0, 25.5, 22.3. MS *m/z* $(2M + Na)^+$ 571.3 found, 571.3 required. Detailed analysis of NMR data is presented in the Supporting Information.

Enrichment of Raloxifene With Oxidative Degradation Product, Raloxifene-N-oxide (2), Isolation and Structural Characterization

To a solution of raloxifene hydrochloride (40 mg, 0.08 mmol) in 2 mL methanol was added 0.2 mL water, followed by 80 μL of 1 N aqueous NaOH solution. To this solution was added aqueous H_2O_2 solution (35%, 0.2 mL) and allowed to stir at room temperature overnight. The reaction solution was then evaporated to dryness on a rotary evaporator. The resulting solid was triturated in methanol-H_2O (2:1 v/v) to yield the desired degradation product 2 as an off-white solid (28 mg, 75%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.62 (d, J = 8.8 Hz, 2H, Ar*H*) 7.32 (m, 1H, Ar*H*), 7.31 (s, 1H, ArH), 7.12 (d, J = 8.6 Hz, 2H, Ar*H*), 6.89 (d, J = 8.8 Hz, 2H, ArH), 6.85, (m, 1H, Ar*H*), 6.63 (d, J = 8.4 Hz, 2H, Ar*H*), 4.56 (t, J = 4.9 Hz, 2H, OCH_2), 3.53 (br s, 2H, NCH_2), 3.30, 3.07 (m, 4H, C*H2*), 2.08, 1.54 (m, 4H, CH2), 1.58, 1.33 (m, 2H, CH_2); ^{13}C (150 MHz, DMSO-d6, ppm): δ 191.9, 161.5, 158.0, 141.0, 131.7, 131.3, 130.2, 129.5, 115.5, 114.7, 114.3, 106.9, 67.3, 61.8, 64.6, 20.8, 19.7; MS *m/z* $(M + H)^+$ 490.2 found, 490.4 required. Detailed analysis of NMR data is presented in the Supporting Information.

Mass Spectrometry Analysis of Raloxifene and Its Oxidation Product, 2

LC-MS analyses of raloxifene (t_{el} = 3.0 min; Supporting Information, Fig. S1A) and 2 (t_{el} = 3.15 min; Supporting Information, Fig. S1B) show mass-to-charge ratios m/z = 474.10 (Supporting Information, Fig. S2A) and m/z = 490.15 (Supporting Information, Fig. S2B), respectively. Comparison of the collision-induced dissociation (CID) spectra of raloxifene and 2 demonstrates the presence of an *N*-oxide moiety in 2 (Fig. 2). The N-oxide can easily be confirmed by the unusual thermal activation/loss of oxygen (deoxygenation of M + H, F1; Fig. 1b). This deoxygenation process is associated with thermal activation and does not result from collisional activation in the desolvation region of the API source and provides evidence for the presence of an N-oxide in the molecule. Such specific loss was previously reported in the literature.[32,33] In addition, the *N*-oxide allows for the presence of a positive charge on the nitrogen of the piperidine, which favors gas-phase fragmentation of the ether bond forming the fragment ion F2 (m/z 389.1, Fig. 2b). In contrary, in the absence of oxidation, the protonation in gas phase of the sulfur atom permits the ring opening of the thiophene moiety and the release of fragment ion F3 (m/z 362.0, Fig. 2a). Fragment ions F4 (m/z 269.0) are the results of the fragmentation at the carbonyl moiety in both raloxifene and 2 (Figs. 2a and 2b). Fragmentation at the carbonyl moiety in 2 is likely to proceed from the deoxygenated ion (M + H, m/z 474.17). Fragment ion F5 (m/z 128.1) is unique to the fragmentation pattern of 2 (Fig. 2b). F5 is likely to be the 1-hydroxy-vinylpiperidinium. Fragment F6 (m/z 112.1) is likely the vinylpiperidinium ion, which results from the protonation of the nitrogen of the piperidine moiety. F6 is common to both the fragmentation patterns of raloxifene and 2 (Figs. 2a and 2b).

To further confirm the presence of an *N*-oxide moiety, 2 is reduced in the presence of $TiCl_3$ (N-oxide moiety is reduced in the presence of TiCl3. Oxidized raloxifene (2) is prepared at 1 mg/mL in 50:50 milliQ H2O:ACN. An aliquot of 200 μL of this solution is mixed with 20 μL of TiCl3/HCl. The solution is vortexed for 1 min. The reaction is quenched by addition of 20 μL of NH_4OH (5 N). The sample is vortexed for 30 s. The latter is diluted by the addition of 400 μL of 50:50 milliQ H2O:ACN solution. The sample is allowed to

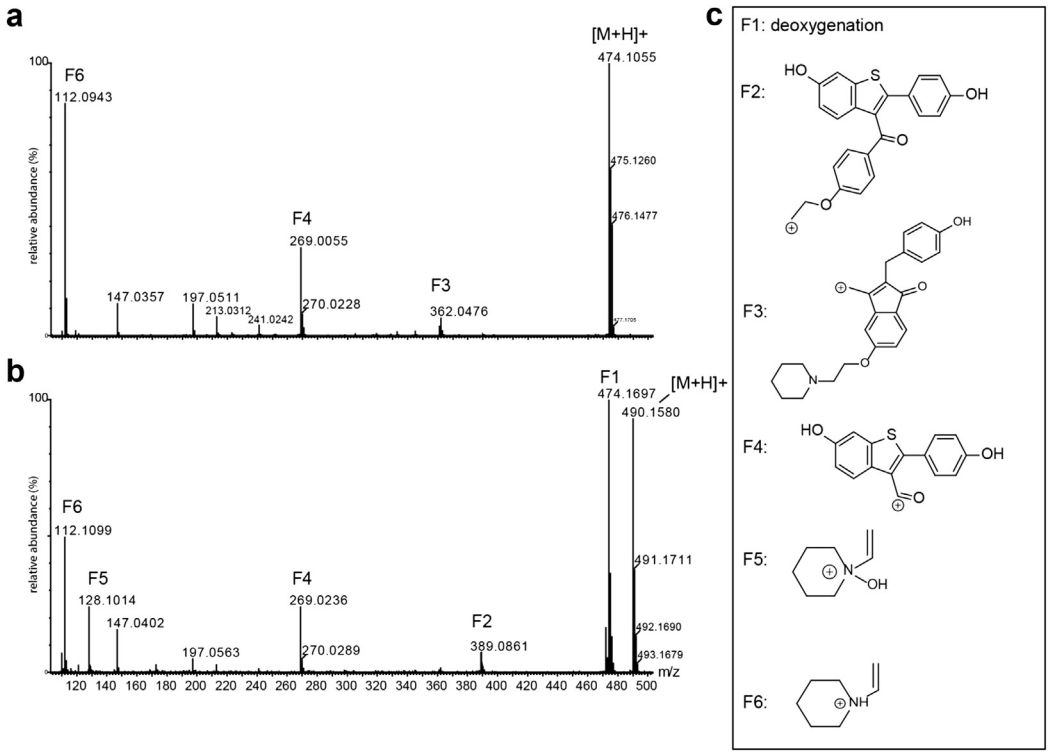


Figure 2. CID spectra of raloxifene (a) and 2 (b). Significant fragment ions are reported as F1-F6 and their tentative structures are presented in panel (c).

stand for 5 min and then the supernatant is decanted. An aliquot of 100 μL of the supernatant is transferred into a new vial for mass spectrometry analysis.) which has been documented as a specific reducing agent of *N*-oxides.[34] The LC-MS data clearly demonstrate the reduction of 2 into the original raloxifene molecule (Supporting Information, Fig. S4A-S4C).

Enrichment of Fluphenazine With Oxidative Degradation Product, Fluphenazine-sulfoxide (3), Isolation and Structural Characterization

To a stirring solution of fluphenazine (22 mg, 0.05 mmol) in water (1 mL) was added 2 drops of concentrated HCl, followed by 2 drops of $NaNO_2$ solution (100 mg/mL in water). Color of the reaction mixture

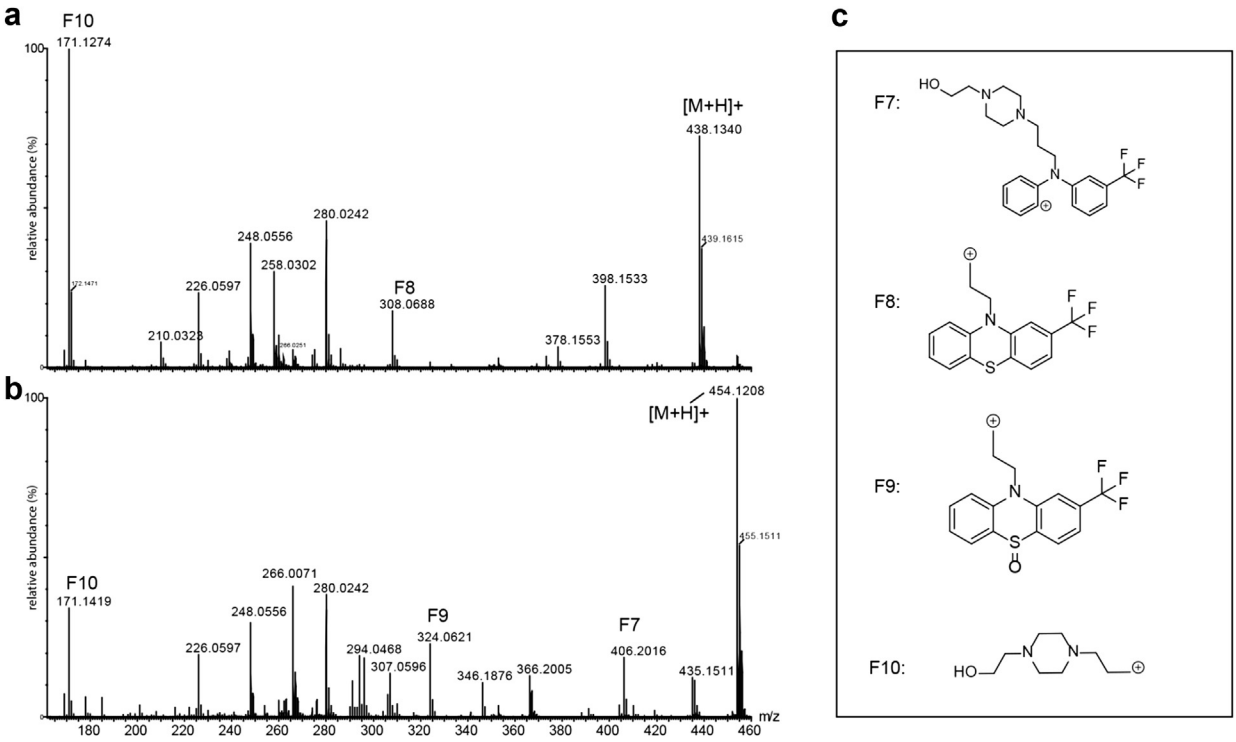


Figure 3. CID spectra of fluphenazine (a) and 3 (b). Significant fragment ions are reported as F7-F10 and their tentative structures are presented in panel (c).

became brown and evolution of gas occurred. After 2 min, concentrated aqueous NH_4OH solution was added dropwise until the reaction solution became basic. Extract this aqueous solution with dichloromethane (2×). Combined organic extracts were dried over Na_2SO_4, filtered, and concentrated. Purified using preparative TLC (eluent: CH_2Cl_2-MeOH-conc. NH_4OH 90:9:1 v/v/v) to yield the desired product 3 as a white solid (16 mg, 70%). ^{1}H NMR (600 MHz, DMSO-d6) d 8.19 (d, J = 7.9 Hz, 1H, Ar*H*), 7.98 (dd, J = 7.7, 1.7 Hz, 1H, ArH), 7.94 (s, 1H, Ar*H*), 7.81 (d, J = 8.5 Hz, 1H, Ar*H*), 7.74 (ddd, J = 8.7, 7.70, 1.70 Hz, 1H,Ar*H*), 7.57 (dd, J = 8.0, 1.6 Hz, 1H, Ar*H*), 7.35 (t, J = 7.3 Hz, 1H, Ar*H*), 4.53, 4.50 (m, 2H, NCH_2), 3.80-3.30 (broad multiplet, 8H, NCH_2), 3.45 (m, 1H, OCH_2), 2.32, 2.24 (m, 2H, NCH_2), 2.31 (m, 2H NCH_2), 2.33 (m, 4H, NCH_2), 2.32 (m, 4H, NCH_2), 1.86 (m, 2H CH_2); ^{13}C NMR (150 MHz, DMSO-d6) δ 139.2, 138.2, 132.9, 132.8, 133.6, 131.3, 124.2, 123.0, 118.0, 117.5, 114.2, 60.8, 59.0, 53.8, 44.6, 23.9; MS *m/z* $(M+H)^+$ 454.4 found, 454.2 required. Detailed analysis of NMR data is presented in the Supporting Information.

Mass Spectrometry Analysis of Fluphenazine and Its Oxidation Product, 3

LC-MS analyses of fluphenazine (t_{el} = 3.0 min; Supporting Information, Fig. S1C) and 3 (t_{el} = 2.50 min; Supporting Information Fig. S1D) show mass-to-charge ratios m/z = 438.13 (Supporting Information, Fig. S3A) and m/z = 454.11 (Supporting Information, Fig. S3B), respectively. The LC-MS data reveal that the oxidation of fluphenazine results in the formation of a sulfoxide moiety (Figs. 3a and 3b). The latter is easily identified by its CID spectrum (Fig. 3b). Indeed, the comparison of the fragment ions (F7-F10) generated during CID of fluphenazine and 3 allows for the localization of the oxidation site at the sulfur atom of fluphenazine. Fragment ion F7 (m/z 406.2, Fig. 3c) corresponds to the loss of sulfoxide. During electrospray ionization, sulfoxide is easily protonated, which ultimately permits the cleavage of the C-S bonds. The latter has been extensively used in proteomic studies to develop sulfoxide-containing cleavable cross-linkers to ease the identification of amino acids during protein-protein interactions.[35-38] Fragment ions F8 (Figs. 3a and 3c, m/z 308.05) and F9 (Figs. 3b and 3c, m/z 324.06) correspond to the loss of 2-(piperazin-1-yl)ethan-1-ol (131.1 Da) moieties during the CID of fluphenazine and 3, respectively. The absence of fragment ion F8 and the presence of fragment ion F9 during CID of 3 (Figs. 3b and 3c) demonstrate the oxidation of the sulfur atom of fluphenazine into sulfoxide. Fragment F10 (m/z 171.1) corresponds to the 3-(4-(2-hydroxyethyl)piperazin-1-yl)propan-1-ylium ion (Fig. 3c). F10 is present during CID of fluphenazine and 3, which demonstrates the absence of oxidation of the piperazine moiety.

Enrichment of Rosuvastatin Calcium With Oxidative Degradation Product, 5-Keto-rosuvastatin (4)

Solid rosuvastatin calcium (50 mg) and 35% aqueous H_2O_2 solution were taken in 2 separate scintillation vials. These 2 vials were then placed in a glass jar. After capping the glass jar, it was placed in a 50°C oven for 1 week. HPLC analysis showed 24% conversion (by peak area) to the desired oxidative degradation product 4.

Enrichment of Ibuprofen With Oxidative Degradation Products, 2-(4-(1-Hydroxy-2-methylpropyl)-phenyl)propanoic Acid (5) and 2-(4-Isobutyrylphenyl)-propanoic Acid (6)

To a solution of ibuprofen (0.9 mL, 500 μM in acetonitrile-MeOH 1:1 v/v) was added a solution of AAPH, 2,2'-azobis(2-aminopropionamide) dihydrochloride (0.1 mL, 10 mM in water). The resulting mixture was incubated at 37°C for 5 h and then analyzed by HPLC. Comparison with the retention time and UV-vis spectra of commercial standards showed that the yield of the degradation products 5 and 6 was 26% and 15%, respectively.

Enrichment of Dextromethorphan With Oxidative Degradation Products, (9α,14α)-3-Methoxy-17-methylmorphinan-10-ol (7) and (9α,13α,14α)-3-Methoxy-17-methylmorphinan-10-one (8)

The enrichment of dextromethorphan with its oxidative degradation products, 7 and 8, was performed according to a previously published[23] method. A 0.5 mg/mL $FeCl_3$ solution (5 mL) in 0.1 N aqueous HCl was mixed with a solution of dextromethorphan (5 mL, 0.1 mg/mL in 0.1 N aqueous HCl-MeCN 75:25 v/v), and the resulting mixture was stirred at room temperature for 48 h to yield 9% of 7 and 16% of 8.

Conclusion

We established here the concept of an oxidation toolkit, a collection of oxidants and methods, for the selective enrichment of API with a desired oxidative degradation product. Selective oxidative transformations of the API have been listed, along with the relevant reagents, with the goal to pursue such transformations in preparative scale. To demonstrate the applicability of this concept, several drug molecules were enriched with relevant oxidative degradation product(s). The toolkit can also be used, as exemplified by the qualification of rosuvastatin-5-keto acid (4), for enriching API with desired oxidative degradation product for the use in animal toxicological studies. The oxidation toolkit presented in this article is not meant to be a static collection of reagents and methods, but a concept to be evolved and enriched over time with new methods and reagents that allow chemoselective, relevant transformations.

Supporting Information

Chromatographic, mass spectrometric, and NMR data.

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