Perspective

Drug Testing and Analysis

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The use of stable isotope labelling for the analytical chemistry of drugs

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This perspective reviews the potential for stable isotope labelling to examine the metabolic transformations of drugs. The increased sensitivity and widespread availability of modern nuclear magnetic resonance (NMR) and high-resolution mass spectrometers will increase the application of stable isotopes to study drug metabolism. Creating mass doublets by mixing a natural isotopic abundance compound with a labelled isotopomer and applying stable isotope filtering to high resolution mass spectrometry allows one to rapidly identify drug metabolites in very complex samples, such as blood or urine. Applying this approach to drug metabolism will require a significant synthesis effort. The relatively small number of ^{13}C, ^{15}N, or $^{17,18}O$-labelled precursors exacerbates this problem, making the synthesis of the labelled drug often more difficult than that of the parent compound. We have developed new strategies for stable isotope labelling of complex molecules based on the rich chemistry of [^{13}C]methyl phenyl sulfide, where the phenylthio group acts as a stable, non-volatile carrier for the valuable ^{13}C-label. For example we have used [^{13}C]methyl phenyl sulfide to prepare the three possible ^{13}C-isotopomers ([1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]) of the two carbon precursors, ethyl 2-(phenylthio) acetate and ethyl N,N-dimethyl oxamate. In each case, these two-carbon labelling precursors are asymmetric and the differential reactivity of the carbons allows for either/or ^{13}C-labelling in the products. We demonstrate the utility of these two carbon precursors in the synthesis of aromatic ring-labelled *N*-(4-hydroxyphenyl)acetamide (acetaminophen or paracetamol). Copyright © 2011 John Wiley & Sons, Ltd.

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Introduction

For the purposes of this perspective stable isotope labelling (SIL) is limited to specific enrichment with the low abundance stable isotopes ^{2}H, ^{13}C, ^{15}N, or $^{17,18}O$. For the convenience of the reader, some properties of these isotopes are listed in Table 1. The contribution of stable isotope labelling in biomedical research has a rich history. In fact, early metabolic tracer experiments were carried out using stable isotopes in combination with mass spectrometry. For example, in 1941, Wood and Nier used $^{13}CO_2$ enriched to 2.1%, to establish that heterotrophs assimilated a fraction of their carbon from CO_2, which led to the discovery of important enzymes such as phosphoenolpyruvate carboxylase.[1] Because radioisotopes could be detected with great sensitivity by relatively inexpensive techniques such as liquid scintillation counting or autoradiography, ^{3}H and ^{14}C displaced stable isotopes as metabolic tracers for many years. With the advancement in the sensitivity and resolution of spectroscopic techniques used to detect stable isotopes, interest in SIL has seen steady growth over the last three decades. The primary experimental advantage of SIL over ^{3}H- or ^{14}C-labelling is that stable isotopes are detected using mass spectrometry (MS), nuclear magnetic resonance (NMR), and vibrational spectroscopic techniques. These methods are sensitive to the chemical environment of the stable isotope label and can be used to determine not only the extent of stable isotope labelling, but in many cases the chemical identity of the labelled products, and the distribution of labels among their atoms. Scalar coupling of the magnetic stable isotopes allows NMR to be used to obtain information about atoms that are chemically bonded. No equivalent information can be derived from radio labelling experiments. Analysis of drugs using SIL, particularly mass spectrometry and NMR spectroscopy, are finding their way into many aspects of the pipeline for improving metabolic and pharmacokinetic properties during the optimization of lead compounds. In particular, SIL coupled with NMR or MS analysis is used effectively in the analysis of the biosynthesis of natural products, hit-to-lead optimization, and drug metabolism. Because the application of isotopes to natural product biosynthesis[2–5] and hit-to-lead optimization[6,7] has been published on extensively, the focus of this perspective is on the application of SIL to drug metabolism.

Stable isotope analysis

NMR Spectroscopy

Over the past several decades, the rapid development of NMR and mass spectrometers has yielded powerful analytical tools for the analysis of cellular components and drugs. The ability to resolve all isotopomers of metabolites in a single spectrum makes NMR analysis useful for examining metabolism in general and drug metabolism in particular. NMR analysis of protein structure has driven the development of NMR instrumentation as well as multidimensional NMR methods. In NMR structure analysis, the

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Table 1. Low abundance stable isotopes of H, C, N, and O.

Isotope	Natural abundance	Nuclear spin	Chemical form of enriched precursor
^{2}H	0.015 %	1	$^{2}H_2O$
^{13}C	1.11 %	1/2	^{13}CO
^{15}N	0.37 %	1/2	^{15}NO, $H^{15}NO_3$, $^{15}NH_3$
^{17}O	0.037 %	5/2	$H_2^{17}O$
^{18}O	0.204 %	0	$H_2^{18}O$

push to higher field has greatly increased both sensitivity and resolution. In addition, the development of heteronuclear *J* correlation NMR pulse sequences allows the observation of ^{13}C and ^{15}N with the higher sensitivity of protons. Recently additional advances in sensitivity were realized with the introduction of cryo-probe technology. By using a small volume cryo-probe, it is now possible to obtain an NMR spectrum of a few milligrams of a small metabolite or drug. Clearly, the revolution in structural NMR required these important instrument improvements as well as the tremendous advances in multi-dimensional NMR.[8,9] Yet, it was the relatively simple realization by Bax *et al*. that uniform labelling of proteins with ^{13}C and ^{15}N introduces a spin 1/2 scalar coupling network along the peptide backbone from the N-terminal ^{15}N-amino group to the ^{13}C-carboxyl terminus.[8,10,11] The fact that this spin-spin network provided a physical basis and greatly simplified the difficult process of making the sequential chemical shift assignments energized the revolution in structural NMR. Stable isotope labelling has the potential to have an energizing effect on analysis of drugs and drug metabolism in particular.

Chemists use similar labelling approaches to unravel the biosynthetic pathways for complex natural products. While it can be a considerable synthetic effort, we now have the capability to synthesize virtually any compound with multiple specific ^{2}H, ^{13}C and/or ^{15}N labels. These labels are essential for establishing metabolic precursor product relationships. By introducing adjacent ^{13}C-labels into directly bonded carbons, we can use ^{13}C-^{13}C scalar coupling and a variety of NMR methods to detect the fate of a carbon-carbon bond throughout complex metabolic transformations. Similarly, NMR analysis of ^{13}C-^{2}H or ^{13}C-^{15}N labelled compounds can be used to trace the metabolic fate of carbon-hydrogen or carbon-nitrogen bonds. This approach is used extensively to unravel the biosynthetic pathways of complex natural products.[2–5,12] By using multiple or uniform ^{13}C-labels and multiple quantum NMR methods it is also possible to eliminate spectral contributions from natural abundance ^{13}C, effectively eliminating the background and allowing NMR analysis of complex mixtures. Although there have been great gains in the sensitivity of the NMR experiment, the fact that many drugs are effective at nanomolar concentrations and their metabolites are present in blood or urine at very low concentrations makes it problematic in to apply SIL/NMR analysis universally to drug metabolism. In cases where samples can be obtained at high enough concentrations, the information content of NMR makes it the method of choice.

Mass spectrometry

In mass spectrometry, new chemical and laser ablation ionization methods coupled to triple quad, ion-trap, time-of-flight (TOF), and Fourier transform ion cyclotron resonance (FT-ICR) mass detectors has increased the sensitivity, speed, mass range, and mass resolving power of mass spectrometers.[13–16] Using an array of mass spectrometers, it is possible to obtain high throughput analysis of molecular ions from essentially all possible drugs and their metabolites with exquisite sensitivity. An array of mass detectors including high resolution TOF, Orbitrap and FT-ICR detect ions with sufficient resolution to extract unique molecular formulas for ions, simplifying identification of drugs and drug metabolites. Recent work in several laboratories has shown that powerful post acquisition data processing filters applied to liquid chromatography-mass spectrometry (LC-MS) data can be used effectively to identify drug metabolites in complex mixtures. Of particular interest here is stable isotope filtering which was originally used to screen for the isotope doublets in natural abundance chlorinated (^{35}Cl 75.53 %, ^{37}Cl 24.47%) and brominated (^{79}Br 50.54 %, ^{81}Br 49.46%) compounds. In this approach an LC-MS data set is filtered to identify co-eluting compounds with mass M and M+2. It is also possible to create mass doublets in non-halogenated compounds by mixing a natural isotopic abundance compound with a stable isotope labelled version. This approach has been applied directly to caffeine metabolism in humans.[17,18] In this case a 50:50 mixture of caffeine and [8-^{13}C, 1,3-$^{15}N_2$]caffeine was administered to human subjects and the urine analyzed for excreted metabolites. Using this approach, the ring opened metabolite 5-acetyl-6-amino-3-methyl uracil of caffeine was identified.

Stable isotope filtered MS analysis

Recently several laboratories have reported closely related SIL/isotope filter-based MS approaches to screen for potential glutathione adducts of drugs.[19–24] While the approach has been demonstrated primarily using *N*-(4-hydroxyphenyl)acetamide (acetaminophen or paracetamol) (Figure 1), it could be used to screen for glutathione adducts of any drug.[23] Using this approach, the drug is incubated with liver microsome preparations in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and a 2:1 mixture of the reduced forms of glutathione (GSH) and [*glycyl*-$^{13}C_2$, ^{15}N]glutathione ([*glycyl*-$^{13}C_2$, ^{15}N]GSH). The ions are analyzed by LC/MS in the positive ion mode. Glutathione adducts were identified by observing mass doublets separated by 3 mass units using a nominal mass detector. Using ion trap, triple quad and Q-TOF mass spectrometers, data dependent MS/MS approaches were used in combination with isotope filters for structural analysis.[19,20] For example, the neutral loss of the glutamyl residue (−129 Da) was used to identify glutathione adducts. In addition to using glutathione, electrophilic drug metabolites have been trapped as isotope doublets using a mixture of potassium cyanide and potassium [^{13}C,^{15}N]cyanide or semicarbazide and [^{13}C, $^{15}N_2$] semicarbazide.[23] More recently this approach has been extended to high-resolution mass detectors (Thermo LTQ Orbitrap and Waters LCT Premier *high resolution* TOF). [21–23] The primary advantage of using high resolution for the isotope filtering approach results from the narrow mass tolerance window that can be applied with accurate mass data, which sharply reduces false positives. A new isotope-pattern-filtering algorithm has been developed to take full advantage of high-resolution data.[25]

The overall goal of the *in vitro* metabolite screens described above is to identify potential drug metabolites and to identify drug scaffolds that will have low bioavailability because of rapid

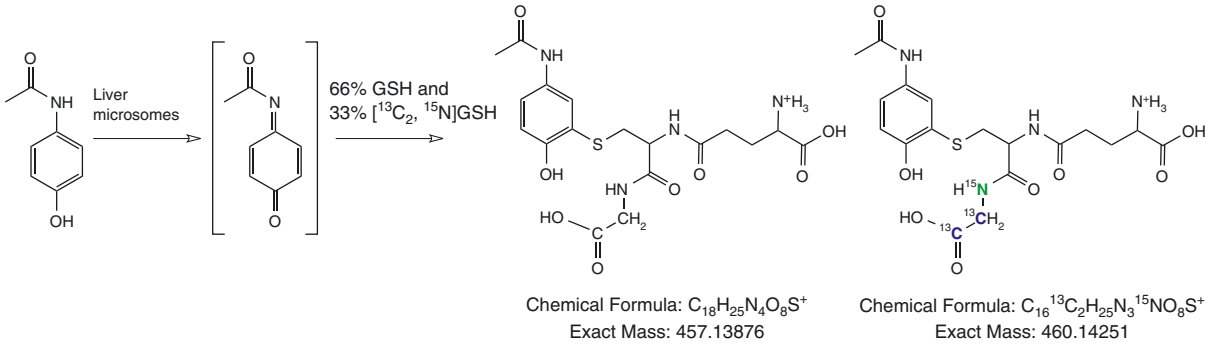

Figure 1. Stable isotope filtered detection of glutathione adducts of drugs. Acetaminophen is incubated with liver microsome preparations in the presence of NADPH and a 2:1 mixture of reduced glutathione (GSH) and reduced [*glycyl*-$^{13}C_2$, ^{15}N]glutathione ([$^{13}C_2$, ^{15}N]GSH). The ions are analyzed by LC-MS in the positive ion mode. Post acquisition, data are Stable Isotope Filtered to identify mass doublets separated by 3 Da.

metabolic clearance during the early stages of drug discovery. The stable isotope filtering approach could also be applied *in vivo* to study drug metabolism, which would be important later in the drug discovery pipeline. Because it would be difficult to isotopically enrich the glutathione pools in animals or humans and impossible to trap electrophilic drug metabolites with cyanide or semicarbazide in humans, it is necessary to create the isotope doublets by incorporation of stable isotopes into the drug itself. The relatively high natural abundance of ^{13}C (1.11 %) leads to a high background of the $^{13}C_1$ isotopomers in natural abundance compounds making it necessary to incorporate three of four isotopic labels. For example, at natural abundance ^{13}C acetaminophen (Molecular formula $C_8H_8N_1O_2$) with 8 carbons is 91.46% of the $^{12}C_8$-isotopomer and 8.21 % of the $^{12}C_7{}^{13}C_1$ isotopomer. However the natural abundance background of the $^{12}C_4{}^{13}C_4$ isotopomer is vanishingly small (0.0001%). In addition, labels must not be exchangeable, ruling out in many cases ^2H or ^{18}O labelling. Finally it is important to incorporate the labels into the core of the drug and not into acetyl groups, for example, which could be lost in the early steps of metabolism. In instances where nothing is understood about the metabolism of a drug it will be useful to synthesize several isotopomers of the drug to be sure to resolve the labels in the metabolites in blood or urine samples. Because of the limited availability of labelled precursors, often the preparation of different isotopomers requires distinct synthetic pathways compounding the synthesis problem. All of this points to a significant synthesis effort with stable isotopes, which is thought to be expensive. Our approach to minimize synthesis cost is outlined below.

Synthesis with stable isotopes

Because carbon-13 and nitrogen-15 are separated from their lighter isotopes by cryogenic distillation of CO and NO, or chemical exchange with HNO_3, all labelled carbons and nitrogens must be derived ultimately from ^{13}CO, $^{15}N^{17,18}O$, $H_2{}^{18}O$ or $^{15}NH_3$. The requirement that all labelled compounds be derived from CO, NO, H_2O and NH_3 rather than the ~20 000 compounds listed in, for example, the Sigma/Aldrich catalogue provides some unique constraints on isotope chemistry. Because of the limited labelling precursor pool, standard synthetic routes are often useless for the preparation of labelled analogs. The highly efficient conversion of CO, NO, or NO_3^- to useful chemical precursors is perhaps the most unique aspect of stable isotope labelling technology. Historically, efficient large-scale methods for the synthesis of [^{13}C]methane, [^{13}C]methanol, [^{13}C]methyl iodide, sodium [^{13}C]formate, potassium [^{13}C]cyanide, and [^{13}C]carbon dioxide formed the foundation of ^{13}C-labelling chemistry. While we make great use of all of the one-carbon precursors listed, we began several years ago to explore [^{13}C]methyl phenyl sulfide (Figure 2) as a labelling precursor and have found it to be remarkably useful. As discussed below, we have found in many cases that [^{13}C]methyl phenyl sulfide significantly shortens chemical synthesis routes and/or significantly increases the recovery of expensive isotope.

[^{13}C]Methyl phenyl sulfide as a one-carbon labelled synthon

Because of its rich chemistry outlined briefly in Figure 2, we have found [^{13}C]methyl phenyl sulfide to be a versatile ^{13}C-labelling precursor.[26] For example, phenylthio [^{13}C]methyl lithium, prepared by treatment of [^{13}C]methyl phenyl sulfide with *sec*-butyl lithium, is used as a nucleophilic synthon. Treatment of the resulting phenylthio [^{13}C]methyl lithium with CO_2 or $^{13}CO_2$ yields 2-(phenylthio) [2-^{13}C]acetic acid or 2-(phenylthio) [1,2-$^{13}C_2$]acetic acid (Figure 2a), a useful two-carbon precursor. [^{13}C]Methyl phenyl sulfide is easily converted to chloro [^{13}C]methyl phenyl sulfide to act as an electrophilic synthon (Figure 2b), which, for example, can be used to produce useful alkoxy derivatives (Figure 2c). In [^{13}C]methyl phenyl sulfide the phenylthio group provides a chemically stable and nonvolatile carrier for the valuable label. The relative acidity of the adjacent methylene protons can be adjusted by oxidizing the sulfide to sulfoxide and then to sulfone, which allows for facile exchange of deuterium for hydrogen. At any point in the synthesis, the phenylthio group can be removed and replaced with hydrogen by treatment with Raney Ni. Similarly, the oxidation of the sulfur activates it for elimination to yield alkenes. In our experience, elimination of the phenyl sulfone under basic conditions yields exclusively the *trans* product. In addition to the synthesis of labelled 2-(phenylthio) acetic acid, we have used [^{13}C]methyl phenyl sulfide to produce a number of labelled 2-carbon precursors including ethyl N,N-dimethyl oxamate (Figure 2d) and phenyl vinyl sulfide (Figure 2h). In all three of these two-carbon precursors, the chemistry allows for production of all three potential ^{13}C-isotopomers (e.g. N'N-dimethyl [1-^{13}C]-, [2-^{13}C]- or [1,2-$^{13}C_2$]oxamate). In addition, these compounds are not symmetrical and the differential reactivity of the carbons allows for either/or labelling in products. We have also extended [^{13}C]methyl phenyl sulfide to prepare a number of useful three- (Figures 2f and 2g) and four carbon precursors (Figures 2h and 2i).

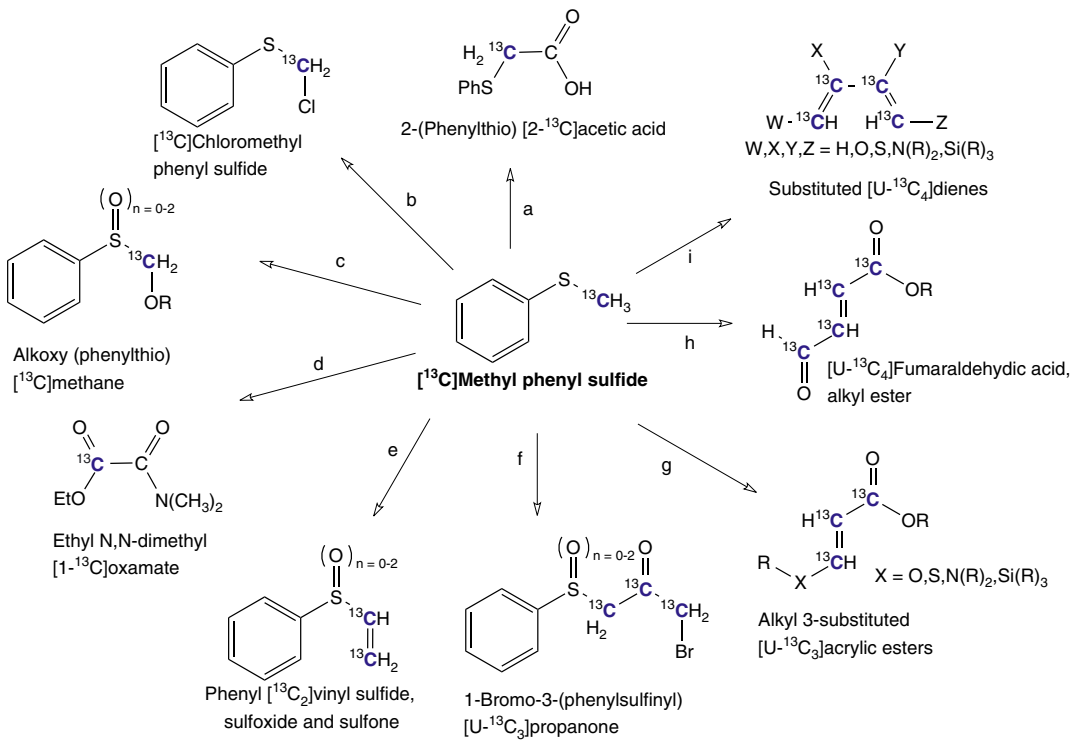

Figure 2. [^{13}C]methyl phenyl sulfide has been used to prepare a large number of ^{13}C-labelled intermediates and products.

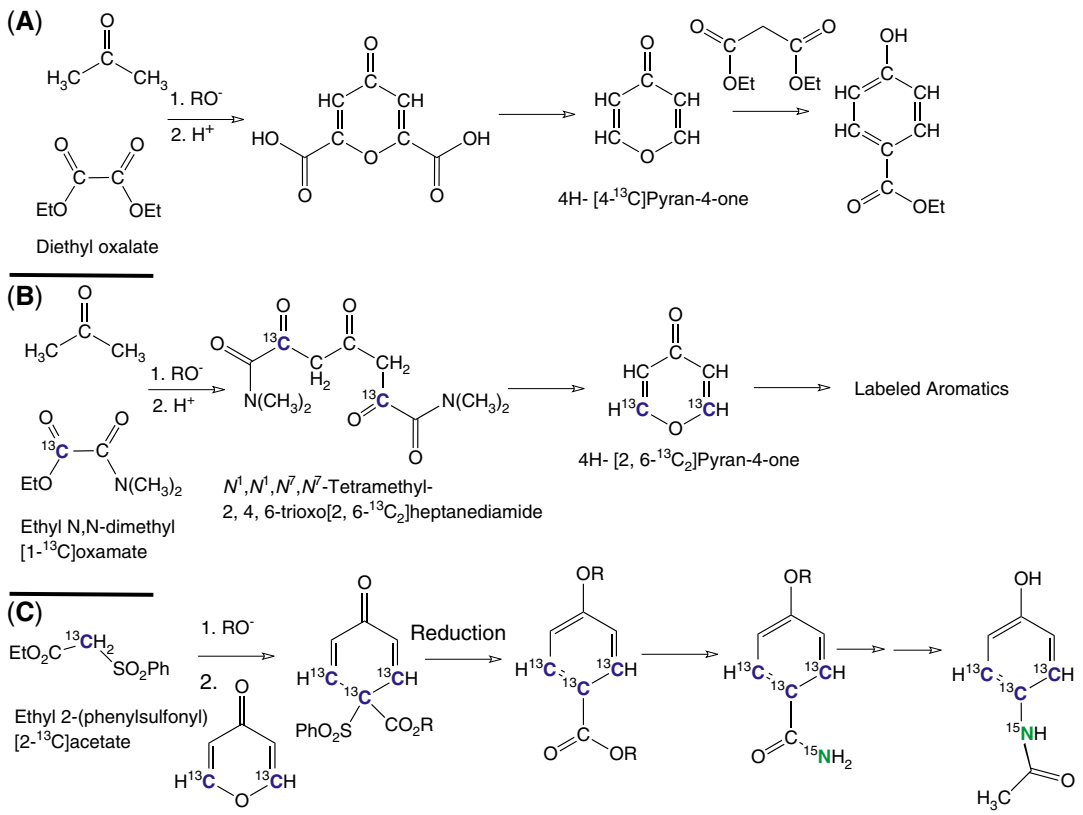

Figure 3. Synthesis of acetaminophen with stable isotopes incorporated into the aromatic core. (a) While the route reported chemical yields for synthesis ethyl 4-hydroxy [4-^{13}C]benzoate are good, the recovery of isotope in the synthesis of many isotopomers is potentially poor because of the symmetrical precursors diethyl oxylate and diethyl malonate. (b,c) This problem is solved using asymmetrical two carbon precursors ethyl N,N-dimethyl [1-^{13}C]oxamate and ethyl 2-(phenylsulfonyl) [2-^{13}C]acetate, both derived from [^{13}C]methyl phenyl sulfide.

Synthesis of labelled drugs

The use of stable isotope labelled 4H-pyran-4-one for the production of labelled aromatics has recently been reported. Takeda *et al*. reported the synthesis of 4H-[2,6-$^{13}C_2$]pyran-4-one from 2-[^{13}C]acetone and diethyl oxalate by way of [4-^{13}C]chelidonic acid (Figure 3a).[27] Marshall *et al*. produced 4H-[2,6-$^{13}C_2$]pyran-4-one from acetone and triethyl[^{13}C]-orthoformate in good yield.[28] By employing the chemistry of [^{13}C]methyl phenyl sulfide (Figure 2) as outlined in Figure 3b, all isotopic combinations for this important aromatic intermediate are possible without loss of any of the expensive label. N^1, N^1, N^7, N^7-tetramethyl-2,4,6-trioxo[2,6-$^{13}C_2$]heptanediamide (Figure 3b) is prepared in near quantitative yield by the reaction of acetone and ethyl N,N-dimethyl [1-^{13}C]oxamate with known regiochemical introduction of the carbon label. Cyclization to 4H-[2,6-$^{13}C_2$]pyran-4-one followed by reaction with ethyl 2-(phenylsulfonyl) [2-^{13}C]acetate can be extended to produce 4-hydroxy[1,2,6-$^{13}C_3$]benzoic acid (Figure 3c). Note that reduction of the ketone leads to elimination of the phenylsulfonyl group to produce the aromatic. This compound can then be converted to [3,4,5-$^{13}C_3$, ^{15}N]acetaminophen by conventional chemistry.

Conclusion

SIL coupled with NMR or MS detection has been the method of choice for unravelling complex natural product biosynthesis pathways. The increased sensitivity and widespread availability of modern NMR and high-resolution mass spectrometers will increase the application of stable isotopes to study drug metabolism. Creating mass doublets by mixing a natural isotopic abundance compound with a labelled isotopomer and applying stable isotope filtering to high resolution MS allows one to rapidly identify drug metabolites in very complex samples like blood or urine. Applying this approach to drug metabolism often requires a significant synthesis effort. The relatively small number of ^{13}C, ^{15}N, or ^{17,18}O-labelled precursors exacerbates this problem, making the synthesis of the labelled drug often more difficult than that of the parent compound. At the National Stable Isotope Resource at Los Alamos, we continue to develop new strategies for SIL complex molecules. Although to date our applications of [^{13}C] methyl phenyl sulfide have largely been reported in the patent literature, we have found it to be very widely applicable as a labelling precursor. In addition, we have found many applications for the ^{13}C-isotopomers of ethyl 2-(phenylthio) acetate, ethyl N,N-dimethyloxamate, and phenyl vinyl sulfide, all derived from [^{13}C]methyl phenyl sulfide. These stable isotope labelled precursors are now commercially available.

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