Drug metabolite synthesis and scale-up

CYP metabolites

Non-CYP phase 1 metabolites

Acyl, *O*-, *N*-glucuronides

Other conjugated metabolites

Isotopically labelled metabolites

www.hyphadiscovery.com

Contents

We provide metabolites to clients for a variety of applications:

- **Structure identification**
- **Authentic standards for bioanalytical method development**
- **Certified standards for clinical quantitative bioanalysis**
- **DMPK / Tox studies**
- **Target / off-target activity testing**
- **Phenotyping assays**
- **Enzyme kinetic and intrinsic clearance assays**
- **As handles for introduction of fluorine into metabolic hotspots for enhancing metabolic stability**
- **Late stage oxidized derivatives of lead compounds for improved potency / solubility / PK properties**

Accessing metabolites of pharmaceutical and agrochemical compounds

Hypha are experts in metabolite synthesis and purification and a trusted supplier of metabolites to support development programs in pharma and agrochemical companies and institutes worldwide, as well as organizations in other sectors.

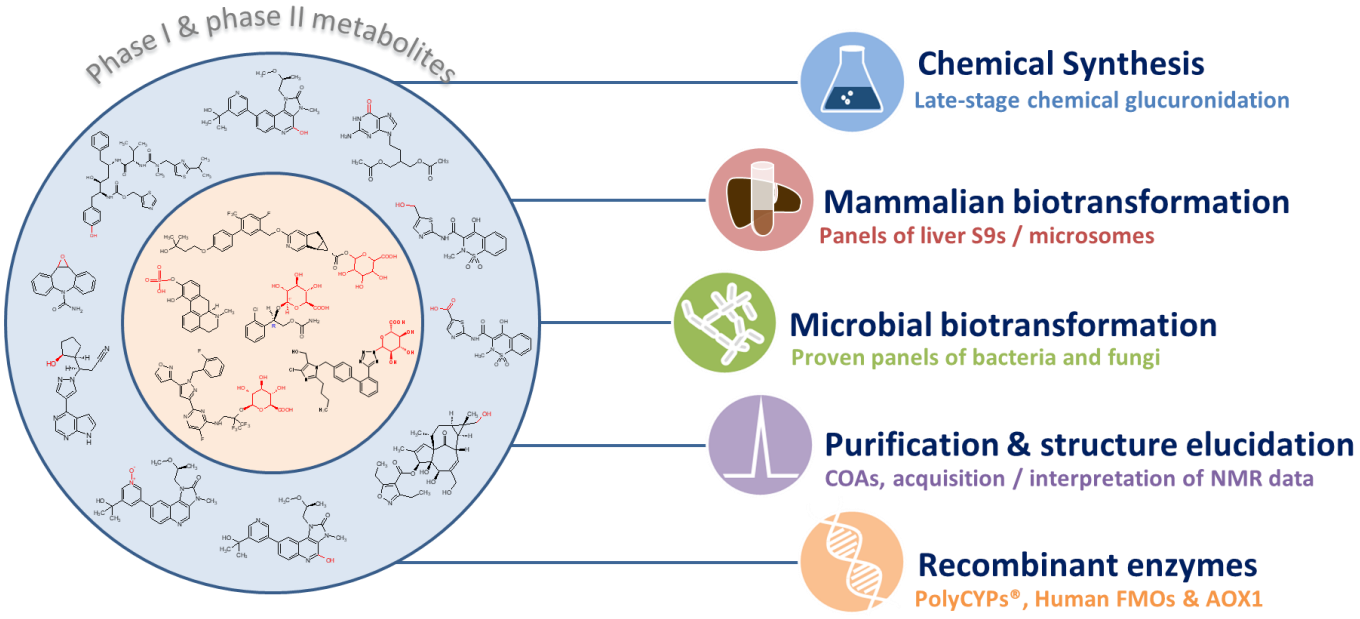
We offer a comprehensive suite of techniques for provision of even the most difficult-to-synthesize metabolites, comprising chemical synthesis, recombinant enzymes, microbial biotransformation, mammalian liver fractions, in addition to the purification of metabolites from biological matrices. Hypha scientists are also experts in structural elucidation via NMR spectroscopy.

Our strategy allows production of phase 1 and 2 metabolites from microgram scale for definitive MetID, and up to multi mg / gram scale for further biological evaluation and provision of certified bioanalytical standards.

> ***"Hypha has exceeded our expectations and is now a 'go to' lab for biosynthesis/synthesis/purification. Hypha's team was a pleasure to work with and our complicated projects were handled with expertise and professionalism. Their excellent scientific communication and project data were extremely comprehensive and we received updates throughout the process. We will definitely be Hypha Discovery clients for life."***
>
> **Head of Toxicology/DMPK , US Pharma Company**

Key features

- Phase 1 CYP and non-CYP metabolites
- Phase 2 metabolites, including *N-*, *O-* & acyl glucuronides, sulfates and other conjugates
- Multiple metabolites captured in a single screen
- No requirement to reveal structural information; the entire process can be conducted blind
- Metabolites purified to >90% (higher purities also possible)
- Optional structure elucidation & COAs
- Scalable to multi-gram amounts
- Formulation know-how for poorly-soluble compounds
- Simple fee-for-service structure, with no downstream terms or royalties



Hypha's one-stop metabolite shop approach for accessing and scaling-up metabolites

Production of phase 1 metabolites

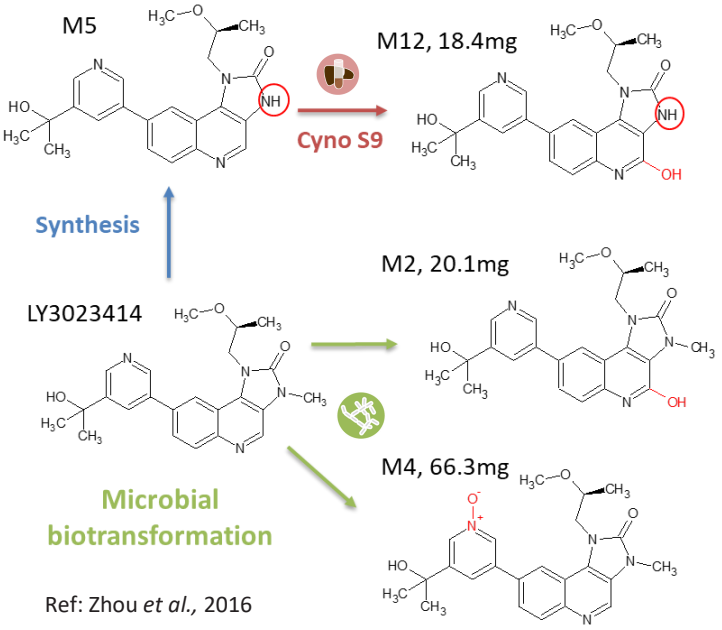
In order to generate phase I metabolites, Hypha can take a number of approaches. Most requirements are met through either application of our recombinant enzyme systems or via whole cell microbial biotransformation. Microbial biotransformation is especially useful where multi-step or mixed pathways are implicated in the biotransformation of a drug to the target metabolite(s). For some challenging metabolites, we can also apply our liver S9 and microsome panels.

Hypha's microbes mimic human and other mammalian CYP and non-CYP phase I metabolic reactions, including aromatic and aliphatic hydroxylation, as well as being effective for conjugative reactions. Using this approach, it is possible to obtain metabolites formed from multiple sequential reactions in a single incubation, e.g. hydroxylation and subsequent glucuronidation.

The microbial biotransformation methods are scalable to enable production of up to gram amounts of pure metabolites without the co-factor costs associated with other methods.

Phase I metabolite synthesis reactions

- Aliphatic and aromatic hydroxylation (single & multiple)
- Heteroatom oxidation (*N* & *S* oxides)
- *N*- & *O*- dealkylation
- Dihydrodiols from phenyls
- Alcohol oxidation/carbonyl reduction
- Others including epoxidation, dehydrogenation, dehydration, hydrogenation, methylation, deacetylation, *N*-acetylation



Ref: Zhou *et al.,* 2016

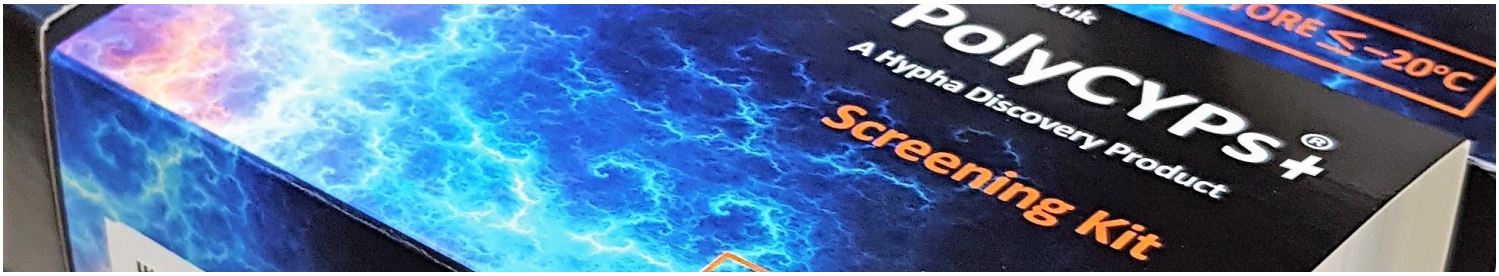
CYP reactions

Metabolites formed from human and other mammalian CYPs can also be made by microbial CYPs. We exploit this ability through the use of whole cell microbial biotransformation, and using our **PolyCYPs® enzyme platform**. PolyCYPs are recombinant microbial CYPs derived from selected talented biotransforming strains in Hypha's collection and cloned into *E. coli*. They are also available in kit form so that scientists can use them in their own laboratories to screen for production of CYP metabolites of interest.

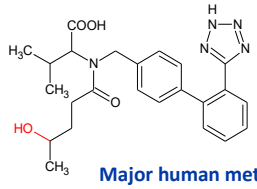
Non-CYP phase 1 reactions

A consequence of the development of drugs that are less susceptible to some mechanisms of CYP metabolism, has been the increase in drugs that undergo metabolism via alternative routes. Non-CYP phase 1 mechanisms involve monoamine oxidases, flavin-containing monooxygenases (FMOs), xanthine oxidases, carboxylesterases and alcohol / aldehyde dehydrogenases.

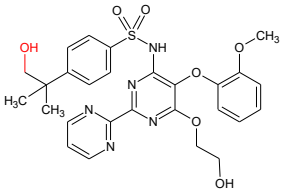
Micro-organisms in Hypha's biotransformation panel are able to undertake non-CYP phase 1 reactions, offering a viable solution to producing metabolites formed by these mechanisms. We also have available human aldehyde oxidase and all the human FMO isoforms for production of metabolites formed by these enzymes.



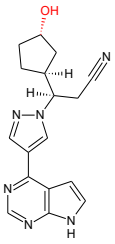
Selected drug metabolites produced by enzymes in Hypha's PolyCYPs+ kit



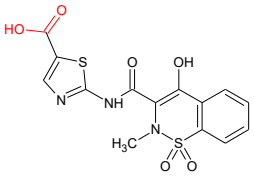
Major human metabolite of valsartan



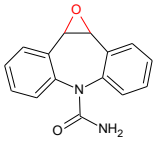
Major human metabolite



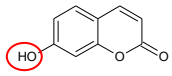
One of several human metabolites of ruxolitinib



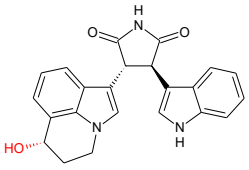
Major human metabolite of meloxicam

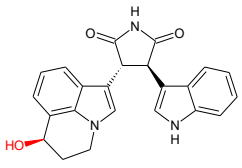


Carbamazepine-10,11-epoxide

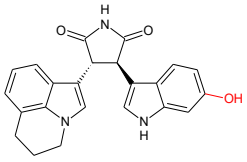


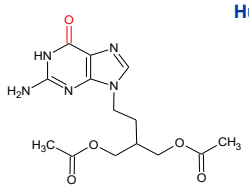
Dealkylated metabolites e.g. of 7-ethoxycoumarin



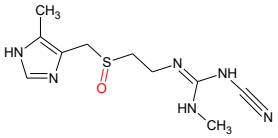


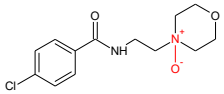
Human metabolites of tivantinib





AOX1 metabolite of famiciclovir





FMO3 mediated *S*– and *N*-oxides of cimetidine and moclobemide

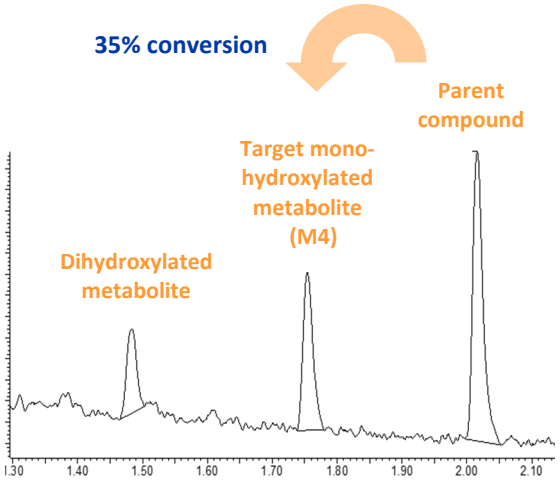
"Hypha Discovery has been a valuable metabolite ID partner. Hypha have provided biotransformation, metabolite purification and structure elucidation answers to some of our most challenging metabolism and metabolite ID problems. We really appreciate the breadth of expertise available at Hypha Discovery and will definitely reach out for future work."

Director of DMPK, US Pharma Company

PolyCYPs+ kits

PolyCYPs®+ kits comprise 18 diverse CYP isoforms cloned from some of Hypha's actinomycete bacteria, as well as two other phase 1 metabolic enzymes - human aldehyde oxidase (AOX1) and flavin monooxygenase 3 (FMO3). Access to any of the other human FMO isoforms is available at Hypha as a service option.

Once a target metabolite or oxidized derivative has been synthesized by one or more of the enzymes in the screening kit, a scale-up reaction with the best performing enzyme can be performed in order to access material for MetID and biological testing. Larger amounts can be generated at Hypha, using either a bulk enzyme extract or through fermentation of a recombinant streptomycete clone expressing the enzyme responsible for the biotransformation.



PolyCYPs scale-up reaction

LC-MS of the reaction of a client compound with PolyCYP 152 scale-up vials at 300mg/L substrate loading. A total of 20.1 mg M4 was purified at >97% purity and supplied to the client with a CoA within 22 days from receipt of order.

Production of conjugated metabolites

We solve the challenges in synthesizing glucuronides and other phase 2 conjugates using multiple tools from our one-stop shop. These include microbial biotransformation, mammalian S9 / microsome preparations and our proprietary late-stage chemical glucuronidation screens.

We can provide up to gram amounts of *O*-, acyl and some *N*-glucuronides, as well as other conjugated metabolites such as sulfates, for a range of applications:

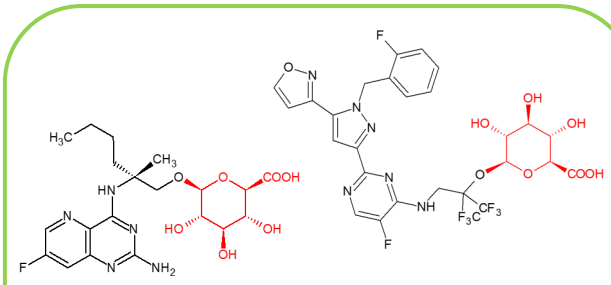
- Drug-drug interaction studies, e.g. investigations with drug transporters and CYP inhibition assays
- Assessment of ring-migration kinetics to check for formation of reactive acyl glucuronides
- Standards to validate stability studies, e.g. quantifying reversion to the aglycone during bioanalytical processing
- Pure analytical reference standards for bioanalysis

"We contacted Hypha Discovery to generate specific phase I and phase II metabolite standards in sufficient quantities and purity to allow structural confirmation and quantitation. Hypha exceeded expectations, providing 60mg of a phase I metabolite and over 100mg of a phase II metabolite at high purity. Hypha's team was a pleasure to work with and communicative and responsive throughout the process. We will undoubtedly be working with Hypha Discovery in the future."

Jason Boer, Director, Incyte Corporation, USA

Phase 2 metabolite synthesis reactions

- *N*-glucuronidation including *N*-carbamoyls
- *O*-glucuronidation
- Acyl-glucuronidation
- Sulfation
- GSH conjugations
- Glycosylations
- *N*-acetylations



***O*-glucuronides of the drugs selgantolimod and praliciguat were produced for clients using microbial biotransformation and purified to > 95% purity**

Refs: Mackman *et al.,* 2020 & Banijamali *et al.,* 2020

Late-stage chemical glucuronidation

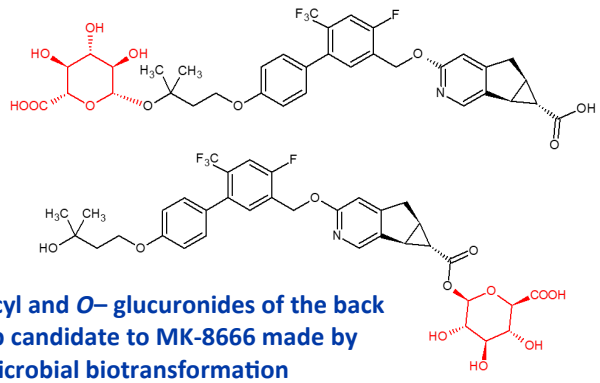
The late-stage chemical glucuronidation screen comprises sets robust and diverse chemical reactions with tailored deprotection strategies compatible with acyl glucuronides and sensitive *N*-glucuronides. All reactions are fully scalable to supply up to gram amounts. The screens were validated using a panel of 27 commercially available drugs from which glucuronides are known to be formed, resulting in >90% of substrates being converted to at least one glucuronide. These methods are a proven and cost-effective way to access multiple different glucuronides. We have also introduced new methods for making acyl glucuronides for the rare occasions when stability or yield issues necessitate an alternative approach.

Scalable solutions for your metabolite needs

Acyl glucuronides

Acyl glucuronides formed from carboxylic acid containing drugs can be a risk for drug induced liver injury (DILI). They can also inhibit transporters and CYPs and in fact, several classes of glucuronide conjugates have been shown to interact with CYP2C8 due to its distinctive active site. It is thus important to investigate these metabolites early on.

Hypha made the acyl glucuronide of the back-up candidate to MK-8666 (Salter *et al.,* 2018) which was later found to form protein adducts through a reactive acyl glucuronide and was identified as one of the causes of the observed DILI (Shang *et al.*, 2020).



Acyl and *O*– glucuronides of the back up candidate to MK-8666 made by microbial biotransformation

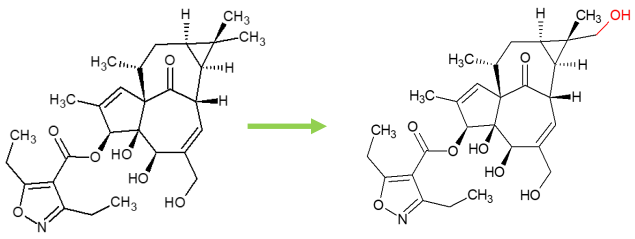
Glycosylated metabolites

Other conjugated metabolites such as sulfates and glycosylated products can also be formed using microbial biotransformation, as illustrated by the sulfate and glucose conjugates formed from apomorphine using our whole-cell system.

New to Hypha's portfolio is the development of recombinant enzymes capable of glycosylating compounds. These glycosyltransferases have been cloned from some of Hypha's biotransformation strains and are able to produce mono-, di– and tri-glucosides of drugs and pesticides.

Structure elucidation

Hypha uses modern NMR instruments and state-of-the-art probe technology for rapid and unambiguous structural identification through access to a 700MHz NMR spectrometer equipped with a 1.7mm micro-cryoprobe. This means only tens of microgram amounts of metabolites are needed to acquire data sets for full structural elucidation. Interpretation can be performed by clients or by our in-house experts.



Hundreds of milligrams of M27, the major disproportionate human metabolite of Leo Pharma's ingenol disoxate, was purified from scale-up of one of Hypha's microbes for MetID and various *in vitro* assays.

Ref: Carlsen *et al.,* 2016

Isotopically labeled metabolites

Through our partnership with the radiolabeling company Selcia, [^{13}C], [^{14}C], [^{2}H], [^{3}H] and [^{15}N]-labeled metabolites can be accessed to support regulatory, development or research projects in the pharma and crop protection industries. Hypha establishes optimized processes using unlabeled or stable labeled parent substrates, which can then be transferred to Selcia's state-of-the-art radiochemistry labs for the production of radiolabeled metabolites.

Multiple metabolites in parallel

Client metabolite projects typically start with a screen to determine the most cost-effective route to produce the target metabolite(s). Once a route to synthesis is identified, this can be readily scaled-up to supply up to multigram amounts of metabolites.

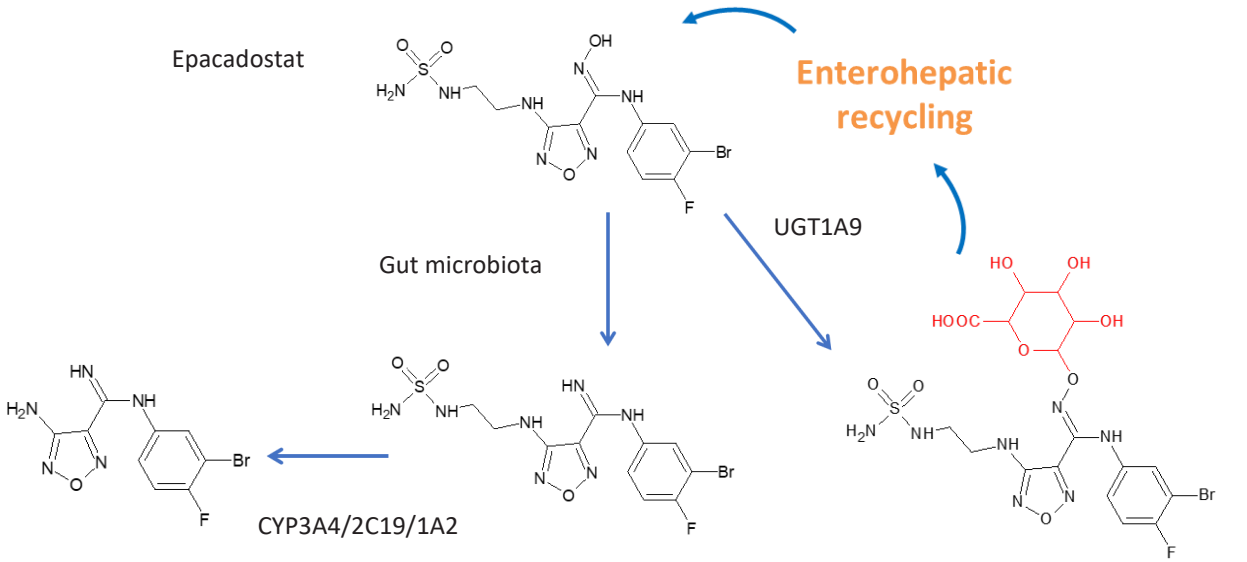
One such project for Incyte Corporation in the US, involved the generation of metabolites of epacadostat (EPA) derived from multiple metabolic pathways. Because of the steady state exposure of these circulating metabolites in humans and the implications in the design of future clinical DDI studies, full characterization of each was necessary, including sourcing of metabolite generation.

Microbes in Hypha's biotransformation panel were able to produce all three metabolites shown in the scheme below including M11, which was derived from the action of gut microbiota.

Three major metabolites are formed from EPA; M9 - a glucuronide formed by the action of UGT1A9 which is subject to enterohepatic recirculation (EHR), M11 - an *N*-dehydroxylated metabolite formed by gut microbiota, and M12 - a secondary metabolite formed from M11 by the action of CYP enzymes (Boer *et al*., 2016).

Several microbial species were capable of producing all 3 metabolites. Incyte requested supply of 25mg each of M9 and M11, for which 2 different species were used for scale-up resulting in 112mg of the glucuronide and 69mg of the reduced gut metabolite supplied at 95% purity.

Transporter study investigations with the metabolites showed a low risk of any DDIs, however it was demonstrated that M9 could potentially influence the PK of epacadostat through EHR (Zhang *et al.,* 2017).



Ref: Boer et al., 2016

When projects are more challenging

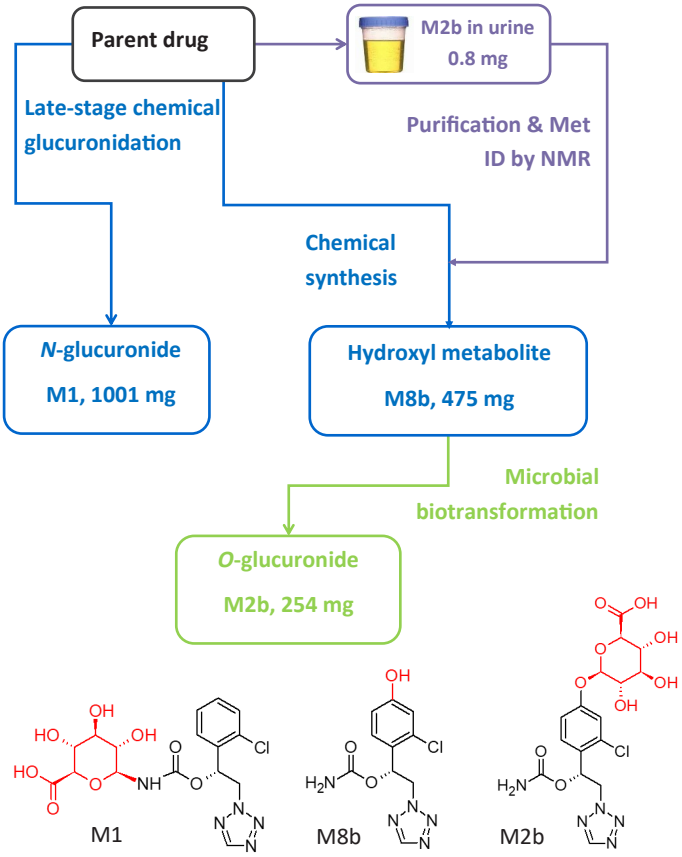
Access to multiple metabolites needed to support clinical development is not always straightforward, and can sometimes mean that more than one technique needs to be applied to fulfil requirements. In one such project, a US pharma client required >200mg of three metabolites of the drug cenobamate; an *N*-glucuronide (M1), an indirect *O*-glucuronide (M2b) and a hydroxylated metabolite (M8b). As part of this project, multiple components of Hypha's one-stop metabolite shop were employed, including chemical synthesis, microbial biotransformation as well as purification and structure elucidation by NMR.

It is our observation that access to *N*-glucuronides is an increasingly common need, as evident in this project where **M1, a major *N*-glucuronide, was accessed using chemical synthesis**. Key to successful synthesis were the mild deprotection conditions used in the late-stage chemical glucuronidation procedure, resulting in the purification of one gram of M1.

In addition to the *N*-glucuronide, an indirect *O*-glucuronide, M2b, and its aglycone, M8b, were also needed. To make M8b, the position of the hydroxyl group first had to be identified. Hypha chemists achieved this by purifying a small amount of M2b from human urine supplied by the client, and elucidating the structure of the conjugate using cryoprobe NMR spectroscopy. Then, knowing the position of hydroxylation from the structure of the phenolic glucuronide, **100s of mgs of M8b were synthesized.** In order to access large amounts of M2b, a different approach was needed as this glucuronide was not amenable to chemical synthesis due to instability and formation of side products.

Instead, **M2b was successfully made through microbial biotransformation of the aglycone M8b**. Following a screen to determine the best microbial catalyst, all 560mg of M8b fed to one of Hypha's biotransforming strains was metabolized, from which 254 mg of M2b was purified.

Hypha supplied the metabolites at >95% organic purity to the client along with Certificates of Analysis.



Scheme for provision of the 3 drug metabolites of cenobamate

"Hypha Discovery came highly recommended and we subsequently contracted with Hypha to extract and purify metabolites from urine. As they were so successful, we requested help for a second program to extract metabolites from human urine and /or synthesize / biosynthesize 3 metabolites. As expected, Hypha has been successful preparing these metabolite reference standards along with structural elucidation and certificates of analysis. In addition, synthesis of one of the metabolites had been attempted at 2 other labs without success; however, Hypha was able to synthesize this difficult metabolite which allowed us to do further evaluations on the metabolite.

Head of Toxicology/DMPK, US Pharma Company

Delivering a first class service to our clients worldwide

Contact us

Our team members are always pleased to answer your questions and help determine if our solutions will meet your requirements.

Hypha attends various US and European conferences throughout the year—check our website www.hyphadiscovery.co.uk to find out what's happening and follow us on LinkedIn. In addition to arranging online meetings, our team is also happy to meet with you at your organization - please let us know if you would appreciate a visit or tailored scientific seminar. We also host visits from clients and prospective clients who may be in the area.

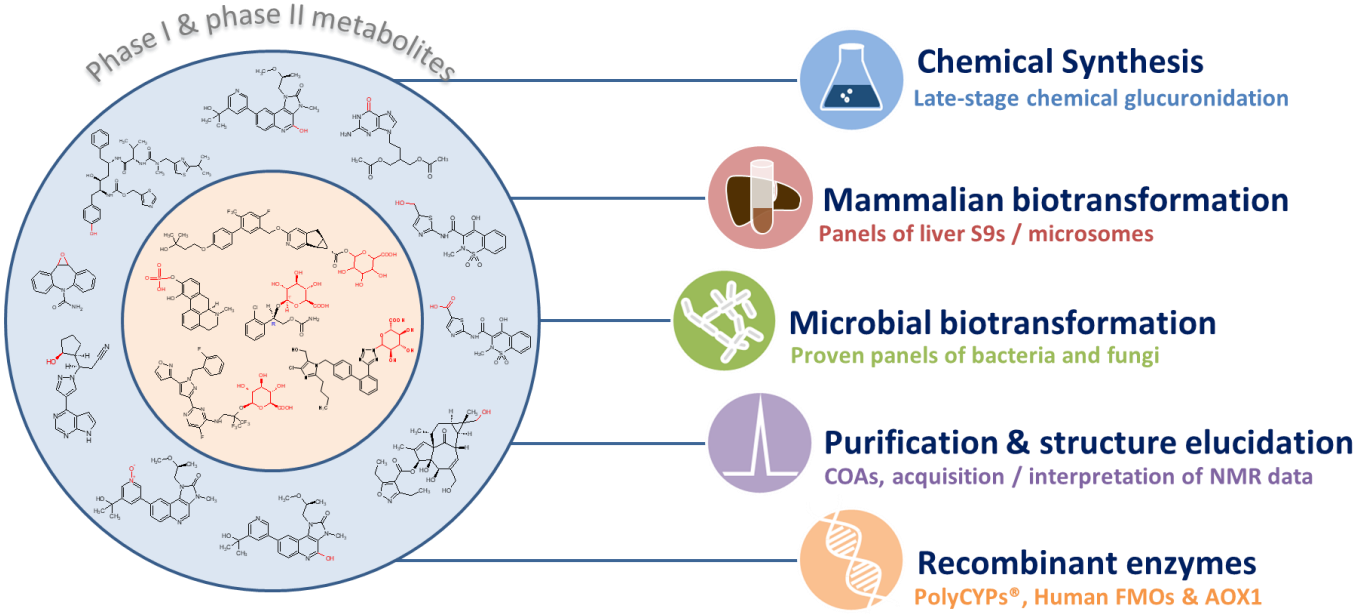
Contact us for a no obligation discussion to find out more about the applications of our technology.

Email: enquiries@hyphadiscovery.com

Phone: +44 (0)1235 824849

www.hyphadiscovery.com

Hypha Discovery Limited
154B Brook Drive, Milton Park,
Abingdon, OX14 4SD, UK



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References in blue are authored or co-authored by Hypha scientists.

"We approached Hypha Discovery for the preparation of 10-50 mg quantities of two API metabolites that had proven difficult to synthesise chemically. The project was highly successful, combining beautifully executed multi-disciplinary science with clear and responsive communications; the collaboration was a genuine pleasure. I have no hesitation in recommending Hypha to others for metabolite generation and scale-up."

Head of CMC, UK Pharma Company