SCALING THE PRODUCTION OF M493, A MAJOR CYP METABOLITE OF XEVINAPANT, BY MICROBIAL BIOTRANSFORMATION

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Abstract: Xevinapant is an oral IAP (inhibitor of apoptosis protein) inhibitor which has been investigated in clinical studies for the treatment of various cancers. The human mass balance study highlighted a previously overlooked major oxidised metabolite, M493 ("oxMET1"), formed via the action of CYP3A4/5. In this study M493 comprised ~9% of total drug-related material, however PK modelling predicted a level above the 10% threshold due to the metabolite's longer half-life in plasma. Further work to initially characterise the drug-drug interaction (DDI) potential of M493 was thus warranted (Lang et al., 2026).

Initially, canine liver microsomes (LMs) were used to generate a small amount of M493 for structure elucidation by NMR spectroscopy, since the LC-MS/MS fragmentation pattern did not permit the exact point of oxidation in the pyrrolo-diazocine core to be pinpointed. NMR data obtained on M493 purified from the LMs and later from additional material produced by microbial biotransformation supported the position of hydroxylation at position 4 in the S-configuration.

Since chemical synthesis of M493 would be challenging, microbial biotransformation provided a scalable and non-animal tissue derived route to generate the quantities of M493 needed for further non-clinical characterisation studies. This poster describes the application of microbial biotransformation to make hundreds of milligrams of M493 from its immediate precursor M477.

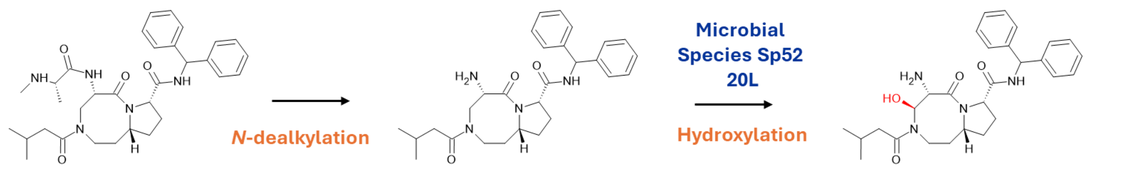
Screening of M477

A prioritised set of 24 microorganisms and a panel of oxidative enzymes (23 microbial CYPs (PolyCYPs®), 25 human and other mammalian recombinant CYPs, AOX1, 5 FMOs and a laccase) were screened for the production of M493 from the intermediate metabolite M477 dosed at 0.1 mg/ml in microtiter plates. Six oxidised metabolites with the mass change of +16 Da were detected across the different reactions.

Analysis by LC-MS indicated production of the target metabolite (492 Da, $C_{28}H_{36}N_4O_4$) in 14 reactions from 5 microbes, 6 PolyCYPs enzymes and 3 human CYPs. The human CYP that best formed M493 from M477 was CYP3A5. Notably CYP3A4 was not observed to form M493 in this screen. Four samples (two microbial strains Sp52 & Sp59, human CYP3A5 and dog CYP3A12) were analysed further by LC-MS/MS and Sp52 found to best produce M493 with little residual parent compound present.

Conversion yields to M493 from the screening stage were estimated by UV peak area as approximately 14%. Since virtually all of the M477 dosed to microbial species Sp52 was used in the screening reaction, this was a good indication that the substrate concentration could be increased to improve volumetric yields of M493 in the scale-up reaction.

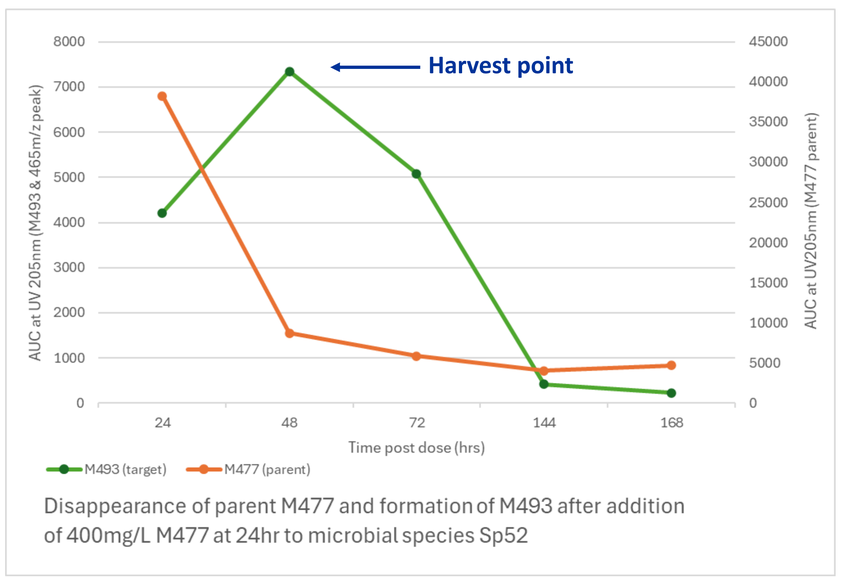
Scale-up of M493



Production

To reduce the volume of the reaction needed to produce ~500 mg of M493, a dose escalation experiment was performed comparing the original 100 mg/L dose with doses up to 400 mg/L M477, each at addition times of 24 h, 42 h and 72 h. Results showed Sp52 was able to tolerate a dose of at least 400 mg/L M477 best at 24h post inoculation. A 20L biotransformation reaction was subsequently performed at this dose, split into 2 x 10L flask batches.

M493 was a relatively transient metabolite which showed significant decreases in concentration once a maximum was observed. The best dose parameters for production of M493 occurred when the production cultures of Sp52 were dosed with 400 mg/L of M477 24 hours after inoculation followed by harvest after a further 48 hours fermentation.



Disappearance of parent M477 and formation of M493 after addition of 400mg/L M477 at 24hr to microbial species Sp52

Purification

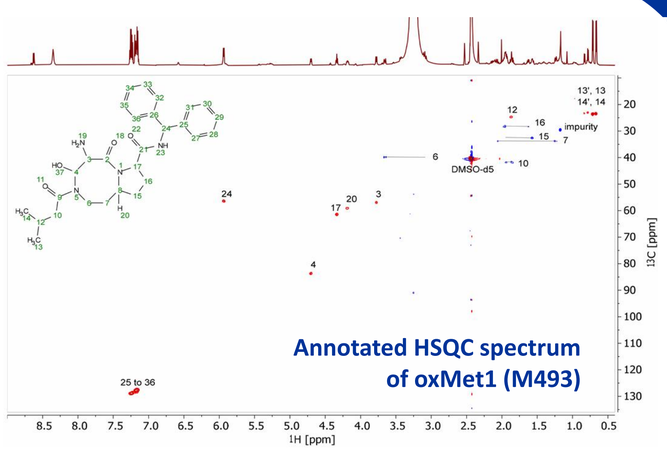
Combined fermentation broth was centrifuged and the supernatant processed through a Diaion HP-20 resin column. Fractions containing M493 were combined, concentrated and separated on a Waters XSelect CSH C_{18} column. M493 (~19% of total M493 produced) was also associated with the biomass which was extracted twice with acetonitrile before being processed in a similar way. Fractions from both the broth and biomass were combined and fractionated using the same column with orthogonal mobile phase conditions.

High purity was ensured by careful fraction cutting of the M493 chromatographic peak, followed by recycling of appropriate off-fractions. Lyophilisation of relevant fractions yielded 597.3 mg of the M493 metabolite as a white powder with purities of 96.2%, 99.2% and 97.7% by LC-UV, LC-ELSD and informal qNMR (Bruker ERETIC2 protocol), respectively.

Analysis of the ^{1}H NMR data showed an unexpected additional set of proton signals at an approximate ratio of 5:1. Because the LC analysis indicated this material was of high purity it was thought that the material appeared in two different conformations rather than being two different isomers. The spectrum from an additional HSQC NMR experiment supported this observation. After analyses, a total of 596 mg M493 was provided to Merck Healthcare KGaA for DDI studies.

Structure assignment of M493 by NMR spectroscopy

To identify and verify the oxidation position in M493 a set of 1D and 2D NMR spectra were recorded. In a first step, a full NMR assignment for M477 was made and used as a reference for M493 structural assignment (Lang et al., 2026). In a second step, NMR spectra of the oxMET1 fraction prepared from incubation in dog liver microsomes were recorded (see HSQC spectrum beneath).



This revealed that position 4 was oxidized, since the diastereotopic CH_2-group in M477 changed to a CH-group in M493, which was further supported by the chemical shifts of the ^{1}H (4.70 ppm) and ^{13}C (83.62 ppm) atom characteristic for a N,O-acetal group.

For stereochemical assignment of the newly formed asymmetric centre the $^3J_{HH}$ vicinal coupling constant between H-4 and H-3 of this conformationally fixed bicyclus was used, which could be easily extracted from the ^{1}H NMR spectrum (8.6 Hz) and used for stereochemical assignment determined by in silico calculation. The ROESY spectrum of the synthesised standard produced by microbial biotransformation supported the identified *S*-configuration (Lang et al., 2026).

Conclusions

- Microbial biotransformation provided a route to stereospecific hydroxylation in the pyrrolo-diazocine moiety of M477 which would have been difficult to achieve by chemical synthesis.
- Unusually CYP3A5 alone rather than CYP3A4/5 formed M493 from M477 at the high substrate dose applied in this screen. CYP3A4/5 is known from previous work to be involved in the metabolism of xevinapant.
- A four-fold increase in substrate dose and early harvest of the biotransformation reaction enabled optimisation of the yield to produce ~600 mg of M493 for further studies at Merck Healthcare KGaA.
- M493 levels decreased significantly after 48 hrs in the biotransformation reaction. Any onwards metabolites or degradants were not monitored or characterised as part of this work but were observed in vitro in hepatocytes and in the radiolabelled mass balance study.

Reference: Lang B, Schieferstein H, Scheible H, et al. Identification, structural elucidation and nonclinical characterisation of oxMET1: a further major metabolite of xevinapant? Xenobiotica. 56(3), 192-203. doi:10.1080/00498254.2026.2630754

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