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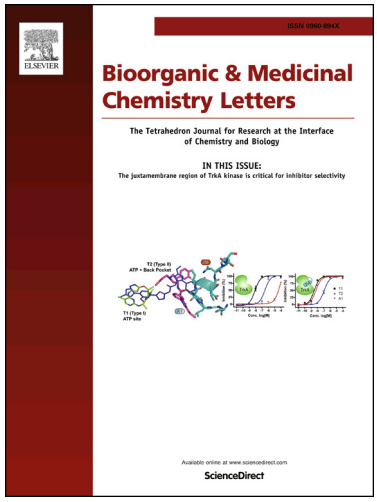

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Substrate and Inhibitor Specificity of Kynurenine Monooxygenase from *Cytophaga hutchinsonii*

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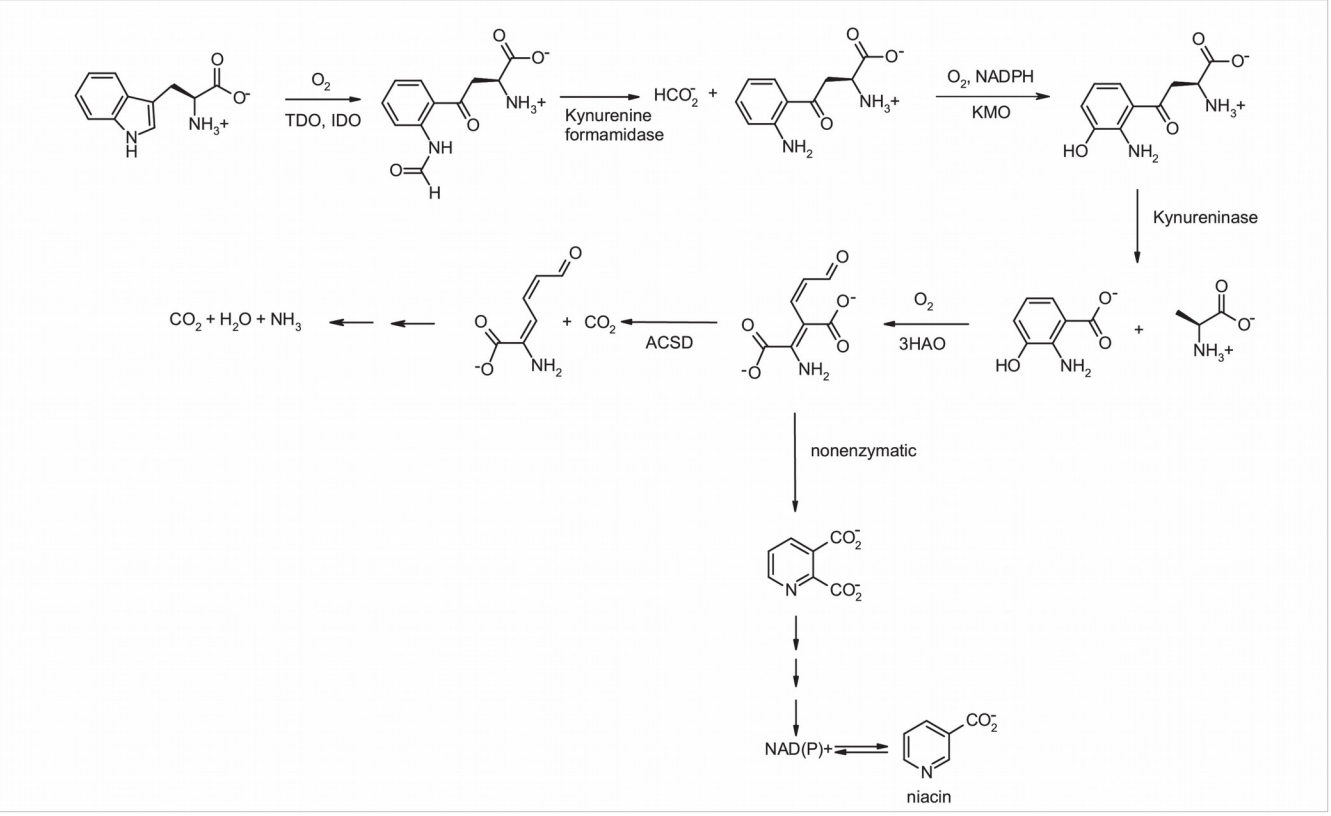
ABSTRACT

Kynurenine monooxygenase (KMO) is a potential drug target for treatment of neurodegenerative disorders such as Huntington's and Alzheimer's diseases. We have evaluated substituted kynurenines as substrates or inhibitors of KMO from *Cytophaga hutchinsonii*. Kynurenines substituted with a halogen at the 5-position are excellent substrates, with values of k_{cat} and k_{cat}/K_m comparable to or higher than kynurenine. However, kynurenines substituted in the 3-position are competitive inhibitors, with K_I values lower than the K_m for kynurenine. Bromination also enhances inhibition, and 3,5-dibromokynurenine is a potent competitive inhibitor with a K_I value of 1.5 μM. A pharmacophore model of KMO was developed, and predicted that 3,4-dichlorohippuric acid would be an inhibitor. The K_I for this compound was found to be 34 μM, thus validating the pharmacophore model. We are using these results and our model to design more potent inhibitors of KMO.

The kynurenine pathway is the major pathway for tryptophan catabolism in many organisms, including man[1], as shown in Scheme 1. Tryptophan undergoes cleavage of the 2,3-double bond catalyzed by a hemoprotein, either tryptophan dioxygenase (TDO) in the liver or indoleamine dioxygenase (IDO) in extrahepatic tissues, to produce N-formyl-L-kynurenine. Subsequent deformylation by kynurenine formamidase gives kynurenine, which then is hydroxylated by a mitochondrial flavoprotein, kynurenine monooxygenase (KMO), to give 3-hydroxy-L-kynurenine. Subsequently, kynureninase, a pyridoxal-5'-phosphate dependent enzyme, catalyzes the hydrolytic cleavage of 3-hydroxy-L-kynurenine to 3-hydroxyanthranilate and L-alanine. 3-Hydroxyanthranilate dioxygenase (3HAO) then produces 2-amino-3-carboxymuconate semialdehyde, which can either cyclize spontaneously to quinolinate[2] or undergo enzymatic decarboxyation by 2-amino-3-carboxymuconate semialdehyde decarboxylase (ACSD) to 2-aminomuconate semialdehyde. The former reaction ultimately leads to $NAD(P)^+$ formation, and the latter reaction leads to complete catabolism via the 2-ketoadipate pathway. Several of the intermediates in the kynurenine pathway are cytotoxic, including 3-hydroxykynurenine and 3-hydroxyanthranilate, which are apoptotic[3], and quinolinate, which is a neurotoxin due to NMDA receptor agonism[4]. Overexpression of the kynurenine pathway has been linked to neurodegenerative diseases such as Huntington's[5], Parkinson's[6] and Alzheimer's diseases[7]. Thus, enzymes in the kynurenine pathway are drug targets, and KMO has emerged as the target of choice, since all downstream metabolites are potentially cytotoxic. As proof of concept, a KMO inhibitor was shown to delay the onset of disease in an Alzheimer's mouse model[8].

Kynurenine analogues were some of the first KMO inhibitors to be examined, and *m*-nitrobenzoylalanine was shown to be an effective KMO inhibitor in the brains of mice[9]. It was recognized that binding to KMO required a substituted aromatic ring and a carboxylate separated by 3 spacer atoms. Later, N-(4-phenylthiazol-2-yl)benzenesulfonamides were found to be potent inhibitors, with K_I values in the nM range[10]. Since the crystal structure of the *Saccharomyces cerevisiae* enzyme



Scheme 1. The kynurenine pathway for tryptophan metabolism.was published,[11] KMO inhibitors have been designed computationally[12]. However, a systematic study of the effects of substituents on kynurenine on the KMO substrate activity and inhibition has not been performed. Recently, we prepared a series of substituted kynurenines and evaluated their activity as substrates for human and *Pseudomonas fluorescens* kynureninase[13]. In the present study, we have evaluated these substituted kynurenines as substrates and inhibitors for KMO from *Cytophaga hutchinsonii,* and we have designed and validated a pharmacophore model for KMO which predicted new inhibitors. The active site residues which contact substrates and inhibitors in the crystal structure of KMO from *Saccharomyces cerevisiae*[11] are conserved in KMO from both *C. hutchinsonii* and humans, so the enzyme from *C. hutchinsonii* is a suitable model to develop lead compounds.

Table 1

Substrates for *C. hutchinsonii* KMO

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m(M^{-1} s^{-1})
L-Kynurenine (**1**)	67±15	2.0 ± 0.2	(5.3 ± 1) x10^4
5-Bromo-L-Kyn (**2**)	4.5±3.0	0.6 ± 0.1	(1.3 ± 0.3) x10^5
5-Chloro-L-Kyn (**3**)	2.0±0.7	0.8 ± 0.1	(4.0 ± 1) x10^5

We tested 5-halo-L-kynurenines as potential inhibitors for KMO, but discovered high blank rates in assays containing the compounds without kynurenine. In addition to NADPH consumption in the presence of 5-bromo-L-kynurenine (**2**), we also observed increasing absorbance in the 370-400 nm region during the reaction, as expected since hydroxylation of kynurenine causes a red shift in the absorption spectrum. Furthermore, HPLC analysis of reaction mixtures of **2** incubated with KMO

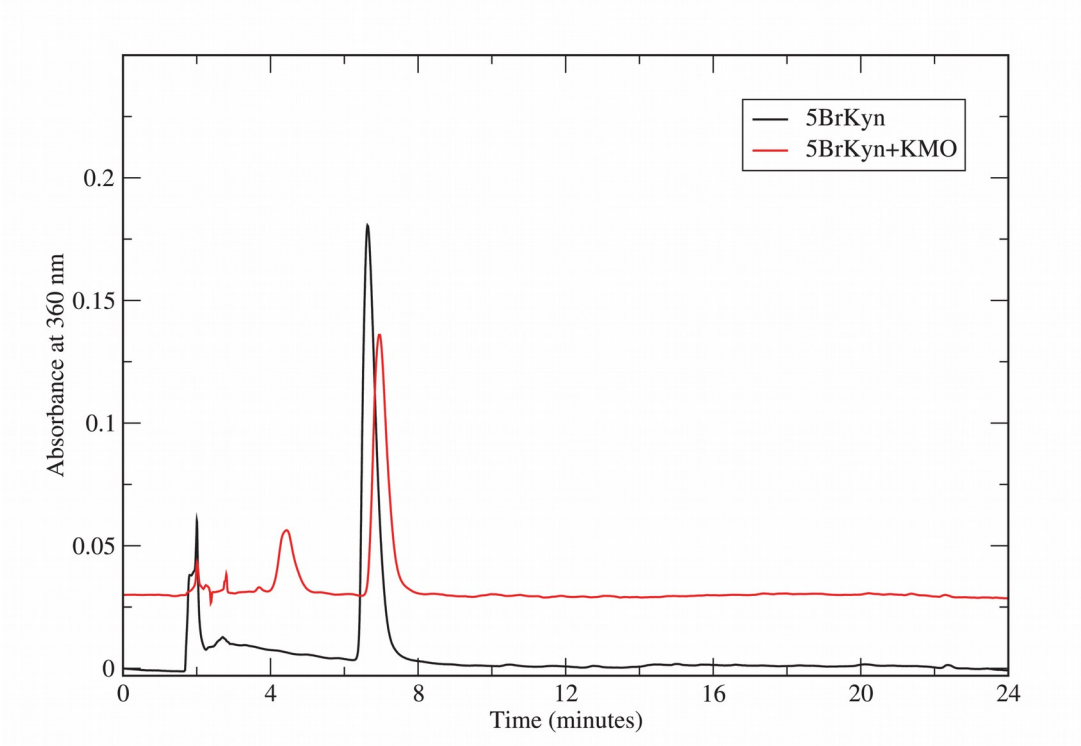


Figure 1. HPLC analysis of 5-bromo-L-kynurenine reaction with KMO. Black line: Reaction mixture without addition of KMO. Red line: Reaction mixture after addition of KMO and incubation at 37 °C for 30 minutes.

shows a new peak which elutes earlier (Figure 1, red line) than kynurenine (Figure 1, black line), and exhibits λ_{max} red-shifted from 380 to 388 nm. ESI-LC/MS analysis of this reaction mixture exhibited new peaks at m/z = 303 and 305 for $(M+1)^+$, as expected for the product, 5-bromo-3-hydroxykynurenine. Thus, these compounds are not acting as uncouplers, consuming NADPH without hydroxylation, although we found previously that β-benzoylalanine is an uncoupler of KMO from *Pseudomonas fluorescens*[14]. 5-Chloro-L-kynurenine (**3**) is also a substrate based on NADPH consumption (Table 1). Both kynurenines halogenated in the 5-position are excellent substrates for *C. hutchinsonii* KMO (Table 1), with k_{cat}/K_m values greater than kynurenine itself. The 2-3-fold decrease in k_{cat} for these compounds is more than compensated by the lower values of K_m. It is interesting that we also found previously that these 5-halogenated kynurenines are excellent substrates for both human and *P. fluorescens* kynureninase[13].

In contrast, kynurenines with substitutents in the 3-position were found to be competitive inhibitors of NADPH consumption in the spectrophotometric assay (Table 2). These compounds could be considered product analogues of KMO. 3-Chloro (**5**), and 3-methylkynurenine (**7**) have K_I values lower than the K_m of kynurenine. However, it is not clear if the value of $K_m \approx K_d$ for kynurenine. As these 3-substituted kynurenines are racemic, the K_I value for the L-isomers should be half of the values given in Table 2. Since 5-bromination lowers the K_m for substrates (Table 1), we were interested to see if this also applied to inhibitors. Bromination of 3-methyl- and 3-chloro-DL-kynurenine with Br_2 in acetic acid gave the corresponding 5-bromo-3-substituted kynurenines (see supplemental data). As anticipated, these compounds have comparable or lower K_I values than the 3-substituted kynurenines (Table 2). The most potent inhibitor tested is 3,5-dibromo-L-kynurenine (**4**), with a K_I value of 1.5 μM (Table 2). The 3-halokynurenines do not appear to be efficient uncouplers of hydroxylation, since the blank rates in the presence of the compounds were not significantly higher than normal.

Table 2

Inhibition of KMO by 3-substituted kynurenines

Compound	K_I (μM)
3, 5-diBr-L-Kyn (**4**)	1.5 ± 0.6
3-Cl-DL-Kyn (**5**)	13.8 ± 8.4
5-Br-3-Cl-DL-Kyn (**6**)	11.0 ± 3.6
3-Me-DL-Kyn (**7**)	8.6 ± 5.8
5-Br-3-Me-DL-Kyn (**8**)	10.2 ± 4.7

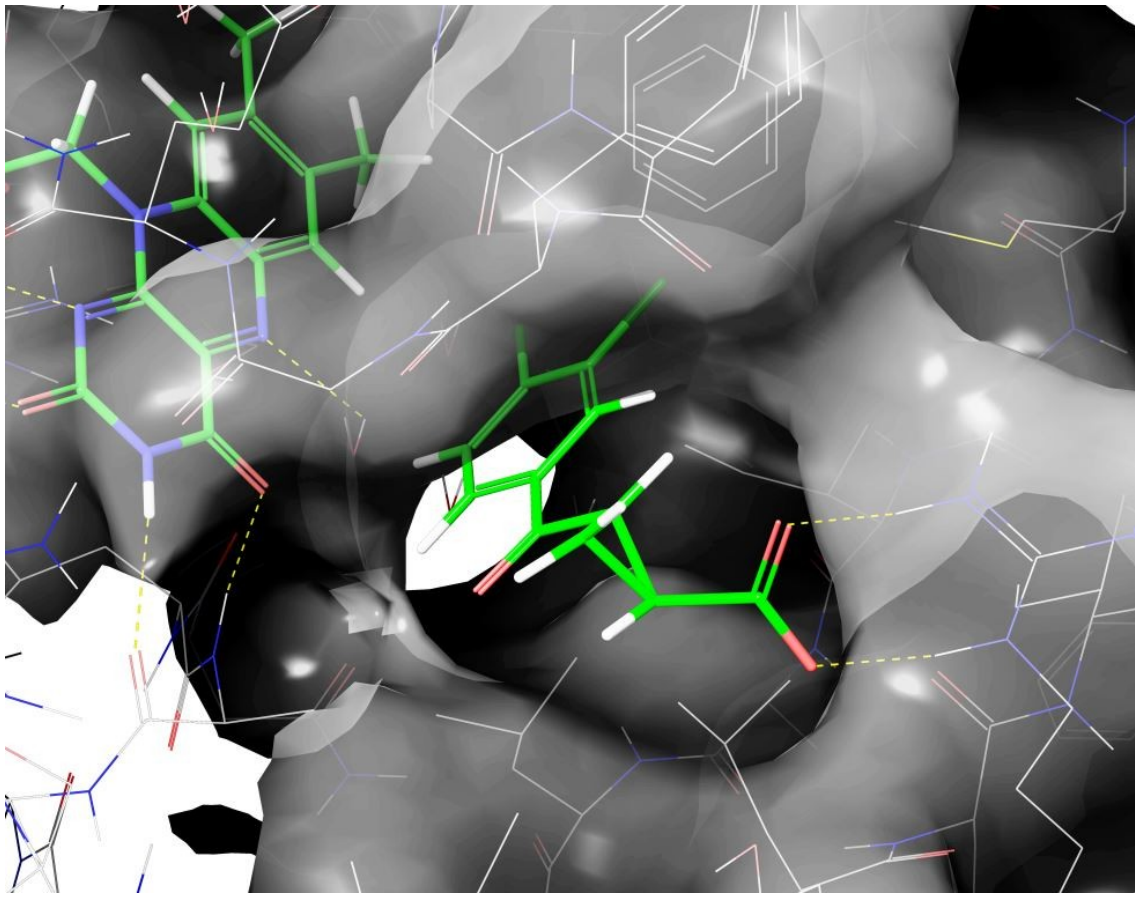


Figure 2. Hydrogen bonding interaction with Arg83. The x-ray crystal structure complex of UPF648 bound to yeast KMO (pdb code: 4J36). The important hydrogen bonding interactions with Arg83 are shown at the right, along with other interactions.

Based on these results and the published crystal structure of *Saccharomyces cerevisiae* KMO, we developed a pharmacophore model. Pharmacophore modeling has been an extremely successful

approach for lead identification and modification[15-17]. There are two broadly defined approaches for pharmacophore modeling: (i), ligand-based pharmacophore development, where there is no drug-target structural information and small ligands with different binding affinities are used, and (ii), structure-based pharmacophore development where specific binding conformations of ligands are used. For the work reported herein, we have adopted primarily the latter approach.

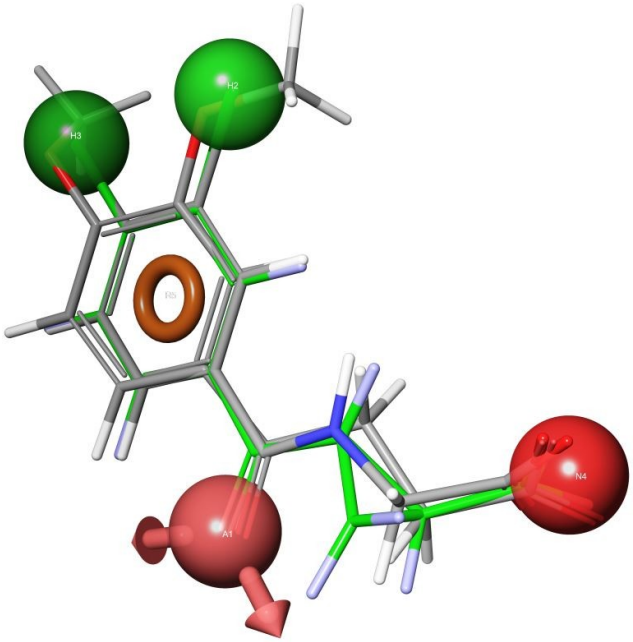


Figure 3. Receptor-based pharmacophoric models of 3,4-dimethoxyhippuric acid aligned with the conformation of UPF648 as bound to yeast KMO (pdb code: 4J36). The green spheres are hydrophobic centers, the red spheres are acidic centers, the pink spheres with arrows are H-bond acceptors, and the orange rings are aromatic planes.

Our pharmacophore modeling concentrated on lead identification. Multiple models were generated using the Schrödinger software package[18], and each was scored in search of novel biologically active chemotypes. We carried out pharmacophore modeling of the bioactive conformation of UPF648 bound yeast KMO[19, 20].

The approach we used is structure-based modeling using the bound conformation of known active compounds in KMO X-ray crystal structures. A good understanding of the SAR of KMO is

desirable for the success of the design and development of novel inhibitors for KMO. If we have a reliable description of the inhibitor-specific structural changes in the KMO catalytic site, we can efficiently enrich compound libraries from which we can identify hits with minimized false positives and negatives. Our proof-of-concept work was performed on a single bound conformation of a small molecule KMO inhibitor, UPF648, bound to yeast KMO[19, 20] (Fig. 2). On the periphery, UPF648 has a pair of hydrogen bonding interactions with Arg83 while the rest of the UPF648 is placed in the larger lipophilic pocket formed by Leu221, Ile232 and Leu234. The pharmacophore centers based on the bound conformation of UPF648 with KMO were generated (Fig. 3) and a number of compounds were retrieved through virtual screening of a database of commercially available chemicals. Two compounds with a high fit value to the pharmacophore model, 3,4-dichlorohippuric acid and 3,4-dimethoxyhippuric acid (Chart 1), have been identified as high scoring hits from this initial screening.

Table 3

Inhibition of KMO by substituted hippuric acids

Compound	K_I (μM)
3,4-Dimethoxyhippuric acid (9)	$(1.0 \pm 0.6) \times 10^3$
3, 4-Dichlorohippuric acid (10)	34 ± 20

3,4-Dimethoxyhippuric acid (**9**) and 3,4-dichlorohippuric acid(**10**) were then tested, with the results shown in Table 3. Although **9** is a weak inhibitor, with a K_I value of about 1 mM, **10** is much better, with a K_I value of 34 μM, thus validating the pharmacophore model in Figure 3. We were surprised that the 3,4-dimethoxy substitution is not tolerated for binding of hippuric acids, because it is found in one of the N-(4-phenylthiazol-2-yl)benzenesulfonamides with an IC_{50} value of 37 nM[10]. This suggests different binding modes for these two compounds. 3,4-Dichloro substitution was also found to be optimal for **11**[12], which can be considered as a cyclized analog of **10**. It should be noted that the 3-position in these hippuric acids is equivalent to the 5-position in the kynurenines (see Chart 1), so the effect of the halogen is similar in increasing affinity of both substrates and inhibitors of KMO. A recent

paper used molecular modeling to screen a series of arylpyrimidine carboxylic acids related to **11** as potential inhibitors of KMO[21]. A similar compound, GSK-180 (**12**) was found to be a potent inhibitor of KMO and is active against a rodent model of acute pancreatitis[22]. KMO inhibitors were the subject of a recent comprehensive review[23].

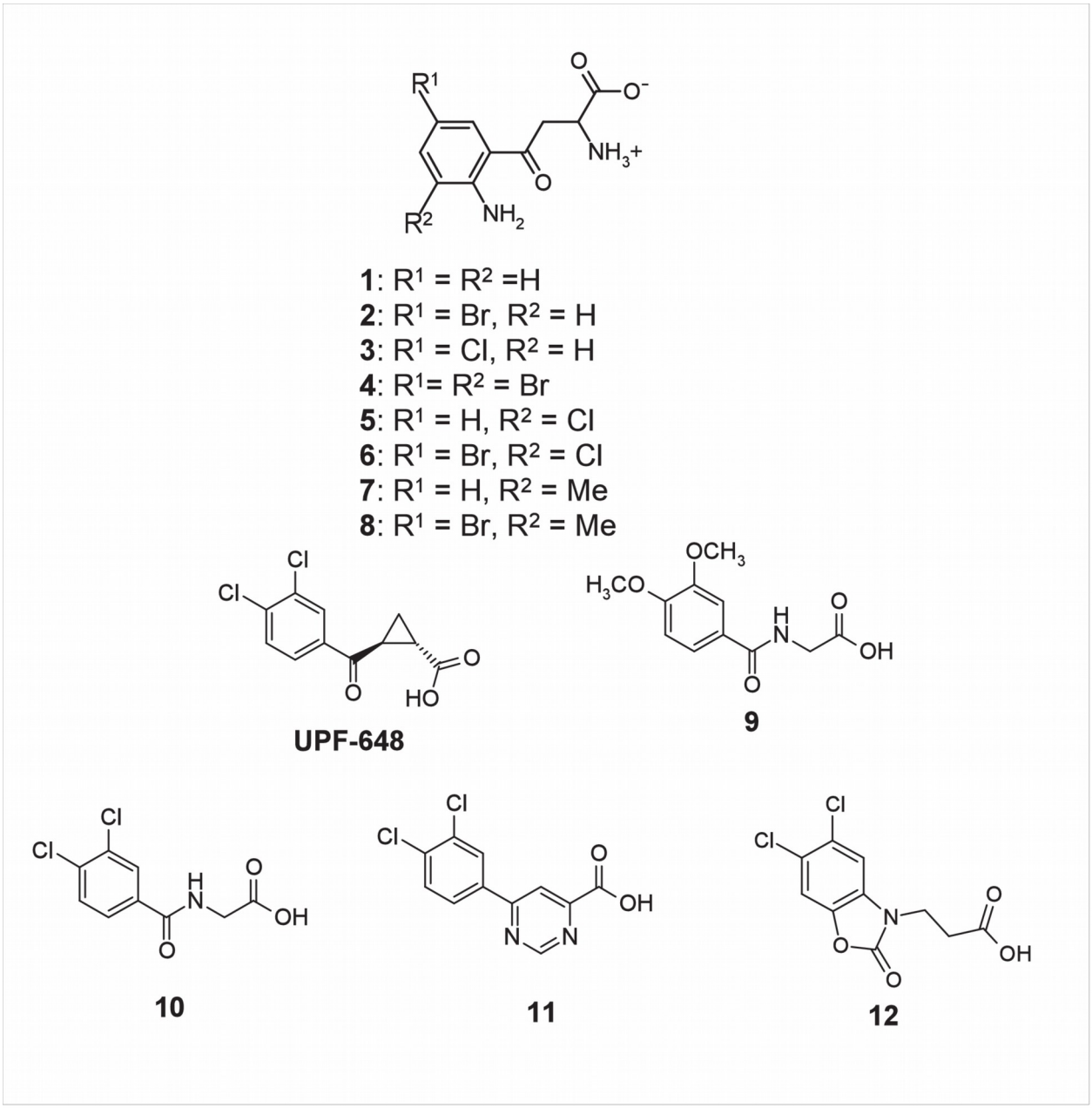


Chart 1. Compounds examined in this study and that were identified by pharmacophore methods using the bound conformation of UPF 648, and other active compounds, **11** and **12**.

In conclusion, kynurenines substituted at the 3-position are competitive inhibitors of KMO, and halogen substitution at C-5 increases both substrate and inhibitor activity. The α-amino group is not necessary for inhibitor binding, since halogen-substituted hippuric acids have K_I values comparable to kynurenines. The pharmacophore modeling results provide a preliminary validation of our approach and set the stage for future structure-based modeling. We have begun to explore in greater detail the binding modes of several key inhibitors within the KMO catalytic site to design more potent inhibitors of KMO.

Supplementary data. The supplementary data contains the details of enzyme purification and assay, and the synthesis and characterization of kynurenine derivatives.

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