Rational Design of 4-Aryl-1,2,3-Triazoles for Indoleamine 2,3-Dioxygenase 1 Inhibition

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Ⓢ *Supporting Information*

ABSTRACT: Indoleamine 2,3-dioxygenase 1 (IDO1) is an important therapeutic target for the treatment of diseases such as cancer that involve pathological immune escape. Starting from the scaffold of our previously discovered IDO1 inhibitor 4-phenyl-1,2,3-triazole, we used computational structure-based methods to design more potent ligands. This approach yielded highly efficient low molecular weight inhibitors, the most active being of nanomolar potency both in an enzymatic and in a cellular assay, while showing no cellular toxicity and a high selectivity for IDO1 over tryptophan 2,3-dioxygenase (TDO). A quantitative structure−activity relationship based on the electrostatic ligand−protein interactions in the docked binding modes and on the quantum chemically derived charges of the triazole ring demonstrated a good explanatory power for the observed activities.
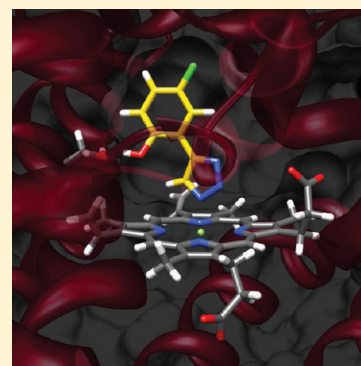
INTRODUCTION

Many tumors develop the capacity to actively suppress a potentially effective immune response.[1] The enzyme indoleamine 2,3-dioxygenase 1 (IDO1, EC 1.13.11.52) is one of the key players in this pathological immune escape and has therefore been selected as a therapeutic target for pharmacological interventions.[2−4] IDO1 catalyzes the initial and rate-limiting step in the catabolism of tryptophan (Trp) along the kynurenine pathway.[5,6] By depleting Trp and accumulating Trp catabolites, IDO1 exerts a local immunosuppressive effect on T-lymphocytes.[7−10] The observations that many human tumors constitutively express IDO1[11] and that increased IDO1 expression in tumor cells is correlated with poor prognosis for survival in several cancer types[12] led to the hypothesis that its inhibition might enhance the efficacy of cancer treatments. Indeed, results from in vitro and in vivo studies have suggested that the efficacy of therapeutic vaccination or chemotherapy may be improved by concomitant administration of an IDO1 inhibitor.[11,13−16] Very recently, it has been shown that the functionally related enzyme tryptophan 2,3-dioxygenase (TDO) may be a complementary anticancer target.[17,18]

The IDO1-like protein Indoleamine 2,3-dioxygenase 2 (IDO2)[19,20] shares 44% of sequence homology with IDO1. However, its physiological role remains unclear due to (i) very low Trp degradation activity, (ii) the presence of polymorphisms abolishing its enzymatic activity in about 50% of Caucasians, and (iii) the presence of multiple splice variants.[21−23]

IDO1 is an extrahepatic heme-containing enzyme that displays less substrate specificity than TDO.[6] In the first step of the catalytic cycle, IDO1 binds both the substrate and molecular oxygen in the distal heme site. The enzyme catalyzes the cleavage of the pyrrole ring of the substrate and incorporates both oxygen atoms before releasing *N*-formyl kynurenine, which is subsequently hydrolyzed to kynurenine by a cytosolic formamidase.[24] The two available crystal structures of IDO1 include the heme-bound ligands cyanide and 4-phenylimidazole (PIM), respectively.[25] Mutant analyses showed that none of the polar amino acid residues in the distal heme site are essential for the activity of the enzyme, suggesting a reaction mechanism involving only the substrate and the dioxygen molecule.[25−27] In the active form of IDO1, the heme iron is in its ferrous state (Fe^{2+}), while in its inactive form, the heme iron is in the ferric (Fe^{3+}) state. Formally, the catalytic cycle of IDO1 does not alter the oxidation state of the
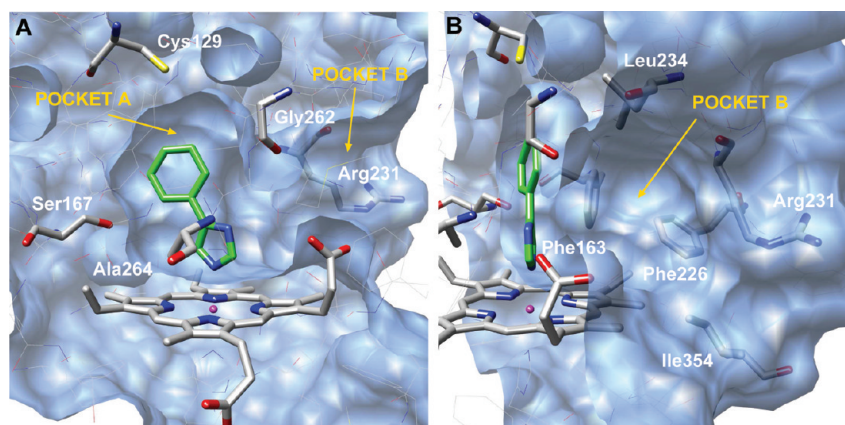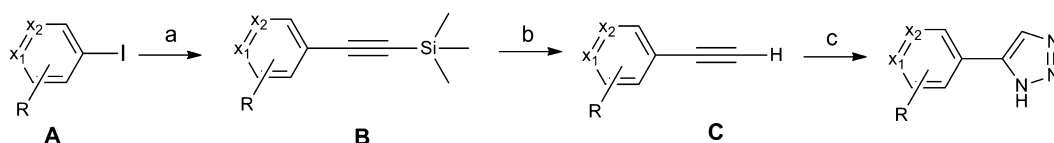
Figure 1. X-ray structure of IDO1: binding site with PIM ligand (green). (A) Two hydrophobic pockets and residues in hydrogen-bonding distance are labeled. (B) Side view, highlighting the residues that form pocket B.

Scheme 1[a]

[a]Reagents, conditions, and yields: (a) TMSA, PdCl$_2$(PPh$_3$)$_2$, Et$_3$N, CuI, dioxane, 45 °C, 5 h, up to 95%; (b) KF, MeOH, rt, 3 h, up to 85%; (c) TMSN$_3$, CuI, DMF/MeOH (9:1), 100 °C, 10–12 h, 30–83%,

heme, but as IDO1 is prone to autoxidation, a reductant is necessary in the enzymatic assay to maintain enzyme activity. Cytochrome b5 has been suggested to be responsible for IDO1 reduction in vivo.[28−30]

The two crystal structures of human IDO1[25] open the way for the in silico design of new IDO1 inhibitors. In the PIM-bound X-ray structure (PDB accession code 2D0T), the ligand is bound in a deep binding site with its phenyl ring inside a hydrophobic pocket (pocket A, Figure 1) and one imidazole nitrogen coordinated to the heme iron at a distance of 2.1 Å. The PIM binding site consists of residues Tyr126, Cys129, Val130, Phe163, Phe164, Ser167, Leu234, Gly262, Ser263, Ala264, and the heme ring. Possible hydrogen-bonding sites are the hydroxyl group of Ser167, the CO group of Gly262, the NH group of Ala264, and the heme 7-propionate group. Ligands larger than PIM may also interact with Phe226, Arg231, Ser235, Phe291, Ile354, and Leu384, which are located at the binding site entrance. Here, additional hydrogen bonds are possible with the side chain of Arg231. A hydrophobic pocket in this region is provided by Phe163, Phe226, Arg231, Leu234, Ile354, and the heme ring (pocket B, Figure 1). The cyanide-bound structure (2D0U) differs from the PIM-bound structure mainly in the access to pocket A, which is hindered by an inward-movement of the backbone of loop 262−266 and the side chain of Phe163, suggesting some flexibility in the active site. In both structures, two buffer molecules (N-cyclohexyltaurine) are bound at the entrance of the active site, hydrogen-bonding to the heme propionate and interacting with pocket B.

The oldest known IDO1 inhibitors with activities in the micromolar range are Trp derivatives and β-carbolines.[6,13,31−36] In recent years, many redox-active IDO1 inhibitors with IC$_{50}$ values of up to 60 nM have been discovered.[37−44] However, there exists the possibility that these act through interference with the chemical reducing agents in the enzymatic assay (methylene blue and ascorbate) rather than through IDO1-specific interactions.[30] Other classes of inhibitors derived from a target-based screen of the NCI diversity set,[45] the 4-phenylimidazole scaffold,[46] the indole scaffold,[47,48] the S-benzylisothiourea scaffold,[49] a high throughput screen,[23] and a virtual screen[50] have been described. The most potent and promising compounds known today are the competitive hydroxyamidine inibitors developed by Incyte,[15,51,52] with enzymatic IC$_{50}$ values of about 60 nM. Compound INCB24360, whose structure has not been disclosed yet, is currently undergoing a phase I clinical trial.

We have previously described a number of new IDO1 inhibitors based on a structure-based in silico design strategy.[53] Among the new scaffolds, we discovered 4-phenyl-1,2,3-triazole (1) to inhibit IDO1 with an IC$_{50}$ value of 60 μM. Some modifications of this scaffold have very recently been described by another group,[54] but improvement of the IC$_{50}$ value was limited to less than a factor of 2 compared to the parent compound. The aim of the present work was to rationally optimize the 4-phenyl-1,2,3-triazole scaffold relying on our in-house drug design tools.[55−61] Substituted 1,2,3-triazoles have been found to be useful drug scaffolds[62−66] and are synthetically accessible under mild conditions through copper(I)-catalyzed azide−alkyne cycloaddition.[67−69]

Here, we computationally designed new ligands and docked them into the IDO1 active site using EADock-DSS[55−59] and AttractingCavities[70] in combination with the parameters derived from SwissParam[60] and a Morse-like metal binding potential (MMBP).[61] Candidates that displayed a good filling of the A pocket and favorable heme interactions were selected for synthesis and tested in an enzymatic assay for IDO1 inhibition. Bioisosteres of the 4-phenyl-1,2,3-triazole scaffold were also investigated. Active ligands were subsequently tested in cellular assays on murine and on human IDO1. Counter-screening against TDO determined their selectivity for IDO1.

To rationalize the observed activities, we then developed a quantitative structure–activity relationship (QSAR) based on the docking results and on quantum chemical calculations.

Following this strategy, we obtained 39 triazole-based IDO1 inhibitors, the most potent having an IC$_{50}$ value in the nanomolar range both in the enzymatic and in the cellular assays while being inactive on TDO and showing no detectable toxicity on the cellular level.

■ CHEMISTRY

The 4-phenyl-1,2,3-triazole derivatives were synthesized using existing protocols or procedures adapted from the literature. The N-unsubstituted 1,2,3-triazoles (**1–42**, **62–64**, and **74**) were synthesized according to the method developed by Yamamoto and co-workers,[71] which involves reaction of ethynyl derivatives with trimethylsilyl azide (Scheme 1). The ethynyl derivatives were synthesized from iodo substrates by the Sonogashira coupling reaction.[72] Compound **33** was synthesized by treatment of **74** with 6 N HCl at room temperature.[73] Compound **34** was derived from compound **30** by treatment with Meldrum's acid in TEAF at 95–100 °C (Scheme 2).[74] Deprotection of the trifluoroacetamide of **41**

Scheme 2[a]
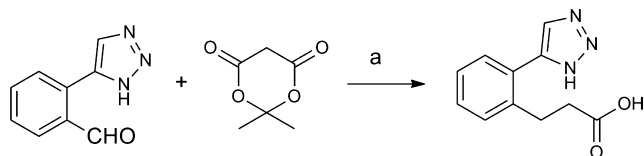
[a]Reagents, conditions, and yields: (a) TEAF, 95–100 °C, 3 h, 45%.
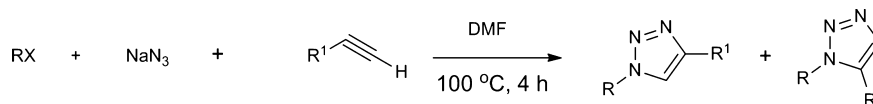
Scheme 3[a]
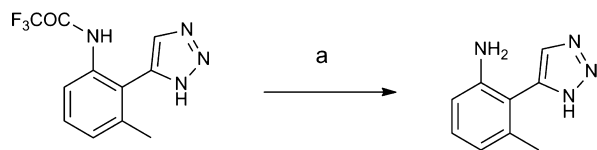
[a]Reagents, conditions, and yields: (a) N$_2$H$_4$·H$_2$O, dioxane, reflux, 2.5 h, 50%.

with N$_2$H$_4$·H$_2$O provided **40** (Scheme 3).[75] All other compounds were synthesized according to literature procedures [**58**,[76] **61**,[77] (**54**, **55**, **56**, and **57**, Scheme 4),[78,79] (**43**, **44**, **45**, **46**, and **47**, Scheme 5),[80,81] (**48**, **49**, and **50**, Scheme 6),[82–84] (**51**, **52**, and **53**, Scheme 6),[82,85,86] and (**59** and **60**, Scheme 7)[82]] or were commercially available (compounds **65–73**).
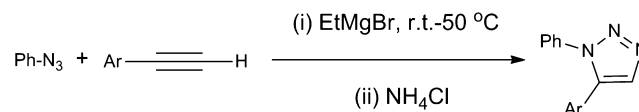
Scheme 5

Ar = Ph, 4F-Ph, 3Me-Ph, 3Cl-Ph, 2-OMe-Ph, 3-Pyridyl

■ RESULTS AND DISCUSSION

Compounds with Aryl Substitutions. Docking PIM into the IDO1 active site using a Morse-like metal binding potential (MMBP),[61] we observe an excellent agreement with the X-ray structure[25] (rmsd 0.3 Å) and an iron–nitrogen bond of 2.1 Å.[53]

Our previously published triazoles[53] (Table 1, compounds **1**, **2**, and **3**) are structurally similar to PIM, with the imidazole ring replaced by a triazole ring. The docked structure of the parent compound **1** closely resembles that of PIM, with one of the nitrogen atoms coordinated to the heme iron (Figure 2A–D). As the triazole ring can occur in three different tautomers (Figure 2A–C) and in a deprotonated form (Figure 2D), we docked all four isomers into the IDO1 active site. Because the MMBP parameters are the same for all nitrogen atoms with a free electron pair,[61] the MMBP cannot distinguish between the binding energies of the deprotonated form and the neutral triazole tautomers. All isomers show similar binding modes (BM) with a conserved position of the phenyl ring, the only difference being the orientation of the triazole ring. In the following, we therefore show only the BM of the deprotonated forms of the N-unsubstituted triazoles.

The *meta*-pyridyl compound (**3**) is more than an order of magnitude less active than the phenyl compound (**1**), while the *para*-pyridyl compound (**2**) retains the activity of **1**. The docked BM of the three compounds do not directly explain this difference in activity, as the pyridyl nitrogen is not making any obvious interaction with the protein (Figure 2E,F).

In the following, we tested different derivatives of **1** with substitutions in *para*, *meta*, and *ortho* positions and combinations of these (Table 1, Figure 2G–O). All substitutions in *para* position of the phenyl ring of the 4-phenyl-1,2,3-triazole scaffold (**1**) reduce the IDO1 inhibitory activity (Table 1, compounds **4**, **5**, **6**, and **7**). The fact that the reduction in activity is correlated with the size of the substituent (F < Cl < CH$_3$ < CF$_3$) as well as results from docking suggest that the protein binding pocket is rather tight in this region and does not provide space for additional groups (Figure 2G).

Substitutions in the *meta* position generally increase the ligand's activity on IDO1, in agreement with the docking results, which show the filling of a small subpocket in this position (Figure 2H,I). Generally, electron-withdrawing and hydrophobic substitutions yield better inhibitors. The only *meta*-substituted compounds with a lower activity than

Scheme 4

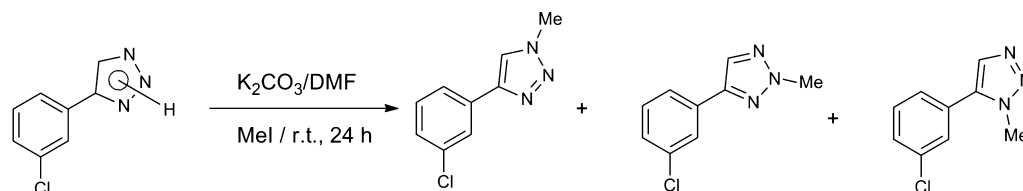(i) R = benzyl chloride, R^1 = Ph
(ii) R = n-butyl iodide, R^1 = Ph

Scheme 6

R = Me or Et
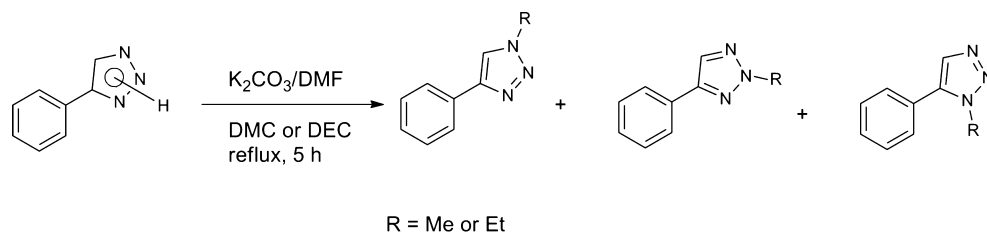
Scheme 7

Table 1. Phenyl-Ring Substituted Derivatives

	X_1	X_2	R	IC$_{50}$ [μM][a]		X_1	X_2	R	IC$_{50}$ [μM][a]
1	C	C		83	22	C	C	2-Br	6.4
2	N	C		85	23	C	C	2-OH	15
3	C	N		1000	24	C	C	2-Cl	32
4	C	C	4-F	190	25	C	C	2-CH$_3$	39
5	C	C	4-Cl	530	26	C	C	2-NH$_2$	60
6	C	C	4-CH$_3$	1000	27	C	C	2-F	80
7	C	C	4-CF$_3$	2300	28	C	C	2-C$_2$H$_5$	230
8	C	C	3-Cl	1.2	29	C	C	2-CF$_3$	650
9	C	C	3-Br	2.0	30	C	C	2-CHO	1300
10	C	C	3-CN	2.8	31	C	C	2-NHCH$_3$	1800
11	C	C	3-CF$_3$	6.0	32	C	C	2-OCH$_3$	NI
12	C	C	3-C$_2$H$_5$	13	33	C	C	2-CH$_2$CH$_2$NH$_2$	NI
13	C	C	3-NO$_2$	15	34	C	C	2-CH$_2$CH$_2$COOH	NI
14	C	C	3-CH$_3$	22	35	C	C	2-OH,5-Cl	0.33[b]
15	C	C	3-F	47	36	C	C	2-CH$_3$,5-Cl	4.3
16	C	C	3-OH	310	37	C	C	2,5-di-Cl	6.4
17	C	C	3-NH$_2$	1500	38	C	C	2,3-di-Cl	23
18	N	C	3-Cl	2.8	39	C	C	2-CH$_3$,3-Cl	72
19	N	C	3-Br	3.3	40	C	C	2-NH$_2$,6-CH$_3$	1200
20	N	C	3-F	7.4	41	C	C	2-NHCOCF$_3$,6-CH$_3$	3000
21	N	C	3-CF$_3$	15	42	C	C	2-CH$_3$,5-NH$_2$	3200

[a]IC$_{50}$ values are the mean of at least two independent assays. [b]IC$_{50}$ value of 0.071 μM at pH 7.4.

compound **1** are the ones with the hydrophilic hydroxyl (**16**, IC$_{50}$ = 310 μM) and amino (**17**, 1500 μM) substitutions. Combination of a favorable *meta* substitution with the *para*-pyridyl substitution (**2**) improves the activity only in the case of the fluoro substitution, the least active compound (**20**), while the activities of the other compounds (**18**, **19**, and **21**) remain approximately constant.

Compounds substituted in the *ortho* position show a wide variability of their IC$_{50}$ values, ranging from 6.4 μM to no detectable activity. Apparently, large substituents are not allowed in the *ortho* position, even though docking results

suggest that these could extend to pocket B (Figure 2M). Hydroxyl (**23**, IC$_{50}$ = 15 μM) and amino (**26**, 60 μM) substitutions in the *ortho* position are much better tolerated than in the *meta* position, and they even lead to increased activity. This could be due to (i) better solvation, (ii) an intramolecular hydrogen bond formed between N3 of the triazole and the *o*-OH or *o*-NH$_2$ group (Figure 2K), or (iii) an intermolecular hydrogen bond formed between the ligand and Ser167. While the last hypothesis has been proposed for the *o*-OH-substituted 4-phenylimidazole analogue[46] and could also explain the inactivity of the *o*-methoxyl substituted ligand (**32**),
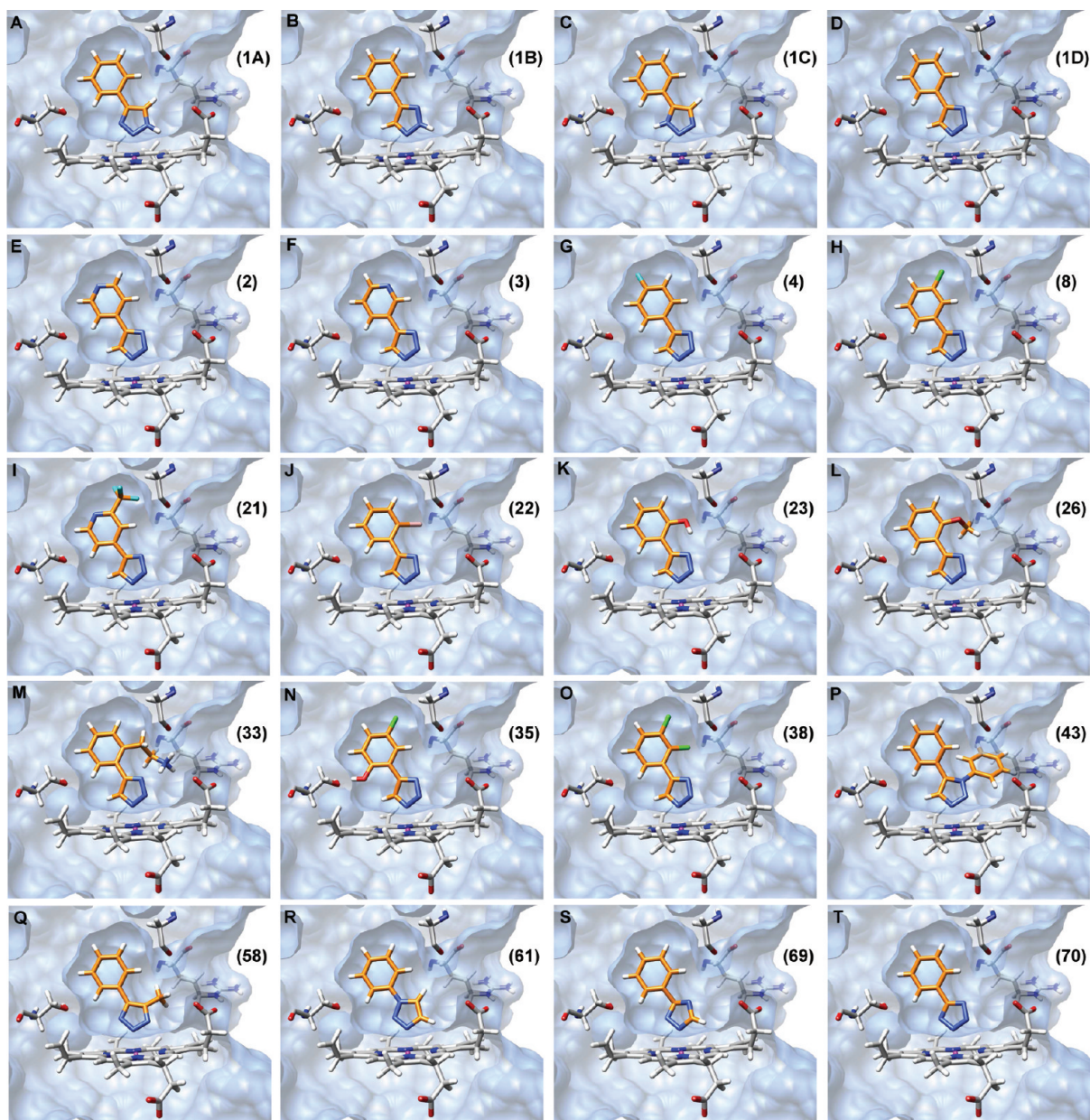
Figure 2. Proposed binding modes from docking results. The compound numbers are given in parentheses. Carbon atoms are shown in orange/gray, nitrogen in blue, oxygen in red, bromine in pink, chlorine in green, fluorine in cyan, iron in magenta, and hydrogen in white.
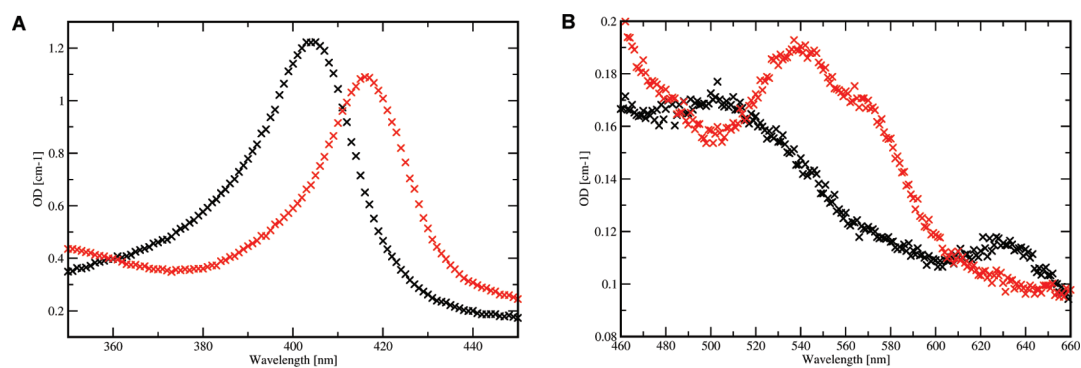
Figure 3. UV spectra of ferric IDO1 without (black) and with 1 mM concentration of compound **35** (red). In the presence of **35**, the Soret peak (A) shifts from 404 to 416 nm and the Q-bands (B) from 499 and 633 nm to 538 and 566 nm, demonstrating a direct binding of the ligand to the heme iron.

it is in discrepancy with the docked BM of compounds **23** and **26** (Figure 2K,L).

When testing doubly substituted compounds, we noted that the substitution effects are not additive. Four of these compounds, all featuring a *meta*-chloro substituent, showed a better activity than the parent compound (**1**). The 2,5-disubstituted compounds (**36** and **37**) were more potent than the corresponding 2,3-disubstituted compounds (**38** and **39**), which is probably due to steric constraints in the IDO1 binding pocket. Docking results favor the 2,3-disubstituted compounds (Figure 2O), as the 2,5-disubstituted compounds (**36** and **37**) fail to bind to the heme, highlighting the lack of binding site flexibility in our model. The only compound displaying a lower IC_{50} value than the *meta*-chloro substitution alone (**8**, 1.2 μM) is the 2-OH,5-Cl substituted compound (**35**, 330 nM, Figure 2N). Again, the *ortho*-hydroxyl substitution leads to an especially favorable activity.

Kinetic experiments with IDO1 inhibitors often yield uncompetitive or noncompetitive modes of inhibition even when there exists strong evidence, as in the case of PIM,[25,32] that they bind directly in the active site and should therefore be competitive with respect to the substrate L-Trp.[32,46,48,54,87] However, the interpretation of IDO1 inhibition kinetics may be complicated by the preferential binding of some inhibitors to the inactive ferric form of IDO1 and by the presence of a second substrate, dioxygen. To obtain a more direct indication of the binding site of our triazole compounds, we measured UV spectra of ferric IDO1 with and without compound **35** (Figure 3). The absorption spectrum of substrate-free ferric IDO1 exhibits a Soret peak at 404 nm and Q-bands at 499 and 633 nm (plus a shoulder at 533 nm) as described in the literature.[88] In presence of compound **35**, the Soret peak strongly shifts to 416 nm together with a hypochromic effect, while the Q-bands shift to 538 and 570 nm. This change in optical absorption is similar to the changes induced by the binding of PIM (peaks at 412.5, 533, and 560 nm) and of pyridine (peaks at 419, 538, and 570 nm) to IDO1, both of which have been confirmed to bind directly to the heme iron.[25,32]
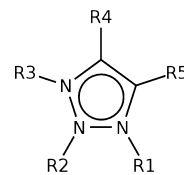
Compounds with Substituted Triazole Rings. Besides the parent compound 4-phenyl-1,2,3-triazole (**1**), we docked its isomer 1-phenyl-1,2,3-triazole (**61**) in the IDO1 active site (Figure 2R) and observed a close similarity to PIM and compound **1**, as all three compounds are sterically almost identical and provide a nitrogen atom with a free electron pair for heme binding. However, **61** does not show any inhibitory activity on IDO1 in the enzymatic assay (Table 2).

Additionally, we observed that any substitution of the triazole ring in position R1, R2, R3, or R5 in combination with the phenyl substitution in position R4 also completely abolished activity (Table 2). This is a significant difference to the imidazole analogues, where, for example, 1-benzyl-5-phenyl-imidazole is more active than PIM.[46] Taking these results together, they hint at the necessity of an ionizable NH group in the triazoles and at the hypothesis that the active form of the triazole for IDO1 inhibition could be its deprotonated, anionic form.

The inactivity of the 4,5-disubstituted compound **58** (Figure 2Q), which preserves the ionizable NH group of the triazole ring, could be related to its increased pK_a value (9.11 vs 8.62 for **1**, Table 4), due to the electron-donating substitution on the triazole ring, or it could be due to steric effects.

Compounds with Modified Scaffolds. Any attempt to replace the 1,2,3-triazole scaffold by bioisosteres such as 1,2,4-

Table 2. Triazole-Ring Substituted Derivatives

	R1	R2	R3	R4	R5	IC_{50} $[\mu M]^a$
1	H			phenyl	H	83
43			phenyl	phenyl	H	NI
44			phenyl	4-F-phenyl	H	NI
45			phenyl	3-CH$_3$-phenyl	H	NI
46			phenyl	3-Cl-phenyl	H	NI
47			phenyl	2-OCH$_3$-phenyl	H	NI
48			methyl	phenyl	H	NI
49	methyl			phenyl	H	NI
50		methyl		phenyl	H	NI
51			ethyl	phenyl	H	NI
52		ethyl		phenyl	H	NI
53	ethyl			phenyl	H	NI
54			n-butyl	phenyl	H	NI
55	n-butyl			phenyl	H	NI
56			benzyl	phenyl	H	NI
57	benzyl			phenyl	H	NI
58	H			phenyl	methyl	NI
59			methyl	3-Cl-phenyl	H	NI
60	methyl			3-Cl-phenyl	H	NI
61	phenyl			H	H	NI

^{a}IC$_{50}$ values are the mean of at least two independent assays.

triazole (**69**), tetrazole (**70**), isoxazole (**71**), pyridine (**72**), or pyrimidine (**73**) completely abolished IDO1 inhibitory activity (Table 3). Also compounds with linkers between the aromatic moieties (**62** and **63**), a cyclohexene analogue (**64**), and benzotriazoles (**65** and **66**) failed to show any activity.

Steric reasons may play a role for some of these compounds. For example, docking results for compounds **63** (filled volume of pocket A: 85%) and **65** (78%) show that these do not fill the IDO1 binding pocket as tightly as the other compounds (**1**: 96%). Compounds with two aromatic six-membered rings (**72** and **73**) apparently need an iron-binding nitrogen in 3-position, as only 3-phenylpyridine has been shown to be active on IDO1 (IC_{50} = 161 μM).[46]

Other compounds (**69**, **70**, and **71**), however, are sterically so similar to compound **1** that their inactivity on IDO1 must be due to electronic effects. Compound **69** (Figure 2S) is a closely related isomer of compound **1**, however, with a higher pK_a value (9.29 vs 8.62), which may prevent its deprotonation under experimental conditions. In contrast, the tetrazole (**70**, Figure 2T) has a very low pK_a value (4.53) and should therefore be deprotonated under experimental conditions. However, quantum chemical atomic point charges show that its electron density is mainly concentrated on N1 and N4 (charge −0.36), which are next to the phenyl ring, while N2 and N3, which are available for heme iron binding, only carry small negative charges (−0.17). In active compounds, the negative charge on the iron-binding nitrogen atom ranges from −0.33 to −0.46. Similarly to compound **70**, in the inactive isoxazole **71**, the nitrogen atom available for iron binding is

Table 3. Modified Scaffolds

	Structure	IC$_{50}$ [μM] ^{a}
62		NI
63		NI
64		NI
65		NI
66		NI
67		1800
68		NI
69		NI
70		NI
71		NI
72		NI
73		NI

^{a}IC$_{50}$ values are the mean of at least two independent assays.

very poor in electrons (charge −0.12) due to the electronegative neighboring oxygen atom.

pK_a Measurements. To better understand the electronic properties of the triazoles, we carried out pK_a measurements of selected compounds (Table 4). The parent compound (**1**) has a pK_a value of 8.62 for deprotonation of its triazole ring, 0.64 pK_a units more acidic than the unsubstituted 1,2,3-triazole (9.26).[89] Compounds with electron-withdrawing substituents on the phenyl ring are more acidic than **1**, because these substituents stabilize the negative charge on the triazole.

In Figure 4A, we plot the measured pK_a values as a function of the Hammett substituent constants σ,[90] assuming (i) that the influence of substituents is additive, $\sigma = \sigma_{ortho} + \sigma_{meta} + \sigma_{para}$, which is a common assumption,[90] and (ii) that the influence of a substituent is equal in *ortho* and in *para* position, $\sigma_{ortho} = \sigma_{para}$, which should be valid if steric hindrance is low and if no special interactions arise in *ortho* position.

For all compounds with *meta* and *para* substitutions (black stars in Figure 4A), a very good linear relationship is obtained:

$$pK_a = 8.607 - 0.787 \times \sigma \qquad (1)$$

with a correlation coefficient of 0.997. Compounds with a chloro substituent in *ortho* position (**24**, **37**, and **38**) follow this correlation quite closely, but compounds with an oxygen-containing substituent in *ortho* position (**24**, **37**, and **38**) are clear outliers of this relationship.

The *ortho*-methoxyl substituent of **32** leads to a more basic pK_a value than predicted from its σ value, suggesting a hydrogen bond between the neutral triazole and the substituent that renders deprotonation of the triazole less favorable. The

opposite is observed for compounds **23** and **35** featuring an *ortho*-hydroxyl substitution, which display more acidic pK_a values than predicted from their σ value, suggesting that here a hydrogen bond between the substituent and the anionic triazole stabilizes the negatively charged form of the triazole. This interpretation is further supported by the strong shift of one pK_a unit from 9.95 to 10.95 for the phenolic pK_a of compound **23**, as this hydrogen now serves to stabilize the monoanion.

All measured pK_a values are higher than the pH at which the enzymatic assay is carried out (6.5). However, the pK_a of a ligand in water does not necessarily reflect its pK_a in the protein-bound state. It has been shown, e.g., that thiols binding to CYP450-CAM show a pK_a shift of 4 units as compared to their values in water.[91] It is therefore possible that the triazoles bind to IDO1 in their deprotonated form. Taking into consideration the inactivity of all triazole compounds lacking an ionizable NH group (Table 2) or having a high pK_a value, this hypothesis becomes the most likely scenario.

We have further tested this hypothesis by carrying out an enzymatic assay at pH 7.4 instead of pH 6.5 for compound **35**. As expected, **35** shows a higher activity (IC$_{50}$ = 71 nM) at pH 7.4 than at pH 6.5 (IC$_{50}$ = 330 nM, see Figure S2 in the Supporting Information).

Quantitative Structure−Activity Relationship. Early on during our efforts to optimize the potency of the triazole ligands, we noticed a correlation between the activities of the *meta*-substituted compounds and the Hammett σ constants (r = 0.62), a correlation that could be even improved by adding the hydrophobicity constant π as a second parameter (r = 0.90, Figure 4B).[90] In this model, the more electron-withdrawing and the more hydrophobic the substituent on the phenyl ring, the better the potency of the triazole compound against IDO1. The influence of the hydrophobicity is not surprising, as docking suggests that the substituent in *meta* position will be placed inside a hydrophobic subpocket of the IDO1 active site (Figure 2H−J). The electronic properties of the substituent, however, control the electronic properties of the triazole ring, as exemplified by our pK_a measurements. Two possible interpretations of the correlation between IDO1 inhibitory activity and σ are therefore that (i) IDO1 inhibition of the triazoles increases with decreasing electron density on the triazole ring, possibly influencing the heme binding strength (heme-binding strength hypothesis), or (ii) IDO1 inhibition increases with decreasing pK_a of the triazole ring, because the ligand must be deprotonated in order to be active (deprotonation hypothesis). A combination of both effects is possible.

We investigated this question by quantum chemical calculations. The direct calculation of binding energies of the triazole compounds to a heme model system is a complex task due to the existence of six possible ligand−heme orientations of the three tautomeric forms and the deprotonated form of the ligands, which, in combination with two possible iron redox states (ferric and ferrous form), leads to 12 different binding energies. Therefore, the complete set of calculations was only carried out for the parent compound **1**, from which favorable ligand−heme orientations were obtained (details given in Supporting Information).

Taking solvation effects into account by a polarizable continuum model (PCM),[92] the neutral parent compound (**1**) binds more strongly to ferric heme (−4.5 kcal/mol, 3H tautomer binding with N1 to iron) than to ferrous heme (−0.3

Table 4. pKa Values and Hammett σ Values

Compound	pKa as Base	pKa as Acid [a]	σ [b]
Imidazole[89]	6.95	14.52	
4-Phenylimidazole (PIM)[89]	6.10	13.42	
1,2,3-Triazole[89]	1.17	9.26	
4-Phenyl-1,2,3-triazole (**1**)[133]	0.40	[c]	
Benzotriazole (**65**)[131]	1.60	8.60	
1,2,4-Triazole[89]	2.19	10.26	
3-Phenyl-1,2,4-triazole (**69**)[89]	2.05	9.29	
Tetrazole[89]		4.79	
5-Phenyltetrazole (**70**)[89]		4.53	
Pyridine[135]	5.23		
Phenol[135]		9.99	
(**1**)		8.62 (0.02)	0.00
(**2**)		7.85 (0.01)	0.94
(**3**)		8.14 (0.01)	0.55
(**58**)		9.11 (0.01)	
(**24**)		8.50 (0.01)	0.23
(**8**)		8.32 (0.01)	0.37
(**5**)		8.46 (0.01)	0.23
(**37**)		8.17 (0.03)	0.60
(**38**)		8.06 (0.01)	0.60
(**32**)		8.99 (0.02)	-0.27
(**15**)		8.38 (0.01)	0.34
(**20**)		7.59 (0.01)	1.28
(**18**)		7.60 (0.01)	1.31
(**16**)		8.46 (0.01, triazole)	0.12
		10.13 (0.02, phenol)	
(**23**)		8.58 (0.02, triazole)	-0.37
		10.95 (0.03, phenol)	
(**35**)		8.25 (0.01, triazole)	0.00
		10.56 (0.04, phenol)	

[a]Standard deviation given in parentheses. [b]The definition of the Hammett σ values is given in the text. [c]The acidic pKa value of 6.32 given in refs 89,134 goes back to a reference dating from the beginning of the 20th century and is obviously wrong.

kcal/mol, 2H tautomer binding with N1 to iron). These binding energies are significantly lower than the binding energies of the deprotonated form of **1** to both ferric (−17.0 kcal/mol, binding with N1 to iron) and to ferrous (−1.4 kcal/mol, binding with N1 to iron) heme. The neutral form of compound **8**, which has an electron-withdrawing *meta*-chloro substituent on the phenyl ring, binds even more weakly to ferric heme (−3.7 kcal/mol) and just as weakly to ferrous heme

(−0.4 kcal/mol) than the parent compound. Its deprotonated form shows similar binding energies for ferric heme (−16.7 kcal/mol) and for ferrous heme (−1.6 kcal/mol) as compound **1**.

In summary, quantum chemical calculations suggest that heme binding strength does not increase with decreasing electron density on the triazole ring but that the deprotonated form of the triazoles binds much more strongly to heme than
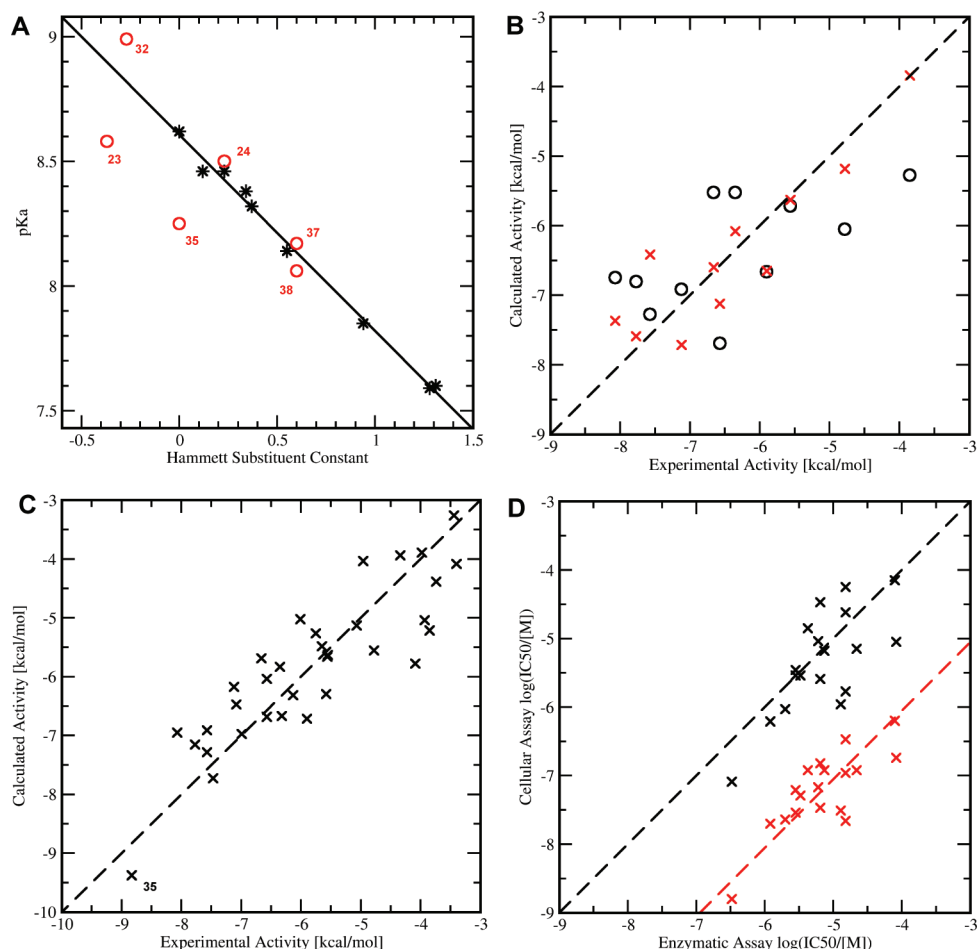
Figure 4. (A) Correlation between the Hammett substituent constant and the measured pK_a values, assuming (i) that the influence of substituents is additive, and (ii) that the influence of a substituent is equal in *ortho* and in *para* position. Compounds with a substitution in *ortho* position are shown as red circles. Compounds without a substitution in *ortho* position are shown as black stars, and the black line is a linear fit to these points ($r = 0.997$). (B) Correlation between experimental and calculated activities of *meta*-substituted compounds using the Hammett substituent constant alone (black, $r = 0.62$) or in combination with the hydrophobicity parameter (red, $r = 0.90$). The black dotted line denotes a perfect correlation. (C) QSAR relationship using the quantum chemical triazole charges and the electrostatic interaction/solvation term from the docking as descriptors (see text). (D) Correlation between the IC_{50} values measured in the enzymatic assay and the IC_{50} values measured in the cellular assays on hIDO1 (black) and on mIDO1 (red).

the neutral form. They support thus the deprotonation hypothesis.

In the following, we developed a quantitative structure−activity relationship (QSAR) for the triazole compounds based on our docking results. All standard terms such as electrostatic interactions, van der Waals interactions, and solvation terms are calculated based on the CHARMM22 classical force field[93,94] and the FACTS implicit solvent model[95] (see Experimental Section). However, this procedure does not include the electronic effects described above. We therefore decided to include an additional electronic term in the QSAR model. As the Hammett constants are only available for a subset of our compounds, we replaced them by an electronic parameter that could be determined for all compounds using quantum chemical calculations. After many trials, we chose to use the sum of atomic charges on the deprotonated triazole ring as calculated by natural population analysis with a large basis set (CH_{triz}) because this parameter proved to be robust and yields a good correlation ($r = 0.96$) with the Hammett σ constants for 15 compounds for which a σ is available[90] (**1**, **4−17**).

Of the 39 active triazoles described in this work, six could not satisfactorily be docked into the IDO1 active site because of

bulky substituents in *para* or *meta* positions (compounds **5**, **6**, **7**, **13**, **36**, and **37**, see filtering conditions in the Experimental Section) and were therefore removed from the test set. For the remaining set of 33 compounds, we obtained the best QSAR model when using two simple parameters in the regression analysis: the sum of the ligand−protein electrostatic interaction energy and the electrostatic desolvation energy on the one hand (V_{elec}), and the quantum chemical charge on the triazole (CH_{triz}) on the other hand. With these two parameters a correlation coefficient of 0.87 and a standard deviation of 0.73 kcal/mol is obtained (Figure 4C).

$$Act = -38.79 + 0.60 \times V_{elec} - 34.48 \times CH_{triz} \qquad (2)$$

where *Act* and V_{elec} are given in kcal/mol and CH_{triz} is given in elementary charge units. Compound **35** has a prominent location on the correlation plot (Figure 4C). However, its omittance from the test set only marginally changes the correlation ($r = 0.85$, stdv = 0.73 kcal/mol). Addition of terms for the van de Waals interactions and hydrophobic desolvation does not improve the correlation, probably because these terms show little variation between the described compounds, which all tightly occupy only the A-pocket of the IDO1 active site.
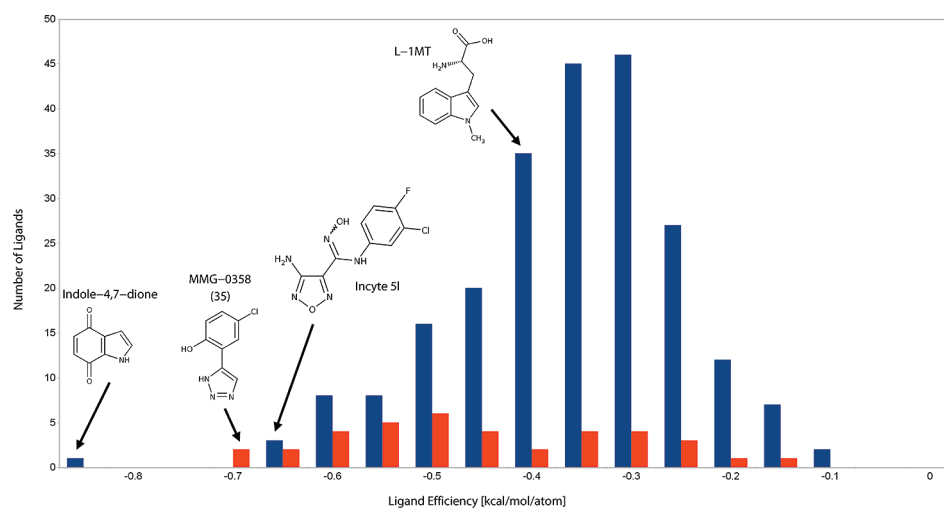
Figure 5. Ligand efficiency [kcal/mol/atom] of known IDO1 ligands (blue) and the compounds described in this work (red). Ligands that probably interact with the cystein residues of IDO1[44,49] were not included. Indole-4,7-dione[41] probably acts through a redox mechanism.

Table 5. Cellular Assays for IDO1 and TDO Inhibition[a]

	enzym IC$_{50}$ [μM]	cell IC$_{50}$ [μM][b]				LD$_{50}$ [μM]
	hIDO1	mIDO1	hIDO1	mTDO	hTDO	
1	83	0.18 (0.05)	8.9 (0.8)	>100	>100	>100
4	190	2.1 (1)	>100	>100	>100	>100
8	1.2	0.020 (0.003)	0.62 (0.01)	>100	>100	>100
9	2.0	0.023 (0.009)	0.94 (0.07)	>100	>100	>100
10	2.8	0.029 (0.006)	2.9 (0.6)	>100	>100	>100
11	6.0	0.07 (0.03)	9 (4)	>100	>100	>100
12	13	0.03 (0.02)	1.1 (0.1)	>100	>100	>100
13	15	0.11 (0.02)	24 (0.1)	>100	>100	>100
14	22	0.12 (0.04)	7 (3)	>100	>100	>100
15	47	1.1 (0.4)	>100	>100	>100	>100
16	310	1.3 (0.6)	>100	>100	>100	>100
17	1500	4 (2)	>100	>100	>100	>100
18	2.8	0.06 (0.02)	3.5 (0.5)	>100	>100	>100
19	3.3	0.05 (0.03)	2.9 (0.4)	>100	>100	>100
20	7.4	0.12 (0.01)	7 (1)	>100	>100	>100
21	15	0.34 (0.05)	56 (2)	>100	>100	>100
22	6.4	0.034 (0.001)	2.6 (0.1)	>100	>100	>100
23	15	0.022 (0.001)	1.7 (0.1)	>100	>100	>100
24	32	1.5 (0.3)	>100	>100	>100	>100
25	39	1.7 (0.1)	>100	>100	>100	>100
26	60	1.7 (0.4)	>100	>100	>100	>100
27	80	0.63 (0.09)	70 (20)	>100	>100	>100
28	230	13 (2)	>100	>100	>100	>100
29	650	20 (1)	>100	>100	>100	>100
32	NI	20 (1)	>100	>100	>100	>100
35	0.33	0.002 (0.002)	0.08 (0.02)	>100	>100	>100
36	4.3	0.12 (0.04)	14 (4)	>100	>100	>100
37	6.4	0.15 (0.04)	34 (2)	>100	>100	>100
38	23	1.7 (0.1)	>100	>100	>100	>100
Incyte **5I**[51]	ND	0.10 (0.07)	0.06 (0.03)	60 (30)	70 (20)	>100
LM10[18]	ND	>100	>100	17	28	>100

[a]IC$_{50}$ values of compounds tested in cells transfected with mouse IDO1 (mIDO1), human IDO1 (hIDO1), mouse TDO (mTDO), or human TDO (hTDO). Standard deviations are given in parentheses. Cytotoxicity of the compounds was tested on Hep G2 cells (LD$_{50}$). For comparison, also the IC$_{50}$ values of the enzymatic assay on hIDO1 are given again. ND: not determined. NI: no inhibition. [b]IC$_{50}$ values are the mean of at least two independent assays.

It is important to notice that the QSAR model does not rely on either the heme-binding strength or the deprotonation hypothesis, but it is valid for both cases.

According to this model, compound **35** is highly active on IDO1 because it displays very good electrostatic interactions with the protein while carrying a low charge on the triazole

ring. Interestingly, in the docked pose (Figure 2N), there is no intramolecular hydrogen bond present, but the hydroxyl group forms a hydrogen bond with Ser167 instead. As MMG-0358 (compound **35**) is is very small, it shows an excellent ligand efficiency[96] of −0.72 kcal/mol/atom, ranking among the most efficient IDO1 inhibitors known to date (Figure 5).

Comparison to Imidazole and Pyrazole Analogues. Although compound **1** is a close analogue of PIM and docks in a similar manner to IDO1, the triazoles and imidazoles differ substantially in their structure−activity relationship for IDO1 inhibition. For example, the disubstituted imidazole compound 1-benzyl-5-phenylimidazole shows a slightly better IC$_{50}$ value than PIM (32 vs 48 μM),[46] while the direct analogue 1-benzyl-5-phenyl-1,2,3-triazole (**56**) is inactive. Replacement of the phenyl ring by a *p*-pyridyl ring in the triazole (**2**) leaves its activity unaffected, while the same replacement in the imidazole strongly reduces its activity (**67**). Despite these dissimilarities, there are also similarities, as the general drop in activity when introducing substituents in *para* position of the phenyl ring, and the much higher activity of compounds with *ortho*-hydroxyl as compared to *meta*-hydroxyl substituted phenyl rings.[46] While the similarities are probably due to the similar binding modes and similar protein interactions in the IDO1 active site, the differences can be explained by distinctive electronic structures and heme interactions. PIM is a much stronger base and a much weaker acid than compound **1** (Table 4), which correlates with the higher charge density on the heme-binding nitrogen of the imidazole (charge −0.46) as compared to 1,2,3-triazole (−0.24 neutral, −0.35 deprotonated). It is therefore active on IDO1 without deprotonation, so that the N3 position is available for additional substitutions, and so that electron-withdrawing substituents on the phenyl ring do not show the same effect as in case of the triazoles.

A close analogue of compound **35** is the previously published 4-fluoro-2-(pyrazol-3-yl)phenol, featuring a pyrazole with a 2-OH,5-F substituted phenyl ring and an IC$_{50}$ value of 26 μM.[46] The about 100-fold higher activity of compound **35** on IDO1 could be explained by the better protein interactions of chloride vs fluoride and by the stronger heme bond of the triazole (−14.8 kcal/mol) vs the pyrazole (−8.4 kcal/mol, see Supporting Information).

Cellular Assays. We tested compounds with a low IC$_{50}$ in the enzymatic assay for their ability to inhibit tryptophan degradation and kynurenine production in cells expressing either murine (mIDO1) or human IDO1 (hIDO1). Such cellular assay is informative for drug development, as it evaluates not only the IDO1 inhibitory effect of the compounds but also their capacity to permeate the cell, their potential cytotoxicity, inhibition of tryptophan and kynurenine transport, and interactions with other proteins as well as the effects of their metabolites. To control specificity, we also tested the inhibitory activity of the compounds on cells expressing murine (mTDO) or human TDO (hTDO). The same murine cell type, stably transfected with murine or human IDO1 or TDO, was used for all assays. Additionally, cell viability was evaluated using Hep G2 cells.

Most compounds active in the enzymatic assay were also active in the cellular assay, usually with lower IC$_{50}$ values (Table 5 and Figure 4D), in line with previous observations.[51,54] In the present case this might be due to the higher pH employed in the cellular assays (7.4) as compared to the enzymatic assays (6.5), favoring deprotonation of the triazole ligands. The IC$_{50}$ values for the triazole compounds are in the micromolar to

nanomolar range, with a good selectivity as no inhibition was observed in TDO-expressing cells. Strikingly, we consistently observed substantially higher inhibitory activities in the cellular assay performed with murine IDO1 as compared to human IDO1, with IC$_{50}$ values on average 77-fold lower. This was not related to the cell type because we used the same murine cell line transfected with mIDO1 or hIDO1 for these assays. We also used human cells HEK293 transfected with hIDO1 and observed similar IC$_{50}$ values as with the murine cells transfected with hIDO1 (data not shown). These differences therefore likely reflect genuine variation in the inhibition of murine versus human IDO1 by this family of compounds. Murine IDO1 shares 62% of sequence identity with human IDO1, and the active site residues mentioned in the Introduction are 100% conserved. However, the orthologues show some distinct biochemical properties, as, for example, mIDO1 displays a 3-fold lower K_m for L-Trp than hIDO1.[97] Despite these differences, an excellent correlation between the cellular assays on mIDO1 and on hIDO1 is observed ($r = 0.95$). Additionally, the observed good correlation between enzymatic and cellular assay results (Figure 4D, $r = 0.71$ for enzym. hIDO1/cell. hIDO1, and $r = 0.79$ for enzym hIDO1/cell mIDO1) enables application of the developed QSAR model also for optimization of cellular activities.

The most promising IDO1 inhibitor reported to date is Incyte compound **5l**,[51] with IC$_{50}$ values of 67 nM in an enzymatic assay and 19 nM in a cellular assay on HeLa cells. In our cellular assays, compound **35** displays a similar inhibitory effect on hIDO1 as **5l** (80 and 60 nM respectively, Table 5) and therefore ranks among the most potent IDO1 inhibitors described so far.

■ SUMMARY

In this work, we have used an in silico driven strategy for the rational optimization of IDO1 inhibitors based on the 4-aryl-1,2,3-triazole scaffold discovered in our earlier work.[53]

New ligands were designed computationally, docked into the IDO1 active site, selected in case of favorable interactions, synthesized, and evaluated for IDO1 inhibition potency in an enzymatic assay. Successful candidates were subsequently tested in cellular assays on mIDO1, hIDO1, mTDO, and hTDO. Bioisosteres of the 4-phenyl-1,2,3-triazole scaffold were also investigated. Using the results of the enzymatic assay, we developed a QSAR model based on the docking results and on quantum chemical calculations. Following this strategy, we obtained 39 triazole-based IDO1 inhibitors, the most potent having an IC$_{50}$ value in the nanomolar range both in the enzymatic and in the cellular assays while being inactive on TDO and showing no detectable toxicity on the cellular level.

Some modifications of the 4-aryl-1,2,3-triazole scaffold have very recently been described independently by another group.[54] Among the 20 compounds described in their work, two were described by us before (**1** and **3**) and seven were also synthesized and tested in the present work (**4**, **5**, **6**, **22**, **24**, **32**, and **65**), while the remaining 11 compounds are either only weakly active or inactive. The enzymatic IC$_{50}$ values measured in our lab generally differ by a factor 2−3 from the values of Huang et al., which is probably due to different experimental conditions. The only outlier is compound **22**, for which the IC$_{50}$ values differ significantly (148[54] vs 7 μM here). The inhibition mode of compounds **1**, **22**, and **24** was determined to be uncompetitive.[54] However, the UV spectra of IDO1 in

presence of compound **35** measured here (Figure 3) support the notion that the triazoles bind directly to the heme iron.

As the compounds described in this work are very small, they might suffer from a low selectivity profile. However, their low cellular toxicity and inactivity on TDO, which shares a common active site architecture with IDO1, support their further development.

In summary, we have used our in-house docking tools to successfully design new IDO1 inhibitors with a good efficacy in enzymatic and in cellular assays. Our most potent compound (MMG-0358, **35**) shows IC$_{50}$ values of 2 nM in a cellular assay on mIDO1, 80 nM in a cellular assay on hIDO1, 330 nM in an enzymatic assay on hIDO1 at pH 6.5, and 71 nM in an enzymatic assay on hIDO1 at pH 7.4. This compound, which additionally has an excellent ligand efficiency[96] of −0.72 kcal/mol/atom, will be evaluated in vivo in the future. The QSAR model derived from the collection of our evaluated ligands will be helpful in the future to design even more potent and specific IDO1 inhibitors.

■ EXPERIMENTAL SECTION

Docking. A consensus docking procedure based on our in-house docking algorithms EADock,[55,56] EADock DSS,[57] and AttractingCavities[70] was used. In the latter, in brief, an extended conformation of the ligand is minimized in the cavities of the protein, starting from different positions and orientations, in an approach similar to that of MCSS.[98] Minimized poses are finally clustered and ranked according to the scoring function of EADock. A detailed description of this algorithm will be the subject of a future communication.

All three algorithms rely on the physical scoring function of the CHARMM22 force field,[93,94] while solvation effects are taken into account by the FACTS model,[95] which has been shown to allow for accurate docking results.[59] Ligand force-field parameters were derived with the SwissParam tool.[60] For all compounds with an acidic pK_a value of 9.5 or below, the deprotonated species was considered. A Morse-like metal binding potential (MMBP) was used to describe the interactions between the heme iron of IDO1 and ligand atoms that display a free electron pair for iron binding.[61] The protein was kept fixed during the docking. We use the PIM-bound X-ray structure (2D0T, chain A) as a target because it has a higher resolution and provides a more suitable induced fit for the triazole ligands than the cyanide-bound structure (2D0U).[25] The two molecules of *N*-cyclohexyltaurine bound at the entrance of the binding site were removed during setup.

For AttractingCavities, the standard parameters with a cubic search space of (20 Å)^3 around the IDO1 active site were used. For EADock DSS, docking results were compared to EADock 2.0 results, and in order to well reproduce the benchmarks, the following custom settings were used: 30000 generated binding modes (BM), 12000 BM evaluated with FullFitness, 100 steepest descent minimization steps, 1000 adopted basis Newton−Raphson minimization steps, 1.5 Å clustering radius, and a cubic search space of (20 Å)^3 around the IDO1 active site. Subsequently, the 250 best BM produced by EADock DSS were fed as input to EADock 2.0, using the same clustering radius and terminating the run when the first three clusters did not change over the course of 100 generations of the evolutionary algorithm.

For analysis, the results of AttractingCavities and EADock 2.0 were merged by comparing the best BM of each cluster and considering them distinct if their mass-weighted rmsd was larger than 0.8 Å. The resulting 10 best distinct BM were filtered to detect energetically favorable poses with a direct bond to the heme iron and a good filling of the A-pocket, criteria that were deemed necessary for IDO1 inhibition by the investigated scaffold. The following filters were applied: (i) a FullFitness that is maximally 5 kcal/mol higher than the one of the globally best BM, (ii) an iron-heteroatom (N, O, S) bond of 3 Å or shorter, (iii) a minimal distance between the ligand and the sulfur atom of Cys129 of less than 5.2 Å, and (iv) a volume filling of

the A-pocket of at least 75%. Finally, for each compound the BM having the lowest FullFitness and meeting all conditions was chosen for analysis.

Density Functional Theory Calculations. Quantum chemical geometry optimizations and charge calculations were carried out in the density functional theory (DFT) framework with the PBE0 hybrid functional[99] using the Gaussian 09 code.[100] Geometry optimizations were carried out with standard settings and the TZVP basis set,[101] while atomic point charges derived from a natural population analysis (NPA)[102] were calculated with the aug-cc-pVTZ basis set[103−105] in order to obtain converged charges.[106] Solvation effects were taken into account by the polarizable continuum model[92] as implemented in Gaussian 09.

For compound **1**, the 2-H tautomer was the most stable both in vacuo and in the PCM. For charge calculations for the QSAR, compounds with an acidic pK_a value of 9.5 or below were considered in their deprotonated form.

The histidine-bound heme complex of IDO1 was modeled by an iron−porphin−imidazole system. Binding energies were calculated by subtracting the energy of the iron−porphin−imidazole system and the energy of the isolated ligand from the energy of the 6-fold coordinated system. For the ferric iron−porphin−imidazole system, the ground state with the hybrid DFT functional PBE0 is a quartet, while for the ferrous system it is a quintuplet. For the 6-fold coordinated systems, a low-spin complex was always assumed, as it has been found experimentally.[32]

Quantitative Structure−Activity Relationship. The binding free energy ΔG of an inhibitor is related to its K_i value by the equation $\Delta G = -RT \ln K_i$. As the K_i of an inhibitor is linearly related to its IC$_{50}$ value and the prefactor only depends on enzyme concentration, substrate concentration, and K_m of the substrate,[107,108] we can calculate the activity of an inhibitor as $Act = -RT \ln$ IC$_{50}$. Differences in the calculated activities of different inhibitors will then be equal to $\Delta\Delta G$.

For the QSAR model, we carried out a linear regression with two variables, using the activities as target values. The first variable is the sum of the NPA charges of all atoms belonging to the deprotonated triazole ring. The second variable, V$_{elec}$, is

$$V_{elec} = \Delta G_{solv,elec} + E_{inter,elec} - E_{corr,elec} \qquad (3)$$

with the electrostatic solvation term

$$\Delta G_{solv,elec} = \Delta G_{solv,elec}^{complex} - \Delta G_{solv,elec}^{ligand} - \Delta G_{solv,elec}^{protein} \qquad (4)$$

as calculated by the FACTS solvation model.[95] $E_{inter,elec}$ is the electrostatic interaction energy between the ligand and the protein calculated with the Coulomb equation and a dielectric constant of 2 as appropriate for the use of FACTS. $E_{corr,elec}$ is a correction term necessary to counterbalance the use of the MMBP and equals the electrostatic interaction energy between the ligand on the one hand and the iron and nitrogen atoms of the heme on the other hand.

Chemistry. *General Remarks.* Materials and reagents were obtained from commercial suppliers and used without further purification. For extraction and chromatography, all solvents were distilled prior to use. Thin layer chromatography for reaction monitoring was performed on silica gel plates (Merck 60 F254) with detection by UV light (254 nm) and charring with KMnO$_4$ or Pancaldi reagent. Flash chromatography was conducted using silica gel 60 Å, 230−400 mesh (Merck 9385). Melting points were measured with a Mettler FP52 apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR spectrometer. Mass spectra were recorded on a Nermag R 10−10C instrument in chemical ionization mode. Electrospray mass analyses were recorded on a Finnigan MAT SSQ 710C spectrometer in positive ionization mode. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker-DPX-400 or Bruker-ARX-400 spectrometer at 400 and 100.6 MHz, respectively. Data for ^{1}H NMR spectra are reported as follows: chemical shift, multiplicity, coupling constant, and integration. Data for ^{13}C NMR spectra are reported in terms of chemical shifts. Chemical shifts are given in parts per million, relative to an internal standard such as

residual solvent signals. Coupling constants are given in hertz. High-resolution mass spectra were recorded via ESI-TOF-HRMS or MALDI-TOF-HRMS. The purity of all novel compounds was confirmed to exceed 95% by NMR and high-resolution mass spectra. Elemental analysis for C, H, and N was performed by Quantitative Technologies Inc.

General Procedures. Representative Procedure for the Preparation of Phenylalkynes.[72] To a stirred solution of iodo derivative (Scheme 1, **A**, 1 mmol, 1 equiv) and Et$_3$N (4 mmol, 4 equiv) in dioxane (4 mL) were added trimethylsilyl acetylene (1.3 mmol, 1.3 equiv), PdCl$_2$(PPh$_3$)$_2$ (0.01 mmol, 0.01 equiv), and CuI (0.02 mmol, 0.02 equiv). The reaction mixture was stirred at 45 °C for 5 h under nitrogen. Diethyl ether (5 mL) and 0.1 N HCl (3 mL) were added, and the organic layer was separated, neutralized with a saturated NaHCO$_3$ (3 mL, twice) solution, washed with brine (3 mL), dried over Na$_2$SO$_4$, and evaporated. The residue (**B**) was added to KF (3.6 mmol, 3.6 equiv) dissolved in MeOH (5 mL), and the reaction mixture was stirred for 3 h at room temperature. The reaction mixture was concentrated, and CH$_2$Cl$_2$ (5 mL) and water (3 mL) were added. The organic layer was collected, dried (MgSO$_4$), and filtered through a short silica plug to afford desired (ethynyl) derivative (**C**) compounds; overall yields up to 85%.

2-Ethynylphenol.[72] Synthesized from 2-iodophenol according to the representative procedure[72] to afford the title compound as a yellowish oil in 85% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.40 (dd, 1H, *J* = 7.5 Hz, 1.7 Hz), 7.33−7.30 (m, 1H), 6.98 (d, 1H, *J* = 8.5 Hz), 6.90 (td, 1H, *J* = 7.5 Hz, 1.0 Hz), 5.81 (s, 1H), 3.50 (s, 1H).

1-Ethyl-2-ethynylbenzene.[109] Synthesized from 1-ethyl-2-iodobenzene according to the representative procedure[72] to afford the title compound as a yellow oil in 80% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.48 (d, 1H, *J* = 7.5 Hz, 1.7 Hz), 7.29 (td, 1H, *J* = 7.4 Hz, 1.5 Hz), 7.23 (d, 1H, *J* = 7.1 Hz), 7.15 (td, 1H, *J* = 7.5 Hz, 1.2 Hz), 3.25 (s, 1H), 2.84 (q, 2H, *J* = 7.5 Hz), 1.26 (t, 3H, *J* = 7.5 Hz).

3-Ethynylbenzonitrile.[110] Synthesized from 2-iodobenzonitrile according to the representative procedure[72] to afford the title compound as a yellow oil in 80% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.79 (s, 1H), 7.73 (dt, 1H, *J* = 7.9 Hz, 1.3 Hz), 7.66 (dt, 1H, *J* = 7.9 Hz, 1.3 Hz), 7.48 (t, 1H, *J* = 7.9 Hz), 3.22 (s, 1H).

1-Ethyl-3-ethynylbenzene.[111] Synthesized from 1-ethyl-3-iodobenzene according to the representative procedure[72] to afford the title compound as yellow oil in 75% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.36−7.30 (m, 2H), 7.25−7.14 (m, 2H), 3.06 (s, 1H), 2.64 (q, 2H, *J* = 7.5 Hz), 1.24 (t, 3H, *J* = 7.6 Hz).

4-Ethynyl-2-fluoropyridine.[112] Synthesized from 2-fluoro-4-iodopyridine according to the representative procedure[72] to afford the title compound as colorless oil in 65% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.21 (d, 1H, *J* = 5.0 Hz), 7.24 (d, 1H, *J* = 5.0 Hz), 7.01 (s, 1H), 3.38 (s, 1H).

2-Chloro-4-ethynylpyridine.[113] Synthesized from 2-chloro-4-iodopyridine according to the representative procedure[72] to afford the title compound as a yellow solid in 60% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.38 (d, 1H, *J* = 5.0 Hz), 7.41 (s, 1H), 7.28 (dd, 1H, *J* = 5.0 Hz, 1.2 Hz), 3.37 (s, 1H).

1,2-Dichloro-3-ethynylbenzene.[114] Synthesized from 1,2-dichloro-3-iodobenzene according to the representative procedure[72] to afford the title compound as brown semisolid in 65% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.46 (d, 2H, *J* = 8.0 Hz), 7.17 (t, 1H, *J* = 8.0 Hz), 3.38 (s, 1H).

1,4-Dichloro-2-ethynylbenzene.[115] Synthesized from 1,4-dichloro-2-iodobenzene according to the representative procedure[72] to afford the title compound as an off-white solid in 73% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.52 (d, 1H, *J* = 2.52 Hz), 7.34 (d, 1H, *J* = 8.5 Hz), 7.26 (dd, 1H, *J* = 8.5 Hz, 2.5 Hz), 3.42 (s, 1H).

2-Ethynyl-N-methylaniline.[116] Synthesized from 2-iodo-N-methyl-aniline according to the representative procedure[72] to afford the title compound as a brown oil in 72% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.34 (dd, 1H, *J* = 7.6 Hz, 1.5 Hz), 7.28−7.23 (m, 1H), 6.65−6.58 (m, 2H), 4.68 (bs, 1H), 3.41 (s, 1H), 2.91 (s, 3H).

2-Ethynylbenzaldehyde.[117] Synthesized from 2-iodobenzaldehyde according to the representative procedure[72] to afford the title

compound as an off-white solid in 85% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 10.55 (s, 1H), 7.94 (d, 1H, *J* = 7.6 Hz), 7.63 (dd, 1H, *J* = 7.2, Hz, 1.1 Hz), 7.58 (td, 1H, *J* = 7.5 Hz, 1.5 Hz), 7.49 (t, 1H, *J* = 7.6 Hz), 3.47 (s, 1H).

3-Ethynyl-4-methylaniline.[118] Synthesized from 3-iodo-4-methyl-aniline according to the representative procedure[72] to afford the title compound as a brown oil in 70% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.01 (d, 1H, *J* = 8.1 Hz), 6.85 (d, 1H, *J* = 2.5 Hz), 6.64 (dd, 1H, *J* = 5.5 Hz, 2.5 Hz), 3.67 (br s, 1H), 3.24 (s, 1H), 2.36 (s, 3H).

4-Chloro-2-ethynylphenol. Synthesized from 4-chloro-2-iodophenol according to the representative procedure[72] to afford the title compound as a colorless oil in 70% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.35 (d, 1H, *J* = 2.5 Hz), 7.24 (dd, 1H, *J* = 8.7 Hz, 2.5 Hz), 6.90 (d, 1H, *J* = 8.8 Hz), 5.76 (s, 1H), 3.52 (s, 1H).

4-Chloro-2-ethynyl-1-methylbenzene.[119] Synthesized from 4-chloro-2-iodo-1-methylbenzene according to the representative procedure[72] to afford the title compound as a yellow oil in 60% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.46 (d, 1H, *J* = 2.2 Hz), 7.24 (dd, 1H, *J* = 6.0 Hz, 2.3 Hz), 7.15 (d, 1H, *J* = 8.1 Hz), 3.33 (s, 1H), 2.44 (s, 3H).

General Procedure for N-Unsubstituted 1,2,3-Triazoles.[71] To a stirred solution of commercially available or synthetically prepared ethynyl substrate (1 mmol, 1 equiv) and CuI (0.05 mmol, 0.05 equiv) in DMF/MeOH solution (2 mL, 9:1) under an argon atmosphere, trimethylsilyl azide was added (1.5 mmol, 1.5 equiv). The resulting solution was stirred at 100 °C for 10−12 h. After consumption of the ethynyl substrate, the mixture was cooled to room temperature and the precipitate was filtered and concentrated under reduced pressure. The crude residue was purified with silica gel column chromatography to obtain the desired product.

General Procedure for the Preparation of Triazoles 48, 49, 50, 51, 52, and 53 by N-Alkylation of 4-Phenyl-1H-1,2,3-triazole.[82−84] To a solution of 4-phenyl-1*H*-1,2,3-triazole (1 mmol, 1 equiv) in DMF (3 mL), K$_2$CO$_3$ (0.25 mmol, 0.25 equiv) was added. The mixture was stirred for 5 min at room temperature. Dimethyl or diethyl carbonate (2.2 mmol, 2.2 equiv) was added, and the mixture was stirred at reflux (around 145 °C) for 5 h. The resulting solution was filtered, and the remaining solid material was washed with Et$_2$O (5 mL). Evaporation of the solvent afforded an oily residue. From the crude oil the different regioisomers were separated by column chromatography on silica gel to give compounds **48**, **49**, **50**, **51**, **52**, and **53** in 7, 60, 20, 6, 19, and 55% yield respectively.

General Procedure for the Preparation of Triazoles 59 and 60 by N-Methylation of 4-(3-Chlorophenyl)-1H-1,2,3-triazole.[82] To a solution of **8** (1 mmol, 1 equiv) in DMF (4 mL), K$_2$CO$_3$ (1 mmol, 1 equiv) was added. The mixture was stirred for 5 min at room temperature. Iodomethane (1 mmol, 1 equiv) was added, and the mixture was stirred at room temperature for 24 h. The resulting solution was filtered, and the remaining solid material was washed with Et$_2$O (4 mL). Evaporation of the solvent afforded an oily residue. From the crude oil, the different regioisomers were separated by column chromatography on silica gel to give compounds **59** and **60** in 7 and 60% yield, respectively.

4-Phenyl-1H-1,2,3-triazole (1).[71] Synthesized from ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **1** as a white solid in 65% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.34 (br s, 1H), 8.00 (s, 1H), 7.84 (d, 2H, *J* = 7.6 Hz), 7.47 (t, 2H, *J* = 7.6 Hz), 7.43−7.37 (m, 1H).

4-(1H-1,2,3-Triazol-4-yl)pyridine (2).[62] Synthesized from 4-ethynylpyridine[71] and TMSN$_3$ according to the general procedure to afford **2** as a white solid in 40% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.60 (d, 2H, *J* = 6.0 Hz), 8.41 (br s, 1H), 7.91 (d, 2H, *J* = 6.0 Hz).

3-(1H-1,2,3-Triazol-4-yl)pyridine (3).[62] Synthesized from 3-ethynylpyridine[71] and TMSN$_3$ according to the general procedure to afford **3** as a white solid in 60% yield. ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$OD): δ 8.96 (s, 1H), 8.51 (d, 1H, *J* = 4.8 Hz), 8.14 (d, 1H, *J* = 7.8 Hz), 7.99 (s, 1H), 7.42−7.38 (m, 1H).

4-(4-Fluorophenyl)-1H-1,2,3-triazole (4).[120] Synthesized from 1-ethynyl-4-fluorobenzene[71] and TMSN$_3$ according to the general procedure to afford **4** as a white solid in 62% yield. ^{1}H NMR (400

MHz, CDCl$_3$): δ 11.65 (s, 1H), 7.95 (s, 1H), 7.85−7.80 (m, 2H), 7.21−7.14 (m, 2H).

4-(4-Chlorophenyl)-1H-1,2,3-triazole (5).[62] Synthesized from 1-chloro-4-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **5** as a white solid in 65% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.66 (s, 1H), 7.98 (s, 1H), 7.79 (d, 2H, J = 8.4 Hz), 7.46 (d, 2H, J = 8.4 Hz).

4-(p-Tolyl)-1H-1,2,3-triazole (6).[62] Synthesized from 1-ethynyl-4-methylbenzene[71] and TMSN$_3$ according to the general procedure to afford **6** as a white solid in 72% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.07 (s, 1H), 7.69 (d, 2H, J = 7.9 Hz), 7.24 (d, 2H, J = 7.9 Hz), 2.35 (s, 3H).

4-(4-(Trifluoromethyl)phenyl)-1H-1,2,3-triazole (7).[121] Synthesized from 1-ethynyl-4-(trifluoromethyl)benzene[71] and TMSN$_3$ according to the general procedure to afford 7 as a white solid in 55% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.77 (s, 1H), 8.06 (s, 1H), 7.98 (d, 2H, J = 8.2 Hz), 7.74 (d, 2H, J = 8.2 Hz).

4-(3-Chlorophenyl)-1H-1,2,3-triazole (8).[121] Synthesized from 1-chloro-3-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **8** as a white solid in 70% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.79 (s, 1H), 8.0 (s, 1H), 7.86 (s, 1H), 7.74 (d, 2H, J = 7.5 Hz), 7.45−7.36 (m, 2H).

4-(3-Bromophenyl)-1H-1,2,3-triazole (9).[62] Synthesized from 1-bromo-3-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **9** as a white solid in 68% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.87 (s, 1H), 8.02 (s, 1H), 8.0 (s, 1H), 7.78 (d, 1H, J = 7.8 Hz), 7.53 (d, 1H, J = 8.1 Hz), 7.35 (t, 1H, J = 8.0 Hz).

3-(1H-1,2,3-Triazol-4-yl)benzonitrile (10).[122] Synthesized from 3-ethynylbenzonitrile[71] and TMSN$_3$ according to the general procedure to afford **10** as a off-white solid in 54% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.73 (s, 1H), 8.16 (s, 1H), 8.09 (d, 1H, J = 7.8 Hz), 8.04 (s, 1H), 7.71−7.65 (m, 1H), 7.60 (t, 1H, J = 7.8 Hz).

4-(3-(Trifluoromethyl)phenyl)-1H-1,2,3-triazole (11).[123] Synthesized from 1-ethynyl-3-(trifluoromethyl)benzene[71] and TMSN$_3$ according to the general procedure to afford **11** as a white solid in 55% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.85 (s, 1H), 8.12 (s, 1H), 8.06 (s, 1H), 8.04 (d, 1H, J = 7.8 Hz), 7.67 (d, 1H, J = 7.9 Hz), 7.61 (t, 1H, J = 7.8 Hz).

4-(3-Ethylphenyl)-1H-1,2,3-triazole (12). Synthesized from 1-ethyl-3-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **12** as a yellow oil in 71% yield. IR (film): ν 3136, 2964, 1613, 1476, 796 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.90 (s, 1H), 8.0 (s, 1H), 7.70 (br s, 1H), 7.65 (d, 1H, J = 7.5 Hz), 7.40 (t, 1H, J = 7.7 Hz), 7.26 (d, 1H, J = 7.7 Hz). ^{13}C NMR (100 MHz, CDCl$_3$): δ 147.0, 145.2, 129.6, 129.1 128.5, 125.8, 123.6, 29.0, 15.6. ESI-TOF-HRMS: m/z calcd for (M + H) C$_{10}$H$_{11}$N$_3$, 174.1031; found, 174.1024.

4-(3-Nitrophenyl)-1H-1,2,3-triazole (13).[124] Synthesized from 1-ethynyl-3-nitrobenzene[71] and TMSN$_3$ according to the general procedure to afford **13** as a off-white solid in 58% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.60 (s, 1H), 8.23−8.11 (m, 2H), 8.02 (s, 1H), 7.62 (t, 1H, J = 8.0 Hz).

4-(m-Tolyl)-1H-1,2,3-triazole (14).[62] Synthesized from 1-ethynyl-3-methylbenzene[71] and TMSN$_3$ according to the general procedure to afford **14** as a white solid in 62% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.88 (s, 1H), 8.0 (s, 1H), 7.68 (s, 1H), 7.64 (d, 1H, J = 7.6 Hz), 7.37 (t, 1H, J = 7.7 Hz), 7.23 (d, 1H, J = 7.6 Hz), 2.45 (s, 3H).

4-(3-Fluorophenyl)-1H-1,2,3-triazole (15).[125] Synthesized from 1-ethynyl-3-fluorobenzene[71] and TMSN$_3$ according to the general procedure to afford **15** as a white solid in 51% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.21 (s, 1H), 7.67 (d, 1H, J = 7.8 Hz), 7.63−7.58 (m, 1H), 7.50−7.43 (m, 1H), 7.10 (td, 1H, J = 8.5 Hz, 2.5 Hz).

3-(1H-1,2,3-Triazol-4-yl)phenol (16).[62] Synthesized from 3-ethynylphenol[71] and TMSN$_3$ according to the general procedure to afford **16** as a light-yellow powder in 20% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.08 (s, 1H), 7.32−7.22 (m, 3H), 6.83−6.77 (m 1H).

3-(1H-1,2,3-Triazol-4-yl)aniline (17).[62] Synthesized from 3-ethynylaniline[71] and TMSN$_3$ according to the general procedure to afford **17** as a light-yellow powder in 55% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.06 (s, 1H), 7.22−7.12 (m, 3H), 6.77−6.72 (m, 1H).

2-Chloro-4-(1H-1,2,3-triazol-4-yl)pyridine (18). Synthesized from 2-chloro-4-ethynylpyridine[71] and TMSN$_3$ according to the general procedure to afford **18** as an off-white solid in 60% yield, mp 211−213 °C. IR (film): ν 3121, 2171, 1604, 1454, 1070, 998, 838 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.45 (br s, 1H), 8.43 (d, 1H, J = 5.6 Hz), 7.96 (s, 1H), 7.86 (d, 1H, J = 5.38 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 151.8, 149.9, 142.9, 142.0, 120.2, 119.0. ESI-TOF-HRMS: m/z calcd for (M + H) C$_7$H$_5$ClN$_4$, 181.0281; found, 181.0275.

2-Bromo-4-(1H-1,2,3-triazol-4-yl)pyridine (19). Synthesized from 2-bromo-4-ethynylpyridine[71] and TMSN$_3$ according to the general procedure to afford **19** as a white solid in 61% yield, mp 206−209 °C. IR (film): ν 3126, 2138, 1601, 1530, 1068, 834 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.44 (s, 1H), 8.39 (d, 1H, J = 5.4 Hz), 8.11 (s, 1H), 7.88 (d, 1H, J = 5.3 Hz). ^{13}C NMR (100 MHz, DMSO): δ 151.6, 142.8, 142.6, 141.6, 124.0, 119.9. ESI-TOF-HRMS: m/z calcd for (M + H) C$_7$H$_5$BrN$_4$, 224.9776; found, 224.9771.

2-Fluoro-4-(1H-1,2,3-triazol-4-yl)pyridine (20). Synthesized from 4-ethynyl-2-fluoropyridine[71] and TMSN$_3$ according to the general procedure to afford **20** as a off-white solid in 62% yield, mp 197−199 °C. IR (film): ν 3138, 2918, 2263, 1618, 1417, 1212, 1074, 830 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$OD): δ 8.23 (d, 1H, J = 5.3 Hz), 8.03 (s, 1H), 7.62−7.51 (m, 1H), 7.37 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 164.5 (d, J = 237.2 Hz), 147.9, 147.7, 144.3 (d, J = 8.8 Hz), 118.1 (d, J = 3.7 Hz), 105.4, 105.0. ESI-TOF-HRMS: m/z calcd for (M + H) C$_7$H$_5$FN$_4$, 165.0576; found, 165.0583.

4-(1H-1,2,3-Triazol-4-yl)-2-(trifluoromethyl)pyridine (21). Synthesized from 4-ethynyl-2-(trifluoromethyl)pyridine[71] and TMSN$_3$ according to the general procedure to afford **21** as a white solid in 51% yield, mp 211−213 °C. IR (film): ν 3147, 3098, 2885, 2160, 1618, 1321, 1112, 707 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.76 (d, 1H, J = 5.0 Hz), 8.53 (br s, 1H), 8.29 (s, 1H), 8.11 (d, 1H, J = 5.2 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 150.4, 148.4 (q, J = 34.16), 143.1, 140.6, 122.7, 121.6 (q, J = 274.7, 116.6 (q, J = 2.89). ESI-TOF-HRMS: m/z calcd for (M + H) C$_8$H$_5$F$_3$N$_4$, 215.0545; found, 215.0548.

4-(2-Bromophenyl)-1H-1,2,3-triazole (22).[126] Synthesized from 1-bromo-2-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **22** as a white solid in 55% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.26 (s, 1H), 7.76 (d, 1H, J = 7.4 Hz), 7.73 (d, 1H, J = 8.0 Hz), 7.46 (t, 1H, J = 7.5 Hz), 7.31 (t, 1H, J = 7.8 Hz).

2-(1H-1,2,3-Triazol-4-yl)phenol (23).[127] Synthesized from 2-ethynylphenol[71] and TMSN$_3$ according to the general procedure to afford **23** as a white solid in 32% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.77 (s, 1H), 8.10 (s, 1H), 7.63 (d, 1H, J = 7.6 Hz), 7.35−7.28 (m, 1H), 7.10 (d, 1H, J = 8.4 Hz), 7.0 (t, 1H, J = 7.8 Hz).

4-(2-Chlorophenyl)-1H-1,2,3-triazole (24).[121] Synthesized from 1-chloro-2-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **24** as a white solid in 59% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.10 (s, 1H), 8.29 (s, 1H), 7.95 (d, 1H, J = 5.9 Hz), 7.53 (dd, 1H, J = 7.6 Hz, 1.5 Hz), 7.43−7.33 (m, 2H).

4-(o-Tolyl)-1H-1,2,3-triazole (25).[62] Synthesized from 1-ethynyl-2-methylbenzene[71] and TMSN$_3$ according to the general procedure to afford **25** as a white solid in 60% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.89 (s, 1H), 7.62 (d, 1H, J = 6.8 Hz), 7.37−7.29 (m, 3H), 2.51 (s,3H)

2-(1H-1,2,3-Triazol-4-yl)aniline (26). Synthesized from 2-ethynylaniline[71] and TMSN$_3$ according to the general procedure to afford **26** as a white solid in 58% yield, mp 128−130 °C. IR (film): ν 3377, 3326, 2528, 1483, 1297, 748 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.10 (s, 1H), 7.54 (d, 1H, J = 7.2 Hz), 7.10 (t, 1H, J = 7.8 Hz), 6.84 (d, 1H, J = 7.8 Hz), 6.73 (t, 1H, J = 7.8 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 145.1, 128.7, 127.8, 117.3, 116.5, 113.8. ESI-TOF-HRMS: m/z calcd for (M + H) C$_8$H$_8$N$_4$, 161.0827; found, 161.0834.

4-(2-Fluorophenyl)-1H-1,2,3-triazole (27).[121] Synthesized from 1-ethynyl-2-fluorobenzene[71] and TMSN$_3$ according to the general procedure to afford **27** as a white solid in 52% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.24 (s, 1H), 8.19−8.05 (m, 2H), 7.43−7.36 (m,1H), 7.32−7.26 (m,1H), 7.25−7.18 (m, 1H).

4-(2-Ethylphenyl)-1H-1,2,3-triazole (28). Synthesized from 1-ethyl-2-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to

afford **28** as yellow oil in 73% yield. IR (film): ν 3139, 2965, 2931, 2873, 1465, 1443, 968, 761 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.70 (br s, 1H), 7.86 (s, 1H), 7.53 (d, 1H, J = 7.6 Hz), 7.42−7.35 (m, 2H), 7.33−7.29 (m, 1H), 2.83 (q, 2H, J = 7.5 Hz), 1.23 (t, 3H, J = 7.5 Hz). ^{13}C NMR (100 MHz, CDCl$_3$): δ 142.6, 129.7, 129.3, 129.1, 128.5, 126.1, 26.6, 15.5. ESI-TOF-HRMS: m/z calcd for (M + H) C$_{10}$H$_{11}$N$_3$, 174.1031; found, 174.1039.

4-(2-(Trifluoromethyl)phenyl)-1H-1,2,3-triazole (29).[63] Synthesized from 1-ethynyl-2-(trifluoromethyl)benzene[71] and TMSN$_3$ according to the general procedure to afford **29** as a white solid in 46% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 11.83 (s, 1H), 7.94 (s, 1H), 7.83 (d, 1H, J = 8.0 Hz), 7.75 (br s, 1H), 7.67 (t, 1H, J = 7.6 Hz), 7.57 (t, 1H, J = 7.7 Hz).

2-(1H-1,2,3-Triazol-4-yl)benzaldehyde (30). Synthesized from 2-ethynylbenzaldehyde[71] and TMSN$_3$ according to the general procedure to afford **30** as a off-white solid in 65% yield, mp 134−137 °C. IR (film): ν 3132, 3089, 2877, 2168, 1684, 1075, 758 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.32 (br s, 1H), 10.40 (s, 1H), 8.09 (d, 1H, J = 7.8 Hz), 8.02 (s, 1H), 7.76−7.69 (m, 2H), 7.63−7.57 (m, 1H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 192.3, 134.1, 133.6, 130.0, 129.2, 128.5, 128.1, 127.6, 125.9. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_7$N$_3$O, 174.0667; found, 174.0675.

N-Methyl-2-(1H-1,2,3-triazol-4-yl)aniline (31). Synthesized from 2-ethynyl-*N*-methylaniline[71] and TMSN$_3$ according to the general procedure to afford **31** as a off-white solid in 72% yield, mp 129−131 °C. IR (film): ν 3405, 3133, 2901, 2268, 1493, 1285, 751 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.11 (s, 1H), 7.56 (d, 1H, J = 7.3 Hz), 7.23 (t, 1H, J = 7.7 Hz), 6.76 (d, 1H, J = 8.3 Hz), 6.71 (t, 1H, J = 7.7 Hz), 2.93 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 146.8, 129.2, 127.7, 115.6, 113.3, 110.2, 29.4. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_{10}$N$_4$, 175.0984; found, 175.0990.

4-(2-Methoxyphenyl)-1H-1,2,3-triazole (32).[62] Synthesized from 1-ethynyl-2-methoxybenzene[71] and TMSN$_3$ according to the general procedure to afford **32** as a white solid in 38% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 12.52 (s, 1H), 8.15 (s, 1H), 7.90 (br s, 1H), 7.43−7.37 (m, 1H), 7.14−7.05 (m, 2H), 4.04 (s, 3H).

2-(2-(1H-1,2,3-Triazol-4-yl)phenyl)ethanamine Hydrochloride (33). Compound **74** (0.20 mmol) was dissolved in diethyl ether (1 mL), and 1 mL of HCl solution (5 N−6 N in isopropyl alcohol)[73] was added dropwise to the stirred solution. The mixture was stirred at ambient temperature for 36 h. The resulting crystals were filtered and washed with diethyl ether to give **33** as a white solid in quantitive yield, mp 202−205 °C, 90% yield. IR (film): ν 3121, 2897, 2248, 1474, 1142, 763 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.11 (s, 1H), 7.60 (d, 1H, J = 6.9 Hz), 7.47−7.39 (m, 3H), 3.28−3.22 (m, 2H), 3.21−3.16 (m, 2H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 143.5, 135.3, 130.4, 130.1, 129.8, 127.5, 127.4, 127.3, 40.2, 30.9. ESI-TOF-HRMS: m/z calcd for (M + H) C$_{10}$H$_{12}$N$_4$, 189.1140; found, 189.1145.

3-(2-(1H-1,2,3-Triazol-4-yl)phenyl)propanoic Acid (34). The mixture of aldehyde **30** (80 mg, 0.46 mmol) and Meldrum's acid[74] (67 mg, 0.46 mmol) in TEAF (1.0 mL) was stirred at 95−100 °C for 3 h and then cooled to room temperature. Some ice and water (2−3 g) was added to the cold reaction mixture before adjusting its pH to 1 with 6 N HCl (1−2 mL) and letting it crystallize at 5−10 °C for a day. After filtering, the crystals were washed with cold water and pentane to afford **34** as a white solid in 45% yield, mp 119−121 °C. IR (film): ν 3124, 2857, 1704, 1407, 1212, 975, 765 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.97 (br s, 1H), 7.54−7.47 (m,1H), 7.41−7.27 (m, 3H), 3.14−3.03 (m, 2H), 2.59−2.51 (m, 2H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 175.2, 139.0, 129.5, 126.2, 34.8, 28.5. ESI-TOF-HRMS: m/z calcd for (M + H) C$_{11}$H$_{11}$N$_3$O$_2$, 218.0930; found, 218.0940.

4-Chloro-2-(1H-1,2,3-triazol-4-yl)phenol (35). Synthesized from 4-chloro-2-ethynylphenol[71] and TMSN$_3$ according to the general procedure to afford **35** as a off-white solid in 60% yield, mp 212−214 °C. IR (film): ν 3140, 2923, 2280, 1474, 1230, 813 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.30 (s, 1H), 7.90 (s, 1H), 7.20 (dd, 1H, J = 7.1 Hz, 2.5 Hz), 6.92 (d, 1H, J = 8.8 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 153.3, 128.6, 126.3, 124.1, 117.3. ESI-TOF-HRMS: m/z calcd for (M + H) C$_8$H$_6$ClN$_3$O, 196.0278; found, 196.0277.

4-(5-Chloro-2-methylphenyl)-1H-1,2,3-triazole (36). Synthesized from 4-chloro-2-ethynyl-1-methylbenzene[71] and TMSN$_3$ according to the general procedure to afford **36** as a white solid in 52% yield, mp 122−124 °C. IR (film): ν 3091, 2869, 2752, 2604, 1480, 1074, 880, 802 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$OD): δ 7.80 (s, 1H), 7.60 (br s, 1H), 7.25−7.16 (m, 2H), 2.40 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 134.5, 132.1, 131.3, 128.2, 127.8, 19.5. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_8$ClN$_3$, 194.0485; found, 194.0480.

4-(2,5-Dichlorophenyl)-1H-1,2,3-triazole (37). Synthesized from 1,4-dichloro-2-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **37** as a white solid in 70% yield, mp 175−176 °C. IR (film): ν 3161, 3061, 2095, 1466, 1054, 803 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$OD): δ 8.35 (s, 1H), 7.96 (s, 1H), 7.49 (d, 1H, J = 8.6 Hz), 7.35 (dd, 1H, J = 7.3 Hz, 2.3 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 142.1, 132.7, 131.4, 130.7, 129.9, 129.3, 129.0. ESI-TOF-HRMS: m/z calcd for (M + H) C$_8$H$_5$F$_3$Cl$_2$N$_3$, 213.9939; found, 213.9945.

4-(2,3-Dichlorophenyl)-1H-1,2,3-triazole (38). Synthesized from 1,2-dichloro-3-ethynylbenzene[71] and TMSN$_3$ according to the general procedure to afford **38** as a white solid in 71% yield, mp 168−170 °C. IR (film): ν 3170, 3071, 2642, 2112, 1444, 1073, 779 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.32 (s, 1H), 7.83 (d, 1H, J = 7.7 Hz), 7.56 (d, 1H, J = 7.7 Hz), 7.38 (t, 1H, J = 7.7 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 142.6, 133.5, 131.5, 129.9, 128.5, 127.6. ESI-TOF-HRMS: m/z calcd for (M + H) C$_8$H$_5$Cl$_2$N$_3$, 213.9939; found, 213.9933.

4-(3-Chloro-2-methylphenyl)-1H-1,2,3-triazole (39). Synthesized from 1-chloro-3-ethynyl-2-methylbenzene[71] and TMSN$_3$ according to the general procedure to afford **39** as a white solid in 51% yield, mp 135−137 °C. IR (film): ν 3162, 2907, 2139, 1572, 1438, 1067, 801 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$−CD$_3$OD): δ 7.80 (s, 1H), 7.44−7.38 (m, 2H), 7.20 (t, 1H, J = 7.9 Hz). ^{13}C NMR (100 MHz, CD$_3$OD): δ 135.1, 134.1, 132.8, 129.1, 128.0, 126.6, 16.5. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_8$ClN$_3$, 194.0485; found, 194.0494.

3-Methyl-2-(1H-1,2,3-triazol-4-yl)aniline (40). To a solution of **41** (40 mg, 0.15 mmol) in dioxane (2 mL), N$_2$H$_4$·H$_2$O[75] (38 mg, 0.75 mmol) was added dropwise at room temperature. The mixture was refluxed for 2.5 h, cooled to room temperature, filtered, and evaporated. The residue was dissolved in CH$_2$Cl$_2$ (5 mL), and the solution was washed with brine (2 × 4 mL) and dried over Na$_2$SO$_4$. Evaporation gave **40** as a white solid in 50% yield, mp 146−148 °C. IR (film): ν 3354, 3123, 2922, 1583, 1466, 962, 771 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.82 (s, 1H), 7.13 (t, 1H, J = 7.8 Hz), 6.74 (d, 1H, J = 7.4 Hz), 6.70 (d, 1H, J = 8.0 Hz), 2.21 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 146.3, 137.8, 129.2, 119.5, 113.4, 19.4. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_{10}$N$_4$, 175.0984; found, 175.0978.

2,2,2-Trifluoro-N-(3-methyl-2-(1H-1,2,3-triazol-4-yl)phenyl)-acetamide (41). Synthesized from *N*-(2-ethynyl-3-methylphenyl)-2,2,2-trifluoroacetamide[71] and TMSN$_3$ according to the general procedure to afford **41** as a white solid in 45% yield, mp 181−183 °C. IR (film): ν 3162, 2938 1698, 1555, 1458, 1213, 1184, 1135, 1066, 771 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.90 (s, 1H), 7.53 (d, 1H, J = 7.7 Hz), 7.40 (t, 1H, J = 7.7 Hz), 7.33 (d, 1H, J = 7.7 Hz), 2.30 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 156.0 (q, J = 40.1), 138.7, 133.8, 129.1, 128.9, 125.3, 122.9, 116.0, (q, J = 287.5), 19.7. ESI-TOF-HRMS: m/z calcd for (M + H) C$_{11}$H$_9$F$_3$N$_4$O, 271.0807; found, 271.0808.

4-Methyl-3-(1H-1,2,3-triazol-4-yl)aniline (42). Synthesized from 3-ethynyl-4-methylaniline[71] and TMSN$_3$ according to the general procedure to afford **42** as yellow semisolid in 40% yield. IR (film): ν 3349, 3136, 2925, 1619, 1493, 1242, 1124, 817 cm^{-1}. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.86 (s, 1H), 7.11 (d, 1H, J = 8.1 Hz), 7.0 (d, 1H, J = 2.4 Hz), 6.69 (dd, 1H, J = 5.6 Hz, 2.5 Hz), 2.38 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 144.6, 131.3, 128.6, 125.6, 116.2, 116.0, 18.8. ESI-TOF-HRMS: m/z calcd for (M + H) C$_9$H$_{10}$N$_4$, 175.0984; found, 175.0980.

1,5-Diphenyl-1H-1,2,3-triazole (43).[80,81] To a solution of EtMgBr in THF (1.0 M, 2 mL), phenylacetylene (0.22 mL, 2 mmol) was added at room temperature. The reaction mixture was heated to 50 °C for 15

min. After cooling the mixture to room temperature, a solution of phenylazide (0.24 g, 2 mmol) in THF (0.8 mL) was added. The resulting solution was stirred at room temperature for 30 min and then heated to 50 °C for 1 h before quenching with saturated NH_4Cl (2 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 3 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel to afford **43** as a white solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.48−7.44 (m, 3H), 7.42−7.35 (m, 5H), 7.27−7.24 (m, 2H).

5-(4-Fluorophenyl)-1-phenyl-1H-1,2,3-triazole (44). Using the same procedure[80,81] as that used for **43**, starting from 1-ethynyl-4-fluorobenzene (115 mg, 1 mmol) in the presence of a solution of EtMgBr (1.0 M, 1 mL), product **44** was obtained as a white solid in 55% yield, mp 100−103 °C. IR (film): ν 3122, 1893, 1492, 1229, 1160, 830, 768 cm^{−1}. ^{1}H NMR (400 MHz, CD_3OD): δ 7.97 (s, 1H), 7.54−7.47 (m, 3H), 7.39−7.35 (m, 2H), 7.33−7.28 (m, 2H), 7.13−7.07 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 163.3 (d, J = 249.7 Hz), 137.5, 136,3, 132.7, 130.7 (d, J = 8.7 Hz), 129.5, 129.3, 125.4, 122.6 (d, J = 3.6 Hz), 115.6 (d, J = 22.4 Hz). ESI-TOF-HRMS: m/z calcd for (M + H) $C_{14}H_{10}FN_3$, 240.0937; found, 240.0931.

1-Phenyl-5-(m-tolyl)-1H-1,2,3-triazole (45). Using the same procedure[80,81] as that used for **43**, starting from 1-ethynyl-3-methylbenzene (116 mg, 1 mmol) in the presence of a solution of EtMgBr (1.0 M, 1 mL), product **45** was obtained as a white solid in 35% yield, mp 109−113 °C. IR (film): ν 2920, 1597, 1499, 1456, 1234, 1050, 763 cm^{−1}. ^{1}H NMR (400 MHz, CD_3OD): δ 7.97 (s, 1H), 7.56−7.48 (m, 3H), 7.41−7.36 (m, 2H), 7.25−7.20 (m, 2H), 7.14 (br s, 1H), 7.06−7.01 (m, 1H), 2.28 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 138.7, 138.6, 136.5, 132.5, 129.8, 129.4, 129.2, 128.9, 128.4, 126.2, 125.5, 125.3, 19.8. ESI-TOF-HRMS: m/z calcd for (M + H) $C_{15}H_{13}N_3$, 236.1188; found, 236.1184.

5-(3-Chlorophenyl)-1-phenyl-1H-1,2,3-triazole (46). Using the same procedure[80,81] as that used for **43**, starting from 1-chloro-3-ethynylbenzene (123 mg, 1 mmol) in the presence of a solution of EtMgBr (1.0 M, 1 mL), product **46** was obtained as a white solid in 70% yield, mp 85−87 °C. IR (film): ν 2920, 1597, 1499, 1456, 1234, 1050, 763 cm^{−1}. ^{1}H NMR (400 MHz, CD_3OD): δ 8.01 (s, 1H), 7.55−7.47 (m, 3H), 7.40−7.35 (m, 3H), 7.34−7.29 (m, 2H), 7.19−7.15 (m, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 137.0, 136.2, 134.4, 133.0, 130.1, 129.7, 129.4, 129.1, 128.3, 126.9, 125.4. ESI-TOF-HRMS: m/z calcd for (M + H) $C_{14}H_{10}ClN_3$, 256.0641; found, 256.0644.

5-(2-Methoxyphenyl)-1-phenyl-1H-1,2,3-triazole (47).[80,81] Using the same procedure[80,81] as that used for **43**, starting from 1-ethynyl-2-methoxybenzene (131 mg, 1 mmol) in the presence of a solution of EtMgBr (1.0 M, 1 mL), product **47** was obtained as a white solid in 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H), 7.44−7.33 (m, 5H), 7.28−7.24 (m, 2H), 7.01 (td, 1H, J = 7.5 Hz, 1.0 Hz), 6.86 (d, 1H, J = 8.4 Hz), 3.44 (s, 3H).

1-Methyl-5-phenyl-1H-1,2,3-triazole (48).[83,84] Synthesized from 4-phenyl-1H-1,2,3-triazole and dimethyl carbonate according to the general procedure of N-alkylation[82] to afford **48** as a light yellow oil, 7% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (s, 1H), 7.56−7.49 (m, 3H), 7.46−7.43 (m, 2H), 4.11(s, 3H).

1-Methyl-4-phenyl-1H-1,2,3-triazole (49).[83,84] Synthesized from 4-phenyl-1H-1,2,3-triazole and dimethyl carbonate according to the general procedure of N-alkylation[82] to afford **49** as a white solid, 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86−7.83 (m, 2H), 7.77 (s, 1H), 7.47−7.43 (m, 2H), 7.38−7.33 (m, 1H), 4.18 (s, 3H).

2-Methyl-4-phenyl-2H-1,2,3-triazole (50).[83,84] Synthesized from 4-phenyl-1H-1,2,3-triazole and dimethyl carbonate according to the general procedure of N-alkylation[82] to afford **50** as a white solid, 20% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H), 7.80 (d, 2H, J = 7.5 Hz), 7.45 (t, 2H, J = 7.5 Hz), 7.40−7.35 (m, 1H), 4.27 (s, 3H).

1-Ethyl-5-phenyl-1H-1,2,3-triazole (51).[86] Synthesized from 4-phenyl-1H-1,2,3-triazole and diethyl carbonate according to the general procedure of N-alkylation[82] to afford **51** as a yellow oil, 6% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 1H), 7.56−7.48 (m, 3H), 7.44−7.39 (m, 2H), 4.43 (q, 2H, J = 7.3 Hz), 1.51 (t, 3H, J = 7.3 Hz).

2-Ethyl-4-phenyl-2H-1,2,3-triazole (52).[86] Synthesized from 4-phenyl-1H-1,2,3-triazole and diethyl carbonate according to the general procedure of N-alkylation[82] to afford **51** as a colorless oil, 19% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H), 7.81 (d, 2H, J = 7.5 Hz), 7.45 (t, 2H, J = 7.5 Hz), 7.40−7.34 (m, 1H), 4.54 (q, 2H, J = 7.3 Hz), 1.63 (t, 3H, J = 7.4 Hz).

1-Ethyl-4-phenyl-1H-1,2,3-triazole (53).[86] Synthesized from 4-phenyl-1H-1,2,3-triazole and diethyl carbonate according to the general procedure of N-alkylation[82] to afford **52** as a white solid, 55% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, 2H, J = 7.5 Hz), 7.78 (s, 1H), 7.45 (t, 2H, J = 7.6 Hz), 7.36 (t, 1H, J = 7.5 Hz), 4.49 (q, 2H, J = 7.4 Hz), 1.67 (t, 3H, J = 7.4 Hz).

1-Butyl-5-phenyl-1H-1,2,3-triazole (54).[78,79] Synthesized according to a previously reported procedure.[78,79] Oil, 13% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (s, 1H), 7.55−7.49 (m, 3H), 7.42−7.39 (m, 2H), 4.37 (t, 2H, J = 7.4 Hz), 1.84 (quintet, 2H, J = 7.4 Hz), 1.30 (sextet, 2H, J = 7.5 Hz), 0.88 (t, 3H, J = 7.4 Hz).

1-Butyl-4-phenyl-1H-1,2,3-triazole (55).[78,79] Synthesized according to a previously reported procedure.[78,79] White solid, 40% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88−7.84 (m, 2H), 7.77 (s, 1H), 7.48−7.42 (m, 2H), 7.38−7.33 (m, 1H), 4.44 (t, 2H, J = 7.5 Hz), 1.97 (quintet, 2H, J = 7.5 Hz), 1.43 (sextet, 2H, J = 7.6 Hz), 1.0 (t, 3H, J = 7.5 Hz).

1-Benzyl-5-phenyl-1H-1,2,3-triazole (56).[78,79] Synthesized according to a previously reported procedure.[78,79] Off-white solid, 15% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (s, 1H), 7.48−7.41 (m, 3H), 7.35−7.25 (m, 5H), 7.13−7.08 (m, 2H), 5.58 (s, 2H).

1-Benzyl-4-phenyl-1H-1,2,3-triazole (57).[78,79] Synthesized according to a previously reported procedure.[78,79] White solid, 45% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85−7.80 (m, 2H), 7.68 (s, 1H), 7.45−7.38 (m, 5H), 7.37−7.31 (m, 3H), 5.61 (s, 2H).

4-Methyl-5-phenyl-1H-1,2,3-triazole (58).[76] Synthesized according to a previously reported procedure.[76] White solid, 30% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.53 (s, 1H), 7.74 (d, 2H, J = 7.7 Hz), 7.52−7.47 (m, 2H), 7.44−7.39 (m, 1H), 2.57 (s,3H).

5-(3-Chlorophenyl)-1-methyl-1H-1,2,3-triazole (59). Synthesized from **8** and iodomethane according to the general procedure of N-methylation[82] to afford **59** as a yellow oil, 7% yield. IR (film): ν 2926, 2854, 1732, 1571, 1473, 1454, 1245, 1098, 790 cm^{−1}. ^{1}H NMR (400 MHz, CD_3OD): δ 7.86 (s, 1H), 7.64−7.62 (m, 1H), 7.56−7.49 (m, 3H), 4.12 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 136.0, 133.7, 131.7, 130.6, 129.8, 129.6, 128.1, 30.6. ESI-TOF-HRMS: m/z calcd for (M + H) $C_9H_8ClN_3$, 194.0485; found, 194.0478.

4-(3-Chlorophenyl)-1-methyl-1H-1,2,3-triazole (60). Synthesized from **8** and iodomethane according to the general procedure of N-methylation[82] to afford **60** as a white solid, mp 104−107 °C, 49% yield. IR (film): ν 3142, 2949, 2158, 1421, 1078, 887 cm^{−1}. ^{1}H NMR (400 MHz, CD_3OD): δ 8.26 (s, 1H), 7.80 (br s, 1H), 7.69 (d, 1H, J = 7.7 Hz), 7.38 (t, 1H, J = 7.9 Hz), 7.31 (br.d, 1H, J = 7.9 Hz), 4.13 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 146.1, 134.5, 132.4, 130.2, 127.7, 125.0, 123.5, 122.3, 35.7. ESI-TOF-HRMS: m/z calcd for (M + H) $C_9H_8ClN_3$, 194.0485; found, 194.0484.

1-Phenyl-1H-1,2,3-triazole (61).[77] Synthesized according to a previously reported procedure.[77] Off-white solid, 71% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, 1H, J = 1.0 Hz), 7.88 (d, 1H, J = 1.0 Hz), 7.80−7.76 (m, 2H), 7.59−7.54 (m, 2H), 7.50−7.45 (m, 1H).

4-Benzyl-1H-1,2,3-triazole (62).[128] Synthesized from prop-2-yn-1-ylbenzene[71] and $TMSN_3$ according to the general procedure to afford **62** as a light-yellow solid in 45% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.78 (s, 1H), 7.48 (s, 1H), 7.37−7.31 (m, 2H), 7.30−7.24 (m, 3H), 4.13 (s, 2H).

4-((Phenylthio)methyl)-1H-1,2,3-triazole (63).[120] Synthesized from phenyl(prop-2-yn-1-yl)sulfane[71] and $TMSN_3$ according to the general procedure to afford **63** as an off-white solid in 55% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.73 (s, 1H), 7.57 (s, 1H), 7.40−7.35 (m, 2H), 7.33−7.27 (m, 2H), 7.26−7.22 (m, 1H), 4.25 (s, 2H).

4-(Cyclohex-1-en-1-yl)-1H-1,2,3-triazole (64).[129] Synthesized from 1-ethynylcyclohex-1-ene[71] and $TMSN_3$ according to the general procedure to afford **64** as a white solid in 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.65 (s, 1H), 7.70 (s, 1H), 6.43 (br s, 1H), 2.51−

2.44 (m, 2H), 2.28−2.21 (m, 2H), 1.85−1.77 (m, 2H), 1.76−1.67 (m, 2H).

tert-Butyl *N*-{2-[2-(1H-1,2,3-Triazol-4-yl)phenyl]ethyl}carbamate **(74).** Synthesized from *tert*-butyl 2-ethynylphenethylcarbamate[71] and TMSN₃ according to the general procedure to afford **74** as yellow oil in 45% yield. IR (film): ν 3137, 2977, 2932, 1681, 1514, 1366, 1167, 765 cm^{-1}. ^{1}H NMR (400 MHz, CDCl₃): δ 7.90 (s, 1H), 7.56−7.52 (m, 1H), 7.42−7.31 (m, 3H), 4.98 (br s, 1H) 3.40 (q, 2H, J = 6.8 Hz), 2.99 (t, 2H, J = 7.1 Hz), 1.45 (s, 9H). ^{13}C NMR (100 MHz, CDCl₃): δ 156.7, 137.2, 130.8, 129.9, 128.8, 126.8, 79.7, 41.8, 34.0, 28.5. ESI-TOF-HRMS: m/z calcd for (M + H) C₁₅H₂₀N₄O₂, 289.1664; found, 289.1655.

Protein Expression and Purification of Recombinant Human IDO1. The coding region for human IDO1 (Ala2-Gly403) was cloned into a derivative of plasmid pET9 (Novagen). The recombinant plasmid, pETIDO, encodes a histidine tag at the N-terminus of IDO1. The bacterial strain BL21 AI (Invitrogen) was used for overexpression of IDO1 and transformed with the plasmid pETIDO. The transformed cells were grown on a rotary shaker at 37 °C and 220 rpm to an OD600 of 1.2, in LB medium supplemented with kanamycin 25 μg/mL, L-tryptophan 50 μg/mL, and bovine hemin (Sigma) 10 μM. The culture was cooled in a water/ice bath and supplemented again with L-tryptophan 50 μg/mL, bovine hemin 10 μM. The expression of his-tagged IDO1 was induced by the addition of 1% (w/v) arabinose. Induced cells were grown at 20 °C, 60 rpm for 20 h. Cells (1 L culture) were collected by centrifugation, resuspended in 40 mL of Mes 25 mM, KCl 150 mM, imidazole 10 mM, protease inhibitors (complete EDTA free, Roche Applied Science), pH 6.5, and disrupted with a French press. The extract was clarified by centrifugation and filtration on a 0.22 μm filter. The enzyme was purified by IMAC using Ni^{2+} as ligand and an IMAC HITRAP column (5 mL; GE Healthcare). Briefly, the extract was loaded on the column with buffer Mes 25 mM, KCl 150 mM, imidazole 10 mM, pH 6.5. The column was washed with 50 mL of the same buffer with imidazole adjusted to 100 mM. Finally, the protein was eluted with Mes 25 mM, KCl 150 mM, EDTA 50 mM, pH 6.5. The buffer was then exchanged to Mes 25 mM, KCl 150 mM, pH 6.5 using a HITRAP desalting column (GE Healthcare). The purity of the enzyme was estimated to be >95% based on SDS PAGE gel and Coomassie blue staning. The ratio of absorbance 404 nm/280 nm of the protein was around 1.9.

Enzymatic Assay. The enzymatic inhibition assays were performed as described by Takikawa et al.[130] with some modifications. Briefly, the reaction mixture (100 μL) contained potassium phosphate buffer (100 mM, pH 6.5) ascorbic acid (20 mM), catalase (400 units/mL), methylene blue (10 μM), purified recombinant IDO1 (2.5 ng/μL), L-Trp (100 μM), and DMSO (5 μL). The inhibitors were serially diluted 10-fold from 1000 to 0.1 μM, or, if not soluble at 1000 μM, by 4 orders of magnitude from their highest soluble concentration. The reaction was carried out at ambient temperature for 60 min and stopped by addition of 30% (w/v) trichloroacetic acid (40 μL). To convert *N*-formylkynurenine to kynurenine, the samples were incubated at 50 °C for 30 min, followed by centrifugation at 2500g for 20 min. Lastly, 100 μL of supernatant from each probe was used for HPLC analysis. The mobile phase for HPLC measurements consisted of 50% sodium citrate buffer (40 mM, pH 2.35) and 50% methanol with 400 μM SDS. The flow rate through the S5-ODS1 column was 1 mL/min, and kynurenine was detected at a wavelength of 365 nm. A compound was defined to be "inactive" if it did not show a clear signal on the dose−response curve, typically at a maximal concentration of 1 mM

Cellular Assay. *Cell Lines.* For the assay evaluating inhibition of murine IDO1 and murine TDO, we used cell lines P815-mIDO1 clone 6 and P815-mTDO clone 12, respectively, as described in Röhrig et al.[53] For human IDO1, we transfected mouse mastocytoma line P815B with a plasmid construct encoding human IDO1 and selected clone P815B-hIDO1 clone 6, which we used in the assay. For human TDO, we transfected mouse mastocytoma line P815B with a plasmid construct encoding human TDO and selected clone P815B-hTDO clone 19, which overexpressed hTDO and was used in the cellular assay. To estimate cytotoxicity (LD₅₀), we used Hep G2 cells.

Assay. The assay was performed in 96-well flat bottom plates seeded with 2 × 10⁵ cells in a final volume of 200 μL of IMDM (Iscove's Modified Dulbecco's Medium, Invitrogen) (80 μM L-tryptophan) supplemented with 2% FCS and a titration of the compound ranging from 0.001 to 100 μM. The cells were incubated at 37 °C. Kynurenine production in control wells without inhibitor was monitored periodically during the assay using 4-(dimethylamino)-benzaldehyde (pDMAB) as described below. The reaction was stopped before 50% of the initial amount of tryptophan was converted into kynurenine to ensure linearity of the reaction. The plates were then centrifuged for 10 min at 300g, and 150 μL of the supernatant were collected for HPLC analysis of tryptophan and kynurenine concentrations. Proteins were precipitated by mixing 50 μL of supernatant with an equal volume of TCA 6%. After centrifugation, 70 μL of supernatant were collected, diluted in an equal volume of water, and injected into the HPLC system (C18 column). Tryptophan was detected at an absorption wavelength of 280 nm and kynurenine at 360 nm.

To measure kynurenine production during the assay, 200 μL of supernatant from control wells were mixed with 40 μL TCA 30% and centrifuged at 3000g for 5 min. Then 100 μL of supernatant were mixed with an equal amount of pDMAB 2% in acetic acid and incubated for 10 min at room temperature. Kynurenine concentration was determined by measuring absorbance at 480 nm. A standard curve was made with pure kynurenine (ICN Biochemicals).

To estimate cytotoxicity (LD₅₀), 2 × 10⁴ Hep G2 cells were plated in 96-well flat bottom plates in a final volume of 200 μL of IMDM (Invitrogen) supplemented with 10% FCS and a titration of the compound ranging from 0.001 to 100 μM. After 24 h of incubation at 37 °C, the number of living cells were evaluated with a MTS/PMS assay (Promega) as described by the manufacturer.

Determination of pK_a Values. pK_a values were determined spectroscopically.[131] About 1 mg of triazole compound were dissolved in 100 mL of water under strong agitation and sonication. Then 75 mg of KCl were added to keep the ionic strength weak but approximately constant (10 mM). The filtered solution was brought to pH 2−3 by addition of few drops of 1 M HCl. Then 300 μL were transferred to a Greiner UV-Star 96-well plate, and its absorption spectra was recorded immediately in 1 nm steps between 230 and 350 nm on a Tecan Safire2 microplate reader. A few drops of 1 M NaOH were added to bring the bulk solution to pH 11−12, and another sample of 300 μL was transferred to the plate for an absorbance scan. If the spectra allowed to differentiate between different species, in the following the pH was varied in the region of the expected pK_a values ±1 pH unit in steps of roughly 0.2 pH units by addition of a few drops of a diluted HCl solution. At each step, a spectra was measured in above-mentioned wavelength interval. As no buffer was used, care was taken to measure the spectra immediately after stabilization of the pH in order to avoid pH drifts due to the dissolution of CO₂. The wavelengths with the largest change in optical density A at different pH values were chosen for analysis and fitted with the function

$$pH = pK_a + \log \frac{A - A_I}{A_M - A} \qquad (5)$$

where A_I is the absorbance of the ionized species, and A_M is the absorbance of the molecular species. As the pK_a values of the phenol moiety present in some compounds differs by more than 1.6 pK_a units from the pK_a values of the triazole moiety, and as both moieties give rise to different absorption peaks, these pK_a values could be determined independently.

UV Spectra. UV spectra were recorded on a Tecan Safire2 microplate reader with 10 μM IDO1 in 100 mM phosphate buffer (pH 6.5) and 5% DMSO at room temperature.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Figures of possible binding modes of selected compounds to heme, ligand−heme binding energies calculated with DFT, the values of the terms entering into the QSAR model, results of

the elemental analysis for compounds **12, 18, 19, 20, 21, 26, 28, 30, 31, 35, 36, 37, 38, 39, 41, 44, 46,** and **60,** and dose−response curves of all active compounds in the enzymatic assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#Both authors contributed equally to this work (U.F.R. and S.R.M.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

IDO1, indoleamine 2,3-dioxygenase 1; BM, binding mode; MMBP, Morse-like metal binding potential; PCM, polarizable continuum model; PIM, 4-phenylimidazole; TDO, tryptophan 2,3-dioxygenase

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