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NOC Chemistry for Tuberculosis—Further Investigations on the Structure–Activity Relationships of Antitubercular Isoxazole-3-carboxylic Acid Ester Derivatives

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Tuberculosis (TB) is a frequent infectious disease that primarily involves the lungs and that is spread throughout the world by *Mycobacterium tuberculosis* (*Mtb*). Both morbidity and mortality related to this disease remain high—each year, approximately nine million people are diagnosed with active TB and 1.6 million die of the disease.[1] The rapid emergence of multidrug-resistant *Mtb* (MDR-TB) and extensively-resistant *Mtb* (XDR-TB) strains, along with the deadly synergism of *Mtb* with human immunodeficiency virus (HIV), have further worsened the TB pandemic, especially in developing countries.[2,3] The ability of *Mtb* to persist in a semidormant state is responsible for the 9–12 month treatment regimen,[4] which leads to poor adherence to the therapeutic course and frequent relapses. The development of an efficacious vaccine is still a challenge,[5] thereby chemotherapy remains the main means to control the spread of TB. The current therapeutic arsenal consists of four first-line TB drugs, isoniazid (INH), pyrazinamide (PZA), ethambutol (EMB) and rifampin (RMP), and multiple second-line drugs (e.g., streptomycin, capreomycin, ciprofloxacin, levofloxacin, *p*-aminosalicylate, ethionamide, cycloserine, thiacetazone, and rifapentine), which are generally more toxic than the first-line drugs and have limited availability in the countries where TB is endemic.[1,6] Although a few novel agents are currently in clinical trials,[7–10] the search for new anti-TB compounds remains indispensable.

We have previously reported the discovery and optimization of certain isoxazole-based compounds exhibiting good anti-TB activity against both the replicating *Mtb* (R-TB) and nonreplicating persistent *Mtb* (NRP-TB) (Figure 1).[11,12] These compounds consist of a 3-isoxazolecarboxylic acid ester core, prepared by nitrile oxide cycloaddition (NOC) chemistry, which is connected at the C-5 position to a substituted aromatic or heteroaromatic moiety via an alkenyl, alkyloxy, benzyloxy, benzylamino, or phenoxy linker. The overall structure–activity relationships (SAR) previously obtained for this compound class have revealed that the linker and the aromatic ring play some role in modulating both the potency and metabolic stability of the compound, while the 3-isoxazolecarboxylic acid alkyl ester is essential for anti-TB activity.[12] However, it should be noticed that the carboxylic acid derived from the hydrolysis of the ester, although inactive in vitro, might be the chemical species responsible for the activity, as supported by results we have recently reported.[12e] Therefore, hydrolysis of the ester at its site of action may be essential to obtaining the desired anti-TB effect.

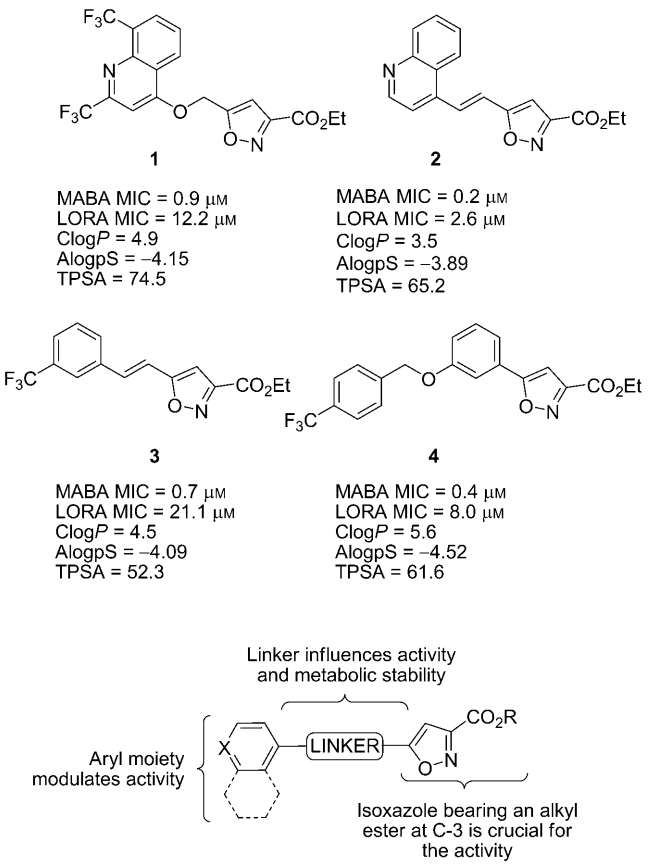

Figure 1. Examples of potent anti-TB isoxazole derivatives, bearing various linker and aromatic moieties, and details of their biological and chemical properties (Top); and the overall construction of the anti-TB compound class (bottom).

Our previous studies have established that, with the exception of the unsubstituted quinoline derivative **2**, lipophilic substituents on the aromatic moiety (e.g., trifluoromethyl, halo-

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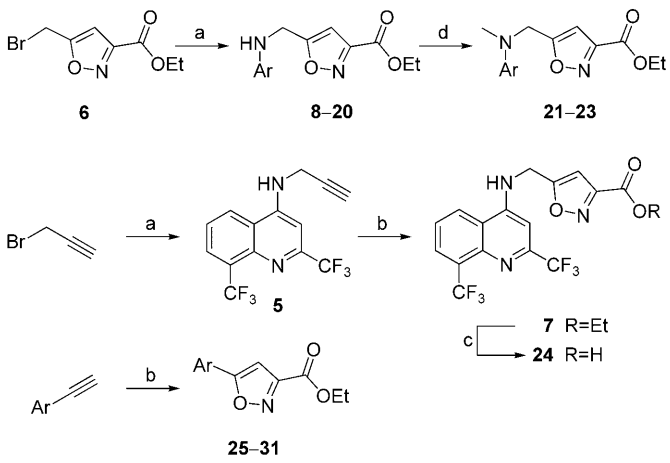
gens and alkyl groups) were generally beneficial to the activity, whereas hydrophilic groups (e.g., hydroxy, amino and *N*-methylpiperazine) had a detrimental effect on the anti-TB potency. However, the better activity is generally coupled with a high Clog*P* and low total molecular polar surface area (TPSA) and predicted water solubility (ALOGpS), features that might represent, in some cases, a hurdle for oral drug administration. To overcome this issue, we chose to investigate whether the linker moiety could be modified so as to improve parameters such as the Clog*P*, TPSA, and ALOGpS, without affecting the anti-TB potency.

Herein, we report the synthesis and anti-TB evaluation of a series of compounds in which the 3-isoxazolecarboxylic acid ester core is attached to the aromatic moiety either directly or via an aminomethylene or an *N*-methylaminomethylene linker. The majority of the new compounds synthesized were found to maintain good anti-TB potency, with the most active compounds having minimum inhibitory concentration (MIC) values in the sub-micromolar range against R-TB and in the low micromolar range against NRP-TB.

The target compounds **7** and **25**–**31**, as well as the key intermediate 5-bromomethyl-3-isoxazolecarboxylic acid ethyl ester (**6**),[13] were synthesized via a dipolar cycloaddition reaction between the nitrile oxide derived from 2-chloro-2-(hydroxyimino)acetate and an appropriate dipolarophile (Scheme 1), as described previously.[14] Compounds **8**–**20**, bearing an aminomethylene linker, were synthesized from bromide **6** and the appropriate aniline under neat conditions at 80 °C. Similarly, the reaction of 4-bromo-2,8-bis(trifluoromethyl)quinoline with an excess of propargylamine afforded the acetylenic intermediate **5**. The *N*-methylated derivatives **21**–**23** were obtained via reductive alkylation of compounds **8**, **13**, and **14**, respectively, with aqueous formaldehyde and $NaBH_3CN$ in acetonitrile with a catalytic amount of acetic acid. Hydrolysis of ester **7** with LiOH produced the corresponding acid **24** in excellent yield.



Scheme 1. *Reagents and conditions:* a) $ArNH_2$ (neat), 80 °C, 3–5 h, (23–75%); b) ethyl 2-chloro-2-(hydroxyimino)acetate, Et_3N (8 h through syringe pump), anhyd Et_2O, RT, 14 h, (42–90%); c) LiOH, THF/MeOH/H_2O (3:1:1), RT, 30 min, (89%); d) HCHO (aq), $NaCNBH_4$, AcOH (cat), CH_3CN, RT, 4–5 h, (50–67%); for complete structures see Tables 1 and 2.

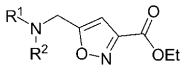
A total of 25 compounds were synthesized and tested for their ability to inhibit the growth of R-TB strain $H_{37}Rv$ in a microplate Alamar Blue assay (MABA).[15] The compounds were also tested in a low oxygen recovery assay (LORA),[16] an in vitro model for the preliminary assessment of the activity against the persistent *Mtb* phenotype. Cytotoxicity was assessed using Vero cells. With the exception of the acid **24** (MIC = > 128 μM), all of the new compounds synthesized were found to inhibit the growth of R-TB in the MABA (Tables 1 and 2).

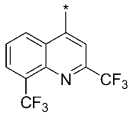
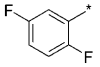
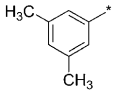
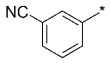
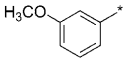
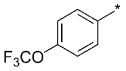
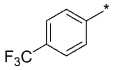
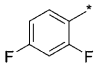
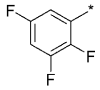
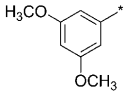
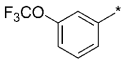
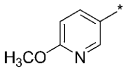
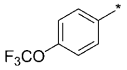
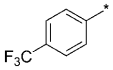
First, we investigated the effect of the aminomethylene linker (compounds **7**–**19**) on the anti-TB activity. The addition of a nitrogen atom in the linker led to the desired reduction in the Clog*P*, and, in addition, the hydrogen-bond donor/acceptor ability of the amino moiety may play a role in the interaction with the active site of the target. The quinoline derivative **7** (MIC = 6.9 μM) was found to be sevenfold less potent than the corresponding oxymethylene derivative **1** (MIC = 0.9 μM) previously reported by us.[11] However, replacement of the substituted quinoline core with an unsubstituted naphthyl ring led to a twofold improvement in activity (compound **8**, MIC = 3.4 μM) as compared to **7**.

Next, in order to further simplify the structure, the naphthyl core was replaced with substituted aromatic rings, i.e., pyridine and benzene, and the effect of various substitution patterns on the activity was investigated. In the first round of modifications, halogens, trifluoromethyl and trifluoromethoxy groups were chosen as substituents since in our earlier studies these groups yielded the best overall activity.[12] Indeed, a trifluoromethoxy group, either in the *para* (**13**, MIC = 0.8 μM) or *meta* (**18**, MIC = 2.4 μM) position of the phenyl ring, was found to be the best substituent in the aminomethylene linker series. Replacement of the trifluoromethoxy group in the *para* position with a trifluoromethyl group (**14**, MIC = 6.5 μM) led to an eightfold decrease in anti-TB potency. Difluoro substitution patterns on the phenyl ring yielded two- to threefold decreased activity as compared to the naphthyl derivative **8** (**9** and **15**, MIC = 7.3 μM and 10.9 μM, respectively), whereas an additional fluorine atom (**16**, MIC = 53.8 μM) significantly decreased the anti-TB potency. Substitution with electron donor alkyl groups, as in the case of 3,5-dimethyl analogue **10** (MIC = 7.0 μM), or with an ethyl group in the *ortho* position of the benzene ring (**20**, MIC = 13.7 μM) yielded an activity comparable to that of the fluorinated compounds.

Other modifications aimed at introducing more hydrophilic groups in the molecule, led to a sharp increase in the MIC values. In particular, a nitrile group in the *meta* position (**11**, MIC = 57.9 μM) yielded the weakest activity in this series. Similarly, replacement of the phenyl ring with a pyridyl ring, as in compound **19** (MIC = 39.6 μM), also seriously undermined the activity. The unsatisfactory anti-TB activity of **19**, as well as of the nitrile derivative **11** and the methoxy substituted compounds **12** and **17**, may partly be due to their very low lipophilicity (Clog*P* = 0.9–1.7), a feature likely to hinder compound penetration through the thick and greasy *Mtb* cell wall. This hypothesis is in part supported by the fact that methylation of the linker nitrogen, which leads to an increase in the Clog*P*,

Table 1. Anti-TB activity of compounds **7**–**23** against *Mtb* strain $H_{37}Rv$.



Compd	$R^{1[a]}$	R^2	MIC [μM] MABA	MIC [μM] LORA	IC_{50}[b] [μM]	Clog P[c]	ALOGpS[d]	TPSA[e]
7		H	6.9	>128	>128	3.7	−4.1	77.3
8		H	3.4	7.6	nd	2.8	−3.8	64.4
9		H	7.3	15.2	nd	2.0	−3.2	64.4
10		H	7.0	14.4	nd	2.7	−3.4	64.4
11		H	57.9	128	nd	1.7	−2.9	88.2
12		H	12.0	126	nd	1.7	−3.1	73.6
13		H	0.8	9.8	>128	2.6	−3.7	73.6
14		H	6.5	9.0	nd	2.5	−3.7	64.4
15		H	10.9	25.3	nd	2.0	−3.2	64.4
16		H	53.8	108.9	nd	2.2	−3.4	64.4
17		H	31.4	28.2	nd	1.7	−3.1	82.8
18		H	2.4	33.3	>128	2.6	−3.7	73.6
19		H	39.6	127.5	nd	0.9	−2.9	86.5
20		H	13.7	31.0	nd	2.6	−3.6	64.4
21		CH_3	1.5	6.5	>128	3.3	−3.7	55.6
22		CH_3	0.7	7.5	>128	3.2	−3.6	64.8
23		CH_3	1.8	6.4	>128	3.1	−3.6	55.6
RMP			0.1	1.0				

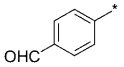
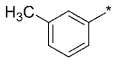
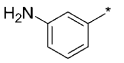
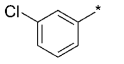
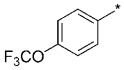
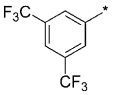
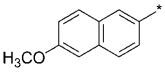
[a] * indicates the point of attachment. [b] Cytotoxicity to Vero cells. [c] Calculated using ChemDraw Ultra 7.0 (CambridgeSoft®). [d] Predicted water solubility calculated using ALOGPS 2.1 (www.vcclab.org/lab/alogps/).[18] [e] Total molecular polar surface area (TPSA) calculated used Molinspiration (www.molinspiration.com/services/psa.html).[19] nd = not determined. MIC values determined by MABA are the mean of replicated experiments (SD < 15%); LORA MIC values represent single measurements.

led to derivatives more potent than the corresponding unmethylated precursors: compounds **21**, **22** and **23** with MIC values of 1.5 μM, 0.7 μM, and 1.8 μM, respectively. Compound **22**, with an MIC value of 0.7 μM, was the most active compound in the series and among the most active of all the isoxazole-based anti-TB compounds reported by our group.[11,12] Moreover, when compared to some of the molecules previously reported by us with a similar range of MIC values (e.g., **1**, **3** and **4**), derivative **22** has a better Clog P and TPSA, and an improved predicted aqueous solubility. To demonstrate the reliability of the predictive models used to drive the design and synthesis of the isoxazole ligands, the aqueous solubility of **22** compared to compounds **4** and **30** was also investigated experimentally (table 2SI in the Supporting Information). The lead compound **22** was indeed found to be more soluble than **30**, which in turn is slightly more soluble than **4**, consistent with the data derived from the computational analysis. The synthesis of compounds **25**–**31**, in which the isoxazole is attached directly to a substituted aromatic moiety, was driven by the evidence that the linker had shown metabolic issues in our earlier studies.[12d]

We have already reported that a phenyl ring directly attached at the C-5 position of the isoxazole ring, when substituted with a benzyloxy, benzylamine or phenoxy side chain, was beneficial for activity as in compound **4** (Figure 1).[12c] We chose to further investigate this finding by introducing small, mainly lipophilic, substituents to the phenyl ring. Indeed, a small set of 5-phenyl-3-isoxazolecarboxylic acid ethyl esters (**25**–**31**) was found to exhibit excellent anti-TB activity with MIC values ranging from 0.7 to 2.2 μM. 3,5-Di(trifluoromethyl)

substitution at the phenyl ring (**30**, MIC = 0.7 μM) yielded the best activity within the small set of compounds synthesized. The trifluoromethoxy substituted compound **29** (MIC = 2.2 μM), which has the same aromatic substitution pattern as **13** (MIC = 0.8 μM), was the least active compound. Surprisingly, a primary amino group at the *meta* position (**27**, MIC = 1.6 μM) was tolerated, whereas in our earlier studies on isoxazole-based anti-TB compounds, primary amino substitution had a detrimental effect on activity.[12]

Table 2. Activity of compounds **25**–**31** against *Mtb* strain $H_{37}Rv$.

Compd	R[a]	MIC [μM] MABA	MIC [μM] LORA	IC_{50}[b] [μM]	Clog *P*[c]	ALOGpS[d]	TPSA[e]
25		2.1	48.7	> 128	2.4	−3.3	69.4
26		1.0	25.1	nd	3.5	−3.3	52.3
27		1.6	41.5	nd	1.9	−2.8	78.4
28		1.0	15.3	> 128	3.7	−3.4	52.3
29		2.2	17.6	nd	4.1	−3.8	61.6
30		0.7	8.1	> 128	4.8	−4.1	52.3
31		0.9	13.2	nd	4.1	−3.9	61.6
RMP		0.1	1.0				

[a] * indicates the point of attachment. [b] Cytotoxicity to Vero cells. [c] Calculated using ChemDraw Ultra 7.0 (CambridgeSoft®). [d] Predicted water solubility calculated using ALOGPS 2.1 (www.vcclab.org/lab/alogps/).[18] [e] Total molecular polar surface area (TPSA) calculated used Molinspiration (www.molinspiration.com/services/psa.html).[19] nd = not determined. MIC values determined by MABA are the mean of replicated experiments (SD < 15%); LORA MIC values represent single measurements.

All of the compounds were also tested for their activity in the LORA. The LORA uses oxygen-deprived conditions simulating the NRP-TB phenotype environment. Among the first-line TB drugs, only RMP and PZA have good activity against NRP-TB. In general, the MIC values against NRP-TB were found to be higher (two- to tenfold) than those against R-TB (Tables 1 and 2). However, several compounds had LORA MIC values in the low micromolar range (< 10 μM). The most active compounds in this assay, namely **21** (LORA MIC = 6.5 μM), **22** (LORA MIC = 7.5 μM), **23** (LORA MIC = 6.4 μM) and **30** (LORA MIC = 8.1 μM), were also among the most potent compounds in MABA. Surprisingly, quinoline derivative **7**, active against the R-TB strain, failed to exhibit any activity in the LORA. Notably, the differences between the MIC values observed in LORA and MABA for each compound in the aminomethylene linker series (**8**–**23**) are, on average, lower than those for compounds where the linker has been removed (**25**–**31**). Therefore, the linker moiety in these isoxazole-based anti-TB agents may play some role in improving the activity against the persistent *Mtb* phenotype.

The lead compounds also maintained good efficacy against RMP and kanamycin single-drug-resistant (SDR) strains (table 3SI in the Supporting Information). The higher MIC values noted with both the INH and streptomycin single-drug-resistant strains suggest differences that are not related to the drug-specific resistance mechanisms of these two strains, but more likely due to differences in compound metabolism or efflux. Although this may be considered a weakness for the further advancement of this compound class, this information can be used to better understand the molecular target or the biochemical pathway inhibited by these molecules, which is as yet undetermined. Finally, when compounds **13**, **22** and **30** were tested in the MABA in the presence of 4% bovine serum albumin (BSA) or 10% fetal bovine serum (FBS), a modest increase in MIC values of 1.5- to 5.8-fold was observed (data not shown). Selected compounds did not show any apparent cytotoxicity against Vero cells (IC_{50} = > 128 μM).

In conclusion, we have synthesized a number of 3-isoxazolecarboxylic acid ester derivatives with the aim of optimizing drug-like properties while maintaining good anti-TB activity. The new compounds synthesized have an aminomethylene linker, an *N*-methylaminomethylene linker, or alternatively, no linker between the isoxazole C-5 position and the substituted phenyl ring. All of the new compounds, with the exception of acid **24**, were active against *Mtb*, with some of them having sub-micromolar MIC values against R-TB and low micromolar MIC values against NRP-TB. The most active compounds against R-TB were 5-[[*N*-[4-(trifluoromethoxy)phenyl]amino]-methyl]-3-isoxazolecarboxylic acid ethyl ester (**13**, MIC = 0.8 μM), along with its *N*-methyl analogue **22** (MIC = 0.7 μM), and 5-[2,5-bis(trifluoromethyl)phenyl]-3-isoxazolecarboxylic acid ethyl ester (**30**, MIC = 0.7 μM). In general, halogenated compounds exhibited good overall anti-TB potency. The compounds bearing an *N*-methylaminomethylene linker were more potent than the corresponding aminomethylene derivatives, following a trend already noticed according to which lipophilicity may, to some extent, affect the activity of these compounds. In addition, the compounds with a substituted phenyl moiety directly attached to the isoxazole C-5 position exhibited good activity against R-TB. The compounds bearing an *N*-methylamino- or aminomethylene linker maintained better ac-

tivity against NRP-TB than the compounds lacking the linker moiety, indicating that the linker may play a role in the activity against the persistent phenotype. Overall, this work highlights the fact that the derivatives of 3-isoxazolecarboxylic acid alkyl esters can be subjected to various modifications at the C-5 position, with the aim of optimizing physicochemical properties, in this case Clog *P*, TPSA, and ALOGpS, while maintaining good anti-TB activity.

Experimental Section

The general procedures for the preparation of compounds **5**–**31** are reported below. ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, HRMS, HPLC purity and a brief description of the biological assays are provided in the Supporting Information.

Chemistry: ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer at 400 MHz and 100 MHz, respectively, with TMS as an internal standard. ^{19}F NMR spectra were recorded on Bruker spectrometer at 376 MHz with trifluoroacetic acid (TFA) as an external standard. Standard abbreviations indicating multiplicity are used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quadruplet, m = multiplet and br = broad. HRMS experiments were performed on Q-TOF-2TM (Micromass). TLC was performed on Merck 60 F_{254} silica gel plates. Column chromatography was performed using CombiFlash® R_f system with RediSep® columns or alternatively using Merck silica gel (40–60 mesh). Preparative HPLC was carried out on a Shimadzu SCL-10A VP instrument with an ACE 5-AQ (21.2 mm × 150 mm) column. Analytical HPLC was carried out on Agilent 1100 HPLC system with a Synergi 4 μ Hydro-RP 80A column, on a variable wavelength detector G1314A. Method 1: Flow rate = 1.4 mL min^{-1}; gradient elution over 20 min, from 30% CH_3CN/H_2O to 100% CH_3CN with 0.05% TFA. Method 2: Flow rate = 1.4 mL min^{-1}; gradient elution over 20 min, from 10% CH_3CN/H_2O to 100% CH_3CN with 0.05% TFA.

***N*-2-propynyl-[2,8-bis(trifluoromethyl)]-4-quinolinylamine (5)**: A stirred solution of 4-bromo-2,8-bis(trifluoromethyl)quinoline (0.2 g, 0.58 mmol) in dioxane (5 mL) was treated with propargylamine (0.074 mL, 1.2 mmol) and the reaction mixture was refluxed overnight. After cooling to RT, the mixture was poured into ice water (15 mL), extracted with EtOAc (3×10 mL), and the organic layers were washed with brine and dried (Na_2SO_4). After filtration, the solvent was removed in vacuo and the crude material was purified by flash chromatography (hexane/EtOAc; 7:3) to give compound **5** as a light yellow powder (88 mg, 45%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.39 (t, J = 2.1 Hz, 1H), 4.22 (dd, J_1 = 2.4 Hz, J_2 = 5.4 Hz, 2H), 5.29 (br s, 1H), 6.92 (s, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 8.09 ppm (d, J = 7.2 Hz, 1H).

General procedure for the synthesis of 6, 7, 25–31: The appropriate acetylene intermediate (1 equiv) and Et_3N (3 equiv) were dissolved in anhyd THF or ether (15 mL mmol^{-1}). Subsequently, ethyl 2-chloro-2-(hydroxyimino)acetate (3 equiv) in anhyd THF or ether (2 mL mmol^{-1}) was added to the solution via syringe pump over an 8 h period, and the reaction mixture was stirred overnight at RT. The reaction mixture was filtered, washed with THF, and the filtrate was concentrated in vacuo. The crude material was purified by flash chromatography using gradient elution (EtOAc/hexane; 0→30–80%). Analytical data for compounds **6**[13] and **27**[17] matched the data published previously.

General procedure for the synthesis of 8–20: The appropriate aniline (1.5 equiv) and 5-bromomethyl-3-isoxazolcarboxylic acid ethyl ester[13] (**6**) (1.0 equiv) were heated at 80 °C until consumption of the limiting reagent. Upon completion, EtOAc was added and the mixture was filtered. The filtrate was concentrated in vacuo and the residue was purified by flash chromatography using gradient elution (EtOAc/hexane; 0→40%) to give the title compounds.

General procedure for the synthesis of 21–23: A stirred solution of amino derivative **8**, **13** or **14** (1 equiv) and formaldehyde (37% aq solution, 0.5 mL mmol^{-1}) in CH_3CN (10 mL mmol^{-1}) at RT $NaBH_3CN$ (3.3 equiv) and glacial AcOH (0.1 mL mmol^{-1}) were added. After 2 h, additional glacial AcOH (0.2 mL mmol^{-1}) was added and the reaction mixture was stirred at RT until consumption of the limiting reagent. The formed precipitate was filtered, washed with water, and purified by flash chromatography using gradient elution (EtOAc/hexane; 0→30%) to give the product.

5-[[[*N*-2,8-bis(trifluoromethyl)-4-quinolinyl]amino]methyl]-3-isoxazolecarboxylic acid (24): $LiOH{\cdot}H_2O$ (7 mg, 0.166 mmol) was added to a solution of **7** (18 mg, 0.042 mmol) in THF/MeOH/H_2O (3:1:1; 5 mL), and the reaction mixture was stirred at RT for 0.5 h. The organic solvents were removed in vacuo, and the resultant aqueous solution was diluted with water and acidified with 1 N HCl to pH 5–6. The precipitate was filtered off to give **24** as a white solid (15 mg, 89%); ^{1}H NMR (400 MHz, CD_3OD): δ = 4.91 (s, 2H), 6.75 (s, 1H), 6.97 (s, 1H), 7.67 (t, J = 8.0 Hz, 1H), 8.13 (d, J = 8.0 Hz, 1H), 8.43 ppm (d, J = 8.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 94.3, 102.3, 124.4, 125.0, 127.8, 143.9, 151.3, 160.6, 170.9 ppm; HRMS-ESI+: m/z $[M+H]^+$ calcd for $C_{16}H_9F_6N_3O_3$: 406.0611, found: 406.0602.

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