Mechanism-Based Design of 3-Amino-4-Halocyclopentenecarboxylic Acids as Inactivators of GABA Aminotransferase

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ABSTRACT: Aminotransferases are pyridoxal 5'-phosphate-dependent enzymes that catalyze reversible transamination reactions between an amino acid and an α -keto acid, playing a critical role in cellular nitrogen metabolism. It is evident that γ -aminobutyric acid aminotransferase (GABA-AT), which balances the levels of inhibitory and excitatory neurotransmitters, has emerged as a promising therapeutic target for epilepsy and cocaine addiction based on mechanism-based inactivators (MBIs). In this work, we established an integrated approach using computational simulation, organic synthesis, biochemical evaluation, and mass spectrometry to facilitate our design and mechanistic studies of MBIs, which led to the identification of a new cyclopentene-based analogue (**6a**), 25-times more efficient as an inactivator of GABA-AT compared to the parent compound (1*R*,3*S*,4*S*)-3-amino-4-fluorocyclopentane carboxylic acid (FCP, **4**).

KEYWORDS: GABA aminotransferase, cyclopentene, deprotonation, rate constant, inactivation efficiency

Aminotransferases (ATs) are essential enzymes that catalyze two coupled transamination reactions between an amino acid and an α -keto acid, thus playing an important role in nitrogen metabolism in cells. All ATs require pyridoxal 5'-phosphate (PLP) as a cofactor, which is linked to a basic lysine residue in the catalytic pocket through a Schiff base, to convert an amino acid into the corresponding carbonyl compound with concomitant conversion of PLP into pyridoxamine 5'-phosphate (PMP) in the first half-reaction. In the second half-reaction, ATs catalyze the reaction of PMP with an acceptor α -keto acid to perform another transfer of an amino group, thereby converting PMP back to PLP.^{1,2} Recent findings have demonstrated that pharmacological inhibition of certain ATs (e.g., γ -aminobutyric acid AT and ornithine AT) is a therapeutic strategy aimed to treat neurological disorders and cancers, respectively.^{3,4}

γ -Aminobutyric acid aminotransferase (GABA-AT, E.C. 2.6.1.19) catalyzes the degradation of the prime inhibitory neurotransmitter GABA to succinic semialdehyde (SSA) with the generation of the major excitatory neurotransmitter L-glutamate (L-Glu) from α -ketoglutarate (α -KG) (Figure 1A).⁴ Normal functioning of the central nervous system (CNS) requires well-balanced levels of inhibitory and excitatory neurotransmitters; a reduction in the level of GABA has been implicated in the symptoms associated with epilepsy,⁵

suggesting that modulation of the deficient level of GABA in the CNS might produce an anticonvulsant effect. Among various approaches to increase the brain concentrations of GABA (e.g., GABA prodrugs and glutamic acid decarboxylase (GAD) activators),⁴ mechanism-based inactivators (MBIs) of GABA-AT are attractive because of their unique inactivation mechanisms and their successful advancement into preclinical/clinical stages.⁴ Unlike other irreversible inhibitors, MBIs are unreactive prior to conversion into an active species in the catalytic site of the target enzyme, thus minimizing unwanted off-target effects.⁶

Vigabatrin (**1**, Sabril, Figure 1B), a MBI of GABA-AT, exhibits anticonvulsant activity and was approved by the FDA in 2009 as an adjunctive therapy for refractory partial seizures.⁷ Mechanistic studies revealed that it irreversibly inhibits GABA-AT by covalent modification through two different mechanisms, a Michael addition pathway (70%) and an enamine pathway (30%), leading to its anticonvulsant effect.^{8,9} It was

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Figure 1. Coupled transamination reactions of GABA-AT (A) and structures of MBI representatives 1–5 (B).

also found to prevent cocaine addiction at a dose of 300 mg/kg in a rat model.¹⁰ However, there are considerable concerns regarding the permanent visual damage associated with long-term vigabatrin administration, which results because of its low inactivation efficiency and poor blood-brain barrier (BBB) permeability, which demand high daily doses (1–3 g per day) that eventually impair its clinical profile.^{11,12} Recent findings identified cyclopentane-based analogue **CPP-115** (**2**, Figure 1B) and cyclopentene-based analogue **OV329** (**3**, Figure 1B), which exhibit several hundred-fold improved inactivation efficiency relative to vigabatrin.^{13,14} GABA-AT crystal structures in complex with **CPP-115/OV329** demonstrated that their difluoromethenyl groups are converted into a carboxylate group in the binding site, and both compounds inactivate the enzyme via tight electrostatic interactions between the two carboxylate groups and two arginine residues

(Arg192 and Arg445).^{14,15} **CPP-115** has been investigated in a Phase I clinical trial,¹⁰ as a compassionate use medication and as a treatment for infantile spasms,¹⁶ while **OV329** suppressed the release of brain dopamine at a dose of 0.1 mg/kg in a rat model of cocaine addiction.¹⁴ Therefore, mechanism-based inactivation of GABA-AT has served as an effective approach to discover novel therapeutic treatments for different neurological disorders.

In 2000, (1*R*,3*S*,4*S*)-3-amino-4-fluorocyclopentane carboxylic acid (**FCP**, **4**, Figure 1B) was reported as an inactivator of GABA-AT.¹⁷ In 2004, crystallography with GABA-AT revealed that **FCP** covalently modifies the Lys329-PLP linkage by forming imine adduct **M5** (Scheme 1) derived from an enamine inactivation mechanism.¹⁸ The proposed inactivation mechanism of **FCP** is initiated by **FCP** acting as a substrate to form Schiff base **M1** with PLP, followed by deprotonation (**M2**) and subsequent elimination of fluoride ion to afford the imine intermediate (**M3**). Subsequent Lys329 attack at the imine moiety of **M3** releases the enamine metabolite (**M4**) and PLP is returned to Lys329. **M4** covalently modifies the Lys329–PLP complex to generate adduct **M5** via an enamine mechanism. However, except for the cocrystal structure, its mechanism was not well supported. Therefore, at the beginning of this work, we resynthesized **FCP** (Scheme S1) and further elucidated its mechanisms of inactivation and alternative turnover using mass spectrometry with the intent of using this as a basis for new inactivator design.

In the present work, the kinetic constants for **FCP** against GABA-AT (Table 1) indicate that **FCP** had a greater binding

Table 1. Kinetic Constants of Analogues **FCP** and **6a–6c** with GABA-AT^a

compd	k_{inact} (min ^{−1})	K_{I} (mM)	$k_{\text{inact}}/K_{\text{I}}$ (min ^{−1} mM ^{−1})
FCP (4)	0.011 ± 0.001	0.053 ± 0.022	0.21
6a	0.132 ± 0.023	0.026 ± 0.011	5.08
6b	0.135 ± 0.010	6.01 ± 0.89	0.022
6c	0.086 ± 0.011	14.26 ± 3.94	0.006
vigabatrin (1)	0.21 ± 0.03	0.29 ± 0.09	0.727

^aData were collected from a time-dependent assay in duplicate according to the experimental protocols in ref 14. k_{inact} and K_{I} values were determined by the equation: $k_{\text{obs}} = k_{\text{inact}} \times [\text{I}]/(K_{\text{I}} + [\text{I}])$ and are presented as means with standard errors.

Scheme 1. Inactivation and Turnover Mechanism of GABA-AT by **FCP**^a

^aCrystal structure (bottom right) of GABA-AT inactivated by **FCP** (green). Ligands and selected residues in GABA-AT are shown in stick representation with atoms colored gray (carbon), red (oxygen), and blue (nitrogen). No PDB code was deposited for the GABA-AT crystal structure, but we obtained coordinates from the authors.¹⁸

Table 2. Intact Protein Mass Results of 6a–6c and FCP with GABA-AT^a

Compound	Structure	Native peak (Da)	Modified peak (Da)	Adduct (Da) ^b
FCP (4)		53080.26 ± 0.93	53437.54 ± 0.60	357.27
6a		53080.51 ± 0.63	53435.93 ± 0.88	355.42
6b		53079.54 ± 0.67	53434.56 ± 0.77	355.02
6c		53079.87 ± 0.74	53435.28 ± 0.87	355.41

^aData are presented as deconvoluted masses (in Daltons) with associated standard deviations around average protein masses. ^bMass shifts were obtained by subtracting average native masses (no PLP attached) from average modified masses.

affinity ($K_i = 0.053$ mM) toward GABA-AT but a lower maximum rate of inactivation (k_{inact}) against GABA-AT (0.011 min^{-1}) than vigabatrin, which eventually led to a modest inactivation efficiency, defined by the k_{inact}/K_i ratio ($0.20 \text{ min}^{-1} \text{ mM}^{-1}$).¹⁹ It was reported that FCP exhibited a partition ratio of 147 with GABA-AT, meaning that 148 equiv of FCP are turned over per active site for each equivalent of compound leading to inactivation.^{17,18} Moreover, 148 equiv of fluoride ion are released per inactivation event, detected with a fluoride ion-selective electrode,¹⁵ indicating that all turnovers require a fluoride ion elimination reaction.^{17,18} Tandem mass spectrometry in the present study of FCP with GABA-AT identified two main metabolites, whose masses fit enamine metabolite **M4** or its imine tautomer **M6** ($[M + H]^+$; theoretical, 128.0706; observed, 128.0705); subsequent hydrolysis gave ketone metabolite **M7** ($[M - H]^-$; theoretical, 127.0401; observed, 127.0391), all with supporting MS² fragmentation (Scheme 1). According to the literature, FCP shows a different turnover mechanism when incubated with aspartate aminotransferase, affording a ketone metabolite without releasing fluoride ion (**S11** in Scheme S2).²⁰ In the current study with GABA-AT, we did not detect this metabolite. Intact protein mass spectrometry of GABA-AT inactivated by FCP produced a mass shift caused by covalent modification, which corresponded to PLP-bound ketone **M8** (Scheme 1; theoretical, 357.06 Da; observed, 357.27 Da; shown in Table 2). Ketone **M8** is the product generated from imine adduct **M5** via hydrolysis under liquid chromatography and mass spectrometry conditions, thereby validating the crystal structure. Therefore, high-resolution intact protein mass spectrometry is an important additional approach to facilitate protein adduct structure determination. The inactivation and alternative turnover mechanisms of FCP are summarized in Scheme 1. After deprotonation to **M2**, exclusive fluoride ion elimination affords **M3**, which is attacked by Lys329 at the iminium group, releasing enamine metabolite **M4**. Most of enamine **M4** tautomerizes to the corresponding imine (**M6**), which undergoes hydrolysis to ketone **M7**, while only a very minor portion of **M4** (0.7% of FCP according to its partition ratio) inactivates the enzyme by enamine addition to PLP, forming **M5**.

With the mechanisms of FCP clarified, we directed our attention to improving its inactivation efficiency. FCP exhibits

good potency against GABA-AT ($K_i = 0.053$ mM), similar to that of CPP-115 ($K_i = 0.059$ mM),¹⁴ a MBI that completed a successful Phase I clinical trial. However, the low maximal rate constant ($k_{\text{inact}} = 0.011 \text{ min}^{-1}$) of FCP with GABA-AT limits its inactivation efficiency. As illustrated in Scheme 1, there are three steps leading to inactivation: deprotonation (**M1** to **M2**), fluoride elimination (**M2** to **M3**), and enamine addition (**M4** to **M5**). The deprotonation step was suggested as the rate-determining step rather than the cleavage of the carbon–fluorine bond in the original FCP article.¹⁷ Our recent findings revealed that OV329 (Figure 1B) was about 10 times more efficient than CPP-115 as an inactivator of GABA-AT.¹⁴ Computational simulations suggested that the incorporation of an endocyclic double bond into the scaffold of CPP-115 is able to bring the difluoromethylenyl group closer to the Lys329 residue, which is responsible for the enhanced binding affinity of OV329 ($K_i = 0.010$ mM).¹⁴ Furthermore, the added double bond led to a 1.5-fold enhancement of the k_{inact} value. Additionally, it was also reported that the incorporation of a double bond into 4-amino-5-fluoropentanoic acid (**5**, Figure 1B), the open-chain analogue of FCP, improved potency against GABA-AT with a comparable rate constant.²¹

To explore the effect of the double bond on the scaffold of FCP, we initially conducted molecular docking studies to predict the binding poses of PLP-bound ligands in the binding site of GABA-AT and quantum mechanical cluster calculations to investigate the reaction profiles of the deprotonation steps.²² The docking pose comparison of **M1** and **M1'** (Figure 2A) suggests that by incorporation of a double bond does not change the salt bridge interactions between Arg192 and the carboxylate group (Figure 2B) and retains the PLP-bound ligand in a similar distance to Lys329, which abstracts the adjacent proton (highlighted with red in Figure 2A). It should be noted that the conformational change does bring the fluorine atom closer to Lys329 (from 4.6 to 3.5 Å) (Figure 2C). Quantum mechanical cluster calculations of the deprotonation step catalyzed by Lys329 (Figure 2D) demonstrate that the ligand-PLP Schiff base **M1'**, containing the added double bond, displays about a 3 kcal/mol lower transition state (TS) energy in going to **M2'** compared with intermediate **M1**, generated from FCP, giving **M2**. This indicates that deprotonation of **M1'** is much easier than of **M1**. The α,β -unsaturated carboxylate of **M1'** leads to a lower pK_a

Figure 2. Computational simulation results of PLP-bound ligands **M1** and **M1'**. (A) The deprotonation reactions of PLP-bound ligand **M1**/**M1'** to **M2**/**M2'**. (B,C) Superimposed docking poses of **M1** (purple) and **M1'** (cyan) with GABA-AT. Ligands and selected residues in GABA-AT are shown in stick representation with atoms colored gray (carbon), red (oxygen), orange (phosphorus), blue (nitrogen), and light cyan (fluorine). (D) Reaction profile of the deprotonation reactions of **M1** to **M2** (green) and **M1'** to **M2'** (blue) in aqueous phase, calculating at a B3LYP/6-31+G(d,p) level of theory.

value for the adjacent proton, relative to that in **M1**, which leads to a more efficient deprotonation step. As deprotonation is considered the rate-determining step, we hypothesized that the lower TS energy from **M1'** to **M2'** should improve the overall inactivation rate, thereby enhancing the k_{inact} value.

To assess the actual effect of this double bond, we synthesized and evaluated several cyclopentene analogues bearing different halogens. The synthetic route to prepare cyclopentene compound **6a** bearing a fluorine atom (Scheme 2) was initiated with (1R)-(-)-2-azabicyclo[2.2.1]hept-5-en-3-one (**7**), which was protected with a *p*-methoxybenzyl (PMB) group (**8**) and epoxidized with *m*-CPBA (**9**). Epoxide rearrangement^{17,23–25} and selective acylation occurred under $\text{BF}_3 \cdot \text{OEt}_2/\text{AcOH}$ conditions to afford the bicyclic key

Scheme 2. Synthetic Route to **6a**^a

^aReagents and conditions: (a) (i) *p*-anisyl alcohol, conc. HCl, rt; (ii) NaH, TBAI, THF/DMF (10:1), 0 °C–rt; (b) *m*-CPBA, CHCl_3 , reflux; (c) $\text{BF}_3 \cdot \text{OEt}_2$, AcOH, DCM, rt; (d) MOMCl, DIPEA, DCM, rt; (e) K_2CO_3 , MeOH/ H_2O , rt; (f) DAST, DCM, -78 °C–rt; (g) 6 N HCl, THF, 80 °C; (h) (i) TsCl, DIPEA, DMAP, CH_3CN , rt, (ii) ceric ammonium nitrate, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, rt; (i) (i) Boc_2O , DIPEA, DMAP, DCM, rt, (ii) K_2CO_3 , MeOH, rt; (j) 4 N HCl, AcOH, 70 °C.

intermediate (**10**), containing a hydroxyl group on the bridgehead, whose structure was confirmed by 2D NMR spectrometry. Methoxymethyl (MOM) protection (**11**), deacylation (**12**), and fluorination with DAST led to the corresponding fluorinated bicycle (**13**) as a single diastereomer. The full retention of relative configuration results from the formation of a transient aziridinium intermediate.²⁴ Subsequent MOM deprotection (**14**) and tosylation with PMB deprotection afforded lactam **15**, containing the tosylate group on the bridgehead to act as a leaving group in the next step. Introduction of a *tert*-butoxycarbonyl (Boc) protecting group on the lactam nitrogen of **15**, followed by a one-pot hydrolysis of the lactam and elimination of the tosylate with $\text{K}_2\text{CO}_3/\text{MeOH}$ afforded the desired cyclopentene key intermediate (**16**). Removal of the Boc group and methyl ester under acidic conditions afforded the final product (**6a**) as a HCl salt, whose structure was confirmed by ^1H , ^{13}C , and 2D NMR spectrometries.

To prepare two other cyclopentene analogues (**6b** and **6c**), bearing chlorine and bromine atoms, respectively, the synthetic routes were initiated from tosylation (**17**) and PMB deprotection of **10** to afford **18** (Scheme 3). After Boc protection (**19**), a one-pot hydrolysis and elimination was carried out with $\text{K}_2\text{CO}_3/\text{MeOH}$ to obtain the desired cyclopentene (**20**). Intermediate **20** was successfully converted to the desired monochloro- (**21a**) or monobromo- (**21b**) substituted compound using NCS or NBS, respectively; the sole diastereomers formed may have been facilitated by neighboring group participation in the halogenation step.^{17,26} Final cyclopentene products **6b** and **6c** were obtained as HCl salts after deprotection.

The kinetic constants shown in Table 1 indicate that (a) Compared to FCP, new cyclopentene analogues **6a–6c** exhibited about a 10-fold enhancement of their rate constants with GABA-AT. (b) Chloro- (**6b**) and bromo-substituted (**6c**) analogues showed much lower binding affinities to GABA-AT,

Scheme 3. Synthetic Route to 6b and 6c^a

^aReagents and conditions: (a) TsCl, DIPEA, DMAP, CH₃CN, rt; (b) ceric ammonium nitrate, CH₃CN/H₂O, rt; (c) Boc₂O, DIPEA, DCM, rt; (d) K₂CO₃, MeOH, rt; (e) NBS or NCS, Ph₃P, DMF, rt; (f) 4 N HCl, AcOH, 70 °C.

corresponding to the larger sizes of the halogens, while the fluoro-substituted analogue (**6a**) showed a 2-fold enhancement of binding affinity compared to **FCP**, thereby establishing that **6a** is about 25-times more efficient than **FCP** as an inactivator of GABA-AT. The correspondence of the inactivation rate constants for **6a–6c** supports the notion that the rate-determining step is deprotonation rather than carbon–halogen bond cleavage.

The irreversibility of the inhibition of GABA-AT by **6a** was assessed by dialysis experiment. After 4 days of dialysis against buffer containing excess PLP and α -KG, there was no regeneration of enzyme activity, confirming that **6a** is an inactivator of GABA-AT (Figure S1A). Consequently, we investigated the potential adduct structures generated from **6a–6c** using intact protein mass spectrometry. The results shown in Table 2 demonstrate that **6a–6c**, bearing different halogens, resulted in identical molecular mass shifts after inactivation of GABA-AT, which match the ketone–PLP adduct structure (**M6'** in Scheme 4; calculated, 355.05 Da; found, 354.02–355.42 Da) generated from an enamine addition pathway. Hydrolysis of the imine in **M5'** to **M6'** might have occurred during chromatography and mass spectrometry. This suggests that they all covalently bind to GABA-AT through the same mechanism as **FCP** (Scheme 1) after forming **M3'** via halide ion elimination (Scheme 4).

Superimposed docking poses in Figure 3A of **6a–6c** exhibit similar binding poses as **FCP** after forming PLP-bound intermediates, such as **M1'**. Docking studies of **FCP** and **6a–6c** in the binding site of GABA-AT show that the amino groups of **FCP** and **6a** display similar orientations toward the Lys329-PLP linkage (Figure 3B), which is beneficial for the reaction with the PLP ligand in the initial binding step, while the amino groups of **6b** and **6c** orient away from the Lys329-bound PLP (Figure 3C). These modeling results suggest that the initial binding step with Lys329-PLP may play the most significant role in influencing their binding affinity, leading to the distinct K_i values.

The partition ratio for **6a** was determined to be 44, and 44 equiv of fluoride ions were released per inactivation event (Figure S1B), indicating that 97.8% of **6a** underwent fluoride ion elimination per inactivation event. Tandem mass spectra of **6a** with GABA-AT identified ketone **M7'** (Scheme 4) ($[M -$

Scheme 4. Inactivation and Turnover Mechanism of GABA-AT by **6a**

H]⁺; calculated, 125.0244; found, 125.0234), generated by fluoride ion elimination and enamine hydrolysis, as the major metabolite with its confirmatory MS² fragmentation pattern. No ketone metabolite containing a fluorine atom was detected, indicating that simple hydrolysis of **M2'** does not occur.

The inactivation and turnover mechanism for **6a** are proposed in Scheme 4. After capturing the PLP ligand from Lys329, PLP-bound **M1'** undergoes deprotonation and fluoride ion elimination to form the imine intermediate (**M3'**). According to the enamine pathway, Lys329 attacks the imine moiety of **M3'**, which leads to the release of enamine metabolite **M4'**, mostly hydrolyzing to **M7'**. A small amount of **M4'** (2.2% of **6a** according to its partition ratio) attaches to the Lys329-PLP linkage, forming **M5'**, and inactivates the enzyme in a manner similar to that by **FCP**. Compared to original inactivator **FCP**, incorporation of the extra double bond decreases the partition ratio (147 for **FCP**¹⁸ vs 44 for **6a**) and leads to a 2-fold greater binding affinity with reduced transition state energy for the rate-determining deprotonation step, which is responsible for a 12-fold enhancement in the rate constant, making **6a** 25-fold more efficient as an inactivator of GABA-AT than **FCP**.

We also assessed the selectivity of **6a** over other ATs, including ornithine aminotransferase (Orn-AT), aspartate aminotransferase (Asp-AT), and alanine aminotransferase (Ala-AT). In a concentration-dependent assay,¹⁴ **6a** showed no inhibitory effect on Asp-AT or Ala-AT up to a concentration of 10 mM. In a time-dependent assay against Orn-AT,²⁷ **6a** displayed a comparable rate constant ($k_{\text{inact}} = 0.143 \pm 0.014 \text{ min}^{-1}$) but 10-fold weaker binding affinity ($K_i = 0.25 \pm 0.06 \text{ mM}$) relative to its effect with GABA-AT.

Figure 3. Superimposed docking poses of FCP (blue), **6a** (cyan), **6b** (yellow), and **6c** (pink) in Schiff bases with PLP from GABA-AT (A). Docking study results for FCP (blue) and **6a** (cyan) prior to reaction with Lys329-PLP (B). Docking study results for **6b** (yellow) and **6c** (pink) prior to reaction with Lys329-PLP (C). Ligands and selected residues in GABA-AT are shown in stick representation with atoms colored gray (carbon), red (oxygen), orange (phosphorus), blue (nitrogen), light cyan (fluorine), green (chlorine), and brown (bromine).

In summary, we have established a GABA-AT MBI discovery strategy using computational simulation, organic synthesis, mechanistic enzymology, and intact protein- and small molecule mass spectrometry. These approaches allowed us to better understand inactivation and alternative turnover mechanisms of a known inactivator, FCP. This strategy facilitated the design of new MBIs, leading to the identification of compound **6a** as a more efficient inactivator of GABA-AT than FCP.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00672>.

Supplementary figures and schemes and details of methods, syntheses, and spectra (PDF)

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Author Contributions

S.S. designed the molecules, carried out most of the experiments, and wrote the initial draft of the manuscript; P.F.D. performed all of the mass spectral experiments and interpreted the data; P.M.W. carried out quantum chemical cluster calculations; W.Z. conducted molecular docking studies; N.L.K. directed the mass spectrometry experiments; and R.B.S. directed the medicinal chemistry work and edited the drafts of the manuscript. All authors have read and edited the manuscript and have given their approval of the final version.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MS, mass spectrometry; MS², tandem mass spectrometry; *m*-CPBA, *meta*-chloroperoxybenzoic acid; DAST, diethylamino-sulfur trifluoride; TBAI, tetra-*n*-butylammonium iodide; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; DIPEA, *N,N*-diisopropylethylamine; DCM, dichloromethane; Ts, tosyl;

DMAP, 4-dimethylaminopyridine; NCS, *N*-chlorosuccinimide; NBS, *N*-bromosuccinimide.

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