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Chiral cyclohexane 1,3-diones as inhibitors of mutant SOD1-dependent protein aggregation for the treatment of ALS

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Abstract

Cyclohexane 1,3-diones were identified as a class of molecules exhibiting a protective effect against mutant SOD1 induced toxicity in PC-12 cells, but an optimized analogue had little or no effect on life extension in the G93A SOD1 mouse model for amyotrophic lateral sclerosis (ALS). Additional testing showed that these compounds were inactive in neurons and further analogue synthesis was carried out to identify compounds with neuronal activity. Starting from two racemic derivatives that were active in cortical neurons, two potent analogues (**1b** and **2b**) were resolved, which were protective against mutant SOD1 induced toxicity in PC-12 cells. Both compounds were found to be active in cortical neurons and presented good ADME profiles in vitro. On the basis of these results, an ALS mouse trial with **1b** was carried out, which showed slightly greater life extension than the FDA-approved ALS drug riluzole, thereby validating cyclohexane 1,3-diones as a novel therapeutic class for the treatment of ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a rare and fatal neurodegenerative disease characterized by progressive motor neuron loss in the central and peripheral neuron systems, leading to clinical muscle atrophy, paralysis, and final death from respiratory failure, generally, in 3–5 years.[1] It is estimated that the incidence of ALS is 1–2 cases per 100,000 people, with an increased risk for military personnel.[2,3] Although there has been progress in the identification of potential targets for the disease, and many new therapeutics have been tested in animals and in clinical trials over the last two decades,[4] no effective treatment is

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ASSOCIATED CONTENT

Supporting Information Available. Detailed description of experimental procedures, biological evaluations and characterization of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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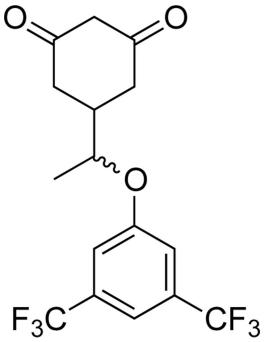
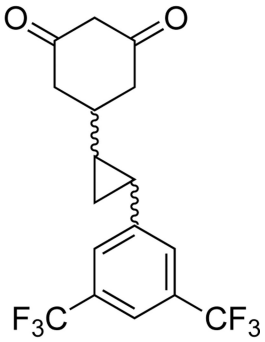
currently available; the only FDA-approved drug, riluzole, a presumptive anti-glutamatergic drug, extends survival by only 2–3 months.[5]

Although ALS is principally a sporadic disease, approximately 10% of all cases are familial (FALS), and over 100 genes are potentially responsible for FALS.[6] Mutations in Cu/Zn superoxide dismutase (SOD1) are the most common cause of FALS.[7] Although mutations in SOD1 account for only 2% of ALS patients, it has recently been shown that astrocytes from both FALS and sporadic ALS (SALS) patients are similarly toxic to motor neurons and that knockdown of SOD1 significantly attenuates astrocyte-mediated toxicity of motor neurons, indicating that SOD1 is a viable target for SALS.[8] Also, because mutant SOD1 leads to oxidative stress, protein misfolding, and aggregation, all of which are associated with ALS pathogenesis,[9] it is reasonable to include inhibitors of mutant SOD1-induced protein aggregation as a viable strategy to identify novel ALS therapeutics.

Three different scaffolds, arylsulfanylpyrazolones (ASP), pyrimidine-2,4,6-triones (PYT), and cyclohexane-1,3-diones (CHD), were identified by a high-throughput cell-based screen[10] developed by Morimoto and co-workers.[11] Extensive modification of the ASP[12, 13] and PYT[14] leads afforded excellent therapeutic candidates, with favorable potency, pharmacokinetics, toxicity, and life extension in the ALS mouse model. However, the most potent of the CHD derivatives did not show any significant extension of life in the ALS mouse model, despite having comparable potency in the PC-12 cell assay and favorable pharmacokinetic properties.[15] Aggregation of mutant G93A SOD1 is induced in the PC-12 assay, which produces a concomitant loss in cell viability. Cell viability is restored through treatment with compounds that reduce protein aggregation. The proposed explanation was the lack of in vitro activity in cortical neurons. Two racemic analogues (**1** and **2**) were identified with enhanced activity in cortical neurons that retained their activity in the PC-12 assay. Here we have synthesized the enantiomers of the active compounds and show that both enantiomers of each scaffold penetrate cortical neurons, that the pharmacokinetics of the eutomers are favorable, and that one of the isomers produces a slightly greater extension of life in the ALS mouse model than riluzole, the only FDA-approved drug for ALS.



(±)-**1**



(±)-*trans*-**2**

Chemistry

As shown in Scheme 1, starting from commercially available ethyl lactate (**3**) and 3,5-ditrifluoromethyl phenol (**4**), the condensed ether (**5**) was formed using a Mitsunobu reaction. Treatment with DIBAL at −78 °C provided aldehyde **6** in a high yield, which was used directly in a Wittig reaction to afford enone **7** in a 5:1 trans to cis ratio. A one-pot procedure, which includes a Michael addition, cyclization, hydrolysis, and decarboxylation, was carried out to give **1** in high yield; starting from chiral ethyl lactates, the two enantiomers (**1a** and **1b**) were readily obtained.

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The route shown in Scheme 2 was used to synthesize **2**. 3,5-Ditrifluoromethyl benzaldehyde (**8**) was treated with triethyl phosphonoacetate and then reduced to obtain mostly *trans*-allyl alcohol **9** in a 67% yield. An enantioselective Simmons-Smith cyclopropanation was performed with bifunctional boron ester **10**[16] in a high yield and excellent enantioselectivity. The alcohol intermediate (**11**) was converted to an enone (**12**) by PCC oxidation and a Wittig reaction. A one-pot procedure of a Michael addition, cyclization, hydrolysis, and decarboxylation was carried out to give *trans*-**2**. This method was used to synthesize the enantiomers of **2**, starting from the enantiomers of **11**.

Mutant SOD1-induced cytotoxicity protection assay and primary cortical neuron protection assay

Compound activity was assessed using a previously described cytotoxicity protection assay.[12] The EC_{50} values of these analogues are summarized in Figure 1. The potencies of the ether linker CHD (**1**) were superior to the cyclopropyl linker compounds (**2**). The enantiomers of the ether linker analogues (**1a** and **1b**) showed a greater potency difference than their cyclopropyl counterparts (**2a** and **2b**); *S*-enantiomer **1b** was 4–5 fold more potent than *R*-enantiomer **1a**, but **2b** was only 1.5–2 fold more potent than **2a**. Ether **1b** was the most potent among all of the CHD analogues tested.[15]

It was previously found that **13** had little or no effect on life extension in the ALS mouse model and was not active with cortical neurons. As shown in Figure 2, all of the compounds in Figure 1 had cortical neuron activity except **13**. Furthermore, compound **1b** exhibited more than 90% neuronal activity at 3 μM, while, as a control, the best ASP compound (**Ref** in Figure 2) required a concentration of 10 μM to reach maximum recovery.

ADME studies in vitro

The aqueous solubility of **1b** and **2b** were evaluated by dilution from a stock solution in DMSO to a final concentration of 1% DMSO in PBS. The solubility limit was the highest concentration with no precipitation. Both compounds were found to have high aqueous solubility[17] (≥100 μM).

The in vitro plasma stability half-life for **1b** was >60 min and for **2b** was 71 min. Human and mouse microsomal stability of these compounds were tested at 1 μM at 37 °C for 1 h in the presence and absence of NADPH (Table 1). Both of the compounds showed moderate clearance with human liver microsomes (31–36 mL/min/kg) and moderate to near high clearance with mouse liver microsomes (45–82 mL/min/kg);[18] both had half-lives in human microsomes greater than an hour. Metabolite identification studies indicated the only metabolic product was insertion of an oxygen atom somewhere other than on the bis(trifluormethyl)phenyl ring (see Supporting Information).

Compounds **1b** and **2b** were further evaluated for their ability to penetrate Caco-2 cell monolayers, which is correlated with intestinal permeability in vivo. As shown in Table 2, both had high permeability from the A side to the B side. Moreover, the low efflux ratio (P_{app} (B→A)/P_{app} (A→B) indicates these compounds are unlikely to be substrates of efflux transport proteins, which is especially important for CNS drugs. Compound **1b** was selected for in vivo testing on the basis of its potency and in vitro predicted pharmacology profile.

In vivo half-life of 1b and activity in the SOD1 G93A ALS mouse model

Maximum blood levels (245 μM) of **1b** by i.p. administration (500 mg/kg) occurred at 12 h, the blood half-life; brain penetration was 8.3 μM with a T_{max} of 12 h. As the commonly

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used ALS animal model, transgenic mice expressing human G93A mutant SOD1 develop a series of similar symptoms to those observed in both familial and sporadic ALS patients.[19] Control and transgenic mice of the same age (± 3 days) and from the same "f" generation were selected from multiple litters to form experimental cohorts. The tolerable dose range for **1b** was determined in wild-type mice by increasing the dose b.i.d., and the maximum tolerated dose was 1280 mg/kg. On the basis of the ADME and MTD studies, the dose levels of 10, 20, and 30 mg/kg were administered daily, starting from 6 weeks of age to the end of life of the G93A mice. Administration of **1b** resulted in a 13% extension in survival at 20 mg/kg compared to untreated G93A mice (Figure 3). This result is slightly better than that observed for the only FDA approved drug riluzole, which showed a lifespan extension of 10–11% at 22 mg/kg in the same animal model.[20]

Conclusion

Previously we had prepared a compound (**13**) that was a potent inhibitor of protein aggregation in PC-12 cells expressing mutant G93A SOD1 with very good pharmacokinetic properties, but which was inactive in vivo in the ALS mouse model. It was found to be inactive in cortical neurons, which led to the design of two racemic compounds (**1** and **2**) that were active in cortical neurons. Chiral syntheses of the enantiomers of **1** and **2** were carried out, and both enantiomers of each compound were found to be active in both the PC-12 and cortical neuron assays. The eutomers of each racemic compound (**1b** and **2b**) had good pharmacokinetic properties, and the more potent of these (**1b**) was shown to extend the life of the ALS mouse by 13%, which is slightly better than that previously reported for riluzole, the only FDA-approved drug for ALS, in the same mouse model. These studies demonstrate the importance of investigating the cortical neuron activity of compounds prior to the expensive and time-consuming task of an ALS mouse trial. They also validate the cyclohexane 1,3-dione class of compounds as a potential therapeutic scaffold for the treatment of ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ADME	absorption, distribution, metabolism, excretion
ALS	amyotrophic lateral sclerosis
CHD	cyclohexane 1,3-dione
CNS	central nervous system
FALS	familial ALS
PBS	phosphate buffered saline
PK	pharmacokinetics
SALS	sporadic ALS
SOD1	Cu/Zn superoxide dismutase

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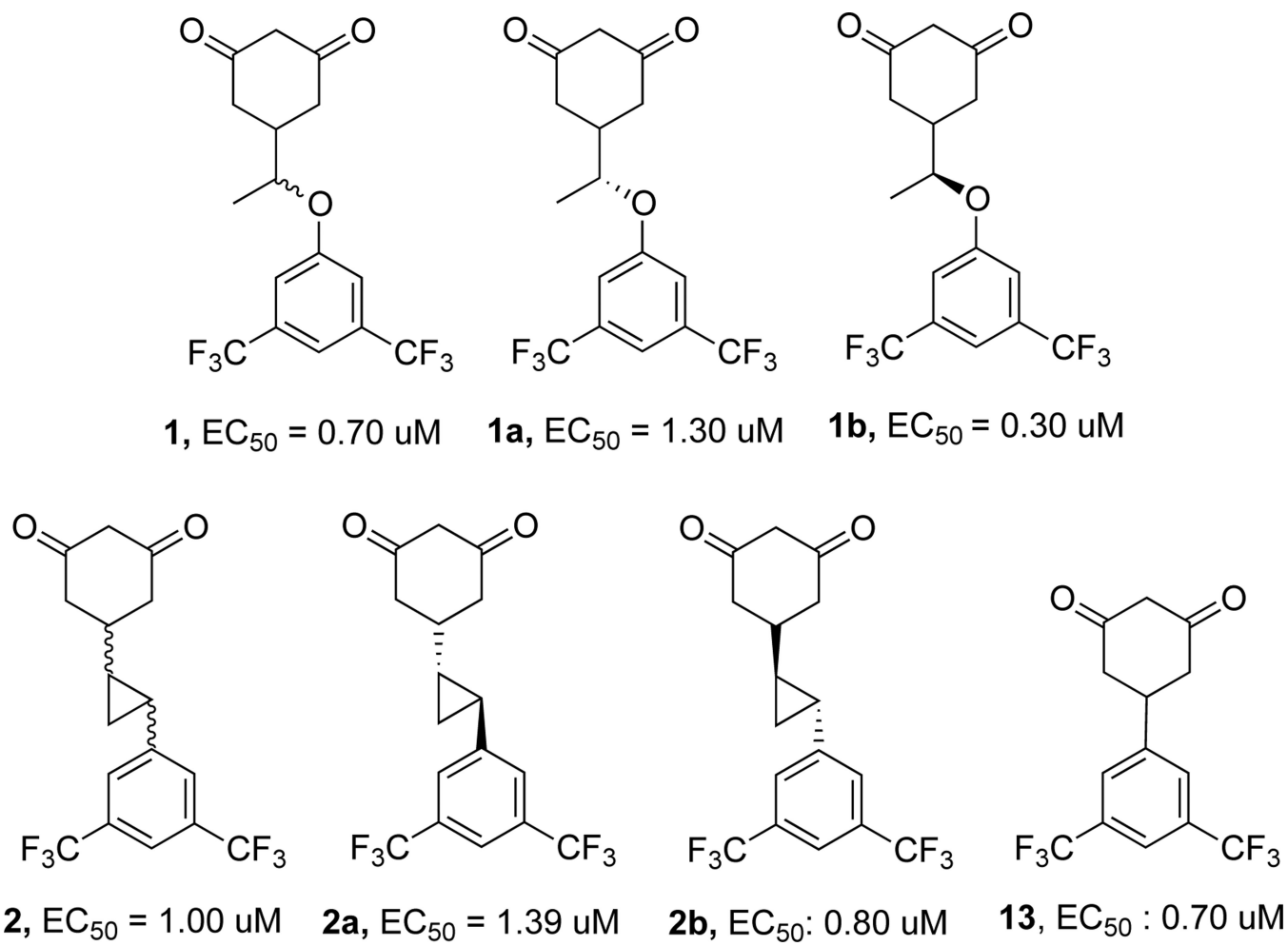

Figure 1. Cytotoxicity protection assay for the CHD analogues

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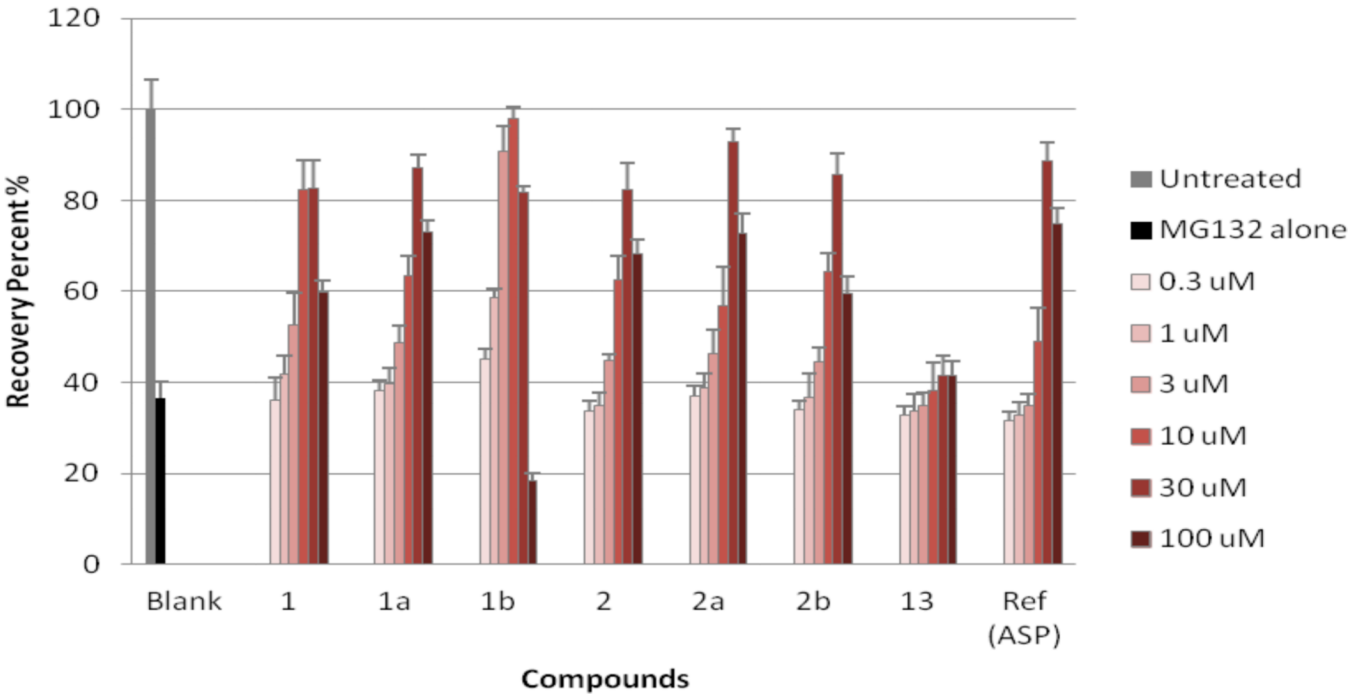

Figure 2. Qualitative primary cortical neurons protection assay
Ref : compound **13** in reference 13.

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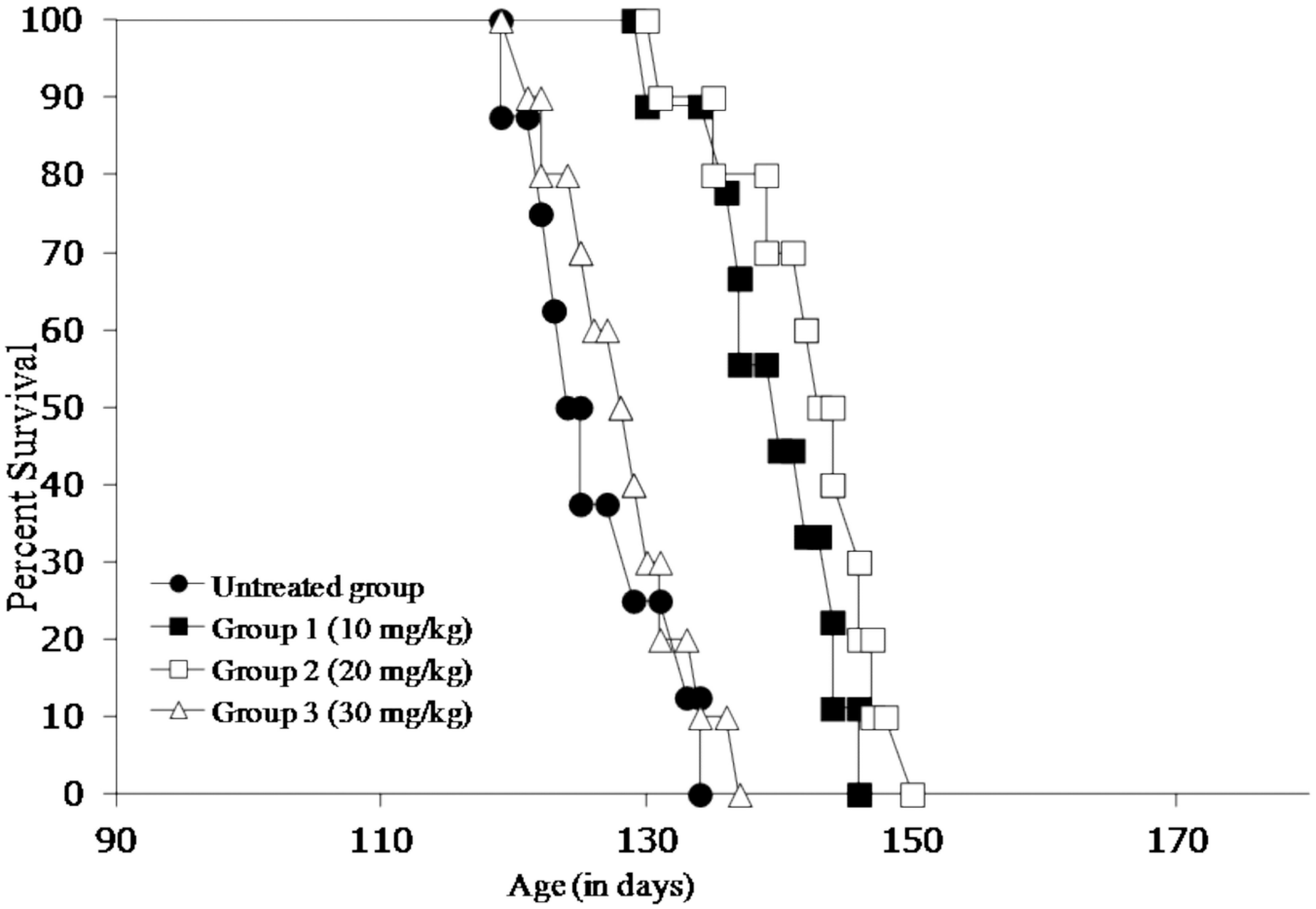

Figure 3. Kaplan-Meier plot of 1b-treated SOD1 G93A ALS mice

untreated group, 125.7 ± 4.3 days; group 1 (10 mg/kg), 139.2 ± 8.3 days; group 2 (20 mg/kg), 142.0 ± ± 9.1 days ($p < 0.03$); group 3 (30 mg/kg), 127.9 ± 4.7 days; $p < 0.63$).

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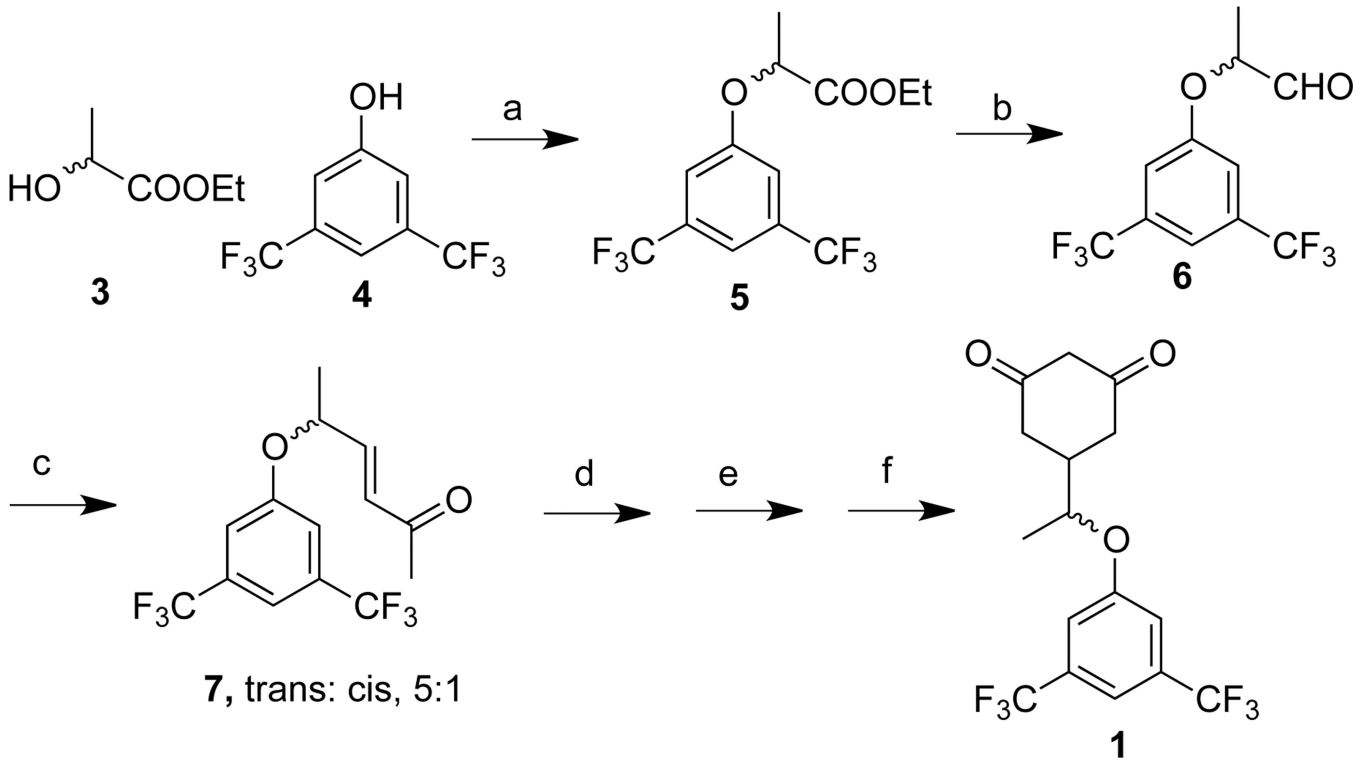
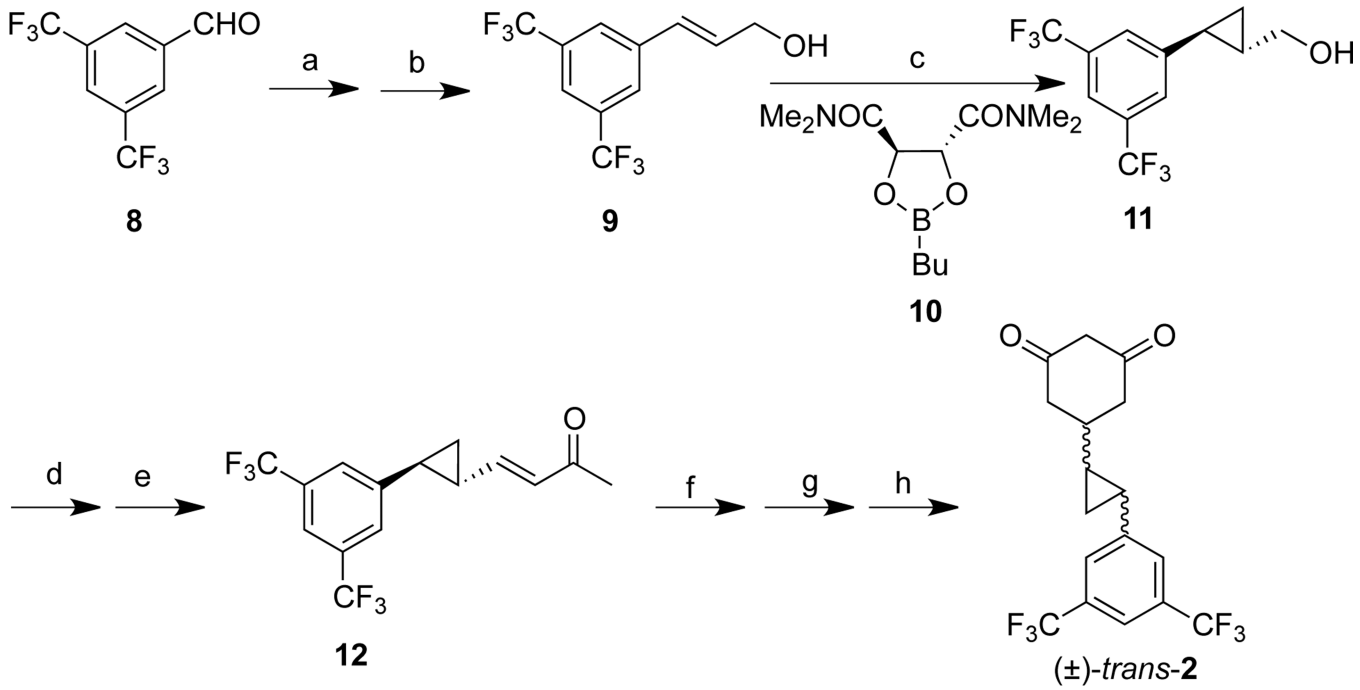


Scheme 1. Synthesis of 1a

[a]Reagents and conditions: (a) PPh_3, DEAD, THF, room temp, overnight, 98%; (b) DIBAL, DCM, −78 °C, 1 h, 96%; (c) 1-(triphenylphosphoranylidene)-2-propanone, THF, room temp, overnight, 82%; (d) diethyl malonate, EtONa, EtOH, room temp, overnight; (e) 2 N NaOH, room temp, 4 h; (f) 1 N HCl, 90 °C, 1 h, 53% for the three steps.



Scheme 2. Synthesis of 2a

[a]Reagents and conditions: (a) Triethyl phosphonoacetate, NaH, THF, 0 °C → room temp, overnight; (b) DIBAL, DCM, 0 °C, 2 h, 67% for the two steps; (c) CH_2I_2, $ZnEt_2$, DCM, 0 °C → room temp, overnight, 88%; (d) PCC, silica gel, DCM, room temp, 3 h; (e) 1-(triphenylphosphoranylidene)-2-propanone, THF, room temperature, overnight, 58% for the two steps; (f) diethyl malonate, EtONa, EtOH, room temp, overnight; (g) 2 N NaOH, room temp, 4 h; (h) 1 N HCl, 90 °C, 1 h, 47% for the three steps.

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Table 1

In vitro microsomal stability of 1b and 2b[a]

	Cmpd	NADPH-dependent		NADPH-absent	
		CL_{int}[b] (mL min^{-1} kg^{-1})	$T_{1/2}$[c] (min)	CL_{int}[b] (mL min^{-1} kg^{-1})	$T_{1/2}$[c] (min)
Human	1b	31	74	10	>180
	2b	36	64	12	>180
Mouse	1b	45	52	45	52
	2b	82	28	63	37

[a]Data were obtained from Apredica.

[b]Microsomal intrinsic clearance.

[c]Half-life.

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Table 2

In vitro Caco-2 permeability of 1b and 2b.[a]

Cmpd	P_{app} (A → B)[b] (10^{-6} cm/S)	P_{app} (B → A)[b] (10^{-6} cm/S)	Efflux ratio (B → A)/ (A→B)
1b	24.1	1.5	0.1
2b	21.7	1.1	0.1

[a] Data were obtained from Apredica.

[b] Apparent permeability.