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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 1991–1995

(*S*)-*N*-[1-(4-Cyclopropylmethyl-3,4-dihydro-2*H*-benzo[1,4]oxazin-6-yl)-ethyl]-3-(2-fluoro-phenyl)-acrylamide is a potent and efficacious KCNQ2 opener which inhibits induced hyperexcitability of rat hippocampal neurons

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Received 4 November 2003; revised 9 January 2004; accepted 14 January 2004

Abstract—(*S*)-*N*-[1-(4-Cyclopropylmethyl-3,4-dihydro-2*H*-benzo[1,4]oxazin-6-yl)-ethyl]-3-(2-fluoro-phenyl)-acrylamide ((*S*)-**2**) was identified as a potent and efficacious KCNQ2 opener. This compound demonstrated significant activity in reducing neuronal hyperexcitability in rat hippocampal slices, and the inhibition mediated by (*S*)-**2** was reversed by the KCNQ blocker linopirdine.

1. Introduction

KCNQ2 and KCNQ3 are voltage-activated potassium channels belonging to the 6-transmembrane domain potassium channel gene family. KCNQ2 and KCNQ3 subunits probably co-assemble in neurons to produce a native M-current, which is an important regulator of neuronal excitability because it determines the excitability threshold, firing properties, and responsiveness of neurons to synaptic inputs. Modulators of the M-channel have been under clinical investigation: blockers such as linopirdine for cognition enhancement[1] and openers (e.g., retigabine, Fig. 1) for epilepsy.[2] Retigabine has been shown to activate KCNQ channels expressed in mammalian cells[3] and native M-currents in rat sympathetic neurons.[2,4] This compound produces a large hyperpolarizing shift in the voltage-dependence of activation of KCNQ currents, and this particular mechanism of action probably underlies at least some of the physiological effects of retigabine. In rat models

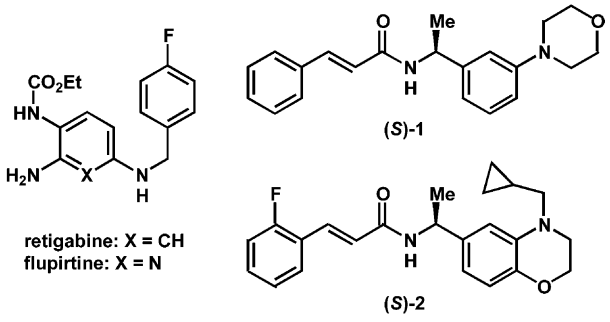

Figure 1.

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doi:10.1016/j.bmcl.2004.01.069

of persistent and neuropathic pain, retigabine has been shown to attenuate nociceptive behaviors.[5] Flupirtine (Fig. 1), a close analogue of retigabine, also shifts the activation curves towards negative voltages, but it is less effective than retigabine.[6] Both retigabine and flupirtine demonstrated significant analgesic activity on tactile allodynia in Chung-lesioned rats.[6] Taken together, this suggests that KCNQ openers may have therapeutic potential in the treatment of neuropathic pain and various chronic pain conditions.

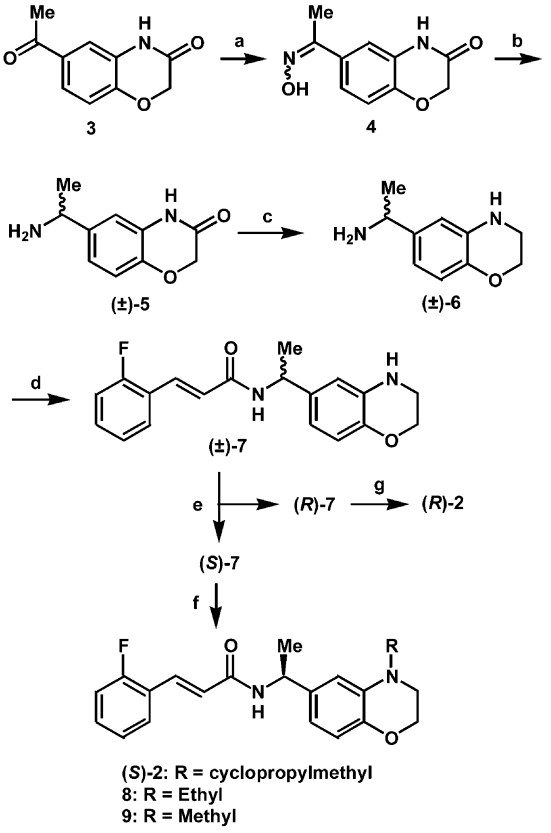
Recently, we reported (*S*)-*N*-[1-(3-morpholin-4-yl-phenyl)-ethyl]-3-phenyl-acrylamide [(*S*)-**1**, Fig. 1] as a novel KCNQ2 opener with excellent oral bioavailability in dogs and rats.[7] This compound demonstrated significant oral activity in the cortical spreading depression model of migraine, suggesting that KCNQ2 openers may also have potential for the treatment of some types of migraine headache.

Despite the recent advances in the KCNQ area, there is still a pressing need for the development of KCNQ channel openers with increased potency compared with retigabine and acrylamide (*S*)-**1**. In an effort to increase the potency of (*S*)-**1**, we retained the morpholine nitrogen of (*S*)-**1** in the same relative orientation, but fused the morpholine onto the phenyl ring. Through this approach we hoped to retain a KCNQ recognition element (the basic nitrogen) while decreasing the entropy of the resulting molecule. The concept of restricting rotatable bonds to improve potency has been employed extensively in medicinal chemistry optimization programs.[8] This investigation led to the identification of (*S*)-*N*-[1-(4-cyclopropylmethyl-3,4-dihydro-2*H*-benzo-[1,4]oxazin-6-yl)-ethyl]-3-(2-fluoro-phenyl)-acrylamide ((*S*)-**2**, Fig. 1) as a potent and efficacious KCNQ2 opener. This report describes the synthesis and abbreviated SAR of acrylamide (*S*)-**2**, its effects on KCNQ-mediated currents, and its activity on neuronal hyperexcitability in rat hippocampal slices.

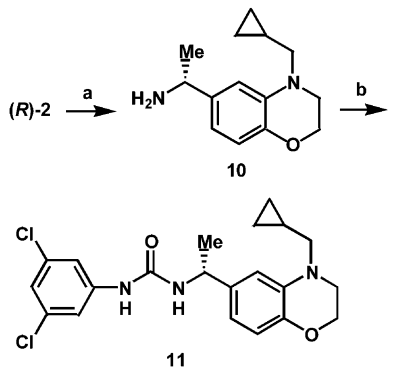
2. Chemistry

The synthesis of (*S*)-**2** and its close analogues started with commercially available 6-acetyl-4*H*-benzo[1,4]-oxazin-3-one (**3**) (Scheme 1). This ketone was converted to oxime **4** through reaction with hydroxylamine hydrochloride. Raney nickel reduction of this oxime furnished amine (±)-**5**, which was further reduced with lithium aluminum hydride to provide diamine (±)-**6**. Direct conversion of **4** to (±)-**6** with lithium aluminum hydride proved to be of much lower yield than the two-step procedure via (±)-**5**. Diamine (±)-**6** was coupled with 3-(2-fluoro-phenyl)-acrylic acid to afford acrylamide (±)-**7**. Chiral HPLC separation provided two enantiomers: (*S*)-**7** and (*R*)-**7**. Compound (*S*)-**7** underwent alkylation with appropriate alkyl halides to afford (*S*)-**2**,[9] **8** and **9**. Compound (*R*)-**2** was prepared from (*R*)-**7** in the same fashion as (*S*)-**2** (Scheme 1).

The absolute configuration of (*S*)-**2**, (*R*)-**2**, **8**, and **9** was established as follows. Treatment of (*R*)-**2** with hydrazine provided amine **10**, which was converted to urea derivative **11** upon reaction with 3,5-dichlorophenyl isocyanate (Scheme 2). The X-ray diffraction analysis of crystalline **11** showed the *R* configuration (Fig. 2), thus indicating the *R* stereochemistry for (*R*)-**2**. Since the amine used for the preparation of (*R*)-**2** was (*R*)-**7**, the amine used for the preparation of (*S*)-**2**, **8**, and **9** must be (*S*)-**7**. Therefore, compounds (*S*)-**2**, **8**, and **9** are assigned with *S* configuration.



Scheme 1. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, NaOH (10 N), THF, reflux, 87%; (b) Raney Ni, H_2 (50 psi), $NH_3{\cdot}H_2O$, MeOH, 94%; (c) $LiAlH_4$, THF, −78 °C, 95%; (d) 3-(2-fluoro-phenyl)-acrylic acid, EDAC·HCl, DMAP, Et_3N, CH_2Cl_2, 91%; (e) chiral HPLC (AD column, 75% hexanes/25% EtOH); (f) K_2CO_3, acetone, cyclopropylmethyl bromide for **2** (78%), EtI for **8** (86%), and MeI for **9** (77%); (g) K_2CO_3, acetone, cyclopropylmethyl bromide, 74%.



Scheme 2. Reagents and conditions: (a) NH_2NH_2, EtOH, 100 °C, 63%; (b) 3,5-dichlorophenyl isocyanate, *i*-Pr_2NEt, CH_2Cl_2, 41%.

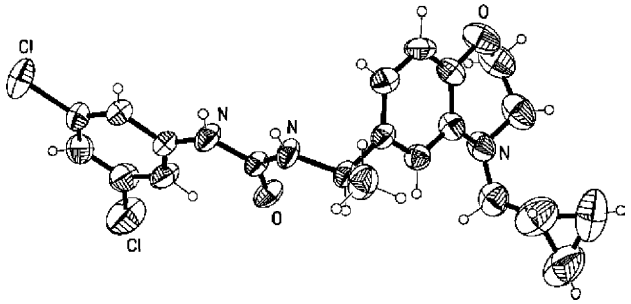


Figure 2. Thermal ellipsoid plot (35% ellipsoids) of crystalline **11**.

3. Results and discussion

Figure 3A and 3B show the effects of (*S*)-**2** on the same HEK 293 cell at a test potential of −30 mV and −110 mV respectively. The very large enhancement of KCNQ2 current measured at −30 mV in the presence of 10 μM (*S*)-**2** is particularly striking because of the change in the kinetic properties of the KCNQ2 current produced by (*S*)-**2**. The control current has the expected sigmoid activation kinetics characteristic of KCNQ currents (lower trace), whereas in the presence of (*S*)-**2**, the KCNQ2 current evoked by stepping from −80 to −30 mV is largely instantaneous with an additional slowly activating current component on top of the instantaneous current fraction. This dramatic change from sigmoid to instantaneous current activation is reflected in the shape of the conductance-voltage G/V relationship of KCNQ2 in the presence of (*S*)-**2** shown in Figure 3C. The current traces in Figure 3B show the large inward KCNQ2 currents produced following a membrane potential step from −80 mV to −110 mV. The KCNQ2 current flows into the cell because the command potential of −110 mV is below the K^+ equilibrium potential, E_{K+} (∼−90 mV). This result indicates that (*S*)-**2** produces a very large and profound hyperpolarizing shift in the voltage-dependence of activation of KCNQ2. The voltage for half-activation ($V_{0.5}$) was −15.9 mV in control conditions and −52.7 mV in the presence of 10 μM (*S*)-**2**, a 36.8 mV shift in the hyperpolarization direction. Note that Figure 3C only shows the normalized outward conductance changes produced by (*S*)-**2**, which is the conventional method for displaying G/V data. The numerical shift in $V_{0.5}$ is actually an underestimate of the total change in voltage-dependence of the conductance.

Figure 4 shows the concentration–response relationship for (*S*)-**2** mediated increase of KCNQ2 current from single HEK 293 cells voltage-clamped at −40 mV. The threshold concentration is ∼10 nM to 30 nM, and the onset of action is a few seconds (time course data not shown) and steady-state activity is reached in approximately 30 s. Following removal of (*S*)-**2**, there is rapid and complete reversal of the enhancement of KCNQ2 current. The EC_{50} for (*S*)-**2** was estimated from the fit to data to be 0.063 ± 0.01 μM with a Hill slope (HS) of 1.8. The HS >1 suggests the mechanism of action of (*S*)-**2** may involve positive cooperative binding, a relatively common observation in drug receptor/channel interactions.[10,11]

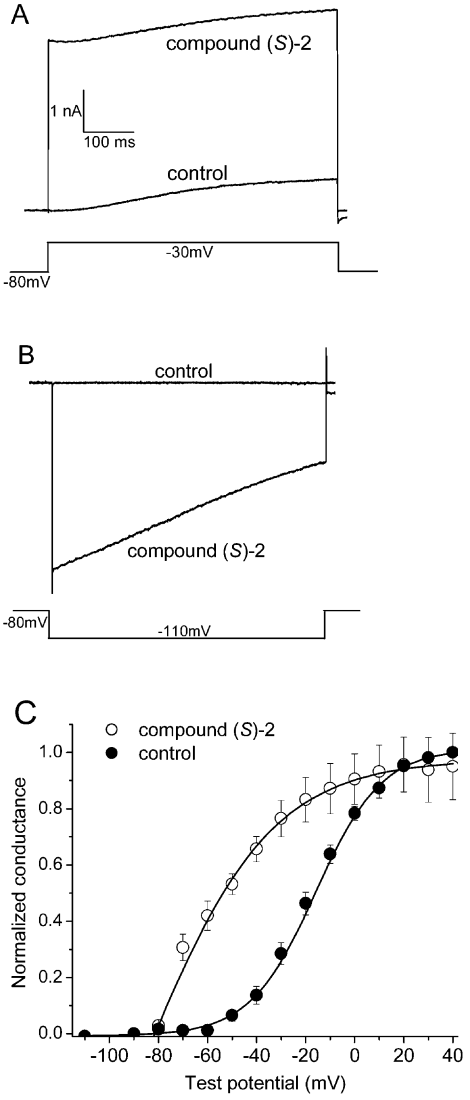


Figure 3. Effect of (*S*)-**2** (10 μM) on mKCNQ2 currents stably expressed in HEK 293 cells measured by the whole-cell patch clamp method. **A** and **B** show representative data from a single cell of the dramatic increase in KCNQ2 current measured at test potentials of −30 mV and −110 mV, respectively, following a step command from −80 mV in the absence and presence of 10 μM (*S*)-**2**. **C** shows the effect of 10 μM (*S*)-**2** on the conductance–voltage relationship for mKCNQ2. Data shown is the mean ±SEM ($n = 6$).

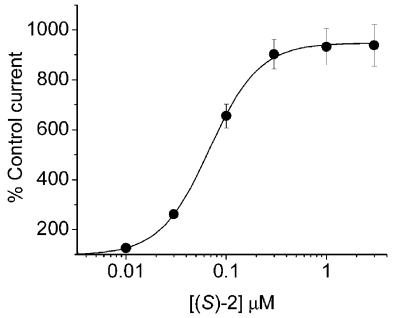


Figure 4. Concentration dependent augmentation effect of mouse KCNQ2 currents by (*S*)-**2**. Data shown is the mean ±SEM for 5 cells.

The mechanism of action of (*S*)-**2** is in large part a hyperpolarizing shift in the voltage dependence of activation of the KCNQ2 conductance. In addition, the instantaneous kinetics of activation observed in the presence of (*S*)-**2** are strikingly different from the activation kinetics of non-treated cells, which implies that (*S*)-**2** converts KCNQ2 channels from being strongly voltage-dependent to a conformational state essentially devoid of voltage-dependence in the physiological membrane potential range. In any given neuron where natively expressed KCNQ2 channels contribute significantly to setting and maintaining the resting membrane potential, one might expect (*S*)-**2** to hyperpolarize and therefore essentially clamp the membrane potential close to E_K^+. The net effect of this would be that any such neurons would become electrically 'silent'. Such a mechanism of action of (*S*)-**2** would be entirely consistent with its ability to strongly inhibit the firing frequency of hippocampal neurons described in this report. Interestingly, the mode switch in activation kinetics produced by (*S*)-**2** is similar to that observed with (*S*)-**1**,[7] but importantly (*S*)-**2**, unlike (*S*)-**1**, does not significantly inhibit KCNQ2 conductance at positive membrane potentials, which could simplify its physiological effects in the CNS. Direct electrophysiological recordings from neurons expressing KCNQ currents should be able to distinguish the functional significance of not inhibiting KCNQ conductance at positive potentials.

Whole-cell patch-clamp evaluation on recombinant mouse KCNQ2 channels expressed in HEK 293 cells demonstrates that (*S*)-**2** is a potent and efficacious opener of KCNQ2 channels. As shown in Table 1, (*S*)-**2** has an improved potency over (*S*)-**1** and retigabine by a factor of ~55-fold and 22-fold, respectively. The efficacies of test compounds were normalized to the efficacy of a specific reference compound,[12] to yield an E/Eref ratio. The ratio is calculated by dividing the maximum current amplitude produced by the test compound by the maximum current amplitude produced by the reference compound. The benefit of using an E/Eref value is that it allows ranking of compound efficacies independent of the amount of basal KCNQ2 current expression. The E/Eref's of (*S*)-**2**, (*S*)-**1** and retigabine are 1.83, 1.40, and 1.60, respectively (Table 1). Thus, (*S*)-**2** is 31% and 14% more efficacious in opening KCNQ2 channels than (*S*)-**1** and retigabine, respectively.

The above EC_{50} measurements were also carried out on several analogues structurally related to (*S*)-**2**. As shown in Table 1, the potency appears to increase as the alkyl substituent attached to the oxazine nitrogen becomes bulkier. Interestingly, the size of the alkyl group has little impact to the efficacy of these analogues with the exception of the unsubstituted analogue (*S*)-**7**. Of special note is that the (*R*)-**2** exhibited no KCNQ2 opener activity when tested at 10 μM, whereas (*S*)-**2** is a highly potent and efficacious KCNQ2 opener, suggesting that the *S* configuration may play an important role in opening KCNQ2 channels for this series of acrylamides.

Table 1. Whole cell patch-clamping data (−40 mV)

Compd	EC_{50} (μM)[a]	E/Eref[a,b]
(*S*)-**1**	3.28 ± 0.05	1.40 ± 0.06
(*S*)-**2**	0.06 ± 0.01	1.83 ± 0.02
8	0.20 ± 0.02	2.1 ± 0.3
9	0.94 ± 0.14	1.8 ± 0.2
(*S*)-**7**	2.0 ± 0.1	1.2 ± 0.1
Retigabine	1.30 ± 0.04	1.60 ± 0.05

[a] These values are the mean ± SEM (n = 2–5).
[b] The efficacy of the test compound relative to the reference compound.[12]

Preliminary studies using thallium(I) influx assay[7] showed that compounds (*S*)-**2**, (*S*)-**7**, **8**, and **9**, like (*S*)-**1**,[7] also activated other members of the KCNQ family. Further electrophysiological characterization is required in order to understand the activation of other KCNQ channels.

Compound (*S*)-**2** was examined for its ability to reduce spontaneous neuronal discharges in rat hippocampal slices. In this assay, the induction of spontaneous neuronal bursting was achieved by bathing slices to an artificial cerebrospinal fluid (CSF) containing zero magnesium ($MgSO_4$) and low calcium ($CaCl_2$ 1.5 mM). The resulting multiple-unit extracellular electrical activity was recorded by advancing an electrode 50–150 μm into the CA1 region of the hippocampus. This electrical activity was then amplified using a differential amplifier, and the number of events per min was collected and analyzed. The analyzed data was expressed in Hz and reported as percent inhibition of compound free control. Figure 5A depicts the multi-unit response of a single hippocampal slice to the application of (*R*)-**2** (inactive enantiomer), (*S*)-**2** (active enantiomer) and (*S*)-**2** in the presence of linopirdine, a KCNQ blocker. Application of (*R*)-**2** (2.5 μM) did not provide any reduction on the neuronal bursting, whereas addition of (*S*)-**2** (2.5 μM) resulted in a significant inhibition on neuronal hyperexcitability. After maximal inhibition by (*S*)-**2** was achieved, the KCNQ blocker linopirdine (10 μM) was applied concurrently with (*S*)-**2** (2.5 μM). Complete reversal of the (*S*)-**2** mediated inhibition was produced by co-application in this example.

In order to establish a concentration–response curve for (*S*)-**2** (Fig. 5B), slices were exposed to a range of concentrations (100 nM–2.5 μM). A minimum of 12 slices were used for each compound concentration. The EC_{50} calculated for (*S*)-**2** was 0.70 μM. It should be noted that (*R*)-**2** had no effect on neuronal discharge when tested at the concentration producing maximal inhibition by (*S*)-**2** (2.5 μM) (Fig. 5B), which is consistent with the inability of this concentration of (*R*)-**2** to activate KCNQ2 expressed in HEK 293 cells (data not shown). Concurrent application of (*S*)-**2** (2.5 μM) and linopirdine (10 μM) significantly reduced the inhibition relative to (*S*)-**2** (2.5 μM) alone (Fig. 5B).

We have hypothesized that a KCNQ opener would be efficacious in reducing the induced neuronal hyperexcitability in rat hippocampal slices, by virtue of increasing K^+ efflux from the cells, stabilizing the cell membrane, and thus making it harder for depolarizing stimuli to

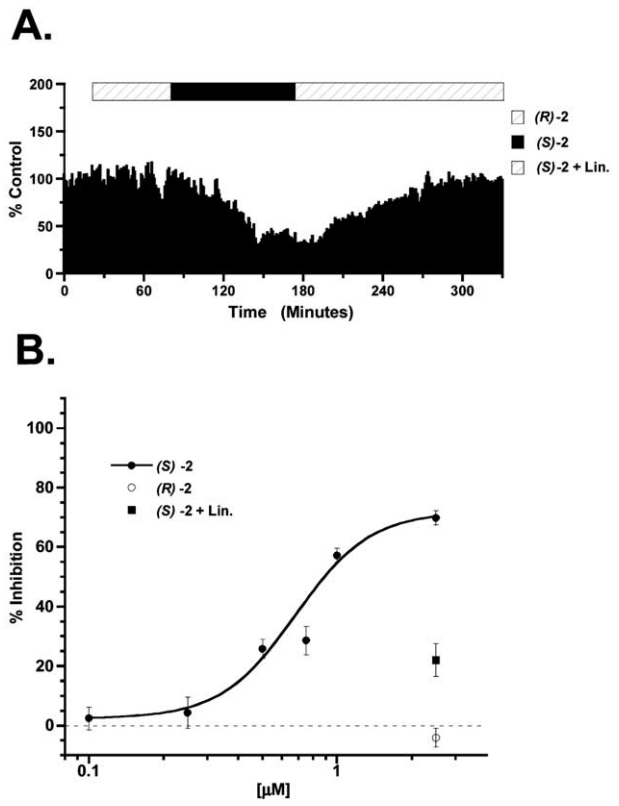


Figure 5. A. Example of the multi-unit response of a single hippocampal slice to the application of (*R*)-**2** (2.5 μM), followed by prolonged application of (*S*)-**2** (2.5 μM). After maximal inhibition by (*S*)-**2** was achieved, linopirdine (Lin., 10 μM) was applied concurrently with (*S*)-**2** (2.5 μM). B. Group concentration–response relationship generated for (*S*)-**2** in hippocampal slices ($n = 12$–36).

spread. Consistent with this hypothesis, the active enantiomer (*S*)-**2** demonstrated a significant inhibition of the neuronal discharge in a concentration-related fashion, while no reduction was observed with the inactive enantiomer (*R*)-**2**. Furthermore, the effect of (*S*)-**2** was mediated specifically via modulation of KCNQ channel function as shown by the reversal of (*S*)-**2** mediated inhibition by the KCNQ blocker linopirdine in this hippocampal slice model.

4. Conclusion

Compound (*S*)-**2** was identified as a potent and efficacious KCNQ2 opener and is expected to be a valuable tool in further studying the pharmacology of KCNQ2 and most probably other KCNQ channels. In vitro testing of (*S*)-**2** in hyperexcited hippocampal slices suggests that KCNQ2 openers may have potential for the treatment of CNS disorders characterized by neuronal hyperexcitability, such as migraine, epilepsy, bipolar disorder and neuropathic pain.

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9. Data for (*S*)-**2**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.68 (d, $J = 16$ Hz, 1H), 7.48–7.43 (m, 1H), 7.34–7.25 (m, 1H), 7.15–7.04 (m, 2H), 6.75 (m, 2H), 6.66–6.59 (m, 1H), 6.50 (d, $J = 16$ Hz, 1H), 5.76 (d, $J = 7.6$ Hz, 1H), 5.17–5.07 (m, 1H), 4.23 (t, $J = 4.4$ Hz, 2H), 3.41 (t, $J = 4.4$ Hz, 2H), 3.14 (d, $J = 6.4$ Hz, 2H), 1.54 (d, $J = 6.8$ Hz, 3H), 0.99–1.04 (m, 1H), 0.52–0.57 (m, 2H), 0.20–0.24 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 3.6 (d, $J = 10$ Hz), 8.1, 21.4, 46.6, 49.2, 55.2, 64.7, 111.1, 114.7, 116.2 (d, $J = 22$ Hz), 116.4, 122.0 (d, $J = 11$ Hz), 123.0 (d, $J = 8$ Hz), 124.4 (d, $J = 4$ Hz), 129.8 (d, $J = 3$ Hz), 130.8 (d, $J = 8$ Hz), 134.1, 135.6, 136.0, 143.5, 161.4 (d, $J = 252$ Hz), 164.7. $[\alpha]_D^{20}$ +5.7 (*c* 3.08, CH_2Cl_2).
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