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Novel immunomodulators based on an oxazolin-2-one-4-carboxamide scaffold ☆

Xinhua He *, Lili Wang, Zhibing Zheng, Junhai Xiao, Wu Zhong, Song Li *

Beijing Institute of Pharmacology & Toxicology, No.27 Taiping Road, Beijing 100850, PR China

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ABSTRACT

A series of oxazolidin-2-one-4-carboxylic amide compounds (**1a–f**) were designed and synthesized as the non-phosphate $S1P_1$ receptor agonists. The single crystal of **1e** was prepared and solved to elucidate the structure of **1a–f**. EC_{50} of **1a–d** were about 1.1–3.6 μM in $S1P_1$ Redistribution® assay, and their cytotoxicity was 8–40-fold lower than FTY720. Though its $S1P_1$ agonist activities in vitro were about 1000-folds weaker than (S)-FTY720-P, at a dose of 10 mg/Kg, the immunosuppressive effects of **1a** were comparable to FTY720. So oxazolidin-2-one-4-carboxylic amide derivatives were found as potential immunomodulator, compound **1a** could be considered as a lead compound, rational modifications of **1a** are anticipated using medicinal chemistry techniques and molecular modeling to obtain analogs with higher affinity and better clinical therapeutic properties.

The need to develop less toxic and more effective immunosuppressants is essential owing to the worldwide growth of the autoimmune disease population. The novel immunosuppressant FTY720,[1,2] unlike conventional immunosuppressive agents, does not inhibit T or B cell activation or proliferation, nor their effector function.[3,4] FTY720 is a prodrug, phosphorylated in vivo and transformed to its active metabolite FTY720-phosphate (FTY720-P) by sphingosine kinase 2.[5] FTY720-P acts as a high-affinity agonist at $S1P_1$-R in lymphocytes, thereby inducing aberrant internalization of the receptor, rendering the lymphocyte cells unresponsive to sphingosine 1-phosphate (S1P, Fig. 1), and depriving them of an obligatory signal to egress from lymphoid organs. Consequently, FTY720 causes the rapid and dramatic depletion of circulating lymphocytes, which reduces and/or inhibits the infiltration of lymphocytes into graft sites and inflamed tissues,[6] thereby exerting powerful immunosuppressive activity. This new mechanism of FTY720 opens a new way to develop novel immunomodulator based on $S1P_1$-R agonist.[7–11] Recently, FTY720 has been approved in United States and other countries for the treatment of patients with relapsing multiple sclerosis; however, it could possibly cause several side effects including potentially fatal infections, bradycardia, skin cancer, macular edema, breathing and liver problems.[12] Therefore an S1P-R agonist with fewer side effects and more powerful immunosuppressive effects would be highly desirable to be developed. Here, we focused on designing innovative $S1P_1$ receptor agonists with more effective immunosuppression but lower toxicity, and we found a series of oxazolidin-2-one-4-carboxylic amide compounds as a novel scaffold of S1P receptor agonists.

FTY720 is converted to (S)-FTY720-P by sphingosine kinase. (S)-FTY720-P acts as an agonist on four S1P receptors ($S1P_{1,3,4,5}$) with IC_{50}s 2.1, 5.9, 23 and 2.2 nM, respectively,[13,14] while the (*R*)-isomer binds with 5–10-fold lower affinity. FTY720 is only phosphorylated partially in vivo, the nonphosphorylated FTY720 has no immunosuppressive effects. In this study, we engaged to develop novel non-phosphate $S1P_1$ receptor agonists which were independent of sphingosine kinase 2 in vivo.

The structures of S1P and FTY720-P consist of a phosphate moiety as 'hydrophilic head' and a linear alkyl group as 'hydrophobic tail'. As previously reported, the 'hydrophilic head' forms ion-pairing interaction with ARG120 and ARG292 of $S1P_1$-R.[15,16] This ion-pairing interaction might be important for proper conformational change of $S1P_1$-R in response to agonist binding.[17] However, this ion-pairing interaction can be replaceable,[18] therefore, we choose amide group as the hydrophilic head to replace the phosphate moiety of FTY720-P. And, we engaged the hydroxyl and amino group of FTY720-P in a carbamatic moiety to form an oxazolinone ring in order to increase the rigidity of the molecule. This oxazolinone ring, which contains two oxygen atoms and a nitrogen atom, has potential ability to form hydrogen-bond network with $S1P_1$-R. In addition, various alkyl chains bearing an oxygen atom were introduced as the 'hydrophobic tail' based on bioisosteric rule to investigate structure–activity relationship of $S1P_1$-R agonists (Fig. 2).

Before we synthesized the molecules, we evaluated the compound's potential to activate $S1P_1$ receptor by computational modeling. We carried out molecular docking of these compounds into the binding site of $S1P_1$-R by means of the affinity module in Insight II program package (Fig. 3). Since the crystal structure of $S1P_1$ receptor has not been published, we constructed the $S1P_1$ receptor model using homology modeling module in InsightII/Homology program. The $S1P_1$ receptor sequence was aligned against the

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* Corresponding authors.
E-mail addresses: hexinhua2007@gmail.com (X. He), lisong_amms@yahoo.cn (S. Li).

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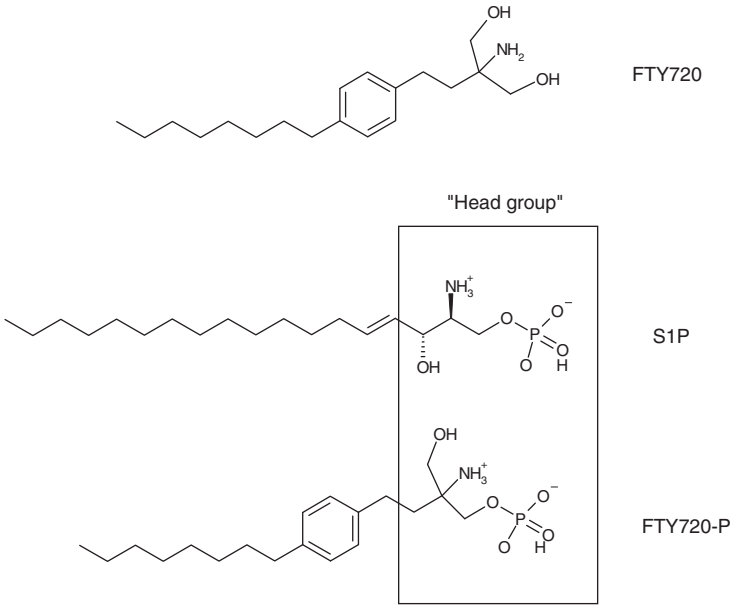


Figure 1. Structure and regions of FTY720, S1P and FTY720-P.

transmembrane helices (THs) of a rhodopsin crystal model (Protein Data Bank code: 1boj).[17] The preliminary model was initially minimized for 5000 iterations of steepest descent, followed by conjugate gradient and Truncated Newton minimizations to a RMSG (root mean square gradient) of 0.01 kcal/mol Å. The active site includes a long and narrow hydrophobic pocket formed by residue Met124, Leu135, Leu136, Leu128, Phe264, Leu271, Phe295, Ala299, as well as the two key residues, Arg120 and Arg292, which are important to interactions of sphingosine 1-phosphate (S1P) with $S1P_1$-R. The molecular docking result indicated that our designed compounds bind to $S1P_1$-R via H-bonds instead of ion-paring interaction between S1P and $S1P_1$-R. The strong hydrogen-bond interactions were found between amino acid Arg120, Phe295 and oxazolidin-2-one ring. Furthermore, the hydrophobic tail of the molecule affected its binding mode. If the R group in Figure 2 was n-C_nH_{2n+1} ($3<n<12$) or p-substituted benzyl, the conformation was linear and fit in the $S1P_1$ receptor active site in a pattern similar to S1P. Based on the binding energy and geometry match, we chose to synthesize the four candidate molecules **1a–d** (Table 1) with lower binding energy of −50 to −69 kcal/mol. However, if the R groups were o-substituted benzyls, the molecules fitted in the $S1P_1$ receptor active site with a different orientation in which the molecules were reversed as showed in Figure 3b. Their binding energy were −20 to −40 kcal/mol, higher than the molecule with linear alkyl group or p-substituted benzyl. For comparison, we also synthesized two molecules with o-substituted benzyl, **1e** and **1f**. In summary, after evaluation by molecular docking, six molecules were chosen to be synthesized to investigate their $S1P_1$-R agonist activity.

The syntheses of compounds **1a–f** are shown in Scheme 1. Commercially available 4-(2-hydroxyl-ethyl)-phenol was transformed to ether 2 by reaction with the corresponding halide. Ether 2 reacted with methanesulfonyl chloride to give intermediate 3. 3 was treated with lithium iodide to give iodide 4 in 96% yield. On the other hands, cyano-acetic acid ethyl ester reacted with sodium nitrite and hydrogen chloride to give cyano-hydroxyimino-acetic acid ethyl ester 5 which was then reduced by Zn/AcOH to give amino-cyano-acetic acid ethyl ester 6 in 60% yields. Subsequently, 6 was reacted with $(BOC)_2O$ to produce C intermediate 7 in good yield. Condensation reaction of 4 and 7 d by NaH in DMF provided 8 in 90% yield. 8 was then reduced by $LiAlH_4$ to get 9. Finally, target product **1a–e** was obtained by hydrolyzing and lactonizing 9 by 'one pot process' under the condition of CF_3COOH in anhydrous CH_2Cl_2. This is the first-time report on a 'one pot process' for the synthesis of oxazolin-2-one-4-carboxylic amide derivatives.[19] In order to confirm the structure of the products, single crystals of **1e** prepared by slow evaporation of a solution of **1e** in ethyl acetate. The molecular structure was solved by using SHELXL97 program. The crystal structure has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number is CCDC 844034.

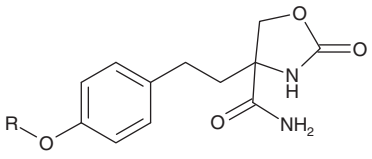


Figure 2. General structure of oxazoline-2-one-4-carboxylic amide derivatives.

Compounds **1a–f** have been evaluated for their in vitro $S1P_1$ receptor (formerly called EDG1) agonist activity using the $S1P_1$ Redistribution® assay (a commercial model with good reproducibility, Fisher BioImage ApS). This assay was designed to screen for $S1P_1$ agonists including internalization by monitoring the translocation of a membrane-localized S1P1-EGFP fusion protein to endosomes.[20,21]

The results are shown in Table 2. EC_{50} of compounds **1a–d** were about 1–4 μM. Compound **1e** showed weak $S1P_1$ agonist activity with an EC_{50} (>100 μM). Compound **1f** did not show any $S1P_1$ agonistic activity in this test. The cytotoxicity was measured by the MTT method with HLF cells (Table 2). It was found that the cytotoxicity of **1a–d** was 8–40-fold lower than FTY720. In particular, TC_{50} of **1b–d** are at same level, while TC_{50} of 1a (121 μM) was about four-fold of the one of **1b–d**. The structure–activity relationship of **1a–f** can be summarized as follow, 1) Introducing a phenyl ring to the lipophilic tail did not affect the compounds $S1P_1$ receptor affinity, but improved its cytotoxicity. 2) Replacing the C8 carbon-chain in tail group with an o-substituted benzyl would lead to significant loss in agonistic activity.

A striking feature of FTY720 is the induction of a marked decrease in the number of peripheral blood lymphocytes (lymphopenia) at doses that display an immunosuppressive effect in

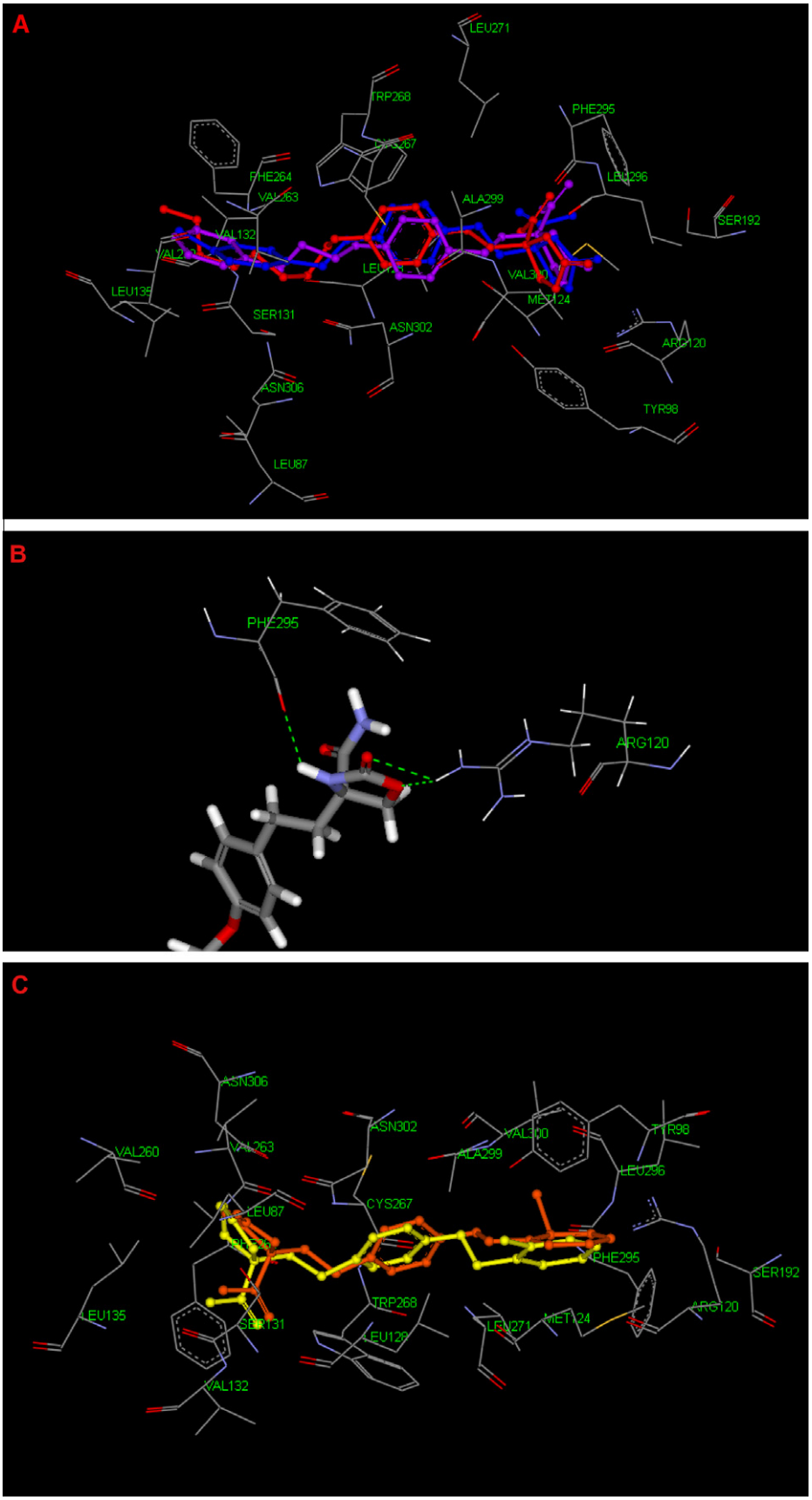


Figure 3. (A) Compound **1a–c** docked in the active site of S1P receptor site: amino acid residues in hydrophobic pocket and H-bond interactions are presented. (B) A close part view of H-bond interactions (green) of **1a** with S1P receptor, the distance between donor Atom and acceptor Atom was presented. (C) Compound **1e** and **1f** docked in the active site of S1P receptor site.

Table 1
Structure of compound **1a–f**

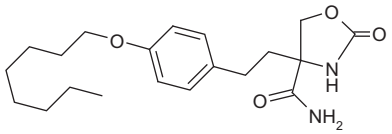
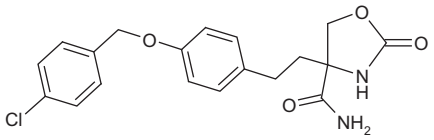
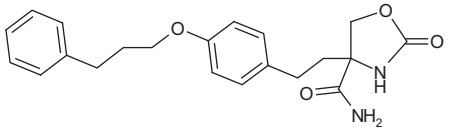
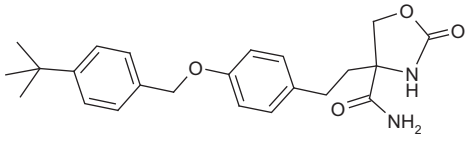
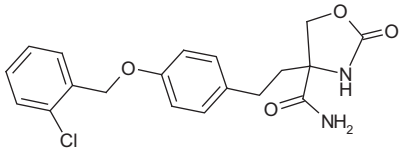
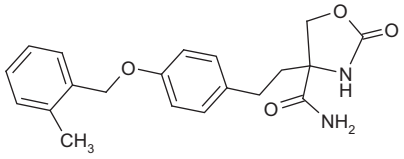
Compound	Structure
1a	
1b	
1c	
1d	
1e	
1f	

Table 2
$S1P_1$ receptor agonist activity and cytotoxicity results for compounds **1a–d**

Compound	EC_{50}[a] (μM)	TC_{50}[a] (μM)	T. I.[b]
FTY720	3.00–10.0[c]	3.15 (±0.06)	
1a	2.84 (±0.37)	121.10 (±29.77)	42.64
(S)-FTY720 P	2.1nM*		
1b	1.10 (±0.89)	27.90 (±4.52)	25.36
1c	3.63 (±4.55)	26.42 (±2.14)	7.28
1d	2.72 (±2.70)	25.44 (±2.20)	9.35
1e	>100	—	
1f	NO	—	

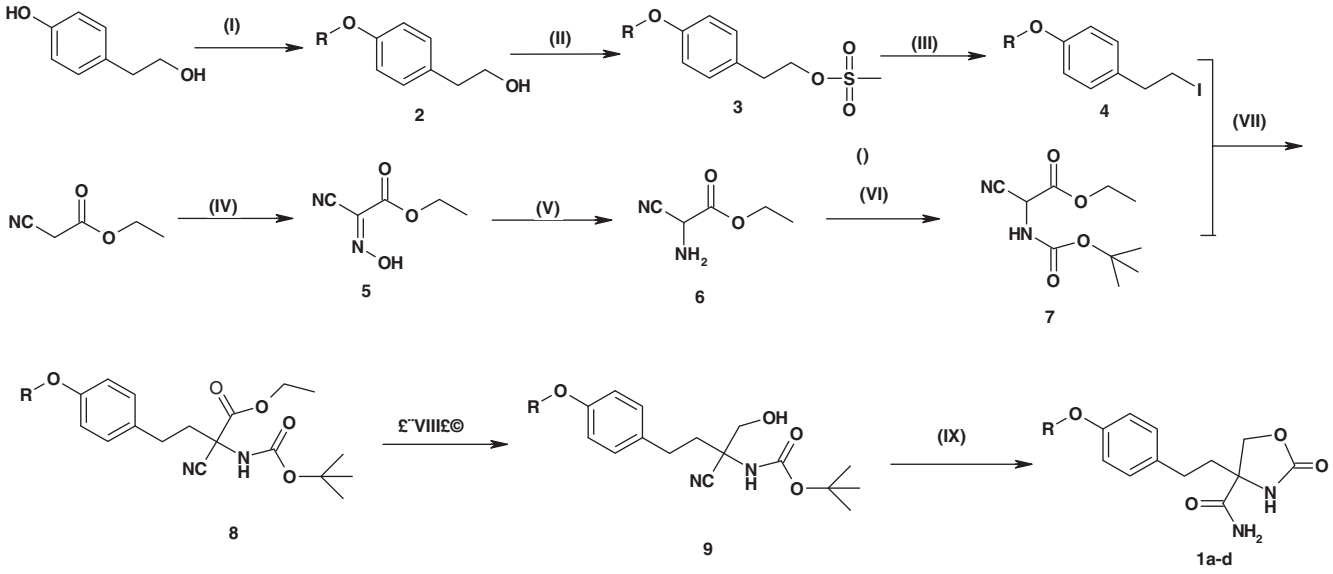
[a] On the basis of triplicate experiments with given standard deviation in parentheses.
[b] In vitro therapeutic index (TC_{50}/EC_{50}).
[c] The EC_{50} of FTY720 cannot be obtained due to its cytotoxicity, and its percent activity (PCTACT) relative to the S1P control at 3.0 μM is 37 ± 3.1% and 10.0 μM is 54 ± 5.6%; —, not test; NO, inactive. *, value of literature.[13,14]

Table 3
Compound **1a** sequesters lymphocytes form circulation

Dose (mg/kg)	Percent of $CD3^+$ cell decrease (%)	
	FTY720	**1a**
0.1	9.7 ± 26.6	—
0.3	47.2 ± 13.7	1.7 ± 5.7
1	79.0 ± 7.6	20.0 ± 6.1
3	80.7 ± 7.8	53.0 ± 6.4
10	86.6 ± 15.8	84.3 ± 4.0

experimental allograft models and autoimmune disease models. Compound **1a** as the most potential agonist was chosen to be tested for its activity of sequester circulating lymphocytes into secondary lymphoid tissues in vivo.[22] Oral administration of **1a** induces a rapid, potent, and dose-dependent decrease of peripheral $CD3^+$ T cell in BALB/C mouse (Table 3). At doses form 0.1 to 3 mg/kg, the efficacy to decrease peripheral blood $CD3^+$ cells was lower for **1a** when compared to FTY720. At a dose of 10 mg/kg, **1a** shows in vivo efficacy comparable to FTY720(at a dose of 10 mg/kg).

In conclusion, a series of oxazolidin-2-one-4-carboxylic amide compounds were designed and synthesized as novel non-phosphate $S1P_1$-R agonists. Though their $S1P_1$ agonist activities in vitro were about 1000-folds weaker than (S)-FTY720-P, the immunosuppressive effect of **1a** in vivo was comparable to FTY720 at a dose of 10 mg/Kg. This result suggested that **1a**, a non-phosphate $S1P_1$ receptor agonist, has its own merit. The T.I. of 1a was 43 in vitro and oral administration of 1a induces a rapid,



Scheme 1. Reagents and conditions: (I) alkyl halide, K_2CO_3, DMF, 70% (average); (II) methanesulfonyl chloride, Et_3N, CH_2Cl_2, 98%; (III) LiI, anhydrous tetrahydrofuran, 96%; (IV) $NaNO_2$, HCl, 81%; (V)Zn, HAc, 61%; (VI) $(BOC)_2O$, 83%; (VII) NaH, DMF, 90%; (VIII) $LiAlH_4$,THF,62%; (IX) CF_3COOH, CH_2Cl_2, 60%.

potent, and dose-dependent decrease of peripheral $CD3^+$ T cell in BALB/C mouse, So compound **1a** could be considered as a candidate for further development of novel immunosuppressive agents, or as a lead compound, rational modifications of **1a** are anticipated using medicinal chemistry techniques and molecular modeling to obtain analogs with higher affinity and better clinical therapeutic properties.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.088.

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22. Selected data for compound **1a**: ^{1}H NMR (400 MHz, $CDCl_3$) MS (EI) [M^+]: m/z = 362; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.24(s,1H), 7.47(s,2H), 7.06–7.08(d, 2H), 6.81–6.83(d, 2H), 4.33–4.35(d, 1H), 4.17–4.19(d, 1H), 2.54–2.60(m, 1H), 2.30–2.38(m, 1H), 1.98–2.06(m, 1H), 1.82–1.90 (m, 1H), 1.64–1.71(m, 2H), 1.25–1.40 (m, 10H), 0.84–0.87 (t, 3H). 1b: MS (EI) [M^+]: m/z = 374; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.14(s, 1H), 7.41–7.45(m, 6H), 7.08–7.10 (d, 2H), 6.90–6.92(d, 2H), 5.00(s, 2H), 4.33–4.35(d, 1H), 4.17–4.19(d, 1H), 2.54–2.60(m, 1H), 2.30–2.38 (m, 1H), 1.98–2.06(m, 1H), 1.82–1.90(m, 1H). **1c**: MS(EI) [M^+]: m/z = 368; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.17(s, 1H), 7.44–7.47(d, 2H), 7.16–7.30(m, 5H), 7.07–7.9(d, 2H), 6.83–6.85(d, 2H), 4.33–4.35(d, 1H), 4.17–4.19 (d, 1H), 3.89–3.91(t, 2H), 2.71–2.73(t, 2H), 2.54–2.60(m, 1H), 2.30–2.38(m, 1H), 1.98–2.06(m, 1H), 1.82–1.90(m, 1H). **1d**: MS(EI) [M^+]: m/z = 396; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.14(s, 1H), 7.33–7.44(m, 6H), 7.08–7.10(d, 2H), 6.90–6.92(d, 2H), 5.00(s, 2H), 4.33–4.35(d, 1H), 4.17–4.19(d, 1H), 2.54–2.60(m, 1H), 2.30–2.38(m, 1H), 1.98–2.06 (m, 1H), 1.82–1.90 (m, 1H), 1.27 (s, 9H). **1e**: EI-MS(m/e): [M^+] = 374; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.14(s,1H), 7.41–7.45(m,6H), 7.08–7.10 (d, 2 H), 6.90–6.92 (d,2H), 5.00(s,2H), 4.33–4.35(d,1H), 4.17–4.19(d, 1H), 2.54–2.60(m, 1H), 2.30–2.38 (m,1H), 1.98–2.06 (m,1H), 1.82–1.90(m, 1H). **1f**: EI-MS(m/e): [M^+] = 355; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.24 (s,1H), 7.44–7.47(d,2H), 7.30–7.32(d,2H), 7.17–7.19(d,2H), 7.07–7.09(d,2H), 6.89–6.91 (d,2H), 5.00(s,2H), 4.33–4.35(d,1H), 4.17–4.19(d, 1H), 2.54–2.60(m, 1H), 2.30–2.38 (m,1H), 2.29(s,3H), 1.98–2.06(m,1H), 1.82–1.90(m, 1H).