European Journal of Medicinal Chemistry 70 (2013) 811–830

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design and synthesis of potent and selective $P2X_3$ receptor antagonists derived from PPADS as potential pain modulators

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ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form
7 October 2013
Accepted 11 October 2013
Available online 18 October 2013

Keywords:
$P2X_3$ receptor
Antagonists
PPADS
Structure-activity relationships
Two-electrode voltage clamp
Pain

ABSTRACT

Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate (**7a**, PPADS), a nonselective P2X receptor antagonist, was extensively modified to develop more stable, potent, and selective $P2X_3$ receptor antagonists as potential antinociceptive agents. Based on the results of our previous report, all strong anionic groups in PPADS including phosphate and sulfonate groups were changed to carboxylic acids or deleted. The unstable azo (−N=N−) linkage of **7a** was transformed to more stable carbon-carbon, ether or amide linkages through the synthesis of the 5-hydroxyl-pyridine moieties with substituents at 2 position via a Diels-Alder reaction. This resulted in the retention of antagonistic activity (IC_{50} = 400 ~ 700 nM) at the $hP2X_3$ receptor in the two-electrode voltage clamp (TEVC) assay system on the *Xenopus* oocytes. Introduction of bulky aromatic groups at the carbon linker, as in compounds **13h**–**n**, dramatically improved the selectivity profiles of $hP2X_3$ when compared with $mP2X_1$ and $hP2X_7$ receptors. Among the substituents tested at the 2-position, the *m*-phenoxybenzyl group showed optimum selectivity and potency at the $hP2X_3$ receptor. In searching for effective substituents at the 4- and 3-positions, we found that compound **36j**, with 4-carboxaldehyde, 3-propenoic acid and 2-(*m*-phenoxy)benzyl groups, was the most potent and selective $hP2X_3$ receptor antagonist with an IC_{50} of 60 nM at $hP2X_3$ and marginal antagonistic activities of 10 μM at $mP2X_1$ and $hP2X_7$. Furthermore, using an *ex-vivo* assay system, we found that compound **36j** potently inhibited pain signaling in the rat dorsal horn with 20 μM **36j** displaying 65% inhibition while 20 μM pregabalin, a clinically available drug, showed only 31% inhibition.

1. Introduction

The purine nucleotide ATP is not only a cellular energy source, but an extracellular messenger that acts by binding to P2 nucleotide receptors [1,2]. The P2 receptors are classified into two families: ionotropic ligand-gated cationic channel (P2X receptors) and metabotropic G-protein coupled receptors (P2Y receptors) [3,4]. P2X receptors are further categorized into seven subtypes $P2X_1$ to $P2X_7$, with common structural features, including two transmembrane (TM1 and TM2) domains, a large extracellular loop containing ATP binding sites, and intracellular N- and C-terminals. Generally, P2X receptors form homo- or hetero-trimers to act as ion channels and the detailed mechanism of channel opening was elucidated by apo and open state crystal structures of zebrafish $P2X_4$ receptors [5]. Activation of P2X receptors, most of which are nonselective cation channels permeable to Na^+, K^+ and Ca^{2+}, results in membrane depolarization and cell excitation [6–8].

P2X receptors are widely expressed throughout the whole body, including in vascular smooth muscles, platelets, the nervous system, and immune system cells [9]. Among the seven P2X receptor subtypes, $P2X_3$ receptors are expressed selectively and at high levels in nociceptive primary sensory neurons in trigeminal, nodose, and dorsal root ganglia (DRG) [10–12]. These $P2X_3$ receptors exist as functional, homomeric ion channels, but can also form heteromultimeric combinations with $P2X_2$ receptors [13–15]. During inflammation and chronic constriction injuries, ATP concentrations and neuronal expression of $P2X_3$ receptors were found to be up-regulated in DRG and spinal cord [16–18]. The restricted expression of $P2X_3$ receptors suggests that they may be crucial in processing pain signal.

A number of studies have evaluated the relationships between $P2X_3$ receptors and pain sensation. Local injection of $P2X_3$ receptor

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http://dx.doi.org/10.1016/j.ejmech.2013.10.026

agonists, ATP or α,β-methyleneATP, into the hindpaws of rats induced nociceptive behaviors and reduced thermal and mechanical thresholds [19–21]. In animal models of inflammatory and neuropathic pain, $P2X_3$ receptor knock-out or downregulation of $P2X_3$ receptor expression by antisense oligonucleotides (ASO) or by short interfering RNA (siRNA) significantly attenuated painful behaviors, including tactile allodynia and mechanical hyperalgesia [22,23]. In addition, the $P2X_3$ receptor antagonist TNP-ATP (2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate) and PPADS (**7a**, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate) reduced the excitability of DRG neurons and neuropathic pain [24]. Taken together, the findings of these studies suggest that $P2X_3$ receptors play important roles in the development and maintenance of chronic pain.

The highly selective, non-nucleotide $P2X_3$-$P2X_{2/3}$ antagonist **1** (A-317491) was developed at Abbott Laboratories in 2002 and its painkilling effects were validated in several animal models [25–28]. Subsequently, the orally active $P2X_3$-$P2X_{2/3}$ antagonists, **2** (RO-4) [29,30], **3** (RO-51) [31], and **4** (RO-85) [32], at Roche and the pyrrolo-pyrimidinone antagonist, **5** at AstraZeneca [33] were developed (Fig. 1). The peptide antagonist, **6** (Spinorphin, $IC_{50} = 8.3$ pM) [34] was reported by our group. Recently, Afferent Pharmaceuticals $P2X_3$ antagonist, AF-219, entered Phase 2 clinical studies in patients with joint pain, visceral pain, and idiopathic chronic cough [35].

In efforts to develop new small chemical antagonists of P2X receptors, we investigated derivatives of the nonselective P2X receptor antagonists, **7a** (PPADS), and **7b** (MRS 2159) [36]. Our preliminary structure-activity relationship (SAR) studies showed that the aldehyde or phosphoric or sulfonic acid group in **7a** could be replaced by carboxylic acid (**7c**) without affecting antagonistic potency at the $mP2X_1$ and $hP2X_3$ receptors [37]. To develop novel antagonists for $hP2X_3$ receptors, we performed extensive modifications of the parent compounds, **7a**-**c**. First, the unstable azo (−N═N−) linkages in **7a**–**c** were replaced with stable carbon-carbon, ether, or amide linkages to increase stability. Second, an acetyl or carboxylic acid group besides the aldehyde group at the 4-position of **7a**–**c** was explored. Third, the 6-azophenyl ring on the 2-position of the pyridine ring was changed to other aromatic rings to improve selectivity at the $P2X_3$ receptors. The antagonistic effects of these compounds were assessed on *Xenopus* oocytes expressing cloned P2X receptors using the two-electrode voltage clamp (TEVC) assay system, and the functional activity of these compounds was evaluated using an *ex-vivo* assay system measuring long-term potentiation (LTP) as a pain signal in rat spinal dorsal horn [38].

2. Results and discussion

2.1. Chemistry

Since the parent compounds (**7a**–**c**) have unstable azo (−N═N−) bonds at the 2-position of the pyridine moiety, we replaced these bonds with stable carbon-carbon or ether bonds using the Diels-Alder reaction with bis-carbonyl based dienophiles and oxazole based dienes (**11a**–**o**). The latter compounds were prepared from compound **8** (L-alanine ethyl ester) using the synthesis strategy shown in Scheme 1. Briefly, **8** was reacted with the appropriate carboxylic acids **9a**–**o** in the presence of general coupling reagents to yield **10a**–**o**, which were cyclized to their corresponding oxazoles, **11a**–**o**, using phosphorous pentoxide. The overall yield of three steps was 92%. The 3,4-dicarboxypyridine derivatives, **12a**–**o**, were synthesized through Diels-Alder reactions of **11a**–**o** with dimethyl maleate, with subsequent hydrolysis using 20% aqueous KOH yielding **13a**–**o** (Scheme 1). To synthesize the 4-acetyl-pyridine derivatives, the dienophile (*E*)-ethyl-4-oxopent-2-enoate (**16**) was reacted with **11a**–**f** in Diels-Alder reactions to give **17a**–**f**, which were hydrolyzed to the corresponding 4-acetyl-3-carboxypyridine analogs, **18a**–**f** (Scheme 2). The acetyl group, a stronger electron-withdrawing group, in **16** oriented the 4 position of pyridine in **18a**–**f** [39,40].

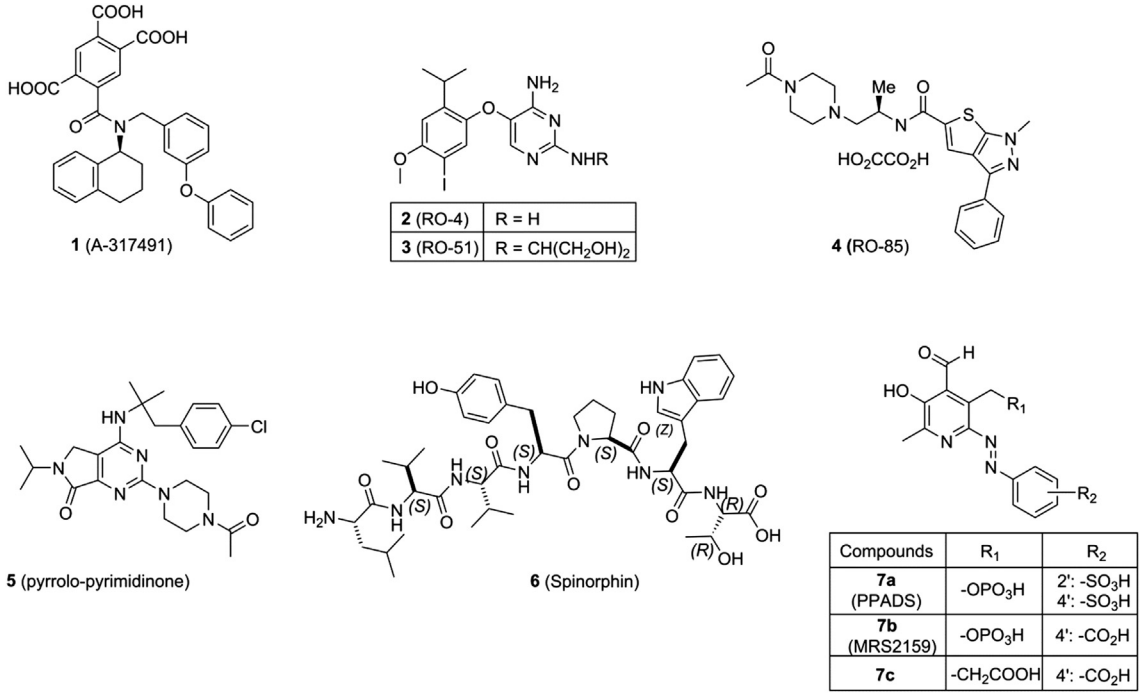
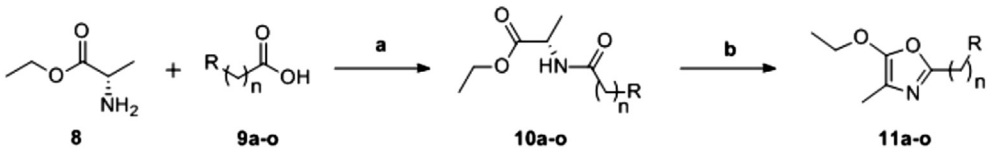


Fig. 1. The structure of $P2X_3$ antagonists.

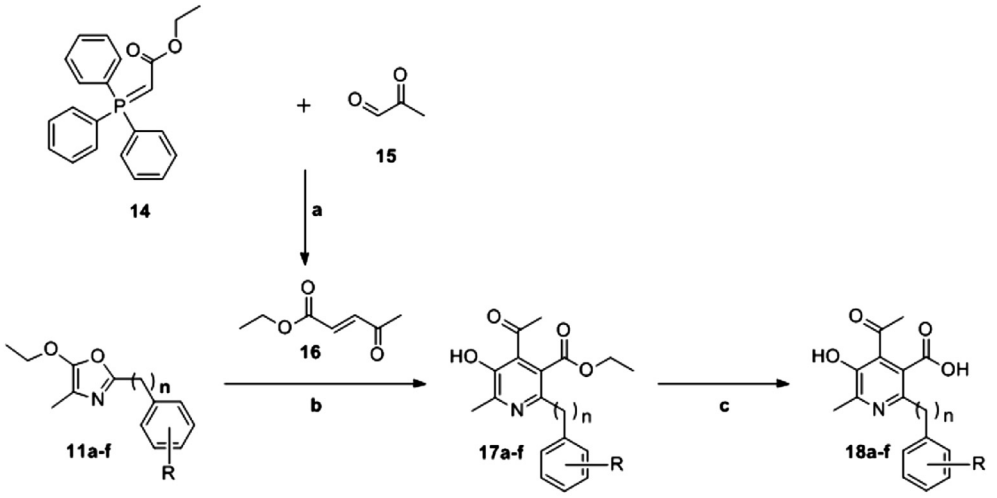


no.	R	n
13a	C_6H_5	1
13b	C_6H_5	2
13c	C_6H_5	2(alkene)
13d	C_6H_4-*p*-OCH_3	2
13e	C_6H_4-*m*-OCH_3	2
13f	C_6H_4-*o*-OCH_3	2
13g	C_6H_4-*p*-COOH (9g, C_6H_4-*p*-CO_2CH_3)	2
13h	$C_{10}H_7$	1
13i	$CH(C_6H_5)_2$	2
13j	C_6H_4-*m*-O-C_6H_5	1
13k	C_6H_4-*m*-O-C_6H_5	2
13l	C_6H_4-*m*-O-C_6H_5	0
13m	C_6H_4-*p*-O-C_6H_5	1
13n	C_6H_4-*o*-O-C_6H_5	1
13o	O-$CH_2C_6H_5$	1

Scheme 1. Synthesis of 3,4-dicarboxypyridine derivatives. Reagents and conditions: (a) EDC, TEA, DCM, RT, 2 h, 65–99%; (b) P_2O_5, $CHCl_3$, reflux, 2 h, 38–99%; (c) dimethyl maleate, neat, 5 h, 21–59%; (d) 20% KOH (aq), RT, 6 h, 27–83%.

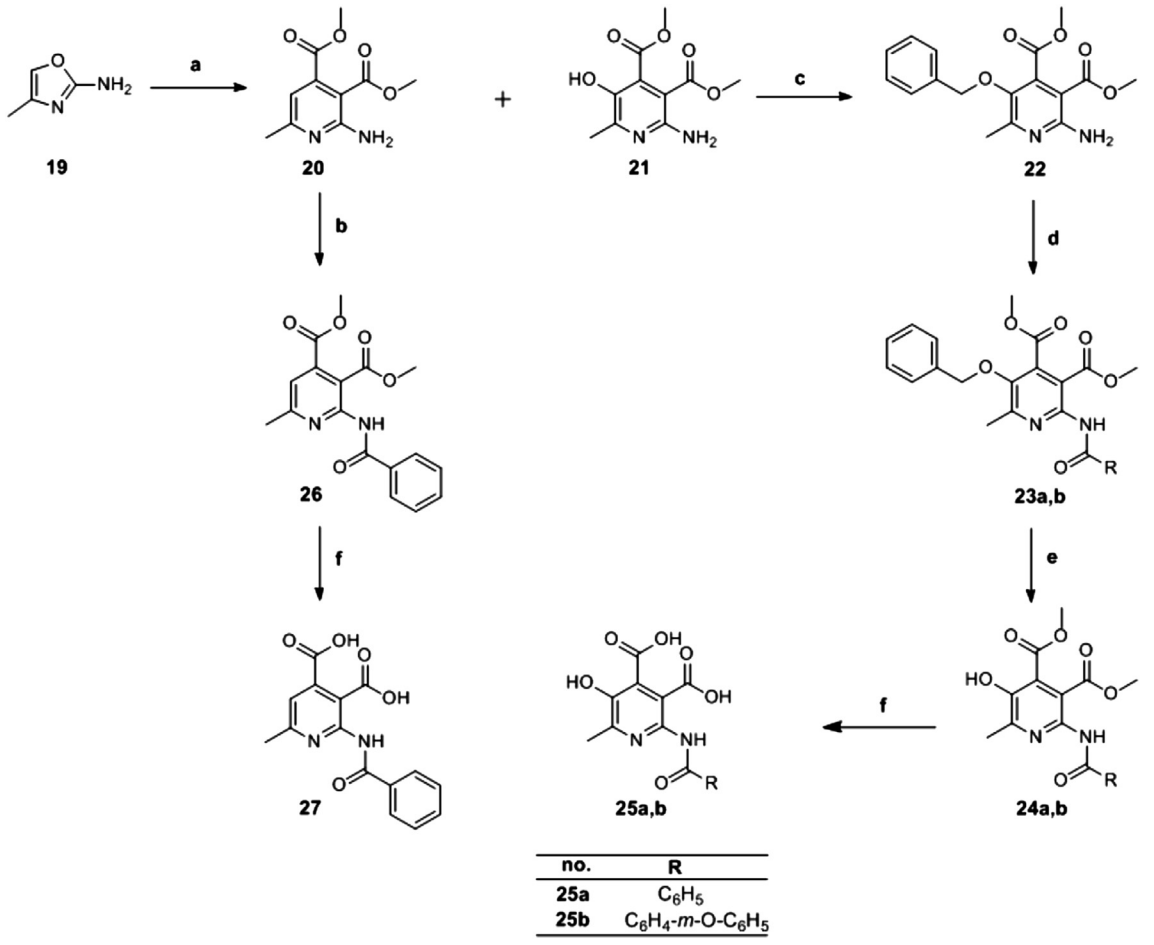
To introduce an amide bond at the 2-position of pyridine derivatives, compound **19** (4-methyl-oxazole-2-amine) was used as a diene in a Diels-Alder reaction. This reaction yielded two compounds, one with a hydrogen (**20**) and the other with a hydroxyl group (**21**) at the 5-position of the pyridine ring. The 5-OH group in **21** was protected by benzylation to selectively acylate the 2-amino group. The 2-amino groups of **20** and **22** were acylated using benzoyl chloride or 3-phenoxybenzoylchloride. The protective group of **23** was selectively removed by hydrogenolysis using Pd/C under H_2 gas to give compound **24**. Lastly, the 3,4-dimethylester groups of **24** and **26** were hydrolyzed using 20% aqueous KOH to give **25** and **27**, respectively (Scheme 3).

The 4-aldehyde-3-propenoic and -3-propionic acid derivatives, **36** and **41**, respectively were synthesized via common intermediates, **33**, which were prepared from the pyridine bis-methylester compounds, **12** as shown in Scheme 4. The 5-OH



no.	R	n
18a	C_6H_5	1
18b	C_6H_5	2
18c	C_6H_5	2(alkene)
18d	C_6H_4-*p*-OCH_3	2
18e	C_6H_4-*m*-OCH_3	2
18f	C_6H_4-*o*-OCH_3	2

Scheme 2. Synthesis of 4-acetyl-3-carboxypyridine derivatives. Reagents and conditions: (a) DMF, DCM, RT, 20 h, 80%; (b) neat, 5 h, HCl (g), EtOH, 0 °C, 10–51%; (c) 20% KOH (aq), RT, 4 h, 70–90%.



Scheme 3. Synthesis of 3,4-dicarboxypyridine derivatives with amide linker at the 2 position. Reagents and conditions: (a) dimethylmaleate, neat, 5 h, 13–17%; (b) benzoyl chloride, DIPEA, toluene, 80 °C, 2 h, 51–67%; (c) benzyl bromide, K_2CO_3, acetone, reflux, 2 h, 99%; (d) 3-phenoxybenzoyl chloride, DIPEA, toluene, 80 °C, 2 h, 77%; (e) Pd/C, H_2 (g), MeOH, RT, 0.5 h, 53–61%; (f) 20% KOH (aq), RT, 4 h, 27–35%.

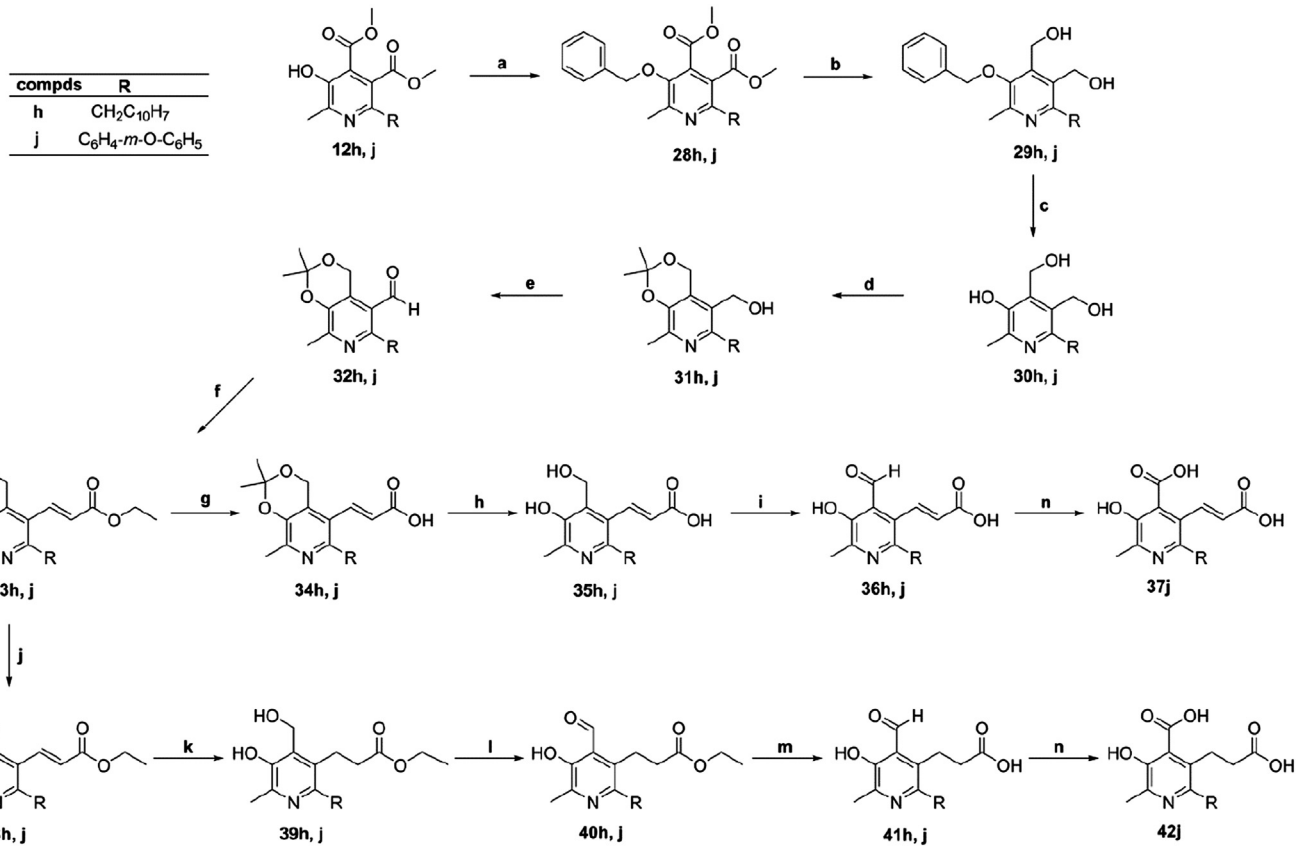
groups of **12** were protected with a benzyl group and the 3,4-bis-methylester group of **28** was reduced to a 3,4-bis-hydroxymethyl group with $LiAlH_4$. The protecting benzyl group was removed with Pd/C under H_2 gas from compound **29**. The 4-hydroxymethyl group and 5-phenolic alcohol of **30** were selectively protected to an acetonide moiety using 2,2-dimethoxypropane and 4 equivalent of *p*-toluenesulfonic acid [41]. The 3-hydroxymethyl group in **31** was oxidized with pyridinium dichromate to yield compound **32**. The Wittig-Honor reaction of **32** with triethyl phosphonoacetate produced only the trans-isomer compound **33**, which was identified by ^{1}H NMR analysis (*J* value = 16.4 Hz). The ethyl ester group of **33** was hydrolyzed with 5% methanolic KOH and the acetonide group of **34** was removed with 10% formic acid followed by manganese dioxide oxidation, yielding the 4-aldyhyde-3-propenoic acid compounds **36**. In the case of the synthesis of 4-aldehyde-3-propionic acid compounds **41**, a different order of procedures for the hydrolysis and oxidation steps was employed to achieve the best chemical yields. The acetonide group of **33** was first deprotected with 10% formic acid to yield **38**, of which α,β-unsaturated double bond was subsequently reduced under hydrogenation conditions to give **39**. The 4-hydroxymethyl group of **39** was converted to an aldehyde group by manganese dioxide in dichloromethane, and hydrolysis of the ester group of **40** using 5% methanolic KOH finally afforded 4-aldehyde-3-propionic acid derivatives, **41** (Scheme 4). Lastly, two dicarboxylic acid analogs, **37j** and **42j** were synthesized from **36j** and **41j**, respectively, by oxidation reactions of 4-carboxaldehyde groups with Oxone®.

To obtain the compounds having direct substitution of a carboxylic acid at the 3 position, the aldehyde group of **32** was further oxidized to carboxylic acid with Oxone®. The acetonide group was removed from **43** with 10% formic acid, yielding lactone compounds **44**, which were formed by dehydration reactions between the 3-carboxylic acid and 4-hydroxymethyl groups of pyridine. Thus, an additional hydrolysis step was needed to generate compounds with 4-hydroxymethyl group (**45**). However, oxidation reactions of compound **45** with manganese dioxide yielded not the compounds with 4-aldehyde-3-carboxy pyridine compounds, but compounds **46** as pyridine-hydroxylactone derivatives (Scheme 5).

2.2. Biology

The antagonistic activities of the synthesized compounds at the $mP2X_1$ and $hP2X_3$ receptors were evaluated by two-electrode voltage clamp (TEVC) assays on the *Xenopus* oocytes expressing cloned $mP2X_1$ and $hP2X_3$ receptors, respectively, as described previously [42]. $hP2X_7$ receptor antagonistic activity was evaluated using ethidium$^+$ uptake assays. Compound **1**, **7b** and KN-62 [43] were used as positive control antagonists for $P2X_1$, $P2X_3$ and $P2X_7$ receptors, respectively.

Compound **7a** is a potent but nonselective P2X receptor antagonist, with potent activities at $mP2X_1$ (IC_{50} = 99 nM), $hP2X_3$ (IC_{50} = 240 nM) and $hP2X_7$ (IC_{50} = 1180 nM) receptors [36,43]. In our preliminary SAR, the phosphate of **7a** could be replaced by the weak anion carboxylic acid and the number of sulfonic acid groups



Scheme 4. Synthesis of 4-aldehyde-pyridine derivatives with 3-propionic and 3-propenoic acids. Reagents and conditions: (a) benzyl bromide, K_2CO_3, acetone, reflux, 2 h, 85–96%; (b) LAH, ether, 0 °C, 69–88%; (c, k) Pd/C, H_2 (g), MeOH, RT, 0.5 h, 82–91%; (d) 2,2-dimethoxypropane, *p*-TsOH, acetone, RT, 12 h, 90%; (e) PDC, DCM, RT, 12 h, 83–90%; (f) NaH, triethyl phosphonoacetate, THF, RT, 1 h, 96%; (g, m) 5% KOH in MeOH, 53–98%; (h, j) 10% formic acid, reflux, 3 h, 33–71%; (i, l) MnO_2, DCM, RT, 2 h, 66–79%; (n) Oxone®, DMF/ACN (1:5), RT, 2 h, 13–37%.

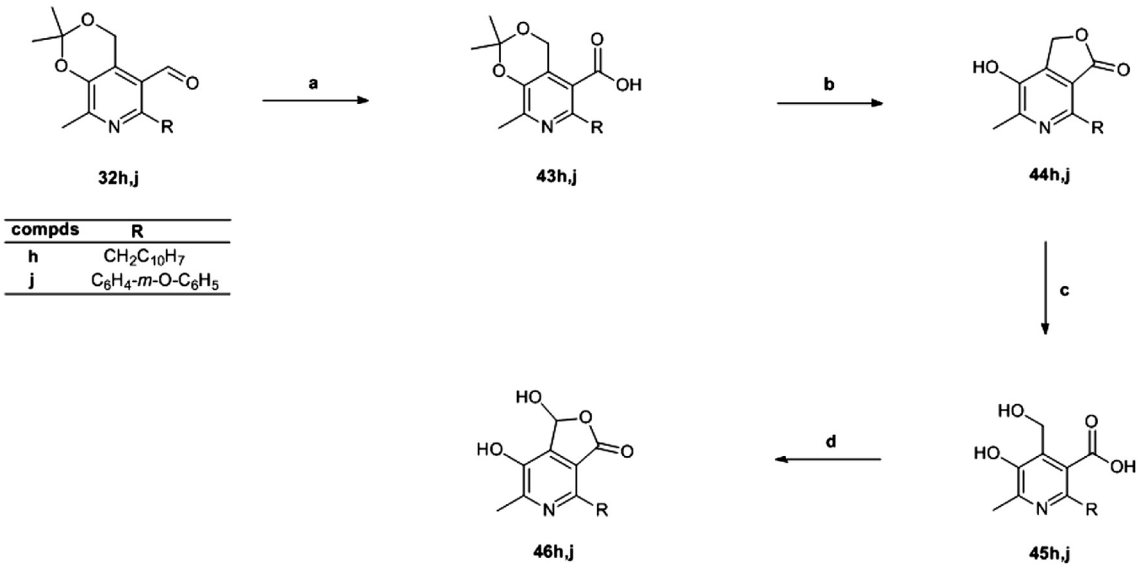
of **7a** could be removed or replaced by carboxylic acid (**7c**) without affecting antagonistic activity of **7a** at $mP2X_1$ and $hP2X_3$ receptors [37]. The unstable azo (−N═N−) linkage of **7c**, however, reduces its chemical stability and bioavailability. We therefore synthesized various 5-hydroxy-pyridine derivatives with stable carbon-carbon, ether, or amide linkages at the 2 position using Diels-Alder reactions. At the 4-position, acetyl, carboxylic acid or aldehyde moieties were introduced.

Table 1 summarizes the antagonistic effects of these 3,4-dicarboxypyridine (**13a**–**g**) and 4-acetyl-3-carboxypyridine derivatives (**18a**–**f**) towards $mP2X_1$, $hP2X_3$, and $hP2X_7$ receptors. Although these analogs were less potent than their parent compounds (**7a**–**c**), and their selectivity for $hP2X_3$ receptor was not improved, we could establish structure-activity relationships of the series of new analogs. We first investigated the effects of the carbon chain at the 2-position of the pyridine moiety. Two carbon linkages, by changing 2-benzyl group (**13a**) to 2-phenethyl group (**13b**), greatly increased the antagonistic activities, showing IC_{50} values of 645 nM at $mP2X_1$ and 797 nM at $hP2X_3$ receptors. The conformational lock of two carbon linkage by employing a *trans* form of double bond (**13c**) did not improve the antagonistic activities, both at $mP2X_1$ (IC_{50} = 768 nM) and at $hP2X_3$ (IC_{50} = 920 nM) receptors.

We next investigated the effects of the methoxy position on the phenyl ring substituted at the carbon linkage, finding that the *p*-position (**13d**) was best rather than the *m*- (**13e**) or *o*- (**13f**) position. For example, compound **13d** had an IC_{50} value of 460 nM at $hP2X_3$ receptors, whereas 10 μM **13e** and **13f** inhibited the receptors only 45.5% and 34.5%, respectively, and the similar activity profile was observed when the compounds were tested at $mP2X_1$ receptors. Replacement of the *p*-methoxy of **13d** with carboxylic acid (**13g**) did not affect the antagonistic activity at the $hP2X_3$ receptor, but enhanced the activity at the $mP2X_1$ receptor, showing increased IC_{50} value of 267 nM (**13g**) from 446 nM (**13d**). This result suggested that the polarity at the *p*-position of the phenyl group may shift selectivity profiles toward $mP2X_1$ rather than toward $hP2X_3$ receptors.

In the case of the 4-acetylcarboxypyridine analogs, **18a**–**f**, the antagonistic potencies markedly decreased at both of $mP2X_1$ and $hP2X_3$ receptors than 3,4-dicarboxypyridine analogs, **13a**–**g**. This result may be due to the lack of affinity of the 4-acetyl group on the pyridine moiety, compared with 4-carboxylic acid group, for the interaction with lysine residues in the ATP binding site of the P2X receptors [8,44]. All compounds in this series showed negligible antagonistic activity for $hP2X_7$ receptor.

To increase potency and selectivity for $hP2X_3$ receptor, we exploited our findings on the effect of polarity of the substituents at the phenyl group connected to the 2-position of pyridine moiety on the selectivity profiling between $mP2X_1$ and $hP2X_3$ receptors. Therefore, we introduced non-polar bulky aromatic groups, such as naphthyl (compound **13h**), diphenyl (compound **13i**), 3-pheonoxyphenyl (compound **13j**–**l**), and 4-pheonoxyphenyl (compound **13m**–**n**) groups at the 2-position of the pyridine moiety. Strikingly, all the compounds in Table 2, **13h**–**n** showed less than 50% inhibition in 10 μM concentrations at $mP2X_1$ receptors, while the compounds antagonized $hP2X_3$ receptors with 400 nM–

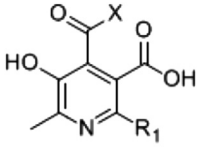


Scheme 5. Synthesis of pyridine-hydroxylactone derivatives. Reagents and conditions: (a) Oxone®, DMF/ACN (1:5), RT, 2 h, 59–94%; (b) 10% formic acid, reflux, 3 h, 70–92%; (c) 5% KOH in MeOH, RT, 4 h, 48–53%; (d) MnO_2, THF/H_2O (3:7), RT, 2 h, 21–26%.

3 μM range of IC_{50} values. The antagonistic potency of these compounds at $hP2X_3$ receptors was increased in the order of 3-phenoxyphenyl > diphenyl > naphthyl (**13j** > **13i** > **13h**) (Table 2). The biological activity of the most potent analog in this series, **13j** (IC_{50} = 474 nM) was parallel to that of the 4-methoxyphenyl derivative, **13d** (IC_{50} = 460 nM), of which antagonistic potency at $mP2X_1$ receptor was also similar (IC_{50} = 446 nM). However, as expected, **13j** displayed dramatically increased selectivity for $hP2X_3$ versus $mP2X_1$ and $hP2X_7$ receptors, at which 10 μM **13j** showed only 42% antagonism and no activity, respectively.

In an attempt to optimize the $hP2X_3$ antagonism of **13j**, the position of phenoxy substituents and carbon-chain length between the pyridine and phenoxyphenyl rings were modified (Table 2). Among the derivatives with phenoxy substituents at various positions of the 2-benzyl moiety, we found that the *m*-phenoxyphenyl compound **13j** was 2- and 6-fold more active than the *p*-phenoxyphenyl compound **13m** (IC_{50} = 1050 nM) and the *o*-phenoxyphenyl compound **13n** (IC_{50} = 2930 nM), respectively. In analyzing the effect of carbon-chain length (*n*) of the phenoxyphenyl moiety, we found that compound **13j** with *n* = 1 was slightly more potent than compound **13k**

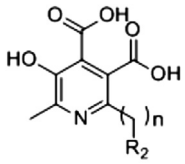
Table 1
Antagonistic effects of 3,4-dicarboxypyridine and 4-acetyl-3-carboxy pyridine derivatives at $mP2X_1$, $hP2X_3$ and $hP2X_7$ receptors.



compounds	X	R_1	$mP2X_1$[a]	$hP2X_3$[a]	$hP2X_7$[d]
1[e]			–	35 ± 4 nM	–
7b[f]			30 ± 13 nM	–	–
KN-62[g]			–	–	158 ± 18 nM
13a	–OH	$-CH_2C_6H_5$	54.2 ± 10.6%[b]	31.4 ± 6.2%[b]	Inactive
13b	–OH	$-CH_2CH_2C_6H_5$	645 ± 23 nM[c]	797 ± 99 nM[c]	Inactive
13c	–OH	$-CH{=}CHC_6H_5$	768 ± 12 nM[c]	920 ± 155 nM[c]	Inactive
13d	–OH	$-CH_2CH_2C_6H_4$-*p*-OCH_3	446 ± 32 nM[c]	460 ± 69 nM[c]	Inactive
13e	–OH	$-CH_2CH_2C_6H_4$-*m*-OCH_3	52.4 ± 6.2%[b]	45.5 ± 6.4%[b]	Inactive
13f	–OH	$-CH_2CH_2C_6H_4$-*o*-OCH_3	49.8 ± 5.4%[b]	34.5 ± 4.3%[b]	Inactive
13g	–OH	$-CH_2CH_2C_6H_4$-*p*-COOH	267 ± 9 nM[c]	458 ± 35 nM[c]	Inactive
18a	$-CH_3$	$-CH_2C_6H_5$	44.2 ± 4.0%[b]	18.3 ± 6.4%[b]	Inactive
18b	$-CH_3$	$-CH_2CH_2C_6H_5$	47.9 ± 8.1%[b]	37.3 ± 10.0%[b]	Inactive
18c	$-CH_3$	$-CH{=}CHC_6H_5$	52.4 ± 6.2%[b]	27.4 ± 5.5%[b]	Inactive
18d	$-CH_3$	$-CH_2CH_2C_6H_4$-*p*-OCH_3	52.0 ± 7.1%[b]	42.2 ± 7.5%[b]	Inactive
18e	$-CH_3$	$-CH_2CH_2C_6H_4$-*m*-OCH_3	29.9 ± 5.7%[b]	15.8 ± 9.0%[b]	Inactive
18f	$-CH_3$	$-CH_2CH_2C_6H_4$-*o*-OCH_3	11.4 ± 3.3%[b]	6.3 ± 2.3%[b]	Inactive

[a]The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and the percentage of inhibition of the ion current by 10 μM[b] or IC_{50} values[c] of compounds was measured for $mP2X_1$ and $hP2X_3$ receptors, respectively (mean ± SEM, $n \geq 3$). [d]The accumulation of ethidium$^+$ was induced by 6 μM BzATP at the $hP2X_7$ receptors expressed in HEK293 cells and % inhibition of the accumulation by 10 μM compounds was measured (mean ± SEM, $n \geq 3$). [e]Reported IC_{50} value is 97 nM at $hP2X_3$ receptors [25]. [f]Reported IC_{50} value is 43 nM at $rP2X_1$ receptors [36]. [g]Reported IC_{50} value is 210 nM at $hP2X_7$ receptors [43].

Table 2
Antagonistic effects of 3,4-dicarboxypyridine derivatives with bulky aromatic groups at 2-position of pyridine moiety for $mP2X_1$, $hP2X_3$ and $hP2X_7$ receptors.



13h-n

compounds	*n*	R_2	$mP2X_1$[a]	$hP2X_3$[a]	$hP2X_7$[d]
13h	1	$-C_{10}H_7$	48.7 ± 3.1%[b]	1250 ± 41 nM	Inactive
13i	1	$-CH(C_6H_5)_2$	42.0 ± 6.6%[b]	824 ± 67 nM	Inactive
13j	1	$-C_6H_4$-*m*-O-C_6H_5	41.8 ± 8.9%[b]	474 ± 23 nM	Inactive
13k	2	$-C_6H_4$-*m*-O-C_6H_5	38.3 ± 2.5%[b]	587 ± 42 nM	Inactive
13l	0	$-C_6H_4$-*m*-O-C_6H_5	49.7 ± 4.1%[b]	3300 ± 640 nM	Inactive
13m	1	$-C_6H_4$-*p*-O-C_6H_5	40.0 ± 2.6%[b]	1050 ± 196 nM	Inactive
13n	1	$-C_6H_4$-*o*-O-C_6H_5	49.6 ± 3.5%[b]	2930 ± 525 nM	Inactive

[a]The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and the percentage of inhibition of the ion current by 10 μM[b] or IC_{50} values[c] of compounds was measured for $mP2X_1$ and $hP2X_3$ receptors, respectively (mean ± SEM, $n \geq 3$). [d]The accumulation of ethidium$^+$ was induced by 6 μM BzATP at the $hP2X_7$ receptors expressed in HEK293 cells and % inhibition of the accumulation by 10 μM compounds was measured (mean ± SEM, $n \geq 3$).

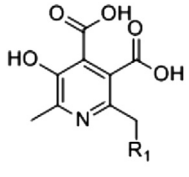
with $n = 2$, whereas the potency of compound **13l** with $n = 0$ was significantly reduced. These results suggest that antagonistic activity for $hP2X_3$ receptors can be optimized by placing the phenoxy group at the *m*-position of the phenyl group with a carbon chain length of 1, linking to the 2 position of pyridine moiety.

We also investigated the effects of other linkages, including ether and amide bonds, at the 2-positon of the pyridine moiety (Table 3). Compound **13o** with an ether linkage showed very weak antagonism for $hP2X_3$ receptors even at 10 μM concentrations. Compound **25a** with amide linkage had similar antagonistic activity (IC_{50} = 723 nM) compared with compound **13b** having carbon-carbon linker. However, the combination of *m*-phenoxyphenyl group and amide linkage (**25b**) showed reduced antagonism at $hP2X_3$ receptors, compared with compound **13k**. Since compounds **20** and **21** were simultaneously obtained in the Diels-Alder reaction with compound **19** in Scheme 3, we could evaluate the importance of 5-hydroxyl group in the pyridine ring for the antagonistic activity (Table 3). As a result, compound **27**, which has no hydroxyl group, was inactive in $hP2X_3$ receptor antagonism, suggesting that an intact hydroxyl group is required for a key interaction, such as hydrogen bonding, with the binding site of $hP2X_3$ receptor.

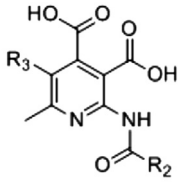
Our previous study showed that an aldehyde group at the 4-position of the pyridine moiety in **7c** resulted in enhanced antagonistic activity at the $hP2X_3$ receptor [37]. Therefore, we employed an aldehyde group at the 4-position (Table 4). Also, modifications were made at the 3-position with propionic acid or propenoic acid to match the corresponding spacer for the phosphate in the parent compound **7a**. Generally, propenoic acid showed dramatically increased antagonistic activity for $hP2X_3$ receptor than propionic acid analogs (**36h** vs **41h**, **36j** vs **41j**). The effect of 2-position substituents was similar to the results shown in Table 2, in which the 3-phenoxyphenyl group showed greater activity than did naphthyl groups (**36j** vs **36h**, **41j** vs **41h**). To assess the effects of aldehyde at the 4-position of pyridine, the aldehyde groups of **36j** and **41j** were further oxidized to prepare **37j** and **42j**, respectively. As expected, the 4-carboxaldehyde derivatives, **36j** and **41j** had 2- and 6-fold greater antagonistic activity than the corresponding carboxylic acid derivatives, **37j** and **42j**, respectively. To investigate the effects of the spacer between the pyridine ring and the 3-position of carboxylic acid, we attempted to synthesize 4-aldehyde-3-carboxypyridine derivatives which had no carbon-chain. However, the oxidation of compound **45** to aldehyde resulted in the generation of pyridine-hydroxylactone analogs, **46** via intramolecular cyclization (Scheme 5). The antagonistic potency of these two compounds, **46h** and **46j**, was significantly reduced (IC_{50} = 856 and 652 nM), respectively.

The subtype selectivity profiles of the series of 4-aldehyde derivatives in Table 4 for $hP2X_3$ receptor vs $mP2X_1$ and $hP2X_7$ receptors were also significantly improved. For example, the most potent antagonists, **36h** and **36j** with IC_{50} values of 145 and 60 nM at $hP2X_3$ receptor, respectively, showed only 15∼48% inhibition towards $mP2X_1$ and $hP2X_7$ receptors at concentrations of 10 μM. Furthermore, the heterogeneously expressed $rP2X_{2/3}$ receptors, which are more physiologically relevant, were also effectively inhibited by compound **36j** with parallel potency at $hP2X_3$ receptor (data shown in Figure S1 of Supporting information). These results indicate that our designing strategies to improve potency and selectivity were successfully employed.

Table 3
Antagonistic effects of 3,4-dicarboxypyridine derivatives with various modifications of pyridine moiety for $hP2X_3$ and $hP2X_7$ receptors.



13b, 13o, 13k

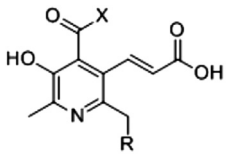


25a, 25b, 27

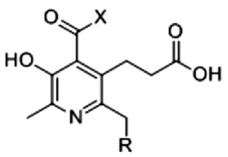
Compounds	R_1	R_2	R_3	$hP2X_3$[a]	$hP2X_7$[d]
13b	$-CH_2C_6H_5$			797 ± 99 nM[c]	Inactive
13o	$-O-CH_2C_6H_5$			23.1 ± 2.9%[b]	Inactive
25a		$-C_6H_5$	−OH	723 ± 100 nM[c]	Inactive
27		$-C_6H_5$	−H	Inactive	Inactive
13k	$-CH_2C_6H_4$-*m*-O-C_6H_5			587 ± 42 nM[c]	Inactive
25b		$-C_6H_4$-*m*-O-C_6H_5	−OH	928 ± 82 nM[c]	Inactive

[a]The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and the percentage of inhibition of the ion current by 10 μM[b] or IC_{50} values[c] compounds was measured for $hP2X_3$ receptors, respectively (mean ± SEM, $n \geq 3$). [d]The accumulation of ethidium$^+$ was induced by 6 μM BzATP at the $hP2X_7$ receptors expressed in HEK293 cells and % inhibition of the accumulation by 10 μM compounds was measured (mean ± SEM, $n \geq 3$).

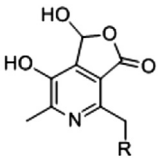
Table 4
Antagonistic effects of 4-aldehyde-3-carboxypyridine derivatives at $hP2X_3$ and $hP2X_7$ receptors.



36h, 36j, 37j



41h, 41j, 42j



46h, 46j

Compounds	X	R	$mP2X_1$[a]	$hP2X_3$[a]	$hP2X_7$[d]
36h	−H	$-C_{10}H_7$	22.0 ± 8.0%[b]	145 ± 41 nM[c]	48.4 ± 3.8%
41h	−H	$-C_{10}H_7$	–	636 ± 86 nM[c]	43.4 ± 5.3%
36j	−H	$-C_6H_4$-*m*-O-C_6H_5	18.3 ± 5.8%[b]	60 ± 25 nM[c]	15.1 ± 5.0%
41j	−H	$-C_6H_4$-*m*-O-C_6H_5	23.0 ± 5.3%[b]	366 ± 63 nM[c]	10.2 ± 6.2%
37j	−OH	$-C_6H_4$-*m*-O-C_6H_5	33.7 ± 3.8%[b]	377 ± 29 nM[c]	Inactive
42j	−OH	$-C_6H_4$-*m*-O-C_6H_5	31.7 ± 5.9%[b]	638 ± 73 nM[c]	Inactive
46h		$-C_{10}H_7$	–	856 ± 76 nM[c]	14.8 ± 6.1%
46j		$-C_6H_4$-*m*-O-C_6H_5	45.0 ± 7.0%[b]	652 ± 60 nM[c]	27.1 ± 10.6%

[a]The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and the percentage of inhibition of the ion current by 10 μM[b] or IC_{50} values[c] of compounds was measured for $mP2X_1$ and $hP2X_3$ receptors, respectively (mean ± SEM, $n \geq 3$). [d]The accumulation of ethidium$^+$ was induced by 6 μM BzATP at the $hP2X_7$ receptors expressed in HEK293 cells and % inhibition of the accumulation by 10 μM compounds was measured (mean ± SEM, $n \geq 3$).

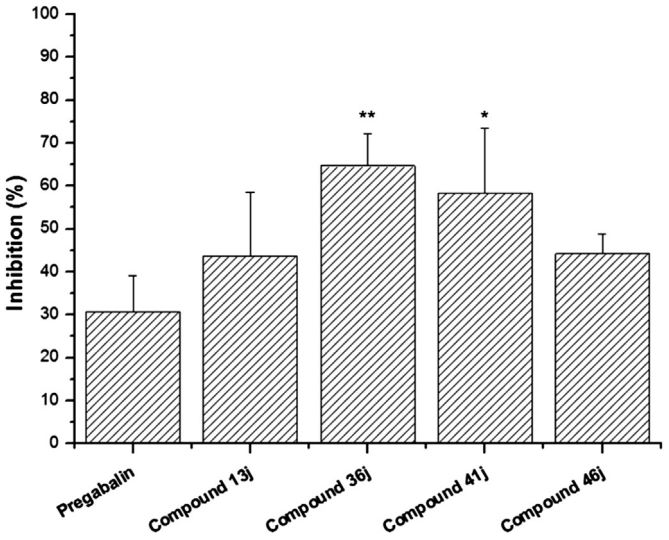


Fig. 2. Inhibition effects of pain signals (Long-Term Potentiation) in rat dorsal horn. The optical signal is monitored in the spinal dorsal horn stained with the voltage sensitive dye (Di-4-ANEPPS). The optical signal is analyzed and calculated as % inhibition of LTP by the compound treatment. Data represents means ± SD with 4 different samples (significant versus Pregabalin/Compound, $^*p < 0.05$, $^{**}p < 0.01$).

As a preliminary animal experiment for the anti-pain signaling activities of new $P2X_3$ receptor antagonists, an *ex-vivo* assay system was employed in the rat dorsal horn, which is involved in neural pain mechanisms. Compounds **13j**, **36j**, **41j**, and **46j** having 3-phenoxyphenyl group at 2-position of pyridine were selected along with pregabalin as a positive control for the *ex-vivo* tests to investigate the relevance between the results of *in-vitro* and *ex-vivo* assay systems. The latter may be affected by the polarity of substituents at 3 and 4 position due to the different permeability in rat dorsal horn. As the results, the profile of $hP2X_3$ receptor antagonism of compounds in the TEVC assay system was similar to their efficacy in the *ex-vivo* assay system. Moreover, most of the antagonists showed greater efficacy than pregabalin at 20 μM concentrations (Fig. 2). Compound **36j**, the most potent and selective of the $hP2X_3$ receptor antagonists, inhibited the pain signal 65%, compared with 31% for pregabalin. These findings show that the optimized $P2X_3$ receptor antagonists, derived from the parent PPADS analogs (**7a**–**c**) have sufficient potential to be antinociceptive agents with *in-vivo* efficacy.

3. Conclusions

In this study, we successfully optimized a nonselective P2X receptor antagonist, **7a** (PPADS), generating potent and selective $hP2X_3$ receptor antagonists, by chemical modifications and SAR studies. These included the replacement of the unstable azo (−N=N−) linker in **7a**–**c** with stable carbon-carbon linkers, and substitutions with 4-carboxaldehyde, 3-propenoic acid and bulky hydrophobic aromatic groups at the 2-positon. SAR analysis suggested that the 5-hydroxyl group of pyridine was essential for the antagonism and that bulky aromatic groups at the 2-position were very important for selectivity toward $hP2X_3$ receptors. The optimized compounds, including the most potent and selective $hP2X_3$ receptor antagonist, **36j**, with an IC_{50} value of 60 nM, also significantly inhibited pain signaling in the rat dorsal horn, suggesting that the carboxypyridine derivatives developed in this study may have the potential to contribute to the development of pain modulators targeting $P2X_3$ receptors.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and used without further purification. Proton nuclear magnetic resonance spectroscopy was performed on a JEOL JNM-LA 300WB spectrometer at 300 MHz or JEOL JNM-ECX 400P spectrometer at 400 MHz, and spectra were taken in $CDCl_3$ or DMSO-d_6. Unless otherwise noted, chemical shifts are expressed as ppm downfield from internal tetramethylsilane, or relative ppm from DMSO (2.5 ppm). Data are reported as follows: chemical shift, integration, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; app., apparent), and coupling constants. Mass spectroscopy was carried out on MALDI-TOF and Electrospray ionization (ESI) instruments.

The purity of all final compounds was determined by HPLC (at least 95% purity unless otherwise noted). The determination of purity was performed on a Shimadzu SCL-10A VP HPLC system using a Shimadzu Shim-pack C18 analytical column (250 mm × 4.6 mm, 5 μm, 100 Å) in two solvent systems. The solvent systems were H_2O: CH_3CN = 70:30 or 0.1% TFA (Trifluoroacetic acid) in H_2O: CH_3CN = 65:35 to 5:95 for 30 min with flow rate 1 mL/min.

Compound **1** and KN-62 were purchased from Sigma-Aldrich. Compound **7b** was prepared according to literature procedures [36].

4.1.1. General procedure for the preparation of compounds (*10a*–*o*)

To a suspension of various carboxylic acid **9a**–**o** (1.0 equiv) and L-alanine ethyl ester (**8**, 1.0 equiv) in dichloromethane was treated with EDC (2.0 equiv). Triethylamine (1.0 equiv) was added drop wise to the mixture for a period of 30 min. After this mixture was stirred for 12 h at room temperature, it was neutralized with saturated aqueous sodium bicarbonate and extracted with dichloromethane twice. The combined organic layer was dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, solvent was removed in vacuum. The residue was purified by silica gel column

chromatography with n-hexanes/ethyl acetate = 1:1 to afford **10a–o** as sticky oil.

*4.1.1.1. Ethyl 2-(2-phenylacetamido)propanoate (**10a**).* Following the general procedure for the synthesis of (**10a–o**), coupling reaction of **8** (20 g, 0.13 mol) and phenyl acetic acid (20 g, 0.15 mol) afforded **10a** (27 g). Yield 96%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.22 (3H, t, J = 7.2 Hz, CH_3), 1.33 (3H, d, J = 7.2 Hz, CH_3), 3.59 (2H, s, CH_2), 4.13 (2H, q, J = 7.2 Hz, CH_2), 4.54 (1H, q, J = 7.2 Hz, CH), 6.12 (1H, d, J = 6.3 Hz, NH), 7.30–35 (5H, m, phenyl); MS (ESI): m/z = 236.1 [M + H].

*4.1.1.2. Ethyl 2-(3-phenylpropanamido)propanoate (**10b**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (7.5 g, 48 mmol) and 3-phenylpropionic acid (11 g, 73 mmol) afforded **10b** (11 g). Yield 92%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.24 (3H, t, J = 7.2 Hz, CH_3), 1.32 (3H, d, J = 7.2 Hz, CH_3), 2.48 (2H, t, J = 7.2 Hz, CH_2), 2.94 (2H, t, J = 7.2 Hz, CH_2), 4.14 (2H, q, J = 7.2 Hz, CH_2), 4.53–4.58 (1H, m, CH), 6.12 (1H, d, J = 6.6 Hz, NH), 7.18–28 (5H, m, phenyl); MS (MALDI-TOF): m/z = 248.4 [M + H].

*4.1.1.3. Ethyl 2-cinnamamidopropanoate (**10c**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (5.0 g, 32 mmol) and cinnamic acid (5.6 g, 38 mmol) afforded **10c** (7.6 g). Yield 95%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.30 (3H, t, J = 7.2 Hz, CH_3), 1.49 (3H, d, J = 6.9 Hz, CH_3), 4.22 (2H, q, J = 7.2 Hz, CH_2), 4.70–4.75 (1H, m, CH), 6.27 (1H, d, J = 6.0 Hz, NH), 6.43 (1H, d, J = 15.6 Hz, =CH), 7.35–7.39 (3H, m, phenyl), 7.48–7.52 (2H, m, phenyl), 7.63 (1H, d, J = 15.6 Hz, =CH); MS (ESI): m/z = 248.2 [M + H].

*4.1.1.4. 2-[3-(4-Methoxy-phenyl)-propionylamino]-propionic acid ethyl ester (**10d**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (5.0 g, 33 mmol) and 3-(4-methoxyphenyl)propionic acid (6.8 g, 37 mmol) afforded **10d** (7.5 g). Yield 81%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (3H, t, J = 7.2 Hz, CH_3), 1.33 (3H, d, J = 6.9 Hz, CH_3), 2.48 (2H, t, J = 5.1 Hz, CH_2), 2.89 (2H, t, J = 5.1 Hz, CH_2), 3.78 (3H, s, CH_3O), 4.15 (2H, q, J = 7.2 Hz, CH_2), 4.52–4.58 (1H, m, CH), 5.95 (1H, d, J = 5.4 Hz, NH), 6.81 (2H, d, J = 7.2 Hz, phenyl), 7.10 (2H, d, J = 7.2 Hz, phenyl); MS (MALDI-TOF): m/z = 279.3 [M + H].

*4.1.1.5. 2-[3-(3-Methoxy-phenyl)-propionylamino]-propionic acid ethyl ester (**10e**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (7.1 g, 46 mmol) and 3-(3-methoxyphenyl)propionic acid (10 g, 56 mmol) afforded **10e** (8.6 g). Yield 67%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.22 (3H, t, J = 7.2 Hz, CH_3), 1.35 (3H, d, J = 7.2 Hz, CH_3), 2.51 (2H, t, J = 6.9 Hz, CH_2), 2.93 (2H, t, J = 6.9 Hz, CH_2), 3.80 (3H, s, CH_3O), 4.17 (2H, q, J = 7.2 Hz, CH_2), 4.54–4.59 (1H, m, CH), 5.99 (1H, d, J = 5.4 Hz, NH), 6.73–6.80 (2H, m, phenyl), 7.17–7.22 (2H, m, phenyl); MS (ESI): m/z = 280.3 [M + H].

*4.1.1.6. Ethyl 2-(3-(2-methoxyphenyl)propanamido)propanoate (**10f**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (3.0 g, 20 mmol) and 3-(2-methoxyphenyl) propionic acid (4.1 g, 22 mmol) afforded **10f** (4.6 g). Yield 82%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.22 (3H, t, J = 7.2 Hz, CH_3), 1.35 (3H, d, J = 7.2 Hz, CH_3), 2.53 (2H, t, J = 6.9 Hz, CH_2), 2.88 (2H, t, J = 6.9 Hz, CH_2), 3.79 (3H, s, CH_3O), 4.15 (2H, q, J = 7.2 Hz, CH_2), 4.52–4.59 (1H, m, CH), 5.96 (1H, d, J = 5.4 Hz, NH), 6.82–7.19 (4H, m, phenyl); MS (ESI): m/z = 280.4 [M + H].

*4.1.1.7. 4-[2-(1-Ethoxycarbonyl-ethylcarbamoyl)-ethyl]-benzoic acid methyl ester (**10g**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (4.9 g, 32 mmol) and 3-(4-(methoxycarbonyl)phenyl)propionic acid (7.9 g, 38 mmol) afforded **10g** (5.8 g). Yield 59%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (3H, t, J = 7.2 Hz, CH_3), 1.33 (3H, d, J = 6.9 Hz, CH_3), 2.48 (2H, t, J = 5.1 Hz, CH_2), 2.89 (2H, t, J = 5.1 Hz, CH_2), 3.78 (3H, s, $COOCH_3$), 4.15 (2H, q, J = 7.2 Hz, CH_2), 4.49–4.52 (1H, m, CH), 5.95 (1H, d, J = 5.4 Hz, NH), 6.81 (2H, d, J = 7.2 Hz, phenyl), 7.10 (2H, d, J = 7.2 Hz, phenyl); MS (ESI): m/z = 307.5 [M + H].

*4.1.1.8. Ethyl 2-(2-(naphthalen-2-yl)acetamido)propanoate (**10h**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (3.0 g, 19 mmol) and 2-naphthylacetic acid (4.0 g, 21 mmol) afforded **10h** (4.1 g). Yield 74%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (3H, t, J = 7.2 Hz, CH_3), 1.31 (3H, d, J = 7.2 Hz, CH_3), 3.74 (2H, s, CH_2), 4.12 (2H, q, J = 7.2 Hz, CH_2), 4.55–4.62 (1H, m, CH), 6.01 (1H, d, J = 6.8 Hz, NH), 7.38–7.50 (3H, m, naphthyl), 7.73–7.85 (4H, m, naphthyl); MS (ESI): m/z = 285.8 [M + H].

*4.1.1.9. Ethyl 2-(3,3-diphenylpropanamido)propanoate (**10i**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (3.0 g, 19 mmol) and 3,3-diphenylpropanoic acid (4.8 g, 21 mmol) afforded **10i** (4.1 g). Yield 65%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.12 (3H, d, J = 7.2 Hz, CH_3), 1.22 (3H, t, J = 7.2 Hz, CH_3), 2.67–2.99 (2H, m, CH_2), 4.12 (2H, q, J = 7.2 Hz, CH_2), 4.42–4.48 (1H, m, CH), 4.55–4.59 (1H, m, CH), 5.80 (1H, d, J = 7.2 Hz, NH), 7.15–7.29 (10H, m, phenyl); MS (ESI): m/z = 325.7 [M + H].

*4.1.1.10. Ethyl 2-(2-(3-phenoxyphenyl)acetamido)propanoate (**10j**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (3.0 g, 19 mmol) and 3-phenoxyphenylacetic acid (5.0 g, 21 mol) afforded **10j** (7.3 g). Yield 95%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.26 (3H, t, J = 7.2 Hz, CH_3) 1.34 (3H, d, J = 6.8 Hz, CH_3), 3.56 (2H, s, CH_2), 4.17 (2H, q, J = 7.2 Hz, CH_2), 4.56 (1H, q, J = 6.8 Hz, CH), 6.04 (1H, d, J = 4.8 Hz, NH), 6.94 (2H, s, phenyl), 7.03 (2H, d, J = 7.6 Hz, phenyl), 7.12 (1H, t, J = 7.6 Hz, phenyl), 7.25–7.35 (4H, m, phenyl); MS (ESI): m/z = 328.3 [M + H].

*4.1.1.11. Ethyl 2-(3-(3-phenoxyphenyl)propanamido)propanoate (**10k**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (3.0 g, 19 mmol) and 3-(3-phenoxyphenyl) propionic acid (5.0 g, 21 mmol) afforded **10k** (6.5 g). Yield 96%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.27 (3H, t, J = 7.6 Hz, CH_3), 1.34 (3H, d, J = 7.2 Hz, CH_3), 2.48 (2H, t, J = 7.2 Hz, CH_2), 2.94 (2H, t, J = 7.2 Hz, CH_2), 4.18 (2H, q, J = 7.6 Hz, CH_2), 4.53–4.56 (1H, m, CH), 5.99 (1H, d, J = 6.0 Hz, NH), 6.85 (2H, s, phenyl), 6.95 (2H, d, J = 7.6 Hz, phenyl), 7.10 (1H, t, J = 7.6 Hz, phenyl), 7.23–7.35 (4H, m, phenyl); MS (ESI): m/z = 342.1 [M + H].

*4.1.1.12. Ethyl 2-(3-phenoxybenzamido)propanoate (**10l**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (2.6 g, 17 mmol) and 3-phenoxybenzoic acid (4.0 g, 19 mmol) afforded **10l** (5.9 g). Yield 99%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (3H, t, J = 7.2 Hz, CH_3), 1.48 (3H, d, J = 7.6 Hz, CH_3), 4.22 (2H, q, J = 7.2 Hz, CH_2), 4.70–4.78 (1H, m, CH), 6.69 (1H, d, J = 6.1 Hz, NH), 7.00 (2H, d, J = 7.6 Hz, phenyl), 7.11 (2H, t, J = 7.6 Hz, phenyl), 7.33–7.41 (3H, m, phenyl), 7.45 (2H, t, J = 7.6 Hz, phenyl); MS (ESI): m/z = 314.1 [M + H].

*4.1.1.13. Ethyl 2-(2-(4-phenoxyphenyl)acetamido)propanoate (**10m**).* Following the general procedure for the synthesis of **10a–o**, coupling reaction of **8** (0.6 g, 4.0 mmol) and 4-phenoxyphenylacetic acid (1.0 g, 4.4 mmol) afforded **10m** (1.4 g). Yield 99%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.26 (3H, t, J = 7.2 Hz, CH_3), 1.36 (3H, d, J = 7.2 Hz, CH_3), 3.55 (2H, s, CH_2), 4.18 (2H, q, J = 7.2 Hz, CH_2), 4.54–4.61 (1H, m, CH), 5.99 (1H, d, J = 6.0 Hz, NH), 6.97–7.02 (4H, m, phenyl), 7.10 (1H, t, J = 7.2 Hz, phenyl), 7.23–7.35 (4H, m, phenyl); MS (ESI): m/z = 342.1 [M + H].

*4.1.1.14. Ethyl 2-(2-(2-phenoxyphenyl)acetamido)propanoate (**10n**).* Following the general procedure for the synthesis of **10a**–**o**, coupling reaction of **8** (3.0 g, 19 mmol) and 2-phenoxyphenylacetic acid (5.0 g, 21 mmol) afforded **10n** (6.8 g). Yield 95%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21 (3H, t, J = 7.2 Hz, CH_3), 1.28 (3H, d, J = 7.2 Hz, CH_3), 3.62 (2H, s, CH_2), 4.13 (2H, q, J = 7.2 Hz, CH_2), 4.46–4.53 (1H, m, CH), 6.34 (1H, d, J = 6.8 Hz, NH), 6.89 (1H, d, J = 8.0 Hz, phenyl), 6.99 (2H, d, J = 8.0 Hz, phenyl), 7.10 (2H, q, J = 6.4 Hz, phenyl), 7.23–7.25 (1H, m, phenyl), 7.31–7.39 (3H, m, phenyl); MS (ESI): m/z = 328.8 [M + H].

*4.1.1.15. Ethyl 2-(2-(benzyloxy)acetamido)propanoate (**10o**).* Following the general procedure for the synthesis of (**10a**–**o**), coupling reaction of **8** (4.2 g, 27 mmol) and benzyloxyacetic acid (5.0 g, 30 mmol) afforded **10o** (6.0 g). Yield 83%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (3H, t, J = 6.8 Hz, CH_3), 1.43 (3H, d, J = 7.2 Hz, CH_3), 4.00 (2H, s, CH_2), 4.23 (2H, q, J = 6.8 Hz, CH_2), 4.57–4.66 (3H, m, CH_2 & CH), 7.13 (1H, d, J = 4.8 Hz, NH), 7.33–7.41 (5H, m, phenyl); MS (ESI): m/z = 266.1 [M + H].

*4.1.2. General procedure for the preparation compounds (**11a**–**o**)*

A mixture of **10a**–**o** (1.0 equiv) and phosphorous pentoxide (2.0 equiv) in anhydrous chloroform was refluxed for 4 h. After reaction mixture was cooled to room temperature, it was treated with 20% KOH aqueous solution and stirred vigorously for 30 min. The mixture was extracted with dichloromethane three times. The organic layers were combined and treated with anhydrous Na_2SO_4. After filtered off, organic layers were concentrated in vacuum and the residue was purified by silica gel column chromatography with hexanes: ethyl acetate = 3:1 to afford **11a**–**o** as sticky oil.

*4.1.2.1. 2-Benzyl-5-ethoxy-4-methyl-oxazole (**11a**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10a** (27 g, 0.11 mol) was reacted with phosphorous pentoxide (62 g, 0.22 mol) to afford **11a** (25 g). Yield 99%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.29 (3H, t, J = 7.2 Hz, CH_3), 2.01 (3H, s, CH_3), 3.94 (2H, s, CH_2), 4.05 (2H, q, J = 7.2 Hz, CH_2), 7.23–34 (5H, m, phenyl); MS (MALDI- TOF): m/z = 216.9 [M + H].

*4.1.2.2. 5-Ethoxy-4-methyl-2-phenethyl-oxazole (**11b**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10b** (7.0 g, 28 mmol) was reacted with phosphorous pentoxide (16 g, 56 mmol) to afford **11b** (6.3 g). Yield 97%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, t, J = 6.0 Hz, CH_3), 2.02 (3H, s, CH_3), 2.45 (2H, t, J = 6.0 Hz, CH_2), 2.90 (2H, t, J = 6.0 Hz, CH_2), 4.02 (2H, q, J = 6.0 Hz, CH_2), 7.18–7.32 (5H, m, phenyl); MS (MALDI-TOF): m/z = 232.0 [M + H].

*4.1.2.3. 5-Ethoxy-4-methyl-2-styryl-oxazole (**11c**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10c** (7.8 g, 32 mmol) was reacted with phosphorous pentoxide (18 g, 64 mmol) to afford **11c** (6.4 g). Yield 90%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.36 (3H, t, J = 7.2 Hz, CH_3), 2.11 (3H, s, CH_3), 4.30 (2H, q, J = 7.2 Hz, CH_2), 6.45 (1H, d, J = 15.9 Hz, =CH), 6.76 (1H, d, J = 15.9 Hz, =CH), 7.28–7.41 (3H, m, phenyl), 7.52–7.54 (2H, m, phenyl); MS (ESI): m/z = 230.3 [M + H].

*4.1.2.4. 5-Ethoxy-2-(4-methoxyphenethyl)-4-methyl-oxazole (**11d**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10d** (7.5 g, 27 mmol) was reacted with phosphorous pentoxide (15 g, 54 mmol) to afford **11d** (6.4 g). Yield 91%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.31 (3H, t, J = 7.2 Hz, CH_3), 2.01 (3H, s, CH_3), 2.83 (2H, t, J = 6.0 Hz, CH_2), 2.90 (2H, t, J = 6.0 Hz, CH_2), 4.05 (3H, s, OCH_3), 4.32 (2H, q, J = 7.2 Hz, CH_2), 6.81 (2H, d, J = 8.7 Hz, phenyl), 7.10 (2H, d, J = 8.7 Hz, phenyl); MS (ESI): m/z = 262.4 [M + H].

*4.1.2.5. 5-Ethoxy-2-[2-(3-methoxy-phenyl)-ethyl]-4-methoxy-oxazole (**11e**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10e** (3.9 g, 14 mmol) was reacted with phosphorous pentoxide (7.9 g, 28 mmol) to afford **11e** (3.6 g). Yield 99%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, t, J = 6.9 Hz, CH_3), 2.04 (3H, s, CH_3), 2.90 (2H, t, J = 6.9 Hz, CH_2), 3.01 (2H, t, J = 6.9 Hz, CH_2), 3.81 (3H, s, CH_3O), 4.31 (2H, q, J = 6.9 Hz, CH_2), 6.74–6.81 (3H, m, phenyl), 7.17–7.24 (1H, m, phenyl); MS (ESI): m/z = 262.3 [M + H].

*4.1.2.6. 5-Ethoxy-2-(2-methoxyphenethyl)-4-methyl-oxazole (**11f**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10f** (4.6 g, 16 mmol) was reacted with phosphorous pentoxide (9.3 g, 32 mmol) to afford **11f** (4.1 g). Yield 95%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.32 (3H, t, J = 6.9 Hz, CH_3), 2.03 (3H, s, CH_3), 2.90 (2H, t, J = 6.9 Hz, CH_2), 3.02 (2H, t, J = 6.9 Hz, CH_2), 3.92 (3H, s, CH_3O), 4.32 (2H, q, J = 6.9 Hz, CH_2), 6.76–7.32 (4H, m, phenyl); MS (MALDI-TOF): m/z = 262.3 [M + H].

*4.1.2.7. 4-[2-(5-Ethoxy-4-methyl-oxazole-2-yl)-ethyl]-benzoic acid methyl ester (**11g**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10g** (5.8 g, 19 mmol) was reacted with phosphorous pentoxide (11 g, 38 mmol) to afford **11g** (2.1 g). Yield 39%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.32 (3H, t, J = 6.0 Hz, CH_3), 2.01 (3H, s, CH_3), 2.91 (2H, t, J = 5.4 Hz, CH_2), 3.04 (2H, t, J = 5.4 Hz, CH_2), 3.91 (3H, s, $COOCH_3$), 4.06 (2H, q, J = 6.0 Hz, CH_2), 7.27 (2H, d, J = 8.1 Hz, phenyl), 7.95 (2H, d, J = 8.1 Hz, phenyl); MS (MALDI-TOF): m/z = 290.4 [M + H].

*4.1.2.8. 5-Ethoxy-4-methyl-2-(naphthalen-2-ylmethyl)oxazole (**11h**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10h** (4.1 g, 14 mmol) was reacted with phosphorous pentoxide (7.9 g, 28 mmol) to afford **11h** (3.8 g). Yield 98%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (3H, t, J = 6.8 Hz, CH_3), 2.01 (3H, s, CH_3), 4.04 (2H, q, J = 6.8 Hz, CH_2), 4.10 (2H, s, CH_2), 7.39–7.46 (3H, m, naphthyl), 7.71–7.81 (4H, m, naphthyl); MS (MALDI-TOF): m/z = 268.4 [M + H].

*4.1.2.9. 2-(2,2-Diphenylethyl)-5-ethoxy-4-methyl-oxazole (**11i**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10i** (4.1 g, 12 mmol) was reacted with phosphorous pentoxide (7.1 g, 24 mmol) to afford **11i** (4.0 g). Yield 99%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (3H, t, J = 7.2 Hz, CH_3), 1.92 (3H, s, CH_3), 3.33 (2H, d, J = 8.4 Hz, CH_2), 3.93 (2H, q, J = 7.2 Hz, CH_2), 4.53 (1H, t, J = 8.4 Hz, CH), 7.15–7.28 (10H, m, phenyl); MS (ESI): m/z = 307.8 [M + H].

*4.1.2.10. 5-Ethoxy-4-methyl-2-(3-phenoxybenzyl)oxazole (**11j**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10j** (7.2 g, 22 mmol) was reacted with phosphorous pentoxide (13 g, 44 mmol) to afford **11j** (6.2 g). Yield 92%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.31 (3H, t, J = 7.2 Hz, CH_3), 2.01 (3H, s, CH_3), 3.92 (2H, s, CH_2), 4.07 (2H, q, J = 7.2 Hz, CH_2), 6.87–6.95 (3H, m, phenyl), 7.02 (2H, d, J = 7.6 Hz, phenyl), 7.12 (1H, t, J = 7.2 Hz, phenyl), 7.25–7.35 (3H, m, phenyl); MS (ESI): m/z = 310.3 [M + H].

*4.1.2.11. 5-Ethoxy-4-methyl-2-(3-phenoxyphenethyl)oxazole (**11k**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10k** (6.5 g, 20 mmol) was reacted with phosphorous pentoxide (12 g, 40 mmol) to afford **11k** (5.6 g). Yield 86%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.33 (3H, t, J = 7.2 Hz, CH_3), 2.00 (3H, s, CH_3), 2.87–2.91 (2H, m, CH_2), 2.99–3.02 (2H, m, CH_2), 4.07 (2H, q, J = 7.2 Hz, CH_2), 6.85–6.87 (2H, m, phenyl), 6.94 (1H, d, J = 7.6 Hz, phenyl), 6.98 (2H, d, J = 7.6 Hz, phenyl), 7.11 (1H, t, J = 7.2 Hz, phenyl), 7.23–7.26 (2H, m, phenyl), 7.32 (1H, t, J = 7.6 Hz, phenyl); MS (ESI): m/z = 324.1 [M + H].

*4.1.2.12. 5-Ethoxy-4-methyl-2-(3-phenoxyphenyl)oxazole (**11l**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10l** (5.9 g, 19 mmol) was reacted with phosphorous pentoxide (11 g, 38 mmol) to afford **11l** (2.4 g). Yield 43%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.38 (3H, t, J = 7.2 Hz, CH_3), 2.10 (3H, s, J = 7.6 Hz, CH_3), 4.21 (2H, q, J = 7.2 Hz, CH_2), 7.02 (3H, d, J = 8.0 Hz, phenyl), 7.12 (1H, t, J = 7.6 Hz, phenyl), 7.33–7.39 (3H, m, phenyl), 7.57 (1H, m, phenyl), 7.66 (1H, d, J = 7.6 Hz, phenyl); MS (ESI): m/z = 296.0 [M + H].

*4.1.2.13. 5-Ethoxy-4-methyl-2-(4-phenoxybenzyl)oxazole (**11m**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10m** (1.4 g, 4.3 mmol) was reacted with phosphorous pentoxide (2.4 g, 8.6 mmol) to afford **11m** (1.1 g). Yield 84%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (3H, t, J = 7.2 Hz, CH_3), 2.02 (3H, s, CH_3), 3.92 (2H, s, CH_2), 4.01 (2H, q, J = 7.2 Hz, CH_2), 6.94–7.01 (4H, m, phenyl), 7.11 (1H, t, J = 7.6 Hz, phenyl), 7.23 (2H, d, J = 8.4 Hz, phenyl), 7.28 (2H, t, J = 7.6 Hz, phenyl); MS (ESI): m/z = 310.1 [M + H].

*4.1.2.14. 5-Ethoxy-4-methyl-2-(2-phenoxybenzyl)oxazole (**11n**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10n** (6.8 g, 21 mmol) was reacted with phosphorous pentoxide (12 g, 42 mmol) to afford **11n** (4.0 g). Yield 59%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.26 (3H, t, J = 7.2 Hz, CH_3), 1.96 (3H, s, CH_3), 3.97–4.03 (4H, m, 2× CH_2), 6.89 (1H, d, J = 8.4 Hz, phenyl), 6.92 (2H, d, J = 7.6 Hz, phenyl), 7.04–7.11 (2H, d, J = 7.6 Hz, phenyl), 7.20 (1H, m, phenyl), 7.23 (3H, t, J = 8.4 Hz, phenyl); MS (ESI): m/z = 309.8 [M + H].

*4.1.2.15. 2-((Benzyloxy)methyl)-5-ethoxy-4-methyl-oxazole (**11o**).* Following the general procedure for the synthesis of **11a**–**o**, the compound **10o** (5.9 g, 23 mmol) was reacted with phosphorous pentoxide (13 g, 45 mmol) to afford **11o** (4.4 g). Yield 79%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (3H, t, J = 7.2 Hz, CH_3), 2.04 (3H, s, CH_3), 4.16 (2H, q, J = 7.2 Hz, CH_2), 4.44 (2H, s, CH_2), 4.59 (2H, s, CH_2), 7.35–7.37 (5H, m, phenyl); MS (MALDI-TOF): m/z = 248.3 [M + H].

*4.1.3. General procedure for the preparation of compounds (**12a**–**o**)*

To a neat of **11a**–**o** (1.0 equiv) was added dropwise dimethyl maleate (2.0 equiv). The mixture was refluxed for 5 h with stirring and cooled to room temperature. The mixture was diluted using ethanol and saturated with HCl gas and concentrated in vacuum. Water was added to the mixture and mixture was extracted with dichloromethane three times. The organic layers were combined and dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, solvent was removed in vacuum and the residue was purified by silica gel column chromatography with hexanes: ethyl acetate = 7:1 to afford **12a**–**o** as sticky oil.

*4.1.3.1. 2-Benzyl-5-hydroxy-6-methyl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12a**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11a** (9.4 g, 40 mmol) and dimethyl maleate (11 mL, 80 mmol) afforded **12a** (8.0 g). Yield 59%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.84 (3H, s, CH_3), 3.76 (3H, s, CH_3O), 4.00 (3H, s, CH_3O), 4.42 (2H, s, CH_2), 7.24–7.31 (5H, m, phenyl); MS (MALDI-TOF): m/z = 314.9 [M + H].

*4.1.3.2. 5-Hydroxy-6-methyl-2-phenethyl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12b**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11b** (2.8 g, 12 mmol) and dimethyl maleate (3.0 mL, 24 mmol) afforded **12b** (1.8 g). Yield 47%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.64 (3H, s, CH_3), 3.10 (2H, t, J = 7.2 Hz, CH_2), 3.35 (2H, t, J = 7.2 Hz, CH_2), 4.00 (3H, s, CH_3O), 4.10 (3H, s, CH_3O), 7.20–7.24 (3H, m, phenyl), 7.26–7.29 (2H, m, phenyl); MS (ESI): m/z = 330.4 [M + H].

*4.1.3.3. 5-Hydroxy-6-methyl-2-styryl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12c**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11c** (4.0 g, 19 mmol) and dimethyl maleate (4.7 mL, 38 mmol) afforded **12c** (1.4 g). Yield 23%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.49 (3H, s, CH_3), 3.83 (6H, s, 2× CH_3O), 6.82 (1H, d, J = 15.4 Hz, =CH), 7.19–7.25 (3H, m, phenyl), 7.38 (2H, d, J = 5.4 Hz, phenyl), 7.72 (1H, t, J = 15.4 Hz, =CH); MS (ESI): m/z = 328.2 [M + H].

*4.1.3.4. 5-Hydroxy-2-[2-(4-methoxy-phenyl)-ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12d**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11d** (6.0 g, 23 mmol) and dimethyl maleate (5.8 mL, 46 mol) afforded **12d** (3.1 g). Yield 37%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.02 (3H, s, CH_3), 3.04 (2H, t, J = 4.8 Hz, CH_2), 3.32 (2H, t, J = 4.8 Hz, CH_2), 3.79 (3H, s, CH_3O), 4.00 (3H, s, CH_3O), 4.08 (3H, s, CH_3O), 6.83 (2H, d, J = 5.4 Hz, phenyl), 7.27 (1H, d, J = 5.4 Hz, phenyl); MS (ESI): m/z = 360.4 [M + H].

*4.1.3.5. 5-Hydroxy-2-[2-(3-methoxy-phenyl)-ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12e**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11e** (2.0 g, 7.6 mmol) and dimethyl maleate (2.0 mL, 15 mmol) afforded **12e** (0.86 g). Yield 31%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.00 (3H, s, CH_3), 3.10 (2H, t, J = 3.9 Hz, CH_2), 3.35 (2H, t, J = 3.9 Hz, CH_2), 3.86 (3H, s, CH_3O), 3.98 (3H, s, CH_3O), 4.10 (3H, s, CH_3O), 6.78 (1H, dd, J = 7.5 Hz, 8.1 Hz, phenyl), 6.90 (1H, d, J = 7.5 Hz, phenyl), 7.00 (1H, s, phenyl), 7.21 (1H, t, J = 8.1 Hz, phenyl); MS (ESI): m/z = 360.1 [M + H].

*4.1.3.6. Dimethyl 5-hydroxy-2-(2-methoxyphenethyl)-6-methylpyridine-3,4-dicarboxylate (**12f**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11f** (1.5 g, 5.7 mmol) and dimethyl maleate (1.4 mL, 11 mmol) afforded **12f** (0.49 g). Yield 24%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.58 (3H, s, CH_3), 2.92 (2H, t, J = 7.2 Hz, CH_2), 2.99 (2H, t, J = 7.2 Hz, CH_2), 3.83 (3H, s, CH_3O), 3.86 (3H, s, CH_3O), 3.94 (3H, s, CH_3O), 6.84–7.21 (4H, m, phenyl), 10.55 (1H, s, OH); MS (ESI): m/z = 340.4 [M + H].

*4.1.3.7. 5-Hydroxy-2-[2-(4-methoxycarbonyl-phenyl)-ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid dimethyl ester (**12g**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11g** (2.1 g, 7.1 mmol) and dimethyl maleate (1.8 mL, 14 mmol) afforded **12g** (0.92 g). Yield 33%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.57 (3H, s, CH_3), 2.90 (2H, t, J = 9.3 Hz, CH_2), 3.04 (2H, t, J = 9.3 Hz, CH_2), 3.86 (3H, s, CH_3O), 3.91 (3H, s, CH_3O), 3.95 (3H, s, $COOCH_3$), 7.25 (2H, d, J = 8.7 Hz, phenyl), 7.94 (2H, d, J = 8.7 Hz, phenyl); MS (ESI): m/z = 388.4 [M + H].

*4.1.3.8. Dimethyl 5-hydroxy-6-methyl-2-(naphthalen-1-ylmethyl) pyridine-3,4-dicarboxylate (**12h**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11h** (3.8 g, 14 mmol) and dimethyl maleate (3.6 mL, 28 mmol) afforded **12h** (2.7 g). Yield 52%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.54 (3H, s, CH_3), 3.72 (3H, s, CH_3O), 3.92 (3H, s, CH_3O), 4.21 (2H, s, CH_2), 7.35–7.48 (3H, m, naphthyl), 7.62–7.78 (4H, m, naphthyl), 10.57 (1H, s, OH); MS (ESI): m/z = 366.4 [M + H].

*4.1.3.9. Dimethyl 2-(2,2-diphenylethyl)-5-hydroxy-6-methylpyridine-3,4-dicarboxylate (**12i**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11i** (4.0 g, 13 mmol) and dimethyl maleate (3.3 mL, 26 mmol) afforded **12i** (1.1 g). Yield 21%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.46 (3H, s, CH_3), 3.37

(2H, d, J = 8.0 Hz, CH_2), 3.75 (3H, s, CH_3O), 3.88 (3H, s, CH_3O), 4.51 (1H, t, J = 8.0 Hz, CH), 7.10–7.24 (10H, m, phenyl), 10.47 (1H, s, OH); MS (ESI): m/z = 406.0 [M + H].

*4.1.3.10. Dimethyl 5-hydroxy-6-methyl-2-(3-phenoxybenzyl)pyridine-3,4-dicarboxylate (**12j**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11j** (5.5 g, 18 mmol) and dimethyl maleate (4.5 mL, 36 mmol) afforded **12j** (2.3 g). Yield 32%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.52 (3H, s, CH_3), 3.78 (3H, s, CH_3O), 3.93 (3H, s, CH_3O), 4.02 (2H, s, CH_2), 6.81 (1H, d, J = 8.0 Hz, phenyl), 6.91–6.99 (4H, m, phenyl), 7.09 (1H, t, J = 6.8 Hz, phenyl), 7.20 (1H, t, J = 7.6 Hz, phenyl), 7.32 (2H, t, J = 8.0 Hz, phenyl), 10.57 (1H, s, OH); MS (ESI): m/z = 408.4 [M + H].

*4.1.3.11. Dimethyl 5-hydroxy–6-methyl-2-(3-phenoxyphenethyl)pyridine–3,4-dicarboxylate (**12k**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11k** (2.0 g, 6.2 mmol) and dimethyl maleate (1.6 mL, 12 mmol) afforded **12k** (0.80 g). Yield 31%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.54 (3H, s, CH_3), 2.92 (2H, m, CH_2), 2.94 (2H, m, CH_2), 3.83 (3H, s, CH_3O), 3.94 (3H, s, CH_3O), 6.82 (2H, m, phenyl), 6.94–7.00 (3H, m, phenyl), 7.10 (1H, t, J = 7.2 Hz, phenyl), 7.22 (1H, t, J = 8.0 Hz, phenyl), 7.33 (2H, t, J = 8.0 Hz, phenyl), 10.56 (1H, s, OH); MS (ESI): m/z = 422.1 [M + H].

*4.1.3.12. Dimethyl 5-hydroxy-6-methyl-2-(3-phenoxyphenyl)pyridine-3,4-dicarboxylate (**12l**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11l** (1.4 g, 4.7 mmol) and dimethyl maleate (1.2 mL, 9.4 mmol) afforded **12l** (0.46 g). Yield 24%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.61 (3H, s, CH_3), 3.67 (3H, s, CH_3O), 3.96 (3H, s, CH_3O), 7.02 (3H, m, phenyl), 7.11 (1H, t, J = 7.6 Hz, phenyl), 7.17 (1H, m, phenyl), 7.33–7.39 (4H, m, phenyl), 10.77 (1H, s, OH); MS (ESI): m/z = 392.3 [M − H].

*4.1.3.13. Dimethyl 5-hydroxy-6-methyl-2-(4-phenoxybenzyl)pyridine-3,4-dicarboxylate (**12m**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11m** (0.50 g, 1.6 mmol) and dimethyl maleate (0.40 mL, 3.2 mmol) afforded **12m** (0.25 g). Yield 38%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.55 (3H, s, CH_3), 3.91 (3H, s, CH_3O), 3.94 (3H, s, CH_3O), 4.03 (2H, s, CH_2), 6.89 (2H, d, J = 8.4 Hz, phenyl), 6.97(2H, d, J = 7.6 Hz, phenyl), 7.09 (1H, t, J = 7.6 Hz, phenyl), 7.19(2H, d, J = 8.8 Hz, phenyl), 7.30 (2H, t, J = 8.8 Hz, phenyl), 10.56 (1H, s, OH); MS (ESI): m/z = 407.9 [M + H].

*4.1.3.14. Dimethyl 5-hydroxy-6-methyl-2-(2-phenoxybenzyl)pyridine-3,4-dicarboxylate (**12n**).* Following the general procedure for the synthesis of **12a**–**o**, Diels-Alder reaction of **11n** (3.9 g, 12 mmol) and dimethyl maleate (3.1 mL, 24 mmol) afforded **12n** (2.0 g). Yield 40%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.47 (3H, s, CH_3), 3.70 (3H, s, CH_3O), 3.91 (3H, s, CH_3O), 4.12 (2H, s, CH_2), 6.86 (1H, d, J = 8.4 Hz, phenyl), 6.89 (2H, d, J = 8.0 Hz, phenyl), 7.01 (2H, t, J = 8.0 Hz, phenyl), 7.13–7.18(2H, m, phenyl), 7.24–7.29 (2H, m, phenyl), 10.46 (1H, s, OH); MS (ESI): m/z = 407.7 [M + H].

*4.1.3.15. Dimethyl 2-(benzyloxymethyl)-5-hydroxy-6-methyl-pyridine-3,4-dicarboxylate (**12o**).* Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of **11o** (0.50 g, 2.0 mmol) and dimethyl maleate (0.50 mL, 4.0 mmol) afforded **12o** (0.33 g). Yield 47%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.56 (3H, s, CH_3), 3.71 (3H, s, CH_3O), 3.95 (3H, s, CH_3O), 4.52 (2H, s, CH_2), 4.64 (2H, s, CH_2), 7.30–7.34 (5H, m, phenyl), 10.66 (1H, s, OH); MS (ESI): m/z = 346.1 [M + H].

*4.1.4. General procedure for the preparation of compounds (**13a**–**o**)*

To a solution of aqueous 20% KOH was added **12a**–**o** with vigorous stirring for 6 h. The mixture was acidified with concentrated HCl solution. Generated solid product was diluted with water to dissolve the KCl salt. The solid product was filtered with excess water and chloroform. After that, solid product was dried to afford **13a**–**o** as a white solid.

*4.1.4.1. 2-Benzyl-5-hydroxy-6-methyl-pyridine-3,4-dicarboxylic acid (**13a**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12a** (1.0 g, 3.2 mmol) was reacted with aqueous 20% KOH (15 mL) to afford **13a** (0.60 g). Yield 66%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.28 (3H, s, CH_3), 3.91 (2H, s, CH_2), 7.11–20 (5H, m, phenyl); MS (ESI): m/z = 286.1 [M + H].

*4.1.4.2. 5-Hydroxy-6-methyl-2-phenethyl-pyridine-3,4-dicarboxylic acid (**13b**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12b** (40 mg, 0.12 mmol) was reacted with aqueous 20% KOH (2.5 mL) to afford **13b** (26 mg). Yield 70%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.31 (3H, s, CH_3), 2.84–2.87 (4H, m, 2× CH_2), 7.18–7.28 (5H, m, phenyl); MS (ESI): m/z = 300.1 [M + H].

*4.1.4.3. 5-Hydroxy-6-methyl-2-styryl-pyridine-3,4-dicarboxylic acid (**13c**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12c** (0.25 g, 0.76 mmol) was reacted with aqueous 20% KOH (8.0 mL) to afford **13c** (0.18 g). Yield 79%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.42 (3H, s, CH_3), 7.11 (1H, t, J = 16.5 Hz, =CH), 7.30 (1H, t, J = 16.5 Hz, =CH), 7.31–38 (3H, m, phenyl), 7.55 (2H, d, J = 7.2 Hz, phenyl); MS (ESI): m/z = 298.1 [M + H].

*4.1.4.4. 5-Hydroxy-2-[2-(4-methoxy-phenyl)ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid (**13d**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12d** (90 mg, 0.27 mmol) and aqueous 20% KOH (3.0 mL) to afford **13d** (52 mg). Yield 65%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.25 (3H, s, CH_3), 2.80–2.95 (4H, m, 2× CH_2), 3.80 (3H, s, CH_3O), 6.91 (2H, d, J = 8.7 Hz, phenyl), 7.21 (2H, d, J = 8.7 Hz, phenyl); ^{13}C NMR (DMSO-d_6, 400 MHz) 19.07, 35.48, 37.68, 55.43, 114.12, 120.40, 128.32, 129.68, 135.06, 142.99, 144.39, 157.83, 166.88, 171.98, 175.15; MS (ESI): m/z = 332.3 [M + H].

*4.1.4.5. 5-Hydroxy-2-[2-(3-methoxy-phenyl)-ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid (**13e**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12e** (0.50 g, 1.4 mmol) was reacted with aqueous 20% KOH (10 mL) to afford **13e** (0.25 g). Yield 54%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.34 (3H, s, CH_3), 2.87–2.91 (4H, m, 2× CH_2), 3.74 (3H, s, CH_3O), 6.76–6.87 (2H, m, phenyl), 7.23–7.25 (2H, m, phenyl); MS (ESI): m/z = 332.3 [M + H].

*4.1.4.6. 5-Hydroxy-2-[2-(2-methoxy-phenyl)-ethyl]-6-methyl-pyridine-3,4-dicarboxylic acid (**13f**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12f** (0.30 g, 0.84 mmol) was reacted with aqueous 20% KOH (6.0 mL) to afford **13f** (0.25 g). Yield 92%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.59 (3H, s, CH_3), 2.86 (2H, t, J = 6.9 Hz, CH_2), 2.89 (2H, t, J = 6.9 Hz, CH_2), 3.77 (3H, s, CH_3O), 6.88–6.96 (2H, m, phenyl), 7.11–7.19 (2H, m, phenyl); MS (ESI): m/z = 332.3 [M + H].

*4.1.4.7. 2-[2-(4-Carboxy-phenyl)-ethyl]-5-hydroxy-6-methyl-pyridine-3,4-dicarboxylic acid (**13g**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12g** (15 mg, 0.040 mmol) was reacted with aqueous 20% KOH (1.3 mL) to afford **13g** (7.2 mg). Yield 54%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.38 (3H, s, CH_3), 2.94–3.0 (4H, m, 2× CH_2), 7.22 (2H, d, J = 8.1 Hz, phenyl), 7.72 (2H, d, J = 8.1 Hz, phenyl); ^{13}C NMR (DMSO-d_6, 400 MHz) 18.79, 36.46, 37.50, 122.78, 127.80, 129.61, 136.69, 137.48, 143.05, 144.36, 171.21, 175.58; MS (ESI): m/z = 346.3 [M + H].

*4.1.4.8. 5-Hydroxy-6-methyl-2-(naphthalene-1-ylmethyl)pyridine-3,4-dicarboxylic acid (**13h**).* Following the general procedure for the

synthesis of **13a**–**o**, the compound **12h** (0.18 g, 0.49 mmol) was reacted with aqueous 20% KOH (6.0 mL) to afford **13h** (0.12 mg). Yield 73%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.36 (3H, s, CH_3), 4.22 (2H, s, CH_2), 7.03–7.18 (4H, m, naphthyl), 7.23–7.26 (3H, m, naphthyl); MS (ESI): m/z = 337.3 [M + H].

*4.1.4.9. 2-(2,2,-Diphenylethyl)5-hydroxy-6-methylpyridine-3,4-dicarboxylic acid (**13i**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12i** (0.10 g, 0.25 mmol) was reacted with aqueous 20% KOH (4.0 mL) to afford **13i** (40 mg). Yield 43%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.36 (3H, s, CH_3), 3.38 (2H, d, J = 8.0 Hz, CH_2), 4.74 (H, t, J = 8.0 Hz, CH), 7.03–7.26 (10H, m, phenyl); MS (ESI): m/z = 377.4 [M + H].

*4.1.4.10. 5-Hydroxy-6-methyl-2-(3-phenoxybenzyl)pyridine-3,4-dicarboxylic acid (**13j**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12j** (0.47 g, 0.060 mmol) was reacted with aqueous 20% KOH (10 mL) to afford **13j** (0.33 g). Yield 72%; ^{1}H NMR (DMSO-d_6, 400 MHz) 2.57 (3H, s, CH_3), 4.17 (2H, s, CH_2), 6.81 (1H, d, J = 8.0 Hz, phenyl), 6.81 (1H, d, J = 8.4 Hz, phenyl), 6.88 (1H, s, phenyl), 6.93 (2H, d, J = 8.0 Hz, phenyl), 7.08 (1H, t, J = 7.6 Hz, phenyl), 7.24 (1H, t, J = 8.4 Hz, phenyl), 7.32 (2H, t, J = 7.6 Hz, phenyl); ^{13}C NMR (DMSO-d_6, 400 MHz) 14.79, 36.99, 116.75, 118.60, 118.90, 123.14, 129.54, 130.84, 139.45, 140.02, 148.04, 155.31, 157.64, 168.15; MS (ESI): m/z = 380.3 [M + H].

*4.1.4.11. 5-Hydroxy-6-methyl-2-(3-phenoxyphenethyl)pyridine-3,4-dicarboxylic acid (**13k**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12k** (0.30 g, 0.74 mmol) was reacted with aqueous 20% KOH (8.5 mL) to afford **13k** (0.14 g). Yield 50%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.55 (3H, s, CH_3), 2.92–2.95 (2H, m, CH_2), 2.98–3.01 (2H, m, CH_2), 6.82 (2H, t, J = 8.0 Hz, phenyl), 6.93–6.99 (3H, m, phenyl), 7.10 (1H, t, J = 7.2 Hz, phenyl), 7.26–7.36 (3H, m, phenyl); ^{13}C NMR (DMSO-d_6, 400 MHz) 16.66, 34.36, 35.83, 117.00, 118.60, 119.08, 123.67, 126.43, 130.02, 130.65, 132.29, 138.12, 143.36, 149.82, 157.21, 167.63, 168.19; MS (ESI): m/z = 394.2 [M + H].

*4.1.4.12. 5-Hydroxy-6-methyl-2-(3-phenoxyphenyl)pyridine-3,4-dicarboxylic acid (**13l**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12l** (180 mg, 0.45 mmol) was reacted with aqueous 20% KOH (5.0 mL) to afford **13l** (60 mg). Yield 35%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.42 (3H, s, CH_3), 6.98 (3H, d, J = 8.0 Hz, phenyl), 7.09–7.13 (2H, m, phenyl), 7.23 (1H, d, J = 8.0 Hz, phenyl), 7.35–7.42 (3H, m, phenyl); MS (ESI): m/z = 366.3 [M + H].

*4.1.4.13. 5-Hydroxy-6-methyl-2-(4-phenoxybenzyl)pyridine-3,4-dicarboxylic acid (**13m**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12m** (100 mg, 0.24 mmol) was reacted with aqueous 20% KOH (4.0 mL) to afford **13m** (60 mg). Yield 66%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.39 (3H, s, CH_3), 4.06 (2H, s, CH_2), 6.82 (2H, d, J = 8.4 Hz, phenyl), 6.91 (2H, d, J = 8.4 Hz, phenyl), 7.04 (1H, t, J = 7.2 Hz, phenyl), 7.32 (4H, t, J = 7.6 Hz, phenyl); MS (ESI): m/z = 379.9 [M + H].

*4.1.4.14. 5-Hydroxy-6-methyl-2-(2-phenoxybenzyl)pyridine-3,4-dicarboxylic acid (**13n**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12n** (0.50 g, 1.2 mmol) was reacted with aqueous 20% KOH (10 mL) to afford **13n** (0.29 g). Yield 62%; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 2.34 (3H, s, CH_3), 4.26 (2H, s, CH_2), 6.70 (2H, d, J = 8.0 Hz, phenyl), 6.81 (1H, d, J = 8.0 Hz, phenyl), 6.98 (1H, t, J = 7.2 Hz, phenyl), 7.10 (1H, t, J = 7.6 Hz, phenyl), 7.17–7.25 (4H, m, phenyl); MS (ESI): m/z = 379.7 [M + H].

*4.1.4.15. 2-(Benzyloxymethyl)-5-hydroxy-6-methylpyridine-3,4-dicarboxylic acid (**13o**).* Following the general procedure for the synthesis of **13a**–**o**, the compound **12o** (100 mg, 0.28 mmol) was reacted with aqueous 20% KOH (4.0 mL) to afford **13o** (30 mg). Yield 27%; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 2.41 (2H, s, CH_3), 4.56 (2H, s, CH_2), 4.59 (2H, s, CH_2), 7.22–7.37 (5H, m, phenyl); MS (ESI): m/z = 318.1 [M + H].

*4.1.5. General procedure for the preparation of (E)-ethyl 4-oxopent-2-enoate (**16**)*

A solution of **14**, (carbethoxymethylene)triphenylphosphorane, (21 g, 61 mmol) in a small volume of dichloromethane was slowly added aqueous **15**, pyruvalehyde, (47 mL, 300 mmol) at room temperature. The mixture was diluted with *N*,*N*-dimethylformamide (30 mL), and it was stirred for 20 h until the starting material was disappeared by TLC monitoring. The reaction mixture was added water and extracted with dichloromethane. The organic layer was washed successively with water and brine, dried over $MgSO_4$ and evaporated under reduced pressure to give the product as sticky oil. ^{1}H NMR spectroscopy showed the product a mixture of *E* and *Z* isomers (9:1). The isomers were separated by flash column chromatography, eluting with *n*-hexanes-ethyl acetate = 3:1. *E* isomer, compound **16** (8.9 g): R_f 0.80 (hexanes-ethyl acetate = 1:1); Yield 80%; ^{1}H NMR (acetone-d_6, 300 MHz) 1.4 (3H, t, J = 7.2 Hz, CH_3), 2.3 (3H, s, CH_3CO), 4.15 (2H, q, J = 7.2 Hz, CH_2), 6.6 (1H, d, J = 15.0 Hz, =CH), 6.9 (1H, d, J = 15.0 Hz, =CH); *Z* isomer (0.90 g): R_f 0.74 (hexanes-ethyl acetate = 1:1); Yield 8.1%; ^{1}H NMR (acetone-d_6) 1.3 (3H, t, J = 7.2 Hz, CH_3), 2.3 (3H, s, CH_3CO), 4.15 (2H, q, J = 7.2 Hz, CH_2), 5.9 (1H, d, J = 12.0 Hz, =CH), 6.4 (1H, d, J = 12.0 Hz, =CH).

*4.1.6. General procedure for the preparation of compounds (**17a**–**f**)*

The oxazole **11a**–**f** (1.0 equiv) and **16**, ethyl 4-oxopent-2-enoate, (1.5 equiv) were mixed well and heated to 55–60 °C for 10–14 h. The reaction mixture containing intermediate (Diels-Alder adduct) was dissolved in ethanol and cooled to 0 °C. HCl gas was passed through the reaction mixture for 20 min and the reaction mixture was stirred at 0–10 °C for about 30 min and then at room temperature for 1 h. The reaction mixture was poured into 8% aqueous sodium bicarbonate solution and extracted with chloroform. The organic layer concentrated and the crude product was purified by silica gel column to afford **17a**–**f** as sticky oil.

*4.1.6.1. Ethyl 4-acetyl-2-benzyl-5-hydroxy-6-methylnicotinate (**17a**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11a** (1.0 g, 4.6 mmol) with **16** (1.3 g, 9.2 mmol) afforded **17a** (0.86 g). Yield 30%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.17 (3H, t, J = 6.9 Hz, CH_3), 2.55 (3H, s, CH_3), 2.59 (3H, s, CH_3CO), 4.40 (2H, s, CH_2), 4.43 (2H, q, J = 6.9 Hz, CH_2), 7.17–7.45 (5H, m, phenyl), 11.34 (1H, s, OH); MS (FAB+): m/z = 314.3 [M + H].

*4.1.6.2. Ethyl 4-acetyl-5-hydroxy-6-methyl-2-phenethylnicotinate (**17b**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11b** (2.0 g, 8.6 mmol) with **16** (2.4 g, 17 mmol) afforded **7b** (0.65 g). Yield 23%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 (3H, t, J = 6.9 Hz, CH_3), 2.52 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 3.00 (2H, t, J = 7.2 Hz, CH_2), 3.23 (2H, t, J = 7.2 Hz, CH_2), 4.41 (2H, q, J = 6.9 Hz, CH_2), 7.18–7.30 (5H, m, phenyl), 11.42 (1H, s, OH); MS (MALDI-TOF): m/z = 328.4 [M + H].

*4.1.6.3. (E)-Ethyl 4-acetyl-5-hydroxy-6-methyl-2-styrylnicotinate (**17c**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11c** (4.0 g, 17 mmol) with **16** (4.7 g, 34 mmol) afforded **17c** (1.0 g). Yield 18%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20 (3H, t, J = 7.2 Hz, CH_3), 1.89 (3H, s, CH_3), 2.66 (3H, s, CH_3CO), 4.10 (2H, q, J = 7.2 Hz, CH_2), 7.26–7.40 (3H, m, phenyl), 7.65 (1H, d, J = 7.2 Hz, phenyl), 7.67 (1H, d, J = 7.2 Hz, phenyl), 7.96 (1H,

d, J = 15.9 Hz, =CH), 8.01 (1H, d, J = 15.9 Hz, =CH); MS (ESI): m/z = 326.2 [M + H].

*4.1.6.4. Ethyl 4-acetyl-5-hydroxy-2-(4-methoxyphenethyl)-6-methylnicotinate (**17d**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11d** (1.6 g, 6.1 mmol) with **16** (1.7 g, 12 mmol) afforded **17d** (1.1 g). Yield 51%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, t, J = 7.2 Hz, CH_3), 2.52 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 2.94(2H, d, J = 7.2 Hz, CH_2), 3.03 (2H, d, J = 7.2 Hz, CH_2), 3.80 (3H, s, CH_3O), 4.35 (2H, q, J = 7.2 Hz, CH_2), 6.82 (2H, d, J = 8.4 Hz, phenyl), 7.11 (2H, d, J = 8.4 Hz, phenyl), 11.38 (1H, s, OH); MS (ESI): m/z = 358.3 [M + H].

*4.1.6.5. Ethyl 4-acetyl-5-hydroxy-2-(3-methoxyphenethyl)-6-methylnicotinate (**17e**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11e** (2.0 g, 7.7 mmol) with **16** (2.2 g, 15 mmol) afforded **17e** (0.85 g). Yield 31%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.21 (3H, t, J = 7.2 Hz, CH_3), 2.45 (3H, s, CH_3), 2.60 (3H, s, CH_3CO), 2.98 (2H, t, J = 7.2 Hz, CH_2), 3.43 (2H, t, J = 7.2 Hz, CH_2), 3.78 (3H, s, CH_3O), 4.32 (2H, q, J = 7.2 Hz, CH_2), 6.79–6.84 (3H, m, phenyl), 7.31 (1H, s, phenyl), 11.28 (1H, br s, OH); MS (ESI): m/z = 357.4 [M + H].

*4.1.6.6. Ethyl 4-acetyl-5-hydroxy-2-(2-methoxyphenethyl)-6-methylnicotinate (**17f**).* Following the general procedure for the synthesis of **17a**–**f**, Diels-Alder reaction of the compound **11f** (1.6 g, 6.1 mmol) with **16** (1.7 g, 12 mmol) afforded **17f** (0.93 g). Yield 43%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, t, J = 7.2 Hz, CH_3), 2.51 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 2.96 (2H, t, J = 6.9 Hz, CH_2), 3.40 (2H, t, J = 6.9 Hz, CH_2), 3.81 (3H, s, CH_3O), 4.38 (2H, q, J = 7.2 Hz, CH_2), 6.85–7.26 (4H, m, phenyl), 11.35 (1H, br s, OH); MS (ESI): m/z = 357.4 [M + H].

*4.1.7. General procedure for the preparation of compounds (**18a**–**f**)*

Compounds **17a**–**f** were dissolved in 5% methanolic KOH and stirred at room temperature for 2–3 h. The reaction mixture was then acidified with concentrated HCl and extracted with chloroform. Organic layer was dried over anhydrous Na_2SO_4 and concentrated. Crude product was purified by silcagel column using ethyl acetate/n-hexanes solvent system to afford **18a**–**f** as white solid.

*4.1.7.1. 4-Acetyl-2-benzyl-5-hydroxy-6-methylnicotinic acid (**18a**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17a** (1.1 g, 3.4 mmol) was reacted with methanolic KOH (50 mL) to afforded **18a** (0.80 g). Yield 84%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.55 (3H, s, CH_3), 2.59 (3H, s, CH_3CO), 4.42 (2H, s, CH_2), 7.17–7.45 (5H, m, phenyl), 11.30 (1H, s, OH); MS (FAB+): m/z = 286.2 [M + H].

*4.1.7.2. 4-Acetyl-5-hydroxy-6-methyl-2-phenethylnicotinic acid (**18b**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17b** (0.43 g, 1.3 mmol) was reacted with methanolic KOH (20 mL) to afforded **18b** (0.24 g). Yield 62%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.52 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 3.00 (2H, t, J = 7.2 Hz, CH_2), 3.23 (2H, t, J = 7.2 Hz, CH_2) 7.18–7.30 (5H, m, phenyl), 11.42 (1H, s, OH); MS (ESI): m/z = 300.2 [M + H].

*4.1.7.3. (E)-4-Acetyl-5-hydroxy-6-methyl-2-styrylnicotinic acid (**18c**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17c** (50 mg, 0.15 mmol) was reacted with methanolic KOH (8.0 mL) to afforded **18c** (20 mg). Yield 37%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.89 (3H, s, CH_3), 2.66 (3H, s, CH_3CO), 7.26–7.40 (3H, m, phenyl), 7.65 (1H, d, J = 7.2 Hz, phenyl), 7.67 (1H, d, J = 7.2 Hz, phenyl), 7.96 (1H, d, J = 15.9 Hz, =CH), 8.01 (1H, d, J = 15.9 Hz, =CH); MS (ESI): m/z = 298.2 [M + H].

*4.1.7.4. 4-Acetyl-5-hydroxy-2-(4-methoxyphenethyl)-6-methylnicotinic acid (**18d**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17d** (0.22 g, 0.62 mmol) was reacted with methanolic KOH (15 mL) to afforded **18d** (0.18 g). Yield 88%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.52 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 2.94(2H, d, J = 7.2 Hz, CH_2), 3.03 (2H, d, J = 7.2 Hz, CH_2), 3.80 (3H, s, CH_3O), 6.82 (2H, d, J = 8.4 Hz, phenyl), 7.11 (2H, d, J = 8.4 Hz, phenyl), 11.38 (1H, s, OH); MS (ESI): m/z = 330.4 [M + H].

*4.1.7.5. 4-Acetyl-5-hydroxy-2-(3-methoxyphenethyl)-6-methylnicotinic acid (**18e**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17e** (0.38 g, 1.1 mmol) was reacted with methanolic KOH (20 mL) to afforded **18e** (0.18 g). Yield 50%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.85 (3H, s, CH_3), 2.60 (3H, s, CH_3CO), 2.98 (2H, t, J = 7.2 Hz, CH_2), 3.43 (2H, t, J = 7.2 Hz, CH_2), 3.78 (3H, s, CH_3O), 6.79–6.84 (3H, m, phenyl), 7.31 (1H, s, phenyl), 11.28 (1H, br s, OH); MS (ESI): m/z = 330.2 [M + H].

*4.1.7.6. 4-Acetyl-5-hydroxy-2-(2-methoxyphenethyl)-6-methylnicotinic acid (**18f**).* Following the general procedure for the synthesis of **18a**–**f**, the compound **17f** (40 mg, 0.62 mmol) was reacted with methanolic KOH (20 mL) to afforded **18f** (31 mg). Yield 84%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.51 (3H, s, CH_3), 2.56 (3H, s, CH_3CO), 2.96 (2H, t, J = 6.9 Hz, CH_2), 3.40 (2H, t, J = 6.9 Hz, CH_2), 3.81 (3H, s, CH_3O), 6.85–7.26 (4H, m, phenyl), 11.35 (1H, br s, OH); MS (ESI): 330.4 [M + H].

*4.1.8. General procedure for the preparation of compounds (**20** and **21**)*

Following the general procedure for the synthesis of **12a**–**o**, Diels–Alder reaction of the **19** (4-methyl-oxazole-2-amine, 1.0 g, 10 mmol) with dimethyl maleate (2.5 mL, 20 mmol) afforded **20** (0.15 g) and **21** (0.21 g) were obtained as sticky oil.

*4.1.8.1. Dimethyl 2-amino-6-methylpyridine-3,4-dicarboxylate (**20**).* Yield 13%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.41 (3H, s, CH_3), 3.84 (3H, s, CH_3O), 3.89 (3H, s, CH_3O), 6.30 (2H, s, NH_2), 6.51 (1H, s, pyridine); MS (ESI): m/z = 225.1 [M + H].

*4.1.8.2. Dimethyl 2-amino-5-hydroxy-6-methylpyridine-3,4-dicarboxylate (**21**).* Yield 17.2%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.43 (3H, s, CH_3), 3.84 (3H, s, CH_3O), 3.91 (3H, s, CH_3O), 5.31 (2H, s, NH_2), 8.04 (1H, s, OH); MS (ESI): m/z = 241.1 [M + H].

*4.1.9. Dimethyl 2-amino-5-(benzyloxy)-6-methylpyridine-3,4-dicarboxylate (**22**)*

A suspension of **21** (0.15 g, 0.61 mmol), K_2CO_3 (0.25 g, 1.8 mmol), and benzyl bromide (87 μL, 0.73 mmol) in dry acetone (10 mL) was refluxed for 2 h. After reaction mixture was cooled down, it was neutralized with NH_4Cl and extracted with dichloromethane twice. The organic layers were combined and dried with anhydrous Na_2SO_4. After Na_2SO_4 was removed, organic layer was concentrated in vacuum and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 3:1 to afford **22** (0.21 g) as yellow sticky oil. Yield 99%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.41 (3H, s, CH_3), 3.85 (3H, s, CH_3O), 3.86 (3H, s, CH_3O), 4.85 (2H, s, CH_2), 6.31 (2H, s, NH_2), 7.35–7.43 (5H, m, phenyl); MS (ESI): m/z = 330.7 [M + H].

*4.1.10. Dimethyl 2-benzamido-5-(benzyloxy)-6-methylpyridine-3,4-dicarboxylate (**23a**)*

A respective **22** (0.10 g, 0.28 mmol) was dissolved in dry toluene (3.0 mL). Benzoyl chloride (39 μL, 0.34 mmol) was added to solution

of **22** and then diisopropylethylamine (59 μL, 0.34 mmol) was added to the reaction mixture. After being stirred for 2 h at 80 °C, the mixture was neutralized with saturated aqueous NH_4Cl and extracted with dichloromethane (3 times). The organic layers were combined and treated with anhydrous Na_2SO_4. After Na_2SO_4 was filtered off, organic layer was concentrated in vacuum and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 3:1 to afford **23a** (0.10 g) as a sticky oil. Yield 77%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.62 (3H, s, CH_3), 3.87 (3H, s, CH_3O), 3.90 (3H, s, CH_3O), 7.39–7.58 (8H, m, phenyl), 8.02 (2H, m, phenyl), 10.80 (1H, s, NH), MS (ESI): m/z = 435.4 [M + H].

4.1.11. Dimethyl 5-(benzyloxy)-6-methyl-2-(3-phenoxybenzamido) pyridine-3,4-dicarboxylate (**23b**)

Following the procedure for the synthesis of **23a**, the compound **22** (0.14 g, 0.42 mmol) was reacted with 3-phenoxybenzoyl chloride (0.12 g, 0.50 mmol) to afford **23b** (0.17 g) as a sticky oil. Yield 77%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.61 (3H, s, CH_3), 3.87 (3H, s, CH_3O), 3.89 (3H, s, CH_3O), 4.94 (2H, s, CH_2), 7.06 (2H, d, J = 8.0 Hz, phenyl), 7.16 (1H, t, J = 7.6 Hz, phenyl), 7.20–7.23 (1H, m, phenyl), 7.36–7.43 (7H, m, phenyl), 7.47 (1H, t, J = 8.0 Hz, phenyl), 7.64–7.71 (2H, m, phenyl), 10.82 (1H, s, NH); MS (ESI): m/z = 526.6 [M + H].

4.1.12. Dimethyl 2-benzamido-5-hydroxy-6-methylpyridine-3,4-dicarboxylate (**24a**)

A solution of **23a** (100 mg, 0.21 mmol) in methanol was added 10% Pd/C (10 mg, 0.010 mmol) and stirred for 0.5 h under H_2 atmosphere. The mixture was filtered through celatum bed. The filtrate was evaporated in vacuum and purified by silica gel column chromatography with chloroform/methanol = 20:1 to afford **24a** (60 mg) as a sticky oil. Yield 76%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.54 (3H, s, CH_3), 3.76 (3H, s, CH_3O), 3.89 (3H, s, CH_3O), 7.43 (2H, t, J = 7.6 Hz, phenyl), 7.53 (1H, t, J = 7.6 Hz, phenyl), 7.90 (2H, d, J = 7.6 Hz, phenyl), 10.51 (1H, s, NH); MS (ESI): m/z = 345.3 [M + H].

4.1.13. Dimethyl 5-hydroxy-6-methyl-2-(3-phenoxybenzamido) pyridine-3,4-dicarboxylate (**24b**)

Following the procedure for the synthesis of **24a**, the compound **23b** (100 mg, 0.19 mmol) was reacted with 10% Pd/C (21 mg, 0.20 mmol) to afford **24b** (44 mg) as sticky oil. Yield 53%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.56 (3H, s, CH_3), 3.85 (3H, s, CH_3O), 3.97 (3H, s, CH_3O), 7.03 (2H, d, J = 7.6 Hz, phenyl), 7.15–7.21 (2H, m, phenyl), 7.35 (2H, t, J = 7.6 Hz, phenyl), 7.42 (1H, t, J = 7.6 Hz, phenyl), 7.53 (1H, s, phenyl), 7.59 (1H, d, J = 7.6 Hz, phenyl), 8.57 (1H, s, OH), 9.87 (1H, s, NH); MS (ESI): m/z = 436.6 [M + H].

4.1.14. 2-Benzamido-5-hydroxy-6-methylpyridine-3,4-dicarboxylic acid (**25a**)

Following the general procedure for the synthesis of **13a**–**o**, the compound **24a** (60 mg, 0.13 mmol) was reacted with aqueous 20% KOH (5.0 mL) to afford **25a** (11 mg) as a white solid. Yield 27%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.37 (3H, s, CH_3), 7.44 (2H, t, J = 7.6 Hz, phenyl), 7.56 (1H, t, J = 6.8 Hz, phenyl), 7.91 (2H, d, J = 7.6 Hz, phenyl), 10.53 (1H, s, NH)); ^{13}C NMR (DMSO-d_6, 400 MHz) 19.76, 121.41, 128.32, 129.81, 130.63, 132.34, 134.41, 140.04, 146.73, 149.57, 166.41, 166.82, 167.83; MS (ESI): m/z = 315.0 [M − H].

4.1.15. 5-Hydroxy-6-methyl-2-(3-phenoxybenzamido)pyridine-3,4-dicarboxylic acid (**25b**)

Following the general procedure for the synthesis of **13a**–**o**, the compound **24b** (44 mg, 0.10 mmol) was reacted with aqueous 20% KOH (4.0 mL) to afford **25b** (12 mg) as a white solid. Yield 37%; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 2.50 (3H, s, CH_3), 7.01 (2H, d, J = 7.6 Hz, phenyl), 7.12–7.20 (2H, m, phenyl), 7.35 (2H, t, J = 7.6 Hz, phenyl), 7.46 (1H, d, J = 8.0 Hz, phenyl), 7.55 (1H, s, phenyl), 7.66 (1H, d, J = 7.6 Hz, phenyl), 7.88 (1H, s, OH); MS (MALDI-TOF): m/z = 409.1 [M + H].

4.1.16. Dimethyl 2-benzamido-6-methylpyridine-3,4-dicarboxylate (**26**)

Following the procedure for the synthesis of **23a**, the compound **20** (90 mg, 0.27 mmol) was reacted with benzoyl chloride (63 μL, 0.54 mmol) to afford **26** (49 mg) as a sticky oil. Yield 67%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.64 (3H, s, CH_3), 3.89 (3H, s, CH_3O), 3.93 (3H, s, CH_3O), 7.13 (1H, s, pyridine), 7.52 (2H, t, J = 7.6 Hz, phenyl), 7.57 (1H, t, J = 7.2 Hz, phenyl), 7.98 (2H, d, J = 7.6 Hz, phenyl), 10.32 (1H, s, NH); MS (ESI): m/z = 329.0 [M + H].

4.1.17. 2-Benzamido-6-methyl-pyridine-3,4-dicarboxylic acid (**27**)

Following the general procedure for the synthesis of **13a**–**o**, the compound **26** (0.12 g, 0.38 mmol) was reacted with aqueous 20% KOH (6.0 mL) to afford **27** (40 mg) as a white solid. Yield 35%; ^{1}H NMR (DMSO-d_6) δ 2.47 (3H, s, CH_3, 400 MHz), 7.29 (1H, s, pyridine), 7.45 (2H, t, J = 7.6 Hz, phenyl), 7.54 (1H, t, J = 7.2 Hz, phenyl), 7.93 (2H, d, J = 7.6 Hz, phenyl), 10.87 (1H, s, NH); MS (ESI): m/z = 229.1 [M − H].

4.1.18. Dimethyl 5-(benzyloxy)-6-methyl-2-(naphthalen-1-ylmethyl)pyridine-3,4-dicarboxylate (**28h**)

Following the procedure for the synthesis of **22**, the compound **12h** (1.0 g, 2.7 mmol) was reacted with benzyl bromide (0.40 mL, 3.3 mmol) to afford **28h** (1.3 g) as yellow sticky oil. Yield 96%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.56 (3H, s, CH_3), 3.72 (3H, s, CH_3O), 3.80 (3H, s, CH_3O), 4.52 (2H, s, CH_2), 4.96 (2H, s, benzyl-CH_2), 7.34–7.44 (8H, m, naphthyl & phenyl), 7.61–7.78 (4H, m, naphthyl); MS (ESI): m/z = 455.9 [M + H].

4.1.19. Dimethyl 5-(benzyloxy)-6-methyl-2-(3-phenoxybenzyl) pyridine-3,4-dicarboxylate (**28j**)

Following the procedure for the synthesis of **22**, the compound **12j** (2.3 g, 5.6 mmol) was reacted with benzyl bromide (0.80 mL, 6.8 mmol) to afford **28j** (2.4 g) as yellow sticky oil. Yield 85%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.53 (3H, s, CH_3), 3.73 (3H, s, CH_3O), 3.81 (3H, s, CH_3O), 4.32 (2H, s, CH_2), 4.95 (2H, s, benzyl-CH_2), 6.80–7.09 (7H, m, phenyl), 7.29–7.37 (7H, m, phenyl); MS (ESI): m/z = 498.0 [M + H].

4.1.20. (5-(Benzyloxy)-6-methyl-2-(naphthalen-1-ylmethyl) pyridine-3,4-diyl)dimethanol (**29h**)

A solution of **28h** (1.6 g, 3.5 mmol) in diethyl ether was added a suspension of $LiAlH_4$ (0.90 mL, 21 mmol) in dry diethyl ether (20 mL) dropwise over a period of 0.5 h in ice-bath. After the addition was complete, the reaction mixture was stirred at 0 °C under N_2 atmosphere for 1 h. The mixture was neutralized with saturated aqueous NH_4Cl and extracted with dichloromethane 3 times. The organic layers were combined and dried with anhydrous Na_2SO_4. After Na_2SO_4 was removed, organic layer was concentrated in vacuum and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 2:1 to afford **29h** (0.76 g) as a yellow sticky oil. Yield 69%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.56 (3H, s, CH_3), 4.45 (2H, s, CH_2), 4.71 (2H, s, CH_2OH), 4.72 (2H, s, CH_2OH), 4.96 (2H, s, benzyl-CH_2), 7.39–7.44 (8H, m, naphthyl & phenyl), 7.61–7.78 (4H, m, naphthyl); MS (ESI): m/z = 399.8 [M + H].

4.1.21. (5-(Benzyloxy)-6-methyl-2-(3-phenoxybenzyl)pyridine-3,4-diyl)dimethanol (**29j**)

Following the procedure for the synthesis of **29h**, the compound **28j** (1.0 g, 2.1 mmol) was reacted with $LiAlH_4$ (1.0 mL, 25 mmol) to afford **29j** (0.80 g) as yellow sticky oil. Yield 88%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.53 (3H, s, CH_3), 4.26 (2H, s, CH_2), 4.68 (2H, s, CH_2OH),

4.72 (2H, s, CH_2OH), 4.93 (2H, s, benzyl-CH_2), 6.80–7.09 (7H, m, phenyl), 7.29–7.37 (7H, m, phenyl); MS (ESI): $m/z = 441.5$ [M + H].

4.1.22. (5-Hydroxy-6-methyl-2-(naphthalen-1-ylmethyl)pyridine-3,4-diyl)dimethanol (**30h**)

Following the procedure for the synthesis of **24a**, the compound **29h** (0.33 g, 0.82 mmol) was reacted with 10% Pd/C (30 mg, 0.28 mmol) to afford **30h** (0.21 g) as sticky oil. Yield 82%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.47 (3H, s, CH_3), 4.37 (2H, s, CH_2), 4.57 (2H, s, CH_2OH), 4.87 (2H, s, CH_2OH), 7.30–7.43 (4H, m, naphthyl), 7.69–7.76 (3H, m, naphthyl); MS (ESI): $m/z = 309.8$ [M + H].

4.1.23. (5-Hydroxy-6-methyl-2-(3-phenoxybenzyl)pyridine-3,4-diyl)dimethanol (**30j**)

Following the procedure for the synthesis of **24a**, the compound **29h** (1.5 g, 3.4 mmol) was reacted with 10% Pd/C (0.15 g, 1.4 mmol) to afford **30j** (1.1 g) was sticky oil. Yield 92%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.41 (3H, s, CH_3), 4.13 (2H, s, CH_2), 4.46 (2H, s, CH_2OH), 4.90 (2H, s, CH_2OH), 6.74–7.27 (9H, m, phenyl); MS (ESI): $m/z = 351.9$ [M + H].

4.1.24. (2,2,8-Trimethyl-6-(naphthalen-1-ylmethyl)-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)methanol (**31h**)

To a mixture of **30h** (0.20 g, 0.65 mmol) and 2,2-dimethoxypropane (1.3 mL, 11 mmol) was added *p*-toluenesulfonic acid (0.50 g, 2.6 mmol) in dry acetone. This mixture was stirred for 12 h at room temperature and neutralized with saturated aqueous $NaHCO_3$ and extracted with dichloromethane 3 times. The organic layers were combined and dried with anhydrous Na_2SO_4. After Na_2SO_4 was removed, the organic layer were concentrated in vacuum and purified by silica gel column chromatography with chloroform/methanol = 30:1 to afford **31h** (0.22 g) as a sticky oil. Yield 99%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (6H, s, 2× CH_3), 2.49 (3H, s, CH_3), 4.22 (2H, s, CH_2), 4.71 (2H, s, CH_2OH), 4.91 (2H, s, CH_2OH), 7.25–7.27 (4H, m, naphthyl), 7.70–7.73 (3H, m, naphthyl); MS (ESI): $m/z = 350.9$ [M + H].

4.1.25. (2,2,8-Trimethyl-6-(3-phenoxybenzyl)-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)methanol (**31j**)

Following the procedure for the synthesis of **31h**, the compound **30j** (1.2 g, 3.4 mmol) was reacted with 2,2-dimethoxypropane (6.7 mL, 54 mmol) to afford **31j** (0.58 g) as a sticky oil. Yield 96%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.54 (6H, s, $2CH_3$), 2.39 (3H, s, CH_3), 4.17 (2H, s, CH_2), 4.47 (2H, s, CH_2OH), 4.93 (2H, s, CH_2OH), 6.97–7.33 (9H, m, phenyl); MS (ESI): $m/z = 392.1$ [M + H].

4.1.26. 2,2,8-Trimethyl-6-(naphthalen-1-ylmethyl)-4H-[1,3]dioxino[4,5-c]pyridine-5-carbaldehyde (**32h**)

To a stirred solution of **31h** (0.20 g, 0.57 mmol) in dry dichloromethane was added pyridinium dichromate (0.26 g, 0.68 mmol) with vigorous stirring. After being stirred overnight, the mixture was filtered through celatum bed with methanol. The filtrate was evaporated under vacuum and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 3:1 to afford **32h** (0.18 g) as a brown sticky oil. Yield 90%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.54 (6H, s, 2× CH_3), 2.52 (3H, s, CH_3), 4.62 (2H, s, CH_2), 5.11 (2H, s, CH_2OH), 7.25–7.52 (4H, m, naphthyl), 7.74–7.76 (3H, m, naphthyl), 10.48 (H, s, CHO); MS (ESI): $m/z = 348.4$ [M + H].

4.1.27. 2,2,8-Trimethyl-6-(3-phenoxybenzyl)-4H-[1,3]dioxino[4,5-c]pyridine-5-carbaldehyde (**32j**)

Following the procedure for the synthesis of **32h**, the compound **31j** (0.57 g, 1.5 mmol) was reacted with pyridinium dichromate (0.67 g, 1.8 mmol) to afford **32j** (0.52 g) as a brown sticky oil. Yield 90%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.53 (6H, s, 2× CH_3), 2.46 (3H, s, CH_3), 4.43 (2H, s, CH_2), 5.10 (2H, s, CH_2OH), 6.81–7.33 (9H, m, phenyl), 10.38 (H, s, CHO); MS (ESI): $m/z = 389.9$ [M + H].

4.1.28. (E)-Ethyl 3-(2,2,8-trimethyl-6-(naphthalen-1-ylmethyl)-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)acrylate (**33h**)

To a suspension of NaH (13 mg, 0.53 mmol) in dry THF was added dropwise triethyl phosphonoacetate (0.10 mL, 0.53 mmol). The mixture color was changed to clear yellow solution with H_2 gas release. After 5 min being stirred, **32h** (0.18 g, 0.48 mmol) in dry THF was added to the reaction mixture and was stirred for 1 h. The mixture was extracted with dichloromethane and washed with brine. The organic layer was dried over Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 3:1 to afford **33h** (0.20 g) as a sticky oil. Yield 96%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.28 (3H, t, $J = 7.2$ Hz, CH_3), 1.54 (6H, s, 2× CH_3), 2.48 (3H, s, CH_3), 4.19 (2H, q, $J = 7.2$ Hz, CH_2), 4.32 (2H, s, CH_2), 4.77 (2H, s, CH_2OH), 5.85 (1H, d, $J = 16.4$ Hz, =CH), 7.35–7.57 (4H, m, naphthyl), 7.67–7.76 (4H, m, naphthyl & =CH); MS (ESI): $m/z = 418.1$ [M + H].

4.1.29. (E)-Ethyl 3-(2,2,8-trimethyl-6-(3-phenoxybenzyl)-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)acrylate (**33j**)

Following the procedure for the synthesis of **33h** the compound **32j** (0.56 g, 1.4 mmol) was reacted with triethyl phosphonoacetate (0.29 mL, 1.5 mmol) to afford **33j** (0.59 g) as a sticky oil. Yield 92%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (3H, t, $J = 7.2$ Hz, CH_3), 1.54 (6H, s, 2× CH_3), 2.40 (3H, s, CH_3), 4.20 (2H, q, $J = 7.2$ Hz, CH_2), 4.41 (2H, s, CH_2), 4.76 (2H, s, CH_2OH), 5.84 (1H, d, $J = 16.4$ Hz, =CH), 6.83–7.29 (9H, m, phenyl), 7.61 (1H, d, $J = 16.4$ Hz, =CH); MS (ESI): $m/z = 460.1$ [M + H].

4.1.30. (E)-3-(2,2,8-Trimethyl-6-(naphthalen-1-ylmethyl)-4H-[1,3]dioxino[4,5-C]pyridin-5-yl)acrylic acid (**34h**)

Compounds **33h** (90 mg, 0.21 mmol) was dissolved in 5% methanolic KOH (7.0 mL) and stirred at room temperature for 2 h. The reaction mixture was then acidified with 1 N HCl and solvent was evaporated. KCl salt was removed by filtration. After being concentrated, the crude product was purified by silcagel column using chloroform/methanol = 10:1 to afford **34h** (60 mg) as a sticky oil. Yield 71%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.54 (6H, s, 2× CH_3), 2.46 (3H, s, CH_3), 4.31 (2H, s, CH_2), 4.77 (2H, s, CH_2OH), 5.86 (1H, d, $J = 16.4$ Hz, =CH), 7.25–7.55 (4H, m, naphthyl), 7.69–7.73 (3H, m, naphthyl), 7.79 (1H, d, $J = 16.4$ Hz, =CH); MS (ESI): $m/z = 390.8$ [M + H].

4.1.31. (E)-3-(2,2,8-Trimethyl-6-(3-phenoxybenzyl)-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)acrylic acid (**34j**)

Following the procedure for the synthesis of **34h**, the compound **33j** (0.30 g, 0.65 mmol) was reacted with 5% methanolic KOH (20 mL) to afford **34j** (0.25 g) as a sticky oil. Yield 89%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.52 (6H, s, 2× CH_3), 2.40 (3H, s, CH_3), 4.13 (2H, s, CH_2), 4.78 (2H, s, CH_2OH), 5.86 (1H, d, $J = 16.4$ Hz, =CH), 6.82–7.31 (9H, m, phenyl), 7.70 (1H, d, $J = 16.4$ Hz, =CH); MS (ESI): $m/z = 432.4$ [M + H].

4.1.32. (E)-3-(5-Hydroxy-4-(hydroxymethyl)-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)acrylic acid (**35h**)

A solution **34h** (55 mg, 0.14 mmol) in 10% formic acid (5.0 mL) was refluxed for 3 h. When the mixture was cooled to room temperature, it was added saturated $NaHCO_3$ and extracted with chloroform (3 times). The organic layers were combined, dried over Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography with chloroform/methanol = 10:1 to afford **35h** (0.27 mg) as a white solid. Yield 54%; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 2.46 (3H, s, CH_3), 4.28 (2H, s, CH_2), 4.80(2H, s, CH_2OH), 5.93 (1H, d,

J = 16.4 Hz, =CH), 7.25–7.55 (4H, m, naphthyl), 7.69–7.73 (3H, m, naphthyl), 7.88 (1H, d, J = 16.4 Hz, =CH); MS (ESI): m/z = 350.3 [M + H].

*4.1.33. (E)-3-(5-Hydroxy-4-(hydroxymethyl)-6-methyl-2-(3-phenoxybenzyl)pyridin-3-yl)acrylic acid (**35j**)*

Following the procedure for the synthesis of **35h**, the compound **34j** (0.25 g, 0.60 mmol) was reacted with 10% formic acid (10 mL) to afford **35j** (0.19 g) as a white solid. Yield 82%; ^{1}H NMR (MeOH-d_4, 400 MHz) δ 2.41 (3H, s, CH_3), 4.06 (2H, s, CH_2), 4.76(2H, s, CH_2OH), 5.87 (1H, d, J = 16.4 Hz, =CH), 6.66–7.31 (9H, m, phenyl), 7.58 (1H, d, J = 16.4 Hz, =CH); MS (ESI): m/z = 492.4 [M + H].

*4.1.34. (E)-3-(4-Formyl-5-hydroxy-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)acrylic acid (**36h**)*

To a solution of **35h** (20 mg, 0.050 mmol) in dry dichloromethane was added activated manganese dioxide (30 mg, 0.35 mmol) with vigorous stirring for 2 h at room temperature under N_2 atmosphere. The mixture was filtered through celatum bed with dichloromethane and the filtrate was evaporated in vacuum and purified by silica gel column chromatography with n-hexanes/ethyl acetate = 3:1 to afford **36h** (14 mg) as a yellow solid. Yield 78%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.60 (3H, s, CH_3), 4.32 (2H, s, CH_2), 5.92 (1H, d, J = 16.4 Hz, =CH), 7.29–7.56 (4H, m, naphthyl), 7.71–7.77 (3H, m, naphthyl), 7.98 (1H, d, J = 16.4 Hz, =CH), 10.13 (1H, s, CHO), 11.33 (1H, s, COOH); ^{13}C NMR (DMSO-d_6, 400 MHz) 18.38, 40.54, 116.82, 120.06, 124.94, 125.01, 127.96, 129.80, 131.75, 134.35, 147.47, 156.47, 157.13, 167.53, 196.93; MS (ESI): m/z = 347.8 [M + H].

*4.1.35. (E)-3-(4-Formyl-5-hydroxy-6-methyl-2-(3-phenoxybenzyl) pyridin-3-yl)acrylic acid (**36j**)*

Following the procedure for the synthesis of **36h**, the compound **35j** (0.18 g, 0.47 mmol) was reacted with manganese dioxide (0.29 g, 3.4 mmol) to afford **36j** (0.12 g) as a yellow solid. Yield 64%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.55 (3H, s, CH_3), 4.15 (2H, s, CH_2), 5.92 (1H, d, J = 16.0 Hz, =CH), 6.79–7.31 (9H, m, phenyl), 7.92 (1H, d, J = 16.0 Hz, =CH), 10.14 (1H, s, CHO), 11.39 (1H, s, COOH); ^{13}C NMR (DMSO-d_6, 400 MHz) 18.83, 41.00, 116.78, 119.17, 123.34, 128.19, 129.81, 138.27, 140.92, 147.87, 150.82, 157.13, 167.20, 196.96; MS (ESI): m/z = 389.9 [M + H].

*4.1.36. (E)-3-(2-Carboxyvinyl)-5-hydroxy-6-methyl-2-(3-phenoxybenzyl)isonicotinic acid (**37j**)*

To a respective suspension of **36j** (28 mg, 0.070 mmol) in DMF/CAN (1:5) solution (2.0 mL) Oxone® (50 mg, 0.080 mmol) was added. After the mixture was stirred for 2 h at room temperature, the Oxone® was removed by filtration. The filtrate was concentrated in a vacuum and purified by silica gel column chromatography with chloroform/methanol = 5:1 to afford **37j** (4.0 mg) as a red solid. Yield 13%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.31 (3H, s, CH_3), 4.15 (2H, s, CH_2), 6.77–6.82 (2H, m, =CH & phenyl), 6.89–6.94 (3H, m, phenyl), 7.07 (1H, t, J = 7.2 Hz, phenyl), 7.25–7.34 (4H, m, phenyl), 7.63 (1H, d, J = 16.4 Hz, =CH); MS (ESI): m/z = 406.1 [M + H].

*4.1.37. (E)-Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)acrylate (**38h**)*

Following the procedure for the synthesis of **35h**, the compound **33h** (0.10 g, 0.24 mmol) was reacted with 10% formic acid (5.0 mL) to afford **38h** (64 mg) as a white solid. Yield 71%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (3H, t, J = 7.2 Hz, CH_3), 2.49 (3H, s, CH_3), 4.17 (2H, q, J = 7.2 Hz, CH_2), 4.20 (2H, s, CH_2), 4.82 (2H, s, CH_2OH), 5.68 (1H, d, J = 16.4 Hz, =CH), 7.28–7.47 (4H, m, naphthyl), 7.57 (1H, d, J = 16.4 Hz, =CH), 7.67–7.76 (3H, m, naphthyl); MS (ESI): m/z = 378.9 [M + H].

*4.1.38. (E)-Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methyl-2-(3-phenoxybenzyl)pyridin-3-yl)acrylate (**38j**)*

Following the procedure for the synthesis of **35h**, the compound **33j** (110 mg, 0.25 mmol) was reacted with 10% formic acid (5.0 mL) to afford **38j** (55 mg) as a white solid. Yield 52%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.28 (3H, t, J = 7.2 Hz, CH_3), 2.45 (3H, s, CH_3), 4.04 (2H, s, CH_2), 4.18 (2H, q, J = 7.2 Hz, CH_2), 4.90 (2H, s, CH_2OH), 5.70 (1H, d, J = 16.4 Hz, =CH), 6.77–7.31 (9H, m, phenyl), 7.55 (1H, d, J = 16.4 Hz, =CH); MS (ESI): m/z = 420.1 [M + H].

*4.1.39. Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)propanoate (**39h**)*

Following the procedure for the synthesis of **24a**, the compound **38h** (60 mg, 0.16 mmol) was reacted with 10% Pd/C (10 mg, 0.090 mmol) to afford **39h** (40 mg) as sticky oil. Yield 66%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.10 (3H, t, J = 6.8 Hz, CH_3), 2.11 (2H, t, J = 7.6 Hz, CH_2), 2.48 (3H, s, CH_3), 2.77 (2H, t, J = 7.6 Hz, CH_2), 4.17 (2H, q, J = 6.8 Hz, CH_2), 4.27 (2H, s, CH_2), 4.89 (2H, s, CH_2OH), 7.28–7.48 (4H, m, naphthyl), 7.68–7.76 (3H, m, naphthyl); MS (ESI): m/z = 379.4 [M + H].

*4.1.40. Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methyl-2-(3-phenoxybenzyl)pyridin-3-yl)propanoate (**39j**)*

Following the procedure for the synthesis of **24a**, the compound **38j** (52 mg, 0.12 mmol) was reacted with 10% Pd/C (5.0 mg, 0.050 mmol) to afford **39j** (35 mg) as sticky oil. Yield 66%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.18 (3H, t, J = 7.2 Hz, CH_3), 2.16 (2H, t, J = 7.6 Hz, CH_2), 2.44 (3H, s, CH_3), 2.78 (2H, t, J = 7.6 Hz, CH_2), 4.05 (2H, q, J = 7.2 Hz, CH_2), 4.10 (2H, s, CH_2), 4.92 (2H, s, CH_2OH), 6.80–7.31 (9H, m, phenyl), MS (ESI): m/z = 422.4 [M + H].

*4.1.41. Ethyl 3-(4-formyl-5-hydroxy-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)propanoate (**40h**)*

Following the procedure for the synthesis of **36h**, the compound **39h** (37 mg, 0.10 mmol) was reacted with manganese dioxide (60 mg, 0.70 mmol) to afford **40h** (60 mg) as yellow sticky oil. Yield 79%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.13 (3H, t, J = 7.2 Hz, CH_3), 2.30 (2H, t, J = 8.0 Hz, CH_2), 2.54 (3H, s, CH_3), 3.24 (2H, t, J = 8.0 Hz, CH_2), 4.02 (2H, q, J = 7.2 Hz, CH_2), 4.37 (2H, s, CH_2), 7.32–7.42 (4H, m, naphthyl), 7.71–7.78 (3H, m, naphthyl), 10.40 (1H, s, CHO); MS (ESI): m/z = 377.8 [M + H].

*4.1.42. Ethyl 3-(4-formyl-5-hydroxy-6-methyl-2-(3-phenoxybenzyl)pyridin-3-yl)propanoate (**40j**)*

Following the procedure for the synthesis of **36h**, the compound **39j** (33 mg, 0.080 mmol) was reacted with manganese dioxide (48 mg, 0.56 mmol) to afford **40j** (27 mg) as yellow sticky oil. Yield 86%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21 (3H, t, J = 7.2 Hz CH_3), 2.37 (2H, t, J = 8.0 Hz, CH_2), 2.49 (3H, s, CH_3), 2.78 (2H, t, J = 8.0 Hz, CH_2), 4.09 (2H, q, J = 7.2 Hz, CH_2), 4.18 (2H, s, CH_2), 6.78–7.32 (9H, m, phenyl), 10.42 (1H, s, CHO); MS (ESI): m/z = 420.4 [M + H].

*4.1.43. 3-(4-Formyl-5-hydroxy-6-methyl-2-(naphthalen-1-ylmethyl)pyridin-3-yl)propanoic acid (**41h**)*

Following the procedure for the synthesis of **34h**, the compound **40h** (25 mg, 0.070 mmol) was reacted with 5% methanolic KOH (5.0 mL) to afford **41h** (13 mg) as a yellow solid. Yield 63%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.37 (2H, t, J = 8.0 Hz, CH_2), 2.56 (3H, s, CH_3), 3.25 (2H, t, J = 8.0 Hz, CH_2), 4.37 (2H, s, CH_2), 7.25–7.53 (4H, m, naphthyl), 7.72–7.74 (3H, m, naphthyl), 10.40 (1H, s, CHO), 11.54 (1H, s, COOH); MS (ESI): m/z = 349.8 [M + H].

*4.1.44. 3-(4-Formyl-5-hydroxy-6-methyl-2-(3-phenoxybenzyl) pyridin-3-yl)propanoic acid (**41j**)*

Following the procedure for the synthesis of **34h**, the compound **40j** (27 mg, 0.060 mmol) was reacted with 5% methanolic KOH (5.0 mL) to afford **41h** (23 mg) was obtained as a yellow solid. Yield 92%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.37 (2H, t, J = 8.0 Hz, CH_2), 2.49 (3H, s, CH_3), 3.21 (2H, t, J = 8.0 Hz, CH_2), 4.18 (2H, s, CH_2), 6.82–7.31 (9H, m, phenyl), 10.41 (1H, s, CHO), 11.56 (1H, s, COOH); ^{13}C NMR (DMSO-d_6, 400 MHz) 18.45, 21.47, 35.34, 40.51, 116.72, 119.01, 120.80, 123.27, 129.83, 131.02, 141.66, 147.94, 149.40, 153.01, 157.09, 176.54, 196.63; MS (ESI): m/z = 392.4 [M + H].

*4.1.45. 3-(2-Carboxyethyl)-5-hydroxy-6-methyl-2-(3-phenoxybenzyl)isonicotinic acid (**42j**)*

Following the procedure for the synthesis of **37j**, the compound **41j** (35 mg, 0.090 mmol) was reacted with Oxone® (61 mg, 0.10 mmol) to afford **42j** (9.9 mg) as a red solid. Yield 27%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.06–2.12 (2H, m, CH_2), 2.24 (3H, s, CH_3), 3.15–3.21 (2H, m, CH_2), 4.12 (2H, s, CH_2), 6.72–6.75 (2H, m, phenyl), 6.83 (1H, d, J = 8.1 Hz, phenyl), 6.94 (2H, d, J = 8.1 Hz, phenyl), 7.09–7.21 (2H, m, phenyl), 7.32 (2H, t, J = 7.5 Hz, phenyl); MS (ESI): m/z = 406.1 [M − H].

*4.1.46. 2,2,8-Trimethyl-6-(naphthalen-1-ylmethyl)-4H-[1,3]dioxino [4,5-c]pyridine-5-carboxylic acid (**43h**)*

Following the procedure for the synthesis of **37j**, the compound **32h** (80 mg, 0.23 mmol) was reacted with Oxone® (140 mg, 0.25 mmol) to afford **43h** (80 mg) as brown sticky oil. Yield 95%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (6H, s, 2× CH_3), 2.44 (3H, s, CH_3), 4.56 (2H, s, CH_2), 4.93 (2H, s, CH_2OH), 7.27–7.34 (3H, m, naphthyl), 7.59–7.65 (4H, m, naphthyl); MS (ESI): m/z = 363.4 [M + H].

*4.1.47. 2,2,8-Trimethyl-6-(3-phenoxybenzyl)-4H-[1,3]dioxino[4,5-c] pyridine-5-carboxylic acid (**43j**)*

Following the procedure for the synthesis of **37j**, the compound **32j** (0.25 g, 0.64 mmol) was reacted with Oxone® (0.43 g, 0.71 mmol) to afford **42j** (0.15 g) as brown sticky oil. Yield 59%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (6H, s, 2× CH_3), 2.40 (3H, s, CH_3), 4.38 (2H, s, CH_2), 4.90 (2H, s, CH_2OH), 6.67 (1H, d, J = 7.2 Hz, phenyl), 6.87 (4H, d, J = 6.4 Hz, phenyl), 6.98 (1H, t, J = 7.6 Hz, phenyl), 7.04 (1H, t, J = 7.6 Hz, phenyl), 7.18 (2H, d, J = 7.2 Hz, phenyl); MS (ESI): m/z = 406.0 [M + H].

*4.1.48. 7-Hydroxy-6-methyl-4-(naphthalen-1-ylmethyl)furo[3,4-c] pyridin-3(1H)-one (**44h**)*

Following the procedure for the synthesis of **35h**, the compound **43h** (70 mg, 0.19 mmol) was reacted with 10% formic acid (5.0 mL) to afford **44h** (44 mg) as sticky oil. Yield 70%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.57 (3H, s, CH_3), 4.63 (2H, s, CH_2), 5.16 (2H, s, CH_2OH), 7.36–7.53 (3H, m, naphthyl), 7.70–7.82 (4H, m, naphthyl); MS (ESI): m/z = 306.2 [M + H].

*4.1.49. 7-Hydroxy-6-methyl-4-(3-phenoxybenzyl)furo[3,4-c] pyridin-3(1H)-one (**44j**)*

Following the procedure for the synthesis of **35h**, the compound **43j** (0.14 g, 0.34 mmol) was reacted with 10% formic acid (7.0 mL) to afford **44j** (0.11 g) as sticky oil. Yield 92%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.55 (3H, s, CH_3), 4.45 (2H, s, CH_2), 5.18 (2H, s, CH_2OH), 6.76 (1H, d, J = 6.8 Hz, phenyl), 6.96 (2H, d, J = 7.6 Hz, phenyl), 7.05–7.13 (3H, m, phenyl), 7.16 (1H, t, J = 7.6 Hz, phenyl), 7.29 (2H, d, J = 7.2 Hz, phenyl); MS (ESI): m/z = 348.0 [M + H].

*4.1.50. 5-Hydroxy-4-(hydroxymethyl)-6-methyl-2-(naphthalen-1-ylmethyl)nicotinic acid (**45h**)*

Following the procedure for the synthesis of **34h**, the compound **44h** (20 mg, 0.070 mmol) was reacted with 5% methanolic KOH (2.5 mL) to afford **45h** (11 mg) as a white solid. Yield 53%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.45 (3H, s, CH_3), 4.32 (2H, s, CH_2), 4.81 (2H, s, CH_2OH), 7.39–7.45 (3H, m, naphthyl), 7.67–7.74 (4H, m, naphthyl); MS (ESI): m/z = 324.2 [M + H].

*4.1.51. 5-Hydroxy-4-(hydroxymethyl)-6-methyl-2-(3-phenoxybenzyl)nicotinic acid (**45j**)*

Following the procedure for the synthesis of **34h**, the compound **44j** (50 mg, 0.14 mmol) was reacted with 5% methanolic KOH (4 mL) to afford **45j** (30 mg) as a white solid. Yield 57%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.38 (3H, s, CH_3), 4.13 (2H, s, CH_2), 4.85 (2H, s, CH_2OH), 6.73 (1H, d, J = 7.2 Hz, phenyl), 6.90 (3H, d, J = 7.6 Hz, phenyl), 6.97 (1H, d, J = 7.6 Hz, phenyl), 6.90 (1H, t, J = 7.6 Hz, phenyl), 7.15 (1H, t, J = 7.2 Hz, phenyl), 7.26 (2H, t, J = 7.2 Hz, phenyl); MS (ESI): m/z = 366.1 [M + H].

*4.1.52. 1,7-Dihydroxy-6-methyl-4-(naphthalen-1-ylmethyl)furo [3,4-c]pyridin-3(1H)-one (**46h**)*

Following the procedure for the synthesis of **36h**, the compound **45h** (10 mg, 0.030 mmol) was reacted with manganese dioxide (19 mg, 0.21 mmol) to afford **46h** (2.1 mg) as yellow sticky oil. Yield 21%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.48 (3H, s, CH_3), 4.50 (2H, s, CH_2), 7.35–7.45 (4H, m, naphthyl & CH), 7.68–7.73 (4H, m, naphthyl); MS (ESI): m/z = 322.1 [M + H].

*4.1.53. 1,7-Dihydroxy-6-methyl-4-(3-phenoxybenzyl)furo[3,4-c] pyridin-3(1H)-one (**46j**)*

Following the procedure for the synthesis of **36h**, the compound **45j** (28 mg, 0.070 mmol) was reacted with manganese dioxide (47 mg, 0.49 mmol) to afford **46j** (13 mg) as yellow sticky oil. Yield 47%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.51 (3H, s, CH_3), 4.38 (2H, s, CH_2), 6.73 (1H, d, J = 7.6 Hz, phenyl), 6.92 (2H, d, J = 8.0 Hz, phenyl), 7.00–7.07 (4H, m, phenyl & CH), 7.13 (1H, t, J = 8.0 Hz, phenyl), 7.28–7.32 (2H, m, phenyl); ^{13}C NMR ($CDCl_3$, 400 MHz) 19.54, 38.80, 93.08, 116.96, 119.14, 120.01, 123.47, 124.39, 129.84, 130.01, 141.56, 153.99, 157.41, 157.55, 168.42; MS (ESI): m/z = 364.2 [M + H].

4.2. Biological methods

The cRNA of $hP2X_3$ receptor was obtained by reverse transcription of the cDNA of $hP2X_3$ receptor, which was generously provided by Dr. W. Stuhmer and Dr. F. Soto of the Max-Plank Institute. The EST clones containing full-length cDNAs of $mP2X_1$ (clone ID: 4189541) and $hP2X_7$ receptor (clone ID: 5286944) were purchased (Invitrogen, CA, U.S.A.), and their sequences were confirmed by DNA sequencing.

4.2.1. Two-electrode voltage-clamp assay at recombinant $mP2X_1$ and $hP2X_3$ receptors

Xenopus oocytes were harvested and prepared as previously described. $mP2X_1$ and $hP2X_3$ receptor cRNA (40 nL, 1 μg/mL), respectively were injected cytosolically to defolliculated oocytes, which were incubated for 24 h at 18 °C in Barth's solution and kept for up to 12 days at 4 °C until used in electrophysiological experiments. ATP-activated membrane currents (V_h = −70 mV) were recorded from cRNA-injected oocytes using the two-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1–2 MΩ tip resistance) and current-recording microelectrodes (5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 mL/min, at 18 °C) containing (mM) NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5. 2 μM ATP was superfused over the oocytes for 60–120 s then washed out for a period of 20 min. For inhibition curves, data were normalized to the current evoked by ATP, at pH 7.5. Compounds for testing were

added for 20 min prior to the ATP exposure and all compounds were tested for the reversibility of their antagonistic effects. The concentration required to inhibit the ATP response by 50% (IC_{50}) was taken from Hill plots constructed using the formula: log ($I/I_{max} - I$), where I is the current evoked by ATP in the presence of an antagonist. Data are presented as mean ± SEM ($n \geq 3$) for the data from different batches of oocytes.

4.2.2. Ethidium$^+$ accumulation assay at hP2X$_7$ receptors

hP2X$_7$-expressing HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum as monolayer culture at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested with treatment of Trypsin/EDTA solution and collected by centrifugation (200 g for 5 min). The cells were resuspended at 2.5×10^6 cells/mL in assay buffers, consisting of 10 nM HEPES, 140 mM KCl, 5 mM glucose, 1 mM EDTA 1 (pH 7.4), and then 100 μM ethidium bromide was added. Cell suspensions were added to 96 well plates containing the P2X$_7$ receptor agonist, 6 μM BzATP, at 2×10^5 cells/well. Plates were incubated at 37 °C for 120 min and cellular accumulation of ethidium$^+$ was determined by measuring fluorescence with Bio-Tek instrument FL600 fluorescent plate reader (excitation wavelength of 530 nm and emission wavelength of 590 nm).

4.2.3. Ex-vivo test for measuring antinociceptive effect

Ex-vivo test for antinociceptive activity was carried out following the reported procedure [38]. Brief procedure of *ex-vivo* test: Rat spinal dorsal horn which is responsible for neural mechanism, Long-Term Potentiation (LTP), was extracted from rat. Voltage sensitive dye (Di-4-ANEPPS) was treated to slice of spinal cords (0.5 mm) and changes in the optical signal in the dorsal horn are monitored before and after the induction of LTP. The LTP was induced with 2 Hz electrical stimulation (0.5 msec pulse) for 2 min. The antinociceptive effect of compounds was measured through LTP inhibition.

Acknowledgments

We thank to Dr. W. Stuhmer and Dr. F. Soto in the Max-Plank Institute for the generous providing of the cDNA of hP2X$_3$ receptor. This research was supported by a Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (Grant No. 2013023642).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.026.

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