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Design, Synthesis, and Biological Assessment of Novel Vanillin-Isoxazole Derivatives as Positive Allosteric Modulators of α7 Nicotinic Acetylcholine Receptor †

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Abstract: The homomeric α7 nicotinic acetylcholine receptor (α7 nAChR) is a cation-permeable pentameric ligand-gated channel present in the nervous system and in non-neuronal cells. α7 nAChRs are highly expressed in brain regions critical for cognition and memory, such as the hippocampus and cerebral cortex. Therefore, enhancing its function with positive allosteric modulators (PAMs) is a promising therapeutic approach for treating cognitive deficits and neurodegenerative disorders. Continuing with our previous work in the search for novel PAMs of α7 nAChR, this study presents the synthesis and biological evaluation of novel isoxazole-vanillin derivatives exhibiting α7-PAM activity. Isoxazole derivatives with functional α7-PAM activity were identified by determining the effects of the synthetic compounds at different concentrations on the properties of the single channels elicited by ACh (100 μM). We found that only vanillin-derived isoxazoles (containing the 4-hydroxy-3-methoxy fragment) exhibited α7-enhancing activity in comparison to isoxazoles derived from dihydroxy- or dimethoxybenzaldehydes. The use of different substituted phenylacetylenes allowed us to create a small library of compounds with α7-PAM activity.

Keywords: Nicotinic receptors; Allosteric modulation; α7-PAM; Isoxazoles

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1. Introduction

As one of the most abundant nicotinic acetylcholine receptor (nAChRs) subtypes present in the central nervous system, α7 nAChRs are highly expressed in the hippocampus, cortex, and subcortical limbic regions where they play a modulatory role in neural circuits associated with cognition, learning, attention, memory and sensory gating information. α7 nAChRs are also expressed astrocytes, microglia, and several non-neuronal cells, where they are involved in neuroprotection, immunity, and inflammation. Decreased α7 activity is associated to neurological, neurodegenerative and inflammatory disorders, such as schizophrenia, Alzheimer's disease, depression and pain. Therefore, α7 nAChRs potentiation using positive allosteric modulators (PAMs) is a promising therapeutic strategy for the treatment of cognitive deficits and neurodegenerative disorders. The use of PAMs to enhance α7 activity offers certain advantages compared to the use of orthosteric agonists, like the conservation of the temporal and spatial pattern of endogenous activation, fewer side effects, higher selectivity and a wide structural diversity. Among these, the isoxazole core and phenolic compounds (Figure 1) are privileged scaffolds exerting potent α7-PAM activity.[1-3]

Isoxazole derivatives, similar to 1,2,3-triazoles, can be obtained through a copper-catalyzed cycloaddition reaction between alkynes and the corresponding dipole. In previous works, using supported copper nanoparticles (CuNPs) as the catalyst, we were able to access a small library of triazole derivatives with α7-PAM activity.[4,5]

Continuing with our previous work, here we present the synthesis and biological evaluation of novel isoxazole-vanillin derivatives exhibiting α7-PAM activity. The one-pot synthesis of 3,5-disubstituted isoxazoles were carried out through the cycloaddition reaction between in situ generated nitrile oxides and terminal alkynes catalyzed by supported CuNPs.

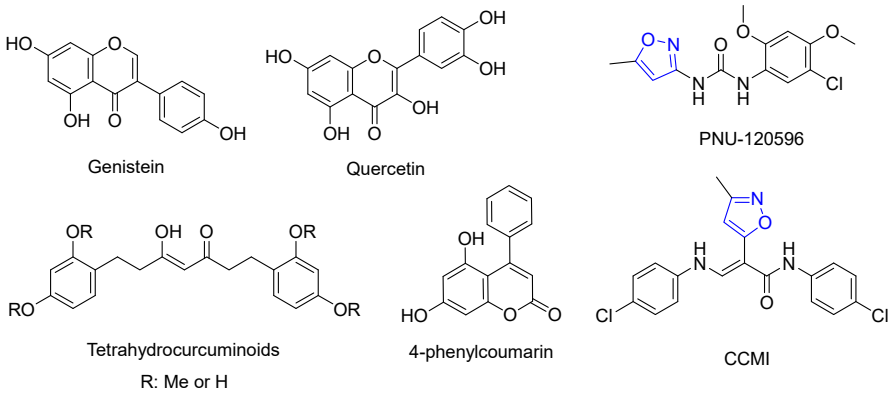


Figure 1. α7-PAMs containing phenolic compounds or the 3,5-isoxazole ring.

2. Experimental Procedures

2.1. General Methods—Chemistry

All starting materials were of the best available grade (Aldrich, Merck) and were used without further purification. The catalyst consisting of copper nanoparticles supported on montmorillonite K10 was prepared according to methods previously reported by our group. [6] Analytical thin-layer chromatography (TLC) was carried out on TLC aluminum sheets with silica gel 60 F254 (Merck) visualized under UV light and/or phosphomolybdic acid solution spray reagent (10% in ethanol), vanillin or ferric trichloride solutions. Column chromatography was performed with Merck silica gel 60 (0.040–0.063 μm, 240–400 mesh) and hexane/ethyl acetate (EtOAc) as eluent. Microwave assisted reactions were carried out using a CEM Discover BenchMate microwave operating at 60W.

2.2. General Procedure for the Synthesis of Isoxazoles [7]

Hydroxylamine hydrochloride (57 mg, 0.825 mmol) and $NaHCO_3$ (63 mg, 0.75 mmol) were added to aldehyde **1** (0.75 mmol) in DMF (2 mL). The reaction mixture was stirred at room temperature until the starting aldehyde was completely converted to the corresponding oxime, as indicated by TLC (30-60 minutes). N-Chlorosuccinimide (NCS, 120 mg, 0.9 mmol) was then added gradually. The mixture was stirred at room temperature until the oxime was fully transformed into the corresponding N-hydroxyimidoyl chloride, as confirmed by TLC using a $FeCl_3$ solution in MeOH as a stain. Subsequently, CuNPs catalyst (20 mg, 1 mol% Cu), the alkyne (0.5 mmol), and $NaHCO_3$ (63 mg, 0.75 mmol) were added, and the reaction mixture was heated under microwave irradiation (standard method, 60W, 80°C) for 30 minutes. Upon completion, the reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The collected organic phases were washed with water (10 mL) and brine (10 mL), and dried over Na_2SO_4. The solvent was evaporated under vacuum, and the resulting product was purified via flash column chromatography (hexane-EtOAc) to yield the corresponding isoxazole **5**.

2.3. Expression of human α7 wild type receptor

In order to achieve *α7* expression, BOSC-23 cells were transfected by the calcium phosphate precipitation protocol with wild type *α7* subunit cDNA together with the *α7* chaperone cDNAs, Ric-3 and NACHO, using a cDNA ratio *α7*:Ric-3:NACHO 1:1:0.2. Green fluorescence protein cDNA was added during the transfection to allow identification of transfected cells. All transfections were carried out for about 8–12 h in DMEM with 10% fetal bovine serum and were terminated by exchanging the medium, as previously described.[8] Cells were used for experiments two to three days after transfection because at that time maximum functional expression levels are achieved.

2.4. Single-Channel Recordings

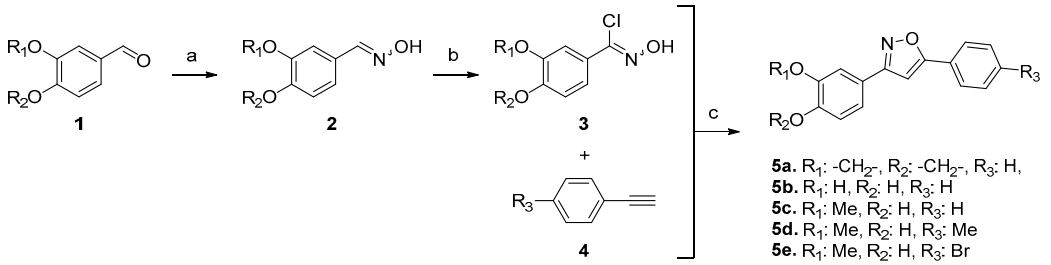
Single channels were recorded in the cell-attached patch configuration as described before.[1, 8, 9] The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). ACh and the tested compounds were included in the pipette solution. DMSO was used to solubilize the compounds and its final concentration was lower than 0.1% (v/v), which does not affect *α7* activation properties.[8] Single-channel currents were digitized at 5–10 μs intervals and low-pass filtered at a cutoff frequency of 10 kHz with an Axopatch 200B patch-clamp amplifier (Molecular Devices, CA, USA). Analysis was done with the program TAC (Bruxton Corporation, Seattle, WA, USA) using the Gaussian digital filter at 9 kHz (Final cut-off frequency 6.7 kHz). Events were detected by the half-amplitude threshold criterion.[8] Using the program TACFit (Bruxton Corporation, Seattle, WA, USA), open-time and closed-time histograms were constructed and fitted by the sum of exponential functions by maximum likelihood criterion. The mean open duration (τ_{open}) was taken from the slowest component of the corresponding histogram. A critical duration (τ_{crit}), which was taken as the point of intersection between closed components, was used to define a burst of channel openings which is identified as a series of closely separated openings preceded and followed by closings longer than τ_{crit}. Each series of opening events spaced by durations briefer than τ_{crit} was considered as a single burst. The mean burst duration (τ_{burst}) was determined from the longest duration component of the open time histogram constructed with the imposed τ_{crit}. Critical durations were defined by the intersection between the first and second briefest components in the closed time histogram for bursts of *α7* activated by ACh (~0.2–0.5 ms) and between the second and the third closed components in the presence of the compounds (~1.5–5 ms).

2.5. Statistical Analysis

For each condition, *n* indicates the number of recordings from different cell patches (independent experiments); and *N* corresponds to the number of cell transfections, each from different days and cell batches. Data are presented as mean ± SD. For pairwise comparisons data sets were analyzed using the two-tailed Student's t-test or Mann–Whitney rank sum test with SigmaPlot 12.0 (Systat Software, Inc.). Statistically significant differences between two groups of data were established at p values < 0.05.

3. Results and Discussion

For the synthesis of 3,5-disubstituted isoxazoles, we employed a one-pot sequential approach (Scheme 1) [7]. Initially, hydroxylamine was added to aldehydes **1** to yield the corresponding aldoximes **2**. These aldoximes were then treated with NCS to generate *N*-hydroxyimidoyl chlorides **3** as the nitrile oxide precursors. Subsequently, the addition of 1 equivalent of base ($NaHCO_3$) generated these 1,3-dipoles, which were subjected to a cycloaddition reaction with terminal alkynes, resulting in the desired 3,5-isoxazoles with complete regioselectivity and very good yields (70-87%).



Scheme 1. One-pot synthesis of 3,5-disubstituted isoxazoles from aldehydes. Reaction conditions: (a) $NH_2OH.HCl$, $NaHCO_3$, DMF, r.t. (b) NCS, r.t. (c) Alkyne, $NaHCO_3$, CuNPs/MK-10, MW (80°C, 30').

The α7 activity was evaluated through high-resolution single-channel recordings in the *cell-attached* patch configuration from BOSC23 cells expressing the receptor. In the control condition, α7 activity induced by 100 µM ACh appeared as brief isolated openings flanked by long closed periods and with lower frequency as activation episodes (bursts). As described in experimental procedures, bursts consist of a few opening events in quick succession, which are evoked by a single receptor molecule (Figure 2). The mean open and burst durations were obtained from the corresponding histograms.

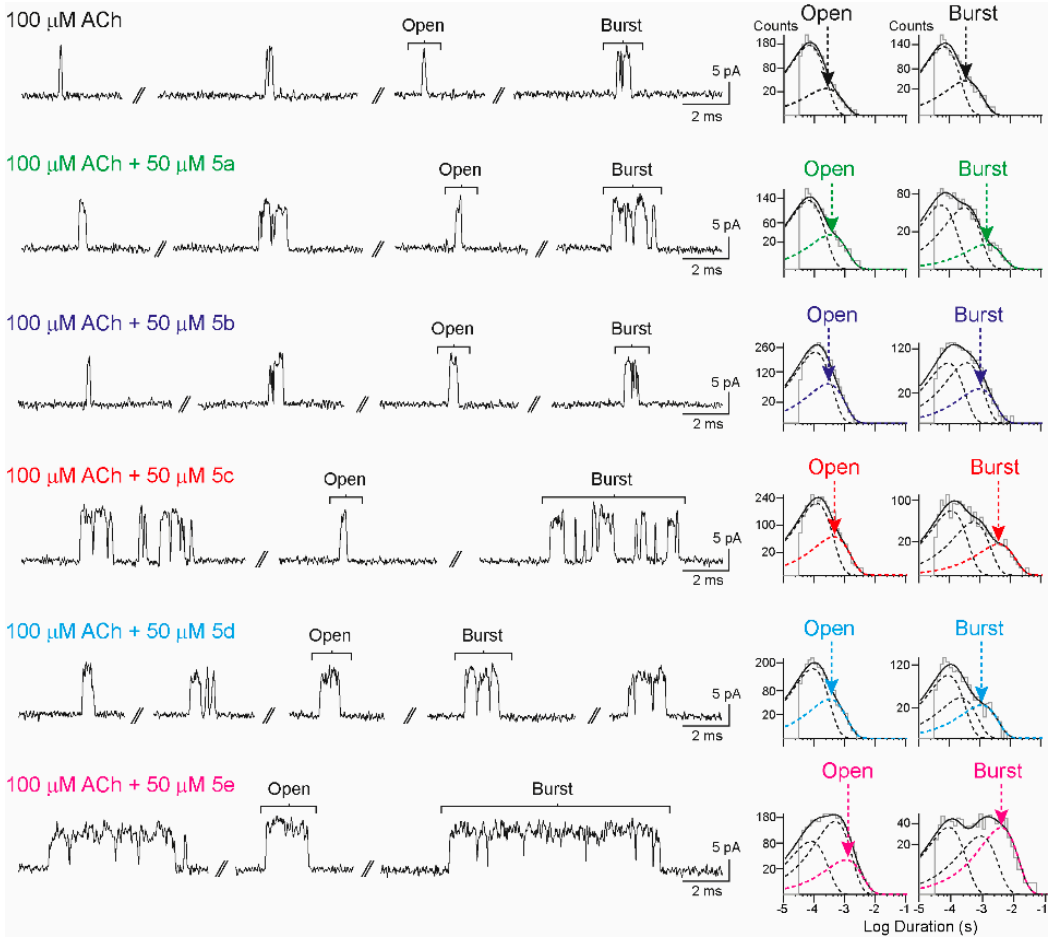


Figure 2. Effects of isoxazoles **5a-e** on α7 receptors at the single-channel level.
(Left) Typical patterns of α7 channel currents in the presence of 100 µM ACh alone or combined with each isoxazole (50 µM). Upward deflections indicate channel openings. (Right) Representative open and burst duration histograms for each condition. Membrane potential = −70 mV. Filter = 9 kHz.

The synthesized compounds were applied together with 100 μM ACh, which is close to the EC_{50} concentration for receptor activation, to evaluate their potential activity as PAMs of $\alpha 7$. 3,5-Isoxazoles were initially screened at 50 μM. Potentiation is typically detected as an increase in burst and open durations. The results are summarized in Table 1.

Table 1. Single-Channel parameters of $\alpha 7$ activated by 100 μM ACh in the absence or presence of the synthetic compounds shown in Scheme 1. Values are mean ± SD.

Condition	Mean Open Duration (ms)	Mean Burst Duration (ms)	n	N
100 μM ACh	0.24 ± 0.03	0.37 ± 0.06	8	7
100 μM ACh+ 50 μM **5a**	0.28 ± 0.04 (ns)	1.69 ± 0.14 (***)	4	2
100 μM ACh+ 50 μM **5b**	0.25 ± 0.04 (ns)	0.81 ± 0.08 (***)	5	3
100 μM ACh+ 50 μM **5c**	0.31 ± 0.06 (*)	3.93 ± 0.68 (***)	5	2
100 μM ACh+ 50 μM **5d**	0.36 ± 0.09 (**)	1.16 ± 0.23 (***)	6	3
100 μM ACh+ 50 μM **5e**	0.87 ± 0.18 (***)	3.88 ± 0.59 (***)	4	2

(*) indicates statistical significance determined by comparing the mean open and burst durations in the presence of each compound with respect to those in the absence. The number of symbols (one, two, or three) indicates different significant p-values ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***). ns: not significant, $p \geq 0.05$.

It is noteworthy that all isoxazoles evaluated showed 7-PAM activity. This can be seen from the significant increase in the mean burst duration in the presence of isoxazoles **5a-e** compared to the control (ACh 100 μM). However, there were differences in the type of potentiation among isoxazoles as only the vanillin derivatives (containing the 4-OH, 3-MeO fragment, compounds **5c-e**) markedly enhanced the mean open duration. The choice of the starting aldehyde seems to be crucial for the 7-PAM activity. Piperonal (**5a**) and 2,3-dihydroxybenzaldehyde (**5b**) derivatives are the least active compounds. Therefore, we continued to work with vanillin derivatives and made variations in the alkyne employed in the cycloaddition reaction. Substitution at position C4 with a methyl group leads to isoxazole **5d**, which shows a decrease in the mean burst duration while maintaining the mean open duration, whereas substitution with bromine leads to **5e**, the most active compound in the series. **5e** presents robust 7-PAM activity with a ~4-fold increase in the mean open duration and a ~10-fold increase in the mean burst duration.

3. Conclusions

A novel series of 3,5-disubstituted isoxazole derivatives was synthesized in high yields via microwaved-assisted [3+2] cycloaddition of in situ generated nitrile oxides and terminal alkynes catalyzed by CuNPs/MK-10. The $\alpha 7$ activity of these compounds, evaluated through high-resolution single-channel recordings in the *cell-attached* patch configuration from BOSC23 cells expressing the receptor, showed that vanillin-isoxazole derivatives exhibited potent $\alpha 7$-PAM activity. The most active isoxazole, **5e**, showed a ~4-fold increase in the mean open duration (0.87 ± 0.18 ms) compared to the control condition (0.24 ± 0.03 ms) and a ~10-fold increase in the mean burst duration (3.88 ± 0.59 ms). These preliminary studies suggest that isoxazole-vanillin derivatives are promising candidates for the development of new families of compounds with α 7-PAM activity. The generation of a small compound library and its biological evaluation are underway.

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Conflicts of Interest: The authors declare no conflict of interest.

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