Communication

Synthesis of Novel Pyrazolo[3,4-b]pyridines with Affinity for β-Amyloid Plaques

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Abstract: Three novel pyrazolo[3,4-b]pyridines were synthesized via the cyclization of 5-amino-1-phenylpyrazole with the corresponding unsaturated ketone in the catalytic presence of ZrCl4. The ketones were afforded by modifying a stabilized ylide facilitated Wittig reaction in fairly high yields. The novel compounds exhibited exciting photophysical properties with the dimethylamine phenyl-bearing pyrazolopyridine showing exceptionally large Stoke’s shifts. Finally, both the dimethylamino- and the pyrene-substituted compounds demonstrated high and selective binding to amyloid plaques of Alzheimer’s disease (AD) patient brain slices upon fluorescent confocal microscopy observation. These results reveal the potential application of pyrazolo[3,4-b]pyridines in the development of AD amyloid plaque probes of various modalities for AD diagnosis.

Keywords: pyrazolo[3,4-b]pyridines; β-amylloid plaque probes; AD diagnosis

1. Introduction

Pyrazolo[3,4-b]pyridines constitute a group of fused heterocyclic systems with outstanding chemical, biological and medicinal significance. This is witnessed by the fact that numerous pharmaceutically active compounds have the pyrazolo[3,4-b]pyridine core incorporated in their structure (Figure 1). They were shown to exhibit a broad range of biological activities and clinical applications. These include antiviral [1], antibacterial [2], antimalarial [3], antitrypanosomal [4], anti-inflammatory [5], anti-hypertension and pulmonary hypertension [6,7] and anti-tumour activity with various mechanisms of action [8–14]. Interestingly, some structurally related pyrazolo[3,4-b]pyridines were reported to exhibit neuroprotective, antidepressant, anxiolytic and anti-Alzheimer’s properties [15–18].

Alzheimer’s disease (AD), the most common type of dementia, is a progressive and irreversible disorder that affects millions of people over the age of 65 and is associated with memory loss, cognitive deficit, behavioral alterations and death [19]. Currently, AD is among the deadly diseases worldwide for which no effective therapeutic treatment is available, and early diagnosis remains a challenge [20]. Consequently, a tremendous effort was made to understand the pathogenesis of the disease for early diagnosis and treatment [21]. One of the major neuropathological characteristics of AD is the deposition of abnormal and misfolded proteins resulting in the formation of extracellular senile plaques...
and intracellular neurofibrillary tangles (NFTs). The primary constituent of senile plaques is the beta-amyloid peptide (Aβ) [22]. An increasing amount of evidence suggests that amyloid plaque imaging can improve diagnostic accuracy, increase diagnostic certainty and result in therapeutic alterations and disease monitoring [23–26]. To this end, Pittsburgh compound B (Figure 1), which exhibited a nanomolar affinity for fibrillar amyloid, was the first amyloid tracer described for Positron Emission Tomography (PET) imaging of amyloid plaques and is the most extensively studied among the clinically used tracers [27,28]. Newer amyloid PET tracers were also developed, including [18F]Florbetapir (trade name: AMYViD), [18F]Florbetaben (trade name: Neuraceq) and [18F]Flutemetamol (trade name: Vizamyl), to provide semi-quantitative information about amyloid deposition in patients [29]. Collectively, these molecules are benzothiazole derivatives and stilbene type of compounds. They are small, planar, rigid and conjugated molecules with interesting fluorescent properties which have readily revealed their binding to amyloid plaques with fluorescent microscopy techniques. Still, their clinical application suffers several limitations such as off-target binding, low signal-to-noise ratio and others that prompt further exploitation of novel tracers [30]. With regard to pyrazolo[3,4-b]pyridines, to the best of our knowledge, there are no reports connecting them with a high affinity to amyloid plaques for either imaging or therapy of AD. Etazolate (Figure 2) is the only pyrazolo[3,4-b]pyridine clinically investigated for its efficacy as an anti-Alzheimer acting agent, as an α-secretase inhibitor for mild to moderate AD [31].

Figure 1. The chemical structures of some clinically used AD tracers.

Figure 2. The structures of pyrazolo[3,4-b]pyridines Etazolate and the target compounds of this work, 5a–c.

As part of our ongoing research on the synthesis of molecules and complexes with high affinity for senile plaques [32–35], we would like to report herein the synthesis of three novel pyrazolo[3,4-b]pyridines (Figure 1) as potential probes for Aβ amyloid plaques. To the best of our knowledge, this particular substitution pattern on the pyrazolo[3,4-b]pyridine core was not previously reported.

Bearing in mind that this type of fused conjugated privileged scaffold exhibits fascinating photophysical properties [36], and some living cell imaging applications were
reported [37]. The absorption and fluorescence properties of the molecules were initially assessed. Consequently, these novel pyrazolopyridines were evaluated for selective binding to amyloid plaques using fluorescence confocal microscopy of human AD brain sections.

2. Results and Discussion

2.1. Synthesis of the Novel Derivatives

The designed pyrazolo[3,4-b]pyridine derivatives (5a–c) were synthesized in a two-step procedure from readily available starting materials and cheap reagents, as depicted in Scheme 1.

More specifically, the synthesis commenced by subjecting aldehydes 6a–c to a high-yielding Wittig reaction to afford the (E)-4-aryl but-3-en-2-ones 8a–c, employing the stabilized ylide 7 [38] in toluene and a catalytic amount of benzoic acid. The latter accelerated the reaction significantly due to the increased aldehyde electrophilicity. The products were readily available by a simple concentration of the reaction mixture and short column chromatography. Moreover, the (E)-geometry of the α,β-unsaturated ketones was confirmed by the high J coupling constants of the protons of the double bond (8a: J = 16.1 Hz, 8b: J = 16.5 Hz, 8c: J = 15.9 Hz). Subsequent condensation of 8a–c with 5-amino-1-phenylpyrazole (9) [39] in the catalytic presence of ZrCl₄ established the pyrazolo[3,4-b]pyridine core and afforded the final compounds 5a–c. ZrCl₄ is a green Lewis acid catalyst of high applicability due to its low toxicity, high availability, low cost, air and water stability and ease of handling. After a series of investigations, the solvent mixture of EtOH/DMF (1:1) was found to be the optimum one to sufficiently dissolve all the reactants and facilitate the reaction progress. Despite the low yields obtained for these derivatives, due to the incomplete condensation of the reactants and the formation of intermediate products, the method allowed the preparation and isolation of the target compounds 5a–c in a pure form via a two-step procedure from readily available starting materials and cheap reagents. It should be noted that although condensation of 9 and its 3-alkyl or aryl derivatives with chalcones and other unsaturated carbonyl compounds are reported in the literature [40–45], no examples of 9 with (E)-4-aryl but-3-en-2-ones were found. Studies towards the deeper understanding of the reaction mechanism and progress, the improvement of the yields and further exploitation of methods for the synthesis of modified derivatives are currently underway.

The final products 5a–c were characterized by NMR spectroscopy (¹H, ¹³C NMR and ¹H-¹H COSY), as well as FTIR and HRMS. In all cases, the signals of the pyrazole phenyl group and the R group originating from ketones 8a–c in the ¹H NMR spectra of all products 5a–c were the primary indication that the pyrazole moiety was incorporated with the monocarbonyl starting material. Moreover, the formation of the pyrazolo[3,4-b]pyridine ring was witnessed by the appearance of characteristic chemical shifts of the singlets corresponding to H-3 at δH 7.26 (5a), 7.28 (5b) and 7.36 (5c) ppm and H-5 at δH 8.48 (5a), 8.39 (5b) and 7.89 (5c) ppm of pyridine and pyrazole moieties, respectively. Additionally,
the signal of the CH$_3$ group as a singlet in the $^1$H NMR spectra of the products at $\delta_H$ 2.87 (5a), 2.79 (5b) and 2.88 (5c) ppm indicated that it is adjacent to C-6.

2.2. Photochemical Properties

The UV-Vis spectra of compounds 5a–c are shown in Figure 3. Compound 5a displays an absorption maximum at 364 nm, and the absorption tail extends to longer than 400 nm. The UV-Vis spectrum of 5c is characterized by the presence of pyrene absorption bands at 348 nm and 356 nm, though they appear red-shifted compared to the pyrene molecule due to the extended conjugation provided via the pyrazolopyridine substitution of the pyrene moiety [46]. Similarly, 5b exhibited three distinct peaks at 350 nm, 368 nm and 388 nm, which are very close to the absorption peaks of the anthracene molecule itself. This result is in good agreement with the observations reported by Becker et al., who investigated the relationship of the molecular geometry of various anthracene molecules with excited-state properties and witnessed the characteristic anthracene pattern in all derivatives tested [47]. The observed lower excitation may be related to some restriction in the charge transfer properties of the molecule [48]. It is very interesting to note that 5a displayed an absorption maximum wavelength of higher magnitude to the polycyclic pyrene and anthracene derivatives. This effect may be attributed to the strongly electron-donating character of the dimethylamine substituent at the para position of the phenyl ring. Indeed, 5a has the highest absorption of the three making it a very efficiently excitable molecule and an attractive candidate for microscopy observation.

![Figure 3](image-url)

**Figure 3.** Absorption spectra of 5a–c in DMSO (50 μM). 5b was only partially dissolved, and 5 μM concentration was used.

The emission spectra of all synthesized compounds are presented in Figure 4. The data revealed 5c emission peaks at 421 nm (blue), whereas 5a and 5b emission peaks occur at 452 and 465 nm (black and red), respectively. It is remarkable that 5a showed very large Stoke’s shifts (101 nm), as presented in Table 1. This may be related to the geometrical differences between the ground state and the emitting excited state, suggesting that the anthracene- and pyrene-substituted molecules are more coplanar to the pyrazolopyridine core compared to the smaller and more flexible dimethylaminobenzene derivative [47].
Table 1. Optical properties of compounds 5a–c. All compounds were excited at their absorbance $\lambda_{\text{max}}$ values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Delta\lambda$ (nm)</th>
</tr>
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<tbody>
<tr>
<td>5a</td>
<td>364</td>
<td>465</td>
<td>101</td>
</tr>
<tr>
<td>5b</td>
<td>368</td>
<td>452</td>
<td>84</td>
</tr>
<tr>
<td>5c</td>
<td>348</td>
<td>421</td>
<td>73</td>
</tr>
</tbody>
</table>

Figure 4. Normalized emission spectra of 5a–c in DMSO. 5a was recorded at 0.5 $\mu$M, and 5b,c were recorded at 5 $\mu$M. Excitation curves are displayed as dashed lines. All compounds were excited at their absorbance $\lambda_{\text{max}}$ values.

2.3. $\beta$-Amyloid Plaque Staining

Taking advantage of the interesting absorption and emission properties, the binding affinity for $\beta$-amyloid plaques of AD was examined following standard staining procedures [35]. Figure 5 show the confocal microscopy images of the in vitro staining of human post-mortem AD fixed brain sections with pyrazolopyridines 5a and 5c. Both compounds bind selectively to the amyloid plaques, allowing clear visualization of both diffused and dense core ones. In the case of anthracene derivative, severe precipitation due to solubility limitations was observed during the staining procedure, which did not allow for any imaging at all. According to the literature, sufficient conjugation and electron-donor substitution on rigid molecules with planar configuration are the common desirable features of most of the molecules serving as A$\beta$ imaging probes with high affinity to amyloid fibrils and plaques [49]. Therefore, our results, in full agreement with the literature, suggest that the dimethylamino- substituted compound 5a and the pyrenyl-bearing 5c exhibit the favorable structural characteristics for high amyloid affinity to enable both selective binding and effective imaging of amyloid deposits. The pyrazolopyridine core is a rigid, flat, conjugated and lipophilic unit. In addition, extensive conjugation is acquired by the pyrene moiety, whereas the electron donor dimethylamino- substitution enhances e-delocalization for $\pi$-stacking.
Figure 5. Confocal Fluorescence microscopy images (DAPI filter, \( \lambda_{ex} = 360 \text{ nm}, \lambda_{em} = 420 \text{ nm} \)), magnification \( \times 20 \), (A) and (B); \( \times 40 \) (C) and (D) of human post-mortem AD fixed brain sections treated with (A,C) compound 5a, (B,D) compound 5c (200 \( \mu \text{M} \)). Dotted circles mark indicative “stained” plaques.

3. Materials and Methods
3.1. General Remarks

All reactions were carried out using dry, freshly distilled solvents unless otherwise noted. Yields refer to chromatographically and spectroscopically (\(^1\text{H NMR}\)) homogeneous materials unless otherwise stated. All reagents were purchased at the highest commercial quality from Sigma-Aldrich (Taufkirchen, Germany) or Alfa-Aesar (Kandel, Germany) and used without further purification unless otherwise stated. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Merck silica gel plates (60 F254), using UV light as visualizing agent and ethanolic phosphomolybdic acid, \( p \)-anisaldehyde or potassium permanganate solutions and heat as developing agents. Purifications with flash column chromatography were carried out using Merck silica gel (60, particle size 0.040–0.063 mm) and elution systems as stated in each experimental procedure. Melting points were determined with a Gallenkamp MFB-595 melting point apparatus (Weiss Gallenkamp, London, UK). NMR spectra were recorded on Bruker Avance DRX-500 or Bruker Advance II 250 MHz instruments (Rheinstetten, Germany) at 25 °C. The following abbreviations were used to explain NMR signal multiplicities: s: singlet, d: doublet, t: triplet, m: multiplet, dd: doublet of doublets, brt: broad triplet. Assignment of \(^1\text{H NMR}\) spectra was based on 2D experiments, and the numbering of final compounds is depicted in the Supplementary Materials. Samples were dissolved in CDCl\(_3\). UV-Vis spectra were recorded with a Hitachi U-3010 spectrophotometer (Mannheim, Germany). Fluorescence spectra were recorded using a HITACHI F-2500 spectrofluorometer at 25 °C in the wavelength range 350–800 nm. Excitation wavelengths of the samples were 320 nm, scan speed was 300 nm/min, and both excitation and emission slit widths were 2.5 nm. HRMS spectra were recorded on UHPLC LC-MSn Orbitrap Velos-Thermo instrument (Thermo Scientific; Bremen, Germany) in the Institute of Biology, Medicinal Chemistry and Biotechnology of the National Hellenic Research Foundation. Fluorescent staining observation was performed with a Leica TCS SP8 MP (Wetzlar, Germany) confocal microscope equipped with an Argon
laser (excitation lines at 458, 476, 488, 496 and 514 nm), a DPSS 561 laser (excitation line at 561 nm) and an IR MaiTai DeepSee Ti:Sapphire laser (Spectra-Physics, Santa Clara, CA, USA) for multiphoton applications. Images were acquired with the LAS X software (Version 5.0.3, Leica Microsystems CMS GmbH, Wetzlar, Germany) and are presented without any postprocessing.

3.2. Synthesis of Compounds

3.2.1. General Procedure for the Preparation of (E)-4-aryl But-3-en-2-ones (8a-c) with Wittig Reaction

To a solution of the aldehyde 6a–c (3.35 mmol) in toluene (7 mL), ylide 7 (4.2 mmol, 1.25 eq) and a catalytic amount of benzoic acid (41 mg, 0.335 mmol) were added, and the reaction mixture was refluxed overnight. Upon completion of the reaction (monitored by TLC), the mixture was concentrated in vacuo. The solid residue was subjected to a short flash column chromatography to afford the target product.

(E)-4-(4-((N,N-dimethylamino)phenyl)but-3-en-2-one (8a) [50]

Light yellow solid. Yield: 86%. Rf: 0.26 (n-hexane:EtOAc 8:2); Mp: 340–341 °C; 1H NMR (500 MHz, CDCl3): δ 7.46 (d, J = 16.1 Hz, 1H; Ar-CH=CH-), 7.44 (d, J = 8.9 Hz, 2H; Ar-H), 6.68 (d, J = 8.9 Hz, 2H; Ar-H), 6.54 (d, J = 16.1 Hz, 1H; Ar-CH=CH-), 3.03 (s, 6H; Ar-N(CH3)2), 2.34 (s, 3H; CH3CO-); ppm; 13C NMR (126 MHz, CDCl3): δ 198.6, 152.1, 144.5, 130.2, 122.6, 122.2, 110.2, 40.2, 27.3 ppm; MS (ESI) [M+H]+ m/z 190.05.

(E)-4-((anthracen-9-yl)but-3-en-2-one (8b) [51]

Yellow solid. Yield: 90%. Rf: 0.35 (n-hexane:EtOAc 9:1); Mp: 109–111 °C; 1H NMR (500 MHz, CDCl3): δ 8.49 (d, J = 16.5 Hz, 1H; Ar-CH=CH-), 8.48 (s, 1H; H10 of anthracene), 8.21 (dd, J = 9.0, 1.8 Hz, 2H; H1, H8 of anthracene), 8.03 (dd, J = 8.1, 1.7 Hz, 2H; H4, H5 of anthracene), 7.51 (m, 4H; H2, H3, H6, H7 of anthracene), 6.72 (d, J = 16.5 Hz, 1H; -CH=CH-CO(CH3)), 2.56 (s, 3H; CH3CO-); ppm; 13C NMR (126 MHz, CDCl3): δ 198.0, 140.6, 136.1, 131.4, 129.5, 129.4, 129.1, 128.63, 126.6, 125.6, 125.2, 28.2 ppm; MS (ESI) [M+H]+ m/z 247.05.

(E)-4-((pyren-1-yl)but-3-en-2-one (8c) [52]

Yellow solid. Yield: 83%. Rf: 0.38 (n-hexane:EtOAc 8:3); Mp: 82–83 °C; 1H NMR (500 MHz, CDCl3): δ 8.69 (d, J = 15.9 Hz, 1H; Ar-CH=CH-), 8.48 (d, J = 9.3 Hz, 1H; Ar-H), 8.30 (d, J = 8.1 Hz, 1H; Ar-H), 8.26–8.11 (m, 5H; Ar-H), 8.10–8.02 (m, 2H; Ar-H), 7.02 (d, J = 15.9 Hz, 1H; Ar-CH=CH-), 2.54 (s, 3H; CH3CO-); ppm; 13C NMR (126 MHz, CDCl3): δ 198.2, 139.9, 133.0, 131.4, 130.8, 130.1, 129.0, 128.8 (2C), 128.3, 127.4, 126.4, 126.2, 126.0, 125.2, 125.0, 124.7, 124.3, 122.4, 28.3 ppm; MS (ESI) [M+H]+ m/z 271.00.

3.2.2. General Procedure for the Preparation of 4-Substituted 6-methyl-1-phenyl-1H-pyrazo[3,4-b]pyridines (5a–c)

To a solution of the α,β-unsaturated ketones 8a–c (0.5 mmol) in DMF (0.5 mL) a solution of 5-amino-1-phenyl-pyrazole (9) (102 mg, 0.5 mmol) to EtOH (0.5 mL) was added at 25 °C. The reaction mixture was degassed, and ZrCl4 (35 mg, 0.15 mmol) was added. The reaction mixture was vigorously stirred at 95 °C for 16 h. After completion of the reaction, the mixture was concentrated in vacuo, and CHCl3 and water were added. The two phases were separated, and the aqueous phase was washed with CHCl3 twice. The combined organic extracts were washed with H2O and brine, dried over Na2SO4 and concentrated in vacuo. The residue was subjected to flash column chromatography to afford the target compounds 5a–c in pure form.

4-(4-(1H-pyrazo[3,4-b]pyridine (5a)

Off-white solid. Yield: 28%. Rf = 0.40 (n-hexane/ EtOAc 8:2); FTIR (KBr, cm⁻¹): 3048, 2982, 2847, 1740, 1567, 1509, 1351, 1193, 956, 812, 740; 1H NMR (500 MHz, CDCl3): δ = 8.48 (d, J = 7.4 Hz, s overlapping, 3H; H1′, H5′, H3), 7.84 (d, J = 7.5 Hz, 2H; H2″, H6″ or H3″, H5″), 7.67 (brt, J = 7.4 Hz, 2H; H2′, H4′), 7.43 (m, 1H; H3′), 7.26 (s overlapping with CDCl3, 1H; H5), 7.00 (d, J = 7.5 Hz, 2H; H2″, H6″ or H3″, H5″), 3.19 (s, 6H; -N(CH3)2), 2.87 (s, 3H; Ar-CH3); ppm; 13C NMR (126 MHz, CDCl3): δ = 159.1 (C), 151.3 (C), 144.4 (C), 140.1 (C), 133.9 (C), 129.5 (CH), 129.1 (CH), 125.8 (CH), 124.9 (C), 121.5 (CH), 115.5 (CH), 113.5
“stained” the amyloid plaques of AD patients’ brain microscopy slices. The results clearly showed the potential of the pyrazolo[3,4-b]pyridine core to act as an AD amyloid plaque probe and prompt us to optimize the synthetic procedure to evaluate the effect of various substitutions on the biological activities of the new compounds.

Supplementary Materials: All the spectroscopic data of the title compounds 5a–c are available online.

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