Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2021, **19**, 2784

Amidino substituted 2-aminophenols: biologically important building blocks for the amidino-functionalization of 2-substituted benzoxazoles†

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Received 5th February 2021,
Accepted 2nd March 2021

DOI: 10.1039/d1ob00235j

rsc.li/obc

Unlike the closely related and widely investigated amidino-substituted benzimidazoles and benzothiazoles with a range of demonstrated biological activities, the matching benzoxazole analogues still remain a largely understudied and not systematically evaluated class of compounds. To address this challenge, we utilized the Pinner reaction to convert isomeric cyano-substituted 2-aminophenols into their amidine derivatives, which were isolated as hydrochlorides and/or zwitterions, and whose structure was confirmed by single crystal X-ray diffraction. The key step during the Pinner synthesis of the crucial carboximidate intermediates was characterized through mechanistic DFT calculations, with the obtained kinetic and thermodynamic parameters indicating full agreement with the experimental observations. The obtained amidines were subjected to a condensation reaction with aryl carboxylic acids that allowed the synthesis of a new library of 5- and 6-amidino substituted 2-arylbenzoxazoles. Their antiproliferative features against four human tumour cell lines (SW620, HepG2, CFPAC-1, HeLa) revealed sub-micromolar activities on SW620 for several cyclic amidino 2-naphthyl benzoxazoles, thus demonstrating the usefulness of the proposed synthetic strategy and promoting amidino substituted 2-aminophenols as important building blocks towards biologically active systems.

Introduction

Aromatic and heteroaromatic diamidines have been extensively studied because of their broad spectrum of antimicrobial activity. Compounds such as pentamidine, berenil, and 4′,6-diamidino-2-phenylindole (DAPI) have been known to be efficient in the treatment of trypanosomiasis and leishmaniasis. Pentamidine, which was discovered over 60 years ago, is still one of the most used drugs for the first stage of human African trypanosomiasis, although treatment with this compound is associated with serious toxicities.[1,2] In order to search for safer and more effective drugs, great efforts have been made in the synthesis and biological evaluation of new dicationic molecules. In particular, diamidino-substituted benzimidazoles, as analogues of DAPI, have emerged as important pharmacophores in the development of antimicrobial agents. Binding in the minor groove of DNA at AT-rich sites is thought to be a key step in the mode of action of these types of dicationic systems, which likely leads to the inhibition of DNA-dependent enzymes or a direct inhibition of transcription.[2] Positively charged extremities are not the sole structural elements for strong DNA binding, but the three-dimensional organization of the molecules is also crucial for sequence-selective DNA binding. One direction in the development of DNA sequence-specific agents is modification of the diamidine structure by including a variety of nitrogen heterocycles into molecules of different shapes.[3–5] With the recent approval of the minor groove binding agent trabectedin for the treatment of patients with soft tissue sarcomas, there has been renewed interest in small molecules as antitumor agents that target the DNA as well.[6,7]

Amidino-substituted benzimidazole derivatives are a well-studied class of compounds with some new recent examples

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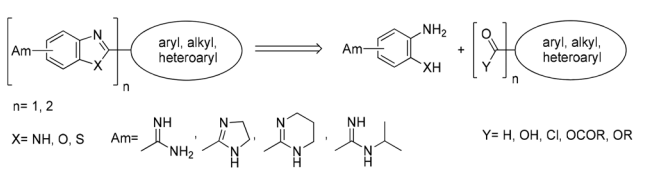
†Electronic supplementary information (ESI) available: Experimental and computational details; single crystal X-ray diffraction analysis; ^{1}H and ^{13}C NMR spectra; Cartesian coordinates for all computed systems and their output files. CCDC 2060884–2060894. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/d1ob00235j

associated with prominent antibacterial,[8] antiparasitic,[9] antifungal,[10] and antiproliferative activities.[11] Besides the benzimidazole core, over the last decade, our scientific focus has been placed on benzothiazole compounds bearing different amidino substituents with an emphasis on their synthesis and the study of anticancer activities and DNA binding properties.[12–20] The obtained results revealed that 2-aryl/heteroaryl benzothiazole derivatives substituted with the cyclic amidino substituent, namely the 2-imidazolinyl group, had the most significant influence on the antiproliferative activity and its enhancement with IC_{50} values in the submicromolar range of concentrations.

Surprisingly, in contrast to a great number of amidino-substituted benzimidazoles and benzothiazoles, structurally related benzoxazoles are still rare. There are only a few reports that deal with the synthesis of amidino-substituted benzoxazoles and their antitumor[21] and antimicrobial[22,23] activities as well as their DNA binding properties.[24] One reason is the lack of a general method for their preparation, which could be based on the condensation reaction of amidino-substituted 2-aminophenols with aldehydes, carboxylic acids or carboxylic acid derivatives since this method is largely and efficiently used for the preparation of the corresponding benzimidazole and benzothiazole derivatives, from amidino-substituted phenylenediamines and 2-aminothiophenols, respectively (Scheme 1).

The most common method for amidine preparation is the nucleophilic addition of amines or ammonia to suitably activated carboxylate equivalents such as imidates, thioimidates, and imidoyl chlorides. Imidates can generally be prepared either by a base-catalysed or acid-catalysed (Pinner synthesis) addition of alcohol to nitrile. The addition of dry hydrochloric acid to a mixture of nitrile and alcohol leads to the hydrochloride salt of an imidate. This salt can further react with ammonia or an amine to form amidine.[25] The key step in the Pinner synthesis is the formation of the imidate functionality which has been recently exploited for the syntheses of diverse classes of N-heterocycles *via* C–N annulation reactions under acid/base/metal-catalysed or radical-mediated reaction conditions.[26]

In this work, we present a novel synthesis of 5- and 6-amidino-substituted 2-arylbenzoxazoles through the condensation reaction of different amidino-substituted 2-aminophenols as the key building blocks, which were unknown to date. We describe the efficient synthetic protocols and structural analysis of the obtained isomeric amidino-substituted 2-aminophenols, while the key step during the Pinner synthesis of the crucial carboximidate intermediate was characterized through mechanistic DFT calculations. Additionally, in order to explore the biological potential of a new small library of amidino-substituted 2-arylbenzoxazoles, the antiproliferative activities of some selected compounds were tested on four human tumour cell lines.



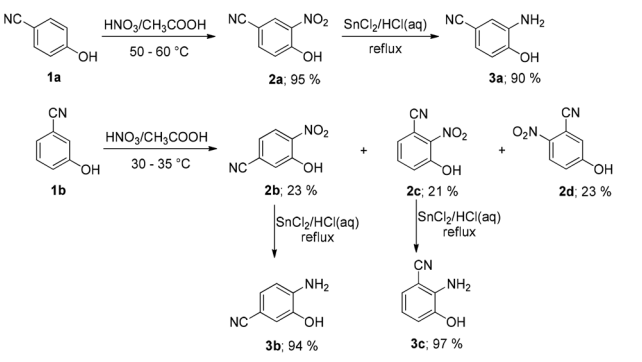
Scheme 1 Retrosynthetic strategy for amidino-functionalization of 2-substituted benzazoles.

Results and discussion

Synthesis of amidino-substituted 2-aminophenols

Commercially available 4-hydroxybenzonitrile (**1a**) and 3-hydroxybenzonitrile (**1b**) were used as starting substrates for the preparation of isomeric cyano-substituted 2-aminophenols in the nitration reaction with fuming nitric acid in glacial acetic acid (Scheme 2).

The nitration reaction of 4-hydroxybenzonitrile (**1a**) was carried out according to the described procedure[27] and 4-hydroxy-3-nitrobenzonitrile (**2a**) was obtained in 95% yield. Our computations confirm the presence of a single product by showing that the stability of the crucial carbocation intermediate is the highest upon the attack of the active nitronium ion (NO_2^+) on position 3, being −17.7 kcal mol^{-1} more stable than isolated reactants, while in the case of position 2, it is only −0.9 kcal mol^{-1}, thus rationalizing why only **2a** was isolated. In addition, kinetic aspects further support this conclusion as the activation barrier to approach position 3 is only $\Delta G^{\ddagger}$ = 1.6 kcal mol^{-1}, being 5.5 kcal mol^{-1} lower than for position 2. Due to the direction of the electrophilic aromatic substitution conditioned by the hydroxy and cyano substituents on the benzene ring, in the nitration reaction of 3-hydroxybenzonitrile (**1b**), more products were formed. An analogous computational analysis revealed that the stability of the carbocation intermediate is −17.6, −17.0 and −21.3 kcal mol^{-1} lower than those of the isolated reactants, when NO_2^+ approaches positions 2, 4 and 6 in **1b**, respectively, while it is only −3.3 kcal mol^{-1} for the attack on position 5. This confirms approximately equal distribution of products **2b–2d**, and the absence of the potential 5-nitro derivative. Still, only those regioisomers in which the hydroxy and nitro groups are on the adjacent C



Scheme 2 Synthesis of nitro- and amino-substituted 2-aminophenols.

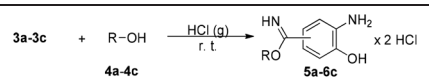
atoms were of interest for further synthesis. The reaction was optimized according to the more favourable ratio of cyano-substituted 2-nitrophenol of interest, where the reaction temperature proved to be the parameter with the most influence on the total yield and the ratio of the resulting products. A reaction temperature of 30–35 °C gave a high total yield of nitrated products where 3-hydroxy-4-nitrobenzonitrile (**2b**) and 3-hydroxy-2-nitrobenzonitrile (**2c**) were isolated by chromatography in 23% and 21% yields, respectively, after the isolation of isomer **2d** in 23% yield by simple crystallization. The next step of the synthesis was the reduction of nitro to the amino group on all prepared 2-nitrophenol isomers of interest, **2a**, **2b** and **2c** (Scheme 2), by our previously optimized method using tin(II) chloride in an ethanol/hydrochloric acid media.[28] Since nitro derivatives **2a**, **2b** and **2c** were soluble in water, the reaction was carried out in dilute hydrochloric acid at reflux with 4 equivalents of tin(II) chloride. All amino derivatives **3a**, **3b** and **3c** were isolated in excellent yields of 90–97%. The structures of the previously reported nitro derivatives **2a**, **2b** and **2d** and amino derivatives **3a** and **3b** were confirmed by ^{1}H NMR and LC-MS. The unknown nitro **2c** and amino **3c** isomers were fully characterized. In addition, the structure of compound **3c** was unequivocally determined by single crystal X-ray diffraction (Fig. S1†).

The classical Pinner reaction was then used for the preparation of amidino-substituted 2-aminophenols. In the first step, the nitrile was converted to the corresponding carboximidate hydrochloride by the addition of alcohol, catalysed by dry hydrogen chloride. For this reaction, it is common to use equimolar ratios of methanol or ethanol and nitrile in a dry solvent or an excess of absolute alcohol in which the corresponding imidates are obtained in high yields.[25,26] Various primary alcohols can be used including different alkoxy- and trihalogen-substituted ethanoles,[29] as well as secondary alcohols such as isopropanol.[30] In the second step of the reaction, the alkoxide group was substituted with an amine to form an amidine group. In order to investigate the reaction yield of this key step of the Pinner reaction, as well as the purity and stability of the corresponding carboximidate hydrochlorides, the use of three primary alcohols was investigated: methanol (**4a**), 2-methoxyethanol (**4b**) and 2-(2′-ethoxyethoxy)ethanol (**4c**), while the mechanistic details and the rationalization of the product distribution were obtained using DFT computations (see later).

The conversion of benzonitrile **3a** to the corresponding imidates **5a–5c** (Table 1) was achieved with all alcohols in excellent yields higher than 89%, with imidate **5b** being obtained quantitatively with very good purity.

Although imidate **5c** was also isolated as a white solid in an almost quantitative yield, it proved to be highly hygroscopic and sensitive to moisture. The structures of **5a–5c** and their purity were confirmed by ^{1}H NMR spectroscopy. Due to their reactivity, they have not been additionally purified and fully characterised. The first step of the Pinner reaction for benzonitrile **3b**, carried out in alcohols **4a** and **4c** (Table 1), did not give a satisfactory result for carboximidate dihydrochlorides **6a** and **6c** because they were isolated in the form of a viscous hygroscopic liquid which was difficult to handle. However, from the reaction carried out in 2-methoxyethanol (Table 1), carboximidate dihydrochloride **6b** was isolated in the form of a stable white solid in an almost quantitative yield of 98% with very good purity confirmed by ^{1}H NMR spectroscopy.

Table 1 Synthesis of carboximidates **5a–6c**



Nitrile	R	Imidate	Isolated yield
3a	CH_3	**5a**	90%
	$CH_2CH_2OCH_3$	**5b**	Quantitative
	$CH_2CH_2OCH_2CH_2OCH_2CH_3$	**5c**	95%
3b	CH_3	**6a**	ND[a]
	$CH_2CH_2OCH_3$	**6b**	98%
	$CH_2CH_2OCH_2CH_2OCH_2CH_3$	**6c**	ND[a]
3c	CH_3	NR[b]	—
	$CH_2CH_2OCH_3$	NR[b]	—

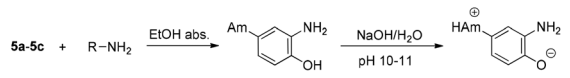
[a] Not determined. [b] No reaction.

It is known from the literature data that the formation of imidates for *ortho*-substituted aromatic nitriles is often impossible.[31] Due to the higher available amounts of *o*-substituted cyano-derivative **3c**, an attempt was made to prepare carboximidates in the first step of the Pinner reaction using methanol (**4a**) and 2-methoxyethanol (**4b**) (Table 1). It was hypothesized that the use of a primary alcohol with a higher molecular weight such as 2-(2′-ethoxyethoxy)ethanol (**4c**) could lead to steric disturbances during alcohol addition; therefore we performed the reaction in alcohols **4a** and **4b**. The result of the reaction showed no conversion of benzonitrile **3c** even with a prolonged reaction time up to 10 days, and we regenerated only the starting substrate **3c**. It was concluded that the formation of imidate from *ortho*-substituted aromatic nitrile **3c** was indeed unsuccessful. Following the described investigation of the first step of the Pinner reaction, 2-methoxyethanol proved to be the best choice due to the obtained quantitative yields of crude carboximidate hydrochlorides **5b** and **6b** with very good purity. Also, they were always obtained as solids which are easy to dry and stable to handle within a few days.

The second step of the Pinner reaction was performed with freshly prepared carboximidates in absolute ethanol with an excess of ammonia at room temperature for 2 days or an excess of primary amine at reflux temperature for 2–4 h (TLC-controlled). Thus, from 2-aminophenol-4-imidate **5a**, **5b** or **5c**, in reaction with gaseous ammonia (**7a**), ethylene-diamine (**7b**), 1,3-propanediamine (**7c**) and isopropylamine (**7d**), 4-amidino-substituted 2-aminophenols were isolated as hydrochlorides **8a**, **8b** and **8d** and/or zwitterions **8a′**, **8b′**, **8c′** and **8d′** in moderate to good yields (Table 2).

Reactions of imidate **5c** with ammonia **7a** afforded amidines **8a** and **8a′**, isolated as hydrochloride and a zwitterion, respectively. It was shown that it is possible to isolate both

Table 2 Synthesis of 4-amidino-substituted 2-aminophenols

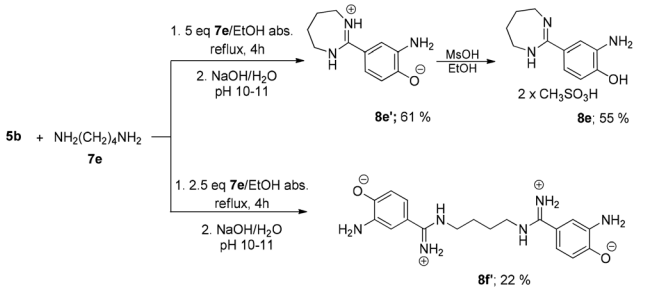


Imidate	Amine	Amidine	Product	Isolated yield
5c	**7a**; R = H	x 1/2 HCl	**8a**	44%
5c	**7a**; R = H		**8a'**	64%
5b	**7b**; R = $(CH_2)_2NH_2$	x 1/2 HCl	**8b**	41%
5c	**7b**; R = $(CH_2)_2NH_2$		**8b'**	78%
5a	**7c**; R = $(CH_3)_2NH_2$		**8c'**	75%
5b	**7d**; R = $CH(CH_3)_2$	x HCl	**8d**	46%
5a	**7d**; R = $CH(CH_3)_2$		**8d'**	51%

forms of amidines, though the isolation of the product was different, as well as the yield of the overall reaction. Hydrochloride **8a** was isolated in a moderate yield while zwitterion **8a′** was isolated in a greater yield after dilution of the reaction mixture with water and basification to pH 10–11 (Table 2). A similar pattern of behaviour was observed in the reaction with 5 eq. of diamine **7b**, where 2-imidazolinyl-substituted 2-aminophenols were prepared as hydrochloride **8b** and zwitterion **8b′** *via* imidate **5b** or **5c**, respectively. Zwitterion **8b′** was isolated in a much greater yield than hydrochloride **8b**, which was isolated in a moderate yield. In the reaction of imidate **5a** with 4 eq. of diamine **7c**, 2-pyrimidinyl-substituted 2-aminophenol **8c′** in a zwitterionic form was prepared in a good yield. From imidates **5b** and **5a**, in the reaction with 2.5 eq. of amine **7d**, *N*-isopropylamidino-substituted 2-aminophenol as hydrochloride **8d** and zwitterion **8d′** were obtained in similar moderate yields. According to these results, it was preferable to isolate the products in the zwitterionic form, since higher yields and cleaner products were obtained with no need for purification.

Unexpectedly, the reaction of imidate **5b** with 1,4-butanediamine (**7e**) gave two different products depending on the amine concentration in solution. Thus, with a higher excess and a higher concentration of amine **7e** (c = 1.25 mol dm^{-3}), the targeted seven-membered cyclic 1,3-diazepinyl amidine **8e′** was isolated as a zwitterion in a good yield (Scheme 3).

In the reaction with a reduced excess and a lower concentration of amine **7e** (c = 0.25 mol dm^{-3}), only the product of the intermolecular Pinner reaction, bis(butane-1,4-diimidamide) **8f′**, was isolated in a modest yield, while **8e′** was not observed by qualitative TLC analysis. This agrees with the literature that a large excess of amines and a prolonged



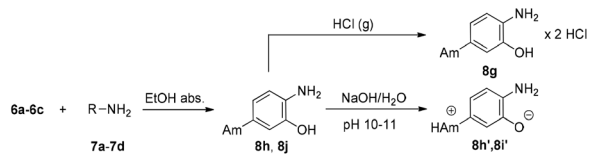
Scheme 3 Reaction of imidate **5b** with 1,4-butanediamine **7e**.

reaction time offer *N*,*N*′-disubstituted and cyclic amidines.[25] The ^{1}H NMR spectra of **8e′** and **8f′** were very similar in the chemical shift and multiplicity of signals and could not be unambiguously attributed to the structure of the product. The key data for determining their structures were obtained from the mass spectra, regarding the values of m/z for the molecular ion of structures **8e′** (206.2) and **8f′** (357.2). To further confirm the formation of the zwitterion **8e′**, a reaction with methanesulfonic acid was carried out in ethanol from which dimethanesulfonate **8e** was isolated, whose molecular structure was confirmed by single-crystal X-ray diffraction.

From the isomeric 2-aminophenol-5-carboximidates **6a–6c** in reaction with ammonia **7a**, diamines **7b** and **7c** and amine **7d**, 5-amidino-substituted 2-aminophenols were isolated as hydrochlorides **8g**, **8h**, and **8j** and/or zwitterions **8h′** and **8i′** in moderate to good yields (Table 3).

After the reaction of imidate **6b** with **7a** and removal of excess of ammonia from the reaction mixture, only precipi-

Table 3 Synthesis of 5-amidino-substituted 2-aminophenols



Imidate	Amine	Amidine	Product	Isolated yield
6b	**7a**; R = H	x 2 HCl	**8g**	72%
6b	**7a**; R = H		**8g'**	0%
6c	**7b**; R = $(CH_2)_2NH_2$	x HCl	**8h**	60%[a]
6b	**7b**; R = $(CH_2)_2NH_2$		**8h'**	81%
6a	**7c**; R = $(CH_3)_2NH_2$		**8i'**	75%[a]
6b	**7d**; R = $CH(CH_3)_2$	x HCl	**8j**	59%

[a] Yields were calculated from nitrile **3b**.

tation of ammonium chloride from ethanol occurred and the expected hydrochloride remained in the mother liquor. An attempt to precipitate the product as zwitterion **8g′** by dilution/basification was also unsuccessful, because of its great solubility in water. Therefore, in a second run, after the completion of the reaction of **6b** with **7a** by saturating the ethanolic mother liquor with dry gaseous hydrogen chloride, the product was precipitated as dihydrochloride **8g** and isolated in a good yield (Table 3). In the reaction of imidate **6c** with 2.5 eq. of diamine **7b**, the hydrochloride of 2-imidazolinyl substituted 2-aminophenol **8h** was afforded in 60% yield. The corresponding zwitterion **8h′** was prepared from imidate **6b** in the reaction with 2.5 eq. of diamine **7b** and isolated in a very good yield. From imidate **6a**, in the reaction with 4 eq. of diamine **7c**, the zwitterionic form of 2-pyrimidinyl-substituted 2-aminophenol **8i′** was obtained in 71% yield. After heating the reaction mixture of imidate **6b** and 2.5 eq. of amine **7d**, *N*-isopropylamidino-substituted 2-aminophenol hydrochloride **8j** was obtained in a moderate yield without the need for a purification step.

The structures of all newly prepared amidino-substituted 2-aminophenols were fully characterised by ^{1}H and ^{13}C NMR spectroscopy, UPLC-MS spectrometry and elemental analysis. NMR analysis based on the values of H–H coupling constants and chemical shifts in the ^{1}H spectra confirmed the structures of compounds. Furthermore, ^{13}C chemical shifts were consistent with the suggested structures. In addition, we succeeded in obtaining nine crystals of 2-aminophenols suitable for X-ray analysis which unequivocally confirm their molecular structures.

Single crystal X-ray studies

Amidino-substituted aminophenols **8a**, **8a′**, **8b**, **8b′**, **8c′**, **8d**, **8e**, **8g** and **8h** crystallized as salts, solvates or salt solvates. X-ray crystallographic study of **8a** (two polymorphic forms, **8a I** and **8a II**, see the ESI†) shows that this compound exists in a zwitterionic form, with the C7–N2 and C7–N3 bonds in the amidinium moiety approximately equal (Table S1†). Such a form is also corroborated by the C1–O1^{-} bond distance being *ca*. 0.02–0.03 Å shorter than in similar structures where the phenol group is not deprotonated (phenol C–O bond length *ca*. 1.36 Å). Crystallizing as a hemihydrochloride monohydrate, the structure of **8a** contains dimeric cations formed by two molecules joined by an O^{-}–H^{+}–O^{-} hydrogen bond bridge (Fig. 1a and Fig. S2†). The structure of **8b** (Fig. S3†), where the amidinium moiety is a part of a dihydroimidazolium ring, exhibits the same cation : anion : water ratio as that of **8a**. The C7–N2, C7–N3 and C1–O1^{-} bonds are of the same lengths as the corresponding ones in **8a**. In contrast, 4-amidino-substituted derivative **8d** (Fig. 1b) and 5-amidino-substituted derivative **8h** (Fig. S4†) did not contain zwitterions forming cationic dimers, but un-ionized phenol groups, resulting in singly charged cations. The C1–O1 bond in **8d** and the C2–O1 bond in **8h** are typical of a phenol C–O bond (Table S1†). Isolation of 4-amidino-substituted 2-aminophenols **8a′** (Fig. 1c), **8b′** and **8c′** (Fig. S5†) from water at pH 10–11 afforded structures in a pure zwitterionic form crystallizing as hydrates. The C7–N2 and C7–N3 bonds of the amidinium moiety as well as the C1–O1^{-} bond in these three structures are all of approximately the same lengths as those of the equivalent ones in previously described zwitterionic salt solvate structures (Table S1†). X-ray crystallographic study also confirmed that **8g** and **8e** crystallized as dications with two chloride or methanesulfonate anions, respectively (Fig. S6†).

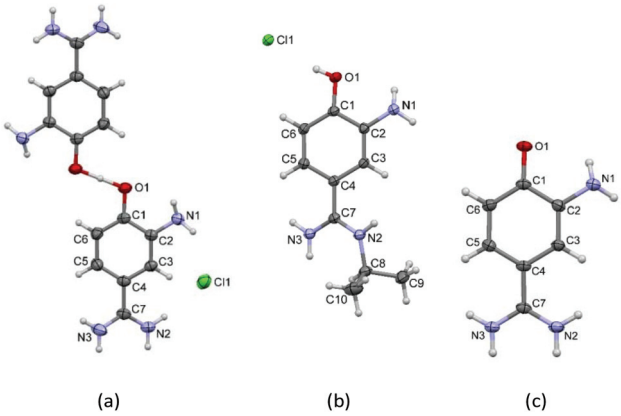


Fig. 1 Molecular structures of (a) **8a I**, (b) **8d** and (c) **8a′**, with atom numbering schemes. Displacement ellipsoids for non-hydrogen atoms are drawn at the 40% probability level. Only the major component of the disordered chloride ion is shown for **8a I**. Solvent molecules are omitted for clarity.

Computational studies

Computational analysis was performed to interpret the reactivity differences among the systems in the Pinner reaction with a particular focus on rationalizing the higher reactivity of **3a** and **3b** towards alcohols relative to **3c**, by calculating the kinetic and thermodynamic parameters for the matching reactions.

Our initial efforts started with neutral un-ionized **3a–3c** and considered the feasibility of solvent-mediated transformations into the corresponding imidates, taking **3a** and MeOH as illustrative examples. As seen in Fig. 2, the reaction starts with

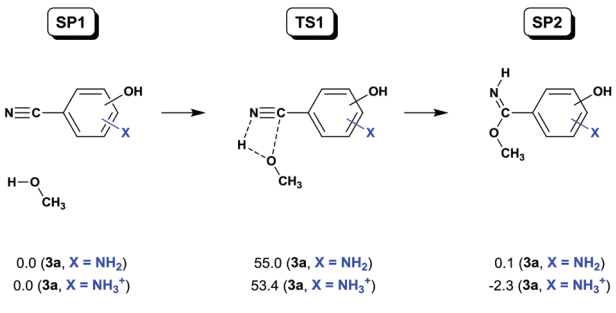


Fig. 2 Reaction mechanism involving neutral and monoprotonated **3a** with methanol. Numbers indicate relative solution-phase Gibbs free energies obtained by the (SMD)/M06-2X/6-31+G(d) model (in kcal mol^{-1}).

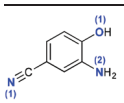
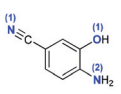
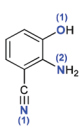
the reactive complex **SP1**, where methanol is aligned for the nucleophilic attack on the cyano C-atom, with the relevant O(methanol)–C_{cyano} (**3a**) bond distance of 3.31 Å. In the transition state **TS1**, the mentioned bond is reduced to 1.60 Å, while the vibration leading to products ($1871i$ cm^{-1}) involves both the O–C bond formation and the proton transfer from the methanol –OH group to the cyano nitrogen. This immediately gives the imidate product, with thermodynamic parameters suggesting a likely process ($\Delta_r G$ = 0.1 kcal mol^{-1}). Still, the reaction has a very high activation barrier of $\Delta G^{\ddagger}$ = 55.0 kcal mol^{-1}, which renders it unfeasible under these conditions. In other words, in neutral derivatives, the matching –CN group does not possess enough electrophilicity to allow the Pinner reaction.

Given that experiments are performed in excess gaseous HCl that allows sequential protonation in solution, we proceeded by inspecting the relative stabilities of various protonation forms of **3a–3c** (Table 4), while evaluating their influence on the Pinner reaction outcomes. In all systems, regardless of the solvent, the first protonation preferentially occurs on the amino nitrogen (N2), being consistent with the typically higher basicity of anilines relative to both phenols and aromatic nitriles.[32] The difference between N2 protonation and other sites is the highest in **3a**, where the –CN group is in the *meta*-position to the protonated amine, and where it least affects its basicity. Specifically, in **3b** and **3c**, the –CN group is at the *para*- and *ortho*-positions, where it lowers the aniline basicity *via* resonance participation,[33] and thus reduces the energy difference between different protonation sites.

Building on that, we analysed the mechanistic aspects of the Pinner reaction starting from the monocationic $\mathbf{3a}^{+}$ (Fig. 2), and again taking MeOH as a typical case. The reaction proceeds through a similar transition state **TS1** and, again, gives the imidate product **SP2** in a single step. Here, the reaction becomes exergonic ($\Delta_r G$ = –2.3 kcal mol^{-1}); however the process is kinetically still very much unfeasible given only a modest reduction in the activation barrier to $\Delta G^{\ddagger}$ = 53.4 kcal mol^{-1}. This indicates that even the protonation of the adjacent amine does not improve the electrophilicity of the –CN moiety to make it susceptible to the nucleophilic approach of MeOH.

The second protonation occurs exclusively on the cyano nitrogen (Table 4) and gives dications $\mathbf{3a}^{++}$–$\mathbf{3c}^{++}$. It turns out that this process activates the –CN group, in line with the literature reports on acidic conditions as an important prerequisite for the Pinner reaction.[34,35] The reactive complexes feature shorter O(solvent)–C_{cyano}($\mathbf{3}^{++}$) bonds, being 2.85, 2.79 and 2.80 Å for $\mathbf{3a}^{++}$–$\mathbf{3c}^{++}$ in methanol, respectively, while 2.95, 2.82 and 2.70 Å in the same order in 2-methoxyethanol. These are shortest in $\mathbf{3c}^{++}$, since the neighbouring *ortho*-NH_3^{+} group promotes the hydrogen bond formation with the solvent (Fig. S7†), which brings the latter closer to the reacting cyano moiety. Yet, it is precisely this hydrogen bonding motif in $\mathbf{3c}^{++}$ that will somewhat hinder an efficient solvent approach towards the –CN group, as the former needs to overcome this stabilizing interaction to initiate the Pinner reaction. Indeed, Fig. 3 shows that the formation of the O(solvent)–C_{cyano}($\mathbf{3}^{++}$) bond occurs through a transition state **TS1** requiring 7.5–8.8 kcal mol^{-1} in MeOH and 7.1–8.5 kcal mol^{-1} in 2-methoxyethanol, being always the highest for $\mathbf{3c}^{++}$. It is interesting to note that the reactivity for this step follows the trend $\mathbf{3b}^{++}$ > $\mathbf{3a}^{++}$ > $\mathbf{3c}^{++}$ in both solvents, while the reaction is kinetically always more feasible in 2-methoxyethanol. This process offers an intermediate with the formed O(solvent)–C_{cyano} bond and the reaction free energies around neutrality. In MeOH, this first step is most favourable for $\mathbf{3b}^{++}$, while in 2-methoxyetha-

Table 4 Relative stability of different protonation forms of **3a–3c** in methanol and 2-methoxyethanol solutions (all values in kcal mol^{-1}). Third protonation always occurs on the hydroxyl oxygen atom **O1**

		Methanol			2-Methoxyethanol		
System	Protonation step	**N1**	**N2**	**O1**	**N1**	**N2**	**O1**
 **3a**	1st protonation (**3a** → $\mathbf{3a}^{+}$)	22.4	**0.0**	30.5	21.4	**0.0**	32.3
	2nd protonation ($\mathbf{3a}^{+}$ → $\mathbf{3a}^{++}$)	**0.0**		14.0	**0.0**		18.0
 **3b**	1st protonation (**3b** → $\mathbf{3b}^{+}$)	17.4	**0.0**	26.6	16.7	**0.0**	28.6
	2nd protonation ($\mathbf{3b}^{+}$ → $\mathbf{3b}^{++}$)	**0.0**		11.0	**0.0**		15.6
 **3c**	1st protonation (**3c** → $\mathbf{3c}^{+}$)	17.7	**0.0**	24.5	17.2	**0.0**	26.5
	2nd protonation ($\mathbf{3c}^{+}$ → $\mathbf{3c}^{++}$)	**0.0**		5.2	**0.0**		8.8

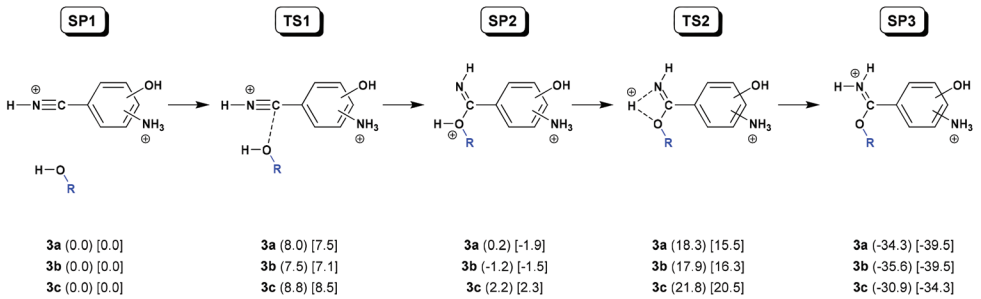


Fig. 3 Reaction mechanism involving diprotonated $\mathbf{3a}^{++}$–$\mathbf{3c}^{++}$ with methanol (in round brackets, R = $-CH_3$) and 2-methoxyethanol (in square brackets, R = $-CH_2CH_2OCH_3$). Numbers indicate relative solution-phase Gibbs free energies obtained by the (SMD)/M06-2X/6-31+G(d) model (in kcal mol^{-1}).

nol, it is for $\mathbf{3a}^{++}$, with a consistent conclusion that the reaction is thermodynamically again least favourable for $\mathbf{3c}^{++}$.

The next step involves the 1,3-proton transfer from the solvent –OH group to the imino N-atom (Fig. 3). Interestingly, this step is kinetically more demanding than the previous one and defines the rate-limiting part of the overall transformation. Still, the process is more feasible by 2–3 kcal mol^{-1} for $\mathbf{3a}^{++}$ and $\mathbf{3b}^{++}$ than for $\mathbf{3c}^{++}$, being in excellent agreement with experiments. Moreover, the obtained results suggest that the reaction proceeds faster in 2-methoxyethanol, which again agrees with experiments. For the latter, we did not observe any notable structural or electronic effect that would help in interpreting the higher reactivity in 2-methoxyethanol over methanol. What likely dominates is a markedly lower dielectric constant of 2-methoxyethanol (ε = 17.2) relative to methanol (ε = 32.6), which likely favours a less polar transition state **TS2** with a delocalized charge, compared to the preceding **SP2** (Fig. 3) which, then, lowers the barrier. The latter stationary point has a more localized and exposed positive charge, thus being more favoured in a more polar MeOH. Such a notion could potentially suggest that alcohols with further lower polarities would provide even more efficient environments for the reaction; yet this has to be considered with certain care, since the utilization of other solvents could exert some structural or electronic effects that would predominate over a simple solvent polarity effect, and, perhaps, reveal different reactivity trends.

Following **TS2**, the reaction offers the desired imidates that are considerably more stable than the initial reactants, thus making the overall reactions highly exergonic and feasible. This is again by 4–5 kcal mol^{-1} more evident in 2-methoxyethanol, which also contributes towards making it a better solvent over methanol, in agreement with experiments.

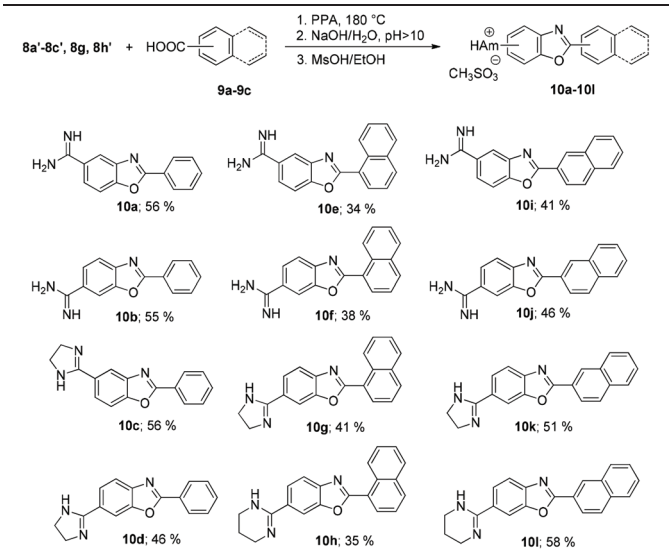
In concluding this section, computations consistently predict a higher reactivity of **3a** and **3b** over **3c** in both solvents, while confirming 2-methoxyethanol as a better environment for the Pinner reaction, likely due to its lower dielectric constant, particularly suitable for a non-polar transition state during the rate-limiting step. To further validate these conclusions, we performed several additional calculations. Given that 2-methoxyethanol also contains a secondary O-atom, we inspected its nucleophilicity towards the –CN group to exclude the possibility of its participation in the reaction. For $\mathbf{3a}^{++}$, this is possible, but the activation barrier is by 0.9 kcal mol^{-1} higher and the reaction free energy is by 4.8 kcal mol^{-1} less exergonic than for the primary hydroxyl O-atom. Even more so, such a route gives an intermediate that hinders further reactions, making this pathway very unlikely. Also, knowing that HCl is used in excess, we studied the reaction of the tricationic $\mathbf{3a}^{+++}$, with the protonated –OH group, in MeOH. The reaction is thermodynamically more favourable than for $\mathbf{3a}^{++}$ by 2.8 kcal mol^{-1}, but is kinetically less feasible by 2.0 kcal mol^{-1}, which likely dominates, or might also contribute towards high reaction yields.

Lastly, given that our analysis was based on inspecting geometric and electronic parameters in the implicit solvation, we also considered a potential effect of the bulk solvation on the reaction outcomes. For that, we performed a set of classical MD simulations with $\mathbf{3a}^{++}$ and $\mathbf{3c}^{++}$ in explicit solvents. The obtained RDF graphs for the corresponding O(solvent)–C_{cyano}($\mathbf{3}^{++}$) bonds (Fig. S8†) show that, in both cases, 2-methoxyethanol solution allows for a closer solvent approach towards the reactive –CN group, which is likely an additional effect contributing towards its higher reactivity over MeOH. Moreover, the obtained peaks in the RDF graphs have the first maximum around 3 Å, which puts our **SP1** geometries presented earlier in good qualitative agreement with these results, thus further confirming the validity of mechanistic calculations.

Synthesis and antiproliferative activity of amidino-substituted 2-arylbenzoxazoles

In order to explore the biological potential of 2-arylbenzoxazoles functionalized with amidines, we performed the synthesis of a new small library of systems. Previously, we developed robust methods for the preparation of a number of 6-amidino-2-aryl/heteroaryl-benzothiazole derivatives by merging 5-amidino-substituted 2-aminothiophenols with carboxylic acids in polyphosphoric acid (PPA).[13,14,18] This method is commonly used for condensation of 2-aminophenols with carboxylic acids, in which cyclization and formation of a benzoxazole ring occur.[36] Thus, novel amidino-substituted 2-arylbenzoxazoles **10a–10l** were prepared from amidino-substituted

Table 5 Synthesis of amidino-substituted 2-arylbenzoxazoles



2-aminophenols **8a′–8c′**, **8g** and **8h′** and aromatic carboxylic acids **9a–9c** in PPA (Table 5). Since it was previously determined that methanesulfonates of amidine derivatives of 2-phenylbenzothiazoles were more stable and soluble in water than chlorides,[16] all amidine benzoxazole derivatives were isolated in the form of methanesulfonates by a simple two-step procedure in moderate overall yields.

Antiproliferative activity was tested *in vitro* on four human tumour cell lines of metastatic colorectal adenocarcinoma (SW620), hepatocellular liver cancer (HepG2), ductal pancreatic adenocarcinoma (CFPAC-1), and cervical cancer (HeLa) by MTT assay. The results are expressed as concentrations of compounds required to inhibit cell growth by 50% (IC_{50}) in Table 6.

Table 6 Antiproliferative activity *in vitro* of amidino-substituted 2-arylbenzoxazole methanesulphonates. 5-Fluorouracil (5-FU) was used as a standard chemotherapeutic compound (internal laboratory control)

	IC_{50}[a] (μM)			
Compound	SW620	HepG2	CFPAC-1	HeLa
10a	25 ± 6.7	25 ± 10	49 ± 1.7	27 ± 6.8
10b	26 ± 6.2	21 ± 10	25 ± 4.2	16 ± 7.6
10c	4.6 ± 1.5	19 ± 1.9	22 ± 0.59	9.1 ± 0.21
10d	8.3 ± 1.6	18 ± 0.24	24 ± 12	9.1 ± 0.37
10e	3.2 ± 0.65	2.7 ± 0.42	4.7 ± 0.41	3.2 ± 1.8
10f	4.3 ± 0.02	3.0 ± 1.4	5.2 ± 0.54	2.7 ± 0.27
10g	0.82 ± 0.58	1.6 ± 0.15	2.8 ± 1.1	0.88 ± 0.37
10h	0.16 ± 0.19	3.5 ± 0.04	4.5 ± 3.3	3.7 ± 0.41
10i	2.8 ± 0.16	2.2 ± 0.98	3.7 ± 0.37	2.2 ± 0.72
10j	2.0 ± 0.81	2.2 ± 1.3	3.4 ± 0.26	2.1 ± 0.63
10k	0.22 ± 0.12	1.4 ± 0.26	1.5 ± 0.72	0.50 ± 0.06
10l	0.18 ± 0.11	1.8 ± 0.73	3.4 ± 1.0	2.1 ± 0.53
5-FU	6.4 ± 0.22	55.22 ± 15.0	6.45 ± 0.66	47.52 ± 1.93

[a] IC_{50} values are concentrations that cause 50% inhibition of cancer cell growth.

Amidino-substituted 2-arylbenzoxazoles **10a–10l** showed strong antiproliferative activity on all tested cell lines at micromolar concentrations, stronger than or comparable to a standard chemotherapeutic 5-fluorouracil. None of the tested systems revealed a significant selectivity towards particular cell lines. The highest activity was observed for **10h**, with a pyrimidinium amidinic substituent at the C-6 position of the benzoxazole skeleton, toward metastatic colorectal adenocarcinoma (SW620) cells with an IC_{50} value of 0.16 μM. In general, benzoxazole **10g** with an imidazolinium amidinic substituent at position C-6 and substituted with naphthalene at position C-2 proved to be the most active on all tested cell lines (IC_{50} = 0.82–2.8 μM). On the other hand, 2-arylbenzoxazoles with the unsubstituted amidinium moiety at the C-5 and C-6 positions had the weakest activity. Comparing the C-5 and C-6 positions of the corresponding amidine substituents on the benzoxazole backbone between derivatives **10a** and **10b**, **10c** and **10d**, **10e** and **10f**, and **10i** and **10j**, no difference in position-dependent antiproliferative activity was observed.

Conclusions

In summary, we developed an efficient synthetic protocol for isomeric amidino-substituted 2-aminophenols from accessible cyano-substituted 2-aminophenols by the Pinner reaction. The key step during the synthesis of the crucial carboximidate intermediate was characterized through mechanistic DFT calculations, which offered insight into the electronic and geometrical aspects determining the higher reactivity of **3a** and **3b** over **3c**, while confirming 2-methoxyethanol as a better solvent over methanol. It also justified the necessity to perform the reaction under excess gaseous HCl, thus affording substrate dications with the protonated cyano group, which allows the conversion into the desired imidates.

X-ray crystal structure study shows that amidino-substituted aminophenols **8a′**, **8b′** and **8c′** crystallized in a pure zwitterionic form, while **8a** and **8b** exist in a zwitterionic form as hemihydrochlorides. On the other side, amidino-substituted derivatives **8d** and **8h** consist of one independent cation and one chloride anion and did not crystallize as zwitterions, as well as two dications, **8e** and **8g**, which contain two methanesulfonate and chloride anions, respectively.

The applicability of the described 2-aminophenols as new building blocks for the preparation of 2-substituted-benzoxazoles by a simple condensation reaction was demonstrated through the synthesis of a small library of amidino-substituted 2-arylbenzoxazoles. Their antiproliferative activities against four human tumour cell lines in micro- and submicro-molar concentrations revealed the biological potential of this thus far vaguely studied class of compounds. The synthesis of a larger library of amidino-functionalized mono- and bis-2-substituted-benzoxazoles by the presented method, together with the evaluation of their antitumor, antitrypanosomal and DNA/RNA binding activities, is currently in progress, and the results will be published in due course.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We greatly appreciate the financial support of the Croatian Science Foundation under the project HRZZ-IP-2018-01-4379 entitled "Exploring the antioxidative potential of benzazole scaffold in the design of novel antitumor agents" and University of Rijeka research grant UNIRI-biomed-18-133. L.H. wishes to thank the Croatian Science Foundation for a doctoral stipend through the Career Development Project for Young Researchers. L.H. and R.V. would like to thank the Zagreb University Computing Centre (SRCE) for granting computational resources on the ISABELLA cluster.

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