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Bioreductively Activated Lysyl Oxidase Inhibitors against Hypoxic Tumours

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Most human cancers consist of solid tumours that are significantly less oxygenated than normal tissues. Once their dimensions reach certain values ($>1\text{ cm}^3$) they contain hypoxic regions generally surrounding necrotic areas. Hypoxic cells are normally resistant to anticancer chemotherapy and radiotherapy. Hypoxia has also been shown to predispose tumours to increased invasion and metastatic processes,[1] leading to poor prognosis.

Lysyl oxidase (LOX) is a copper-dependent amine oxidase, which catalyzes the oxidation of the ϵ -amino group of peptidyl lysine to peptidyl aldehyde, by consuming O_2 and water, and producing ammonia and hydrogen peroxide.[2] The catalytically active site of LOX is characterised by the presence of a Cu^{II} ion, bound to three His residues, and a unique covalently bound organic cofactor, lysine tyrosylquinone (LTQ). This cofactor differentiates LOX from most other known amine oxidases, which are usually associated with the cofactor trihydroxyphenylalanine quinone (TPQ). The catalytic cycle effected by LOX begins with the condensation of the LTQ cofactor with the primary amino group of the substrate (e.g. peptidyl lysine).[2]

The main function of LOX is the initiation of the covalent cross linking of collagen and elastin in extracellular matrices (ECM). LOX overexpression in hypoxic tumour cells has recently been determined and, at the same time, LOX has been proven to play a key role in the promotion of the metastatic process of several solid tumours.[3] In fact, a statistically significant correlation was found between LOX expression and hypoxic tumours in patients affected by breast and head-and-neck cancers. These results confer a clinical relevance on LOX as a potential marker for identifying highly invasive/metastatic and hypoxic tumour phenotypes, which cause the worse outcomes in terms of distant metastasis formation and overall patient survival. LOX mRNA expression was found to be regulated by hypoxia-inducible factor-1 (HIF-1), and its influence on metastasis and survival was assessed in vitro

and in vivo against MDA231 breast and SiHa cervical cancer cells. The results of these studies indicated that *inhibition of LOX activity can prevent, or even treat, metastasis*. [4a] Furthermore, in vitro MDA231 and SiHa cell invasion assays revealed that only *catalytically active LOX that is secreted into the extracellular matrix is responsible for the increased invasion in hypoxic cells*. [4a] LOX was also shown to be required for formation of cellular adhesion interactions, which are essential for cancer cell migration, through activation of focal adhesion kinase. More recently, a key role of LOX in the formation of the premetastatic niche was also demonstrated. [4b] Therefore, this easily accessible extracellular enzyme may constitute an innovative therapeutic target for the treatment and the prevention of metastatic cancer.

β -Aminopropionitrile (BAPN) has been extensively used as the reference LOX inhibitor since the early 1970s ($IC_{50} = 10 \mu M$). [5] The reduction of the metastatic colonisation potential of MDA231 breast cancer cells due to BAPN-promoted LOX inhibition was recently proved in vivo by bioluminescence imaging and microcomputed tomography. [6] Unfortunately, BAPN itself cannot be developed as a therapeutic agent because its extremely simple structure allows many unselective biological interactions, and it is therefore likely to cause numerous unwanted side effects. BAPN is also considered to be the main factor responsible for the syndrome called lathyrism, [7] a pathological condition seen in people and animals after excessive ingestion of sweet pea (*Lathyrus odoratus*), which contains significant amounts of BAPN. [8] Moreover, there are a limited number of LOX inhibitors described in the literature, mainly because only a few therapeutic uses have been envisaged so far. The majority of inhibitors are either primary amino- (BAPN, taurine, benzylamines, allylamines), or hydrazino- (isoniazide, semicarbazide, thiosemicarbazide) derivatives, [9] which are either very small unspecific bioactive compounds, or they possess features that are regarded as undesirable properties in drug candidates (reactive and/or toxic functional groups). [10] As a consequence, their development as antimetastasis drugs has been limited. Two patents have described a series of pyridazinone-based potent LOX inhibitors, [11] however, the systemic inhibition of LOX arising may lead to lathyrism because of the lack of collagen maturation in many organs likely to be targeted after administration in vivo.

In order to selectively deliver BAPN to hypoxic tumour cells, to block the LOX-induced promotion of metastasis invasion, we herein report the design of prodrugs capable of releasing BAPN selectively under hypoxic conditions. There are several masking groups known to be readily removed under hypoxic conditions by means of reductive processes promoted in the total or partial absence of oxygen. [12] Among them, nitroaromatic masking groups have already proved to be sensitive to bioreductive removal since they are efficiently activated by human cellular reductases, particularly when the cells possess low oxygen contents and are rich in reduced cofactors. For these reasons, nitroaryl precursors of active drugs can be included among some of the most efficient hypoxia-activated prodrugs. [13] Hence, we decided to develop a preliminary series of bioreductively activated nitroaromatic prodrugs of BAPN (pro-BAPNs **1–4**) by masking the pharmacophoric primary amino group of BAPN with representative nitroaryl moieties linked to the nitrogen atom by a methyl (**1**, **2**, and **4**) or a methoxycarbonyl (**3**) unit.

Nitro derivatives and, in particular, 5-nitrofurans were previously shown to present a considerable risk of genotoxicity.[14] Nevertheless, several marketed drugs, such as nimesulide and nitrofurantoin, possess these functionalities. In these cases it was also shown that the genotoxic effects found *in vitro* are often attenuated *in vivo* by metabolic detoxification mechanisms.[14] Moreover, the nitroaryl pro-BAPNs presented here are intended for the potential treatment of metastatic cancer, thus reducing the influence that long-term genotoxic effects might have on the risk–benefit balance, as confirmed by the large number of nitroaryl-based anticancer agents currently in clinical trials.[13]

In accordance to previously studied bioreductively activated nitroaryl prodrugs, their anticipated mechanism of activation begins with the transformation of the nitro group to an amine or hydroxylamine under hypoxic conditions.[13] This structural change weakens the bond between the masking portion and BAPN, leading to the facile release of active BAPN (Scheme 1), now able to exert its inhibitory action on LOX.

pro-BAPNs **1–4** were synthesised as shown in Scheme 2. Water-soluble amine hydrochloride salts **1**, **2**, and **4** were easily prepared by a condensation of the corresponding aldehyde precursors (**5**, **6**, or **7**, respectively) with an excess of BAPN to give the imine intermediates. The imine were then reduced with NaBH_4 , and final treatment with $\text{Et}_2\text{O}\cdot\text{HCl}$ gave the desired compounds. In the case of the nitrofurane analogue **4**, very slow addition of NaBH_4 and strict temperature control was required during the reduction step to avoid by-product formation arising through reduction of the furan ring. Carbamate derivative **3** was obtained in a single step by reacting *p*-nitrobenzylchloroformate **8** with an excess of BAPN in dichloromethane.

The ease of activation of bioreductive prodrugs has often been successfully associated with their reduction potential values.[13,15] Therefore, we carried out cyclic voltammetry analyses of compounds **1–4** in isosaline phosphate buffer solutions (pH 7.2) (Figure 1). All the compounds produced a single reduction peak when the potential was scanned from 0.0→−1.8 V (vs. Ag/AgCl), which arises as a result of the four-electron four-proton reduction of the nitro group to hydroxylamine. Nitrobenzyl derivatives **1–3** exhibited similar reduction potentials (E°) with values ranging from −0.77 for the *ortho*-nitro analogue **2** to −0.75 V for the two *para*-nitrobenzyl derivatives **1** and **3**. On the other hand, nitrofuranmethyl derivative **4** displayed an appreciably less negative E° value (−0.61 V), which should account for its increased susceptibility to reductive processes. The voltammetry graph (Figure 1) shows that these reduction peaks are irreversible. This could indicate the fast transformation of the molecule (release of BAPN) following NO_2/NHOH conversion.

Compounds **1–4** were then assessed biologically in LOX inhibition and cell invasion assays (Figure 2). The effectiveness of LOX inhibition was determined under normoxia or hypoxia by measuring the fluorescent activity in conditioned medium (CM) from hypoxic MDA-MB-231 breast cancer cells incubated with BAPN or test compounds (200 μM), as previously described.[16] The *ortho*-nitrobenzyl derivative **2** gave poorly reproducible results, possibly

due to its high photolability causing interference with the fluorescence-based enzyme assay. Therefore, these results were excluded. A first look at the results illustrated in Figure 2a shows that, as expected, compounds **1**, **3**, and **4** are much more active under hypoxic rather than normoxic conditions, thus confirming their activation in reducing environments. Of course, the reference inhibitor, BAPN, showed no significant differences in its activity between air and hypoxia, since it does not need any activation. Among the new pro-BAPNs, the best results were obtained for compounds **3** and **4**. Both compounds exhibited much greater inhibitory activities than BAPN itself selectively under hypoxia, whereas their activity in air is practically equal to the control and DMSO.

The method used for in vitro invasion analysis of breast cancer MDA-MB-231 cells has previously been described,[4a] and the results are shown in Figure 2b. Highly invasive breast cancer MDA-MB-231 cells becomes even more aggressive under oxygen deprivation, as confirmed by the control data, where the invasion under hypoxia (60%) is about twice as much as that in air (30 %). Here again, the best results were obtained with compounds **3** and **4**, both of which induced a six-fold reduction in cell invasion under hypoxic conditions (~10% invasion with **3** and **4** vs. 60% in control experiments). Among them, the most hypoxia-selective anti-invasive agent was pro-drug **4**. In fact, in contrast to nitrobenzyl carbamate **3**, which showed an anti-invasive action even under normoxic conditions, nitrofuranyl **4** did not change the control level of invasion in air (30 %), whereas it noticeably reduced the invasive process under hypoxic conditions. The nonselective anti-invasive effect associated with carbamate **3** suggests that this compound exerts an anti-invasive effect regardless of bioreductive activation, probably following a non-LOX-mediated mechanism. However, this requires further future investigations. Interestingly, the most active and hypoxia-selective compound (**4**) showed the least negative E° value (-0.61 V) when compared to the other active compounds in the series (**1** and **3**; $E^\circ = -0.75$ V for both of them). Although this database is too small to demonstrate a definitive correlation between the electrochemical reduction potentials (E°) of the compounds and their in vitro activity under hypoxic conditions, it appears evident that the hypoxia-enhanced activities displayed by compound **4**, both in LOX inhibition and cell invasion assays, may be associated to the ease of reductive activation of the nitroaryl masking portion, which turns out to be more straightforward with the nitrofuranyl moiety of **4** than with the nitrophenyl portions of **1** and **3**.

The promising biological activity of these compounds may also be attributed in part to the masking portions of **3** and **4**, which, after detaching from BAPN under hypoxic conditions, are likely to interact with LOX in a synergistic fashion with the active BAPN. In fact, literature reports have shown that bioreduction of the nitro group of nitrobenzyl and nitrofuranylmethyl prodrugs gives the corresponding hydroxylamine intermediates, which then undergo a spontaneous 1,6-elimination to release the active molecule, BAPN in this case, and corresponding short-lived electrophilic species **9** and **10** (Scheme 3).[13a] These unstable intermediates are highly reactive towards nucleophiles and might actually contribute to the enzyme inhibition.

Attempts to demonstrate this involvement of species **9** and **10**, derived from the masking portions, in a synergistic manner to LOX inhibition have so far failed. Model compounds such as commercially available *p*-nitrobenzyl and 5-nitrofuryl alcohols showed no significant effects in these assays, probably because of the different nature of the OH leaving group present in the model compounds, when compared to the carbamic or protonated amine moieties present in **3** and **4**, respectively. Nevertheless, future efforts to verify this hypothesis may be successful, for example, by MS characterisation of adducts of LOX with these elusive electrophilic species.

To the best of our knowledge, the results presented here indicate, for the first time, that a bioreductively activated inhibitor of a new tumour target (LOX) is able to exert a noticeable anti-invasive effect on a highly metastatic hypoxic cancer cell line. This research has remarkable potential since it may lead to the development of drugs against both early and late stage metastasis. Since the target (LOX) is extracellular, it is particularly accessible even to drugs that do not efficiently cross the cell membranes. This constitutes a further advantage, because higher concentrations of drugs in the tumour stroma are more likely to be reached than inside the cancer cell. Finally, unspecific side effects due to systemic inhibition of LOX should be limited by the use of prodrugs, such as compound **4**, that are designed to selectively deliver the LOX inhibitors to hypoxic tumour tissues. Further development of this class of prodrugs, both by optimisation of the masking groups or the use of different LOX inhibitors, are currently underway.

Experimental Section

All the chemicals were purchased from commercially available sources (Sigma–Aldrich, AlfaAesar) and used without further purifications. Electrochemical measurements of compounds **1–4** dissolved in isotonic PBS (pH 7.2) were performed using a Princeton Applied Research (PAR) 273A potentiostat/galvanostat employing PAR M270 electrochemical software, carried out in a three-electrode cell comprised of a glassy carbon disk electrode together with a counter electrode (Pt wire) and a reference electrode (Ag/AgCl). NMR spectra were obtained on a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) and adjusted according to residual solvent peaks. Electron impact (EI, 70 eV) mass spectra were obtained on a HP-5988A mass spectrometer. Chromatographic separations were performed on silica gel by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity columns (Kieselgel 60, 0.063–0.200 mm; Merck). Reactions were followed by thin-layer chromatography (TLC) on aluminium-backed silica gel sheets (60 F₂₅₄, Merck) visualised under a UV lamp.

3-(4-Nitrobenzylamino)propanenitrile hydrochloride (**1**)

A solution of 4-nitrobenzaldehyde (0.65 g, 4.3 mmol) in anhyd CH₂Cl₂ (30 mL) was treated with BAPN (0.95 g, 13 mmol) and stirred at RT overnight before being concentrated in vacuo. The crude product was redissolved in CH₃OH (30 mL) and treated slowly and cautiously with NaBH₄ (1.2 g, 32 mmol) at 0 °C under nitrogen. After 1 h the reaction was diluted with ice water and extracted with CH₂Cl₂. The combined organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The crude product was purified by flash chromatography (*n*-Hex/EtOAc, 4:6) affording 3-(4-nitrobenzylamino)propanenitrile (0.76 g, 3.7 mmol, 86% yield): *R*_f=0.18 (Hex/EtOAc, 4:6); ¹H NMR (200 MHz, CDCl₃): δ =2.55 (t, *J*=6.4 Hz, 2H), 2.95 (t, *J*=6.4 Hz, 2H), 3.96 (s, 2H), 7.53 (AA'XX', *J*_{AX}=8.8 Hz, *J*_{AA'/XX'}=2.1 Hz, 2H), 8.20 ppm (AA'XX', *J*_{AX}=8.6 Hz, *J*_{AA'/XX'}=2.1 Hz, 2H); ¹³C NMR (50 MHz, CDCl₃): δ =19.1, 44.6, 52.5, 118.6, 123.2, 123.9, 128.7, 147.2 ppm; MS (EI, 70 eV): *m/z*: 206 [*M*+H]⁺. This compound was dissolved in CH₃OH and treated with a saturated Et₂O·HCl solution. Compound **1** precipitated from the resulting mixture as a white solid (0.84 g, 3.5 mmol, 95% yield): ¹H NMR (200 MHz, D₂O): δ =3.06 (t, *J*=6.8 Hz, 2H), 3.53 (t, *J*=6.8 Hz, 2H), 4.47 (s, 2H), 7.75 (AA'XX', *J*_{AX}=8.8 Hz, *J*_{AA'/XX'}=2.2 Hz, 2H), 8.35 ppm (AA'XX', *J*_{AX}=8.8 Hz, *J*_{AA'/XX'}=2.3 Hz, 2H); ¹³C NMR (50 MHz, D₂O): δ =15.5, 43.3, 51.0, 118.0, 124.9, 131.5, 138.0, 149.0 ppm; *E*^o (vs. Ag/AgCl)= −0.75 V; Anal. calcd for C₁₀H₁₂ClN₃O₂·H₂O: C 46.25, H 5.43, N 16.18, found: C 45.93, H 5.53, N 16.39.

3-(2-Nitrobenzylamino)propanenitrile hydrochloride (2)

A solution of 2-nitrobenzaldehyde (0.33 g, 2.2 mmol) in anhyd CH_2Cl_2 (20 mL) was submitted to the same reaction procedure as described for **1**. Flash chromatography (*n*-Hex/EtOAc, 1:1) gave the intermediate 3-(2-nitrobenzylamino)propanenitrile (0.39 g, 1.9 mmol, 86% yield): $R_f=0.19$ (Hex/EtOAc, 1:1); ^1H NMR (200 MHz, CDCl_3): $\delta=2.54$ (t, $J=6.6$ Hz, 2 H), 2.96 (t, $J=6.6$ Hz, 2 H), 4.10 (s, 2 H), 7.44 (ddd, $J=8.0, 6.3, 2.5$ Hz, 1 H), 7.56–7.68 (m, 2 H), 7.96 ppm (dd, $J=7.8, 1.1$ Hz, 1H); ^{13}C NMR (50 MHz, CDCl_3): $\delta=19.0, 44.8, 50.2, 118.6, 123.1, 124.9, 128.3, 131.1, 133.4, 149.1$ ppm; MS (EI, 70 eV): m/z : 206 $[M+H]^+$. The intermediate was dissolved in CH_3OH and treated with a saturated $\text{Et}_2\text{O}\cdot\text{HCl}$ solution. Compound **2** precipitated from the resulting mixture as a white solid (0.39 g, 1.6 mmol, 85% yield): ^1H NMR (200 MHz, D_2O): $\delta=3.11$ (t, $J=6.9$ Hz, 2 H), 3.64 (t, $J=6.9$ Hz, 2H), 4.60 (s, 2H), 7.71–7.88 (m, 3 H), 8.32 ppm (dd, $J=8.1, 1.2$ Hz, 1H); ^{13}C NMR (50 MHz, D_2O): $\delta=15.1, 43.4, 49.3, 117.7, 125.7, 126.3, 131.9, 134.0, 135.5, 148.4$ ppm; E° (vs. Ag/AgCl) = -0.77 V; Anal. calcd for $\text{C}_{10}\text{H}_{12}\text{ClN}_3\text{O}_2\cdot 0.5\text{H}_2\text{O}$: C 47.91, H 5.23, N 16.76, found: C 48.15, H 5.32, O 16.87.

4-Nitrobenzyl 2-cyanoethylcarbamate (3)

A solution of 4-nitrobenzylchloroformate (1.95 g, 9.04 mmol) in anhyd CH_2Cl_2 (50 mL) was treated at RT under nitrogen with BAPN (1.9 g, 27 mmol). After 1 h at RT the reaction mixture was diluted with water and extracted with CH_2Cl_2 . The combined organic phase was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by flash chromatography (*n*-Hex/EtOAc, 4:6) to afford **3** as a white solid (2.0 g, 8.0 mmol, 89% yield): $R_f=0.26$ (Hex/EtOAc 4:6); ^1H NMR (200 MHz, CDCl_3): $\delta=2.64$ (t, $J=6.2$ Hz, 2 H), 3.50 (q, $J=6.2$ Hz, 2H), 5.22 (s, 2 H), 5.30 (br s, 1H), 7.51 (AA'XX', $J_{AX}=8.8$ Hz, $J_{AA'}/XX'=2.1$ Hz, 2 H), 8.23 ppm (AA'XX', $J_{AX}=8.8$ Hz, $J_{AA'}/XX'=2.2$ Hz, 2H); ^{13}C NMR (50 MHz, CDCl_3): $\delta=19.0, 37.5, 65.7, 117.9, 123.9, 128.3, 143.5, 147.8, 155.8$ ppm; MS (EI, 70 eV): m/z : 249 $[M]^+$; E° (vs. Ag/AgCl) = -0.75 V; Anal. calcd for $\text{C}_{11}\text{H}_{11}\text{N}_3\text{O}_4$: C 53.01, H 4.45, N 16.86, found: C 53.17, H 4.32, O 17.11.

3-[(5-Nitrofuranyl)methylamino]propanenitrile hydrochloride (4)

A solution of 5-nitrofuran-1-carbaldehyde (1.02 g, 7.23 mmol) in CH_2Cl_2 (50 mL) was submitted to the same reaction procedure as described for **1** (*CAUTION: in this case, it is extremely important to maintain the temperature below 0 °C during the addition of NaBH_4 , which must be very slow*). Flash chromatography (*n*-Hex/EtOAc 2:8) gave the intermediate 3-[(5-nitrofuran-2-yl)methylamino]propanenitrile (0.83 g, 4.3 mmol, 59% yield): $R_f=0.23$ (Hex/EtOAc 2:8); ^1H NMR (200 MHz, CDCl_3): $\delta=2.55$ (t, $J=6.5$ Hz, 2H), 2.98 (t, $J=6.5$ Hz, 2 H), 3.94 (s, 2H), 6.51 (d, $J=3.7$ Hz, 1H), 7.28 ppm (d, $J=3.7$ Hz, 1H); MS (EI, 70 eV): m/z : 196 $[M+H]^+$. This compound was dissolved in CH_3OH and treated with a saturated $\text{Et}_2\text{O}\cdot\text{HCl}$ solution. Compound **4** precipitated from the resulting mixture as a white solid (0.88 g, 3.8 mmol, 88% yield): ^1H NMR (200 MHz, D_2O): $\delta=3.09$ (t, $J=6.8$ Hz, 2 H), 3.57 (t, $J=6.8$ Hz, 2H), 4.57 (s, 2H), 7.02 (d, $J=3.8$ Hz, 1H), 7.59 ppm (d, $J=3.8$ Hz, 1H); ^{13}C NMR (50 MHz, D_2O): $\delta=15.2, 42.8, 43.3, 113.7, 116.6, 117.5, 147.7, 154.9$ ppm; E° (vs. Ag/AgCl) = -0.61 V; Anal. calcd for $\text{C}_8\text{H}_{10}\text{ClN}_3\text{O}_3\cdot 0.5\text{H}_2\text{O}$: C 39.93, H 4.61, N 17.46, found: C 39.64, H 4.82, O 17.34.

Cell culture and oxygen deprivation

Cell lines were obtained from ATCC and routinely cultured and oxygen deprived for 18 h as previously reported.[17] In brief, cells were maintained under hypoxic conditions (1–2% O_2) at 37°C within a modular incubator chamber filled with 5% CO_2 and 1–2% O_2 balanced with N_2 .

LOX activity assay

Following a previously described method,[4a,16] conditioned phenol-red-free medium (50 μ L) was taken from cells incubated for 24 h under hypoxic conditions. Samples were incubated overnight at 37°C with 200 μ M BAPN or compounds **1–4**. Fluorescent reading was set=0 for 500 μ M BAPN.

In vitro invasion analysis

The method used was as previously reported.[18] Briefly, cells were serum deprived for 24 h, then 2.5×10^4 cells were seeded in triplicate on both Matrigel-coated and uncoated inserts, moved to chambers containing 750 μ L of 10% FBS as a chemo-attractant and incubated under normoxic or oxygen-deprived conditions for 24 h. Treatment with 200 μ M LOX inhibitors (BAPN, **1**, **3**, or **4**) was performed 24 h before serum deprivation, and continued throughout the experiment.

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Figure 1. Cyclic voltammograms of compounds **1–4** (0.5 mM solutions) recorded at glassy carbon electrode in a 0.15M NaCl pH 7.2 phosphate buffer solution. Sweep rate=0.1 Vs⁻¹; **1**: (-----); **2**: (.....); **3**: (-.-.); **4**: (—).

Figure 2.

a) LOX inhibition (fluorescence) assay for LOX activity on CM from MDA-MB-231 cells incubated in air (□) or hypoxia (2% O₂; ■) with 200 μM LOX inhibitors. Results are the mean of 3 experiments ±SEM; b) cell (trans-well) invasion assay to test ability of compounds **1**, **3** and **4** to inhibit in vitro invasion of cells incubated in air (□) or hypoxia (2% O₂; ■) with 200 μM LOX inhibitors.

Scheme 1.
Mechanism of activation of pro-BAPNs **1–4** in hypoxic cancer cells.

Scheme 2.

Reagents and conditions: a) β -aminopropionitrile (3 equiv), anhyd CH_2Cl_2 , RT, 16 h; b) NaBH_4 (8 equiv), anhyd MeOH, 0 °C, 1 h; c) $\text{Et}_2\text{O}\cdot\text{HCl}$, anhyd MeOH, RT, 5 min, 85–95 %, 3 steps (89% in the case of **3**).

Scheme 3. Mechanism of formation of short-lived electrophilic species **9** and **10**.