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Aldehyde detection by chromogenic/fluorogenic oxime bond fragmentation †

Syed Salahuddin, Olivier Renaudet and Jean-Louis Reymond *

Department of Chemistry & Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland. E-mail: jean-louis.reymond@ioc.unibe.ch; Fax: + 41 31 631 80 57; Tel: 41 31 631 43 25

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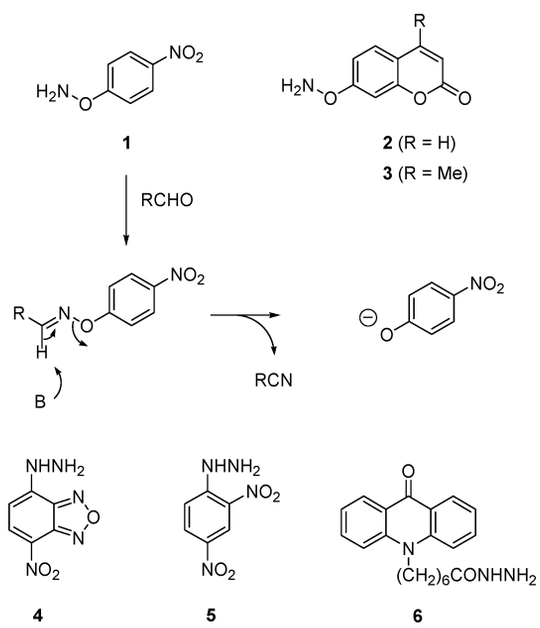
Amination of 4-nitrophenol, umbelliferone and 4-methylumbelliferone gave the corresponding oxyamines **1**–**3**. These oxyamines react with aldehydes and ketones to form oximes. In the case of aliphatic aldehydes and electron-poor aromatic aldehydes, the oximes undergo base-catalyzed fragmentation in aqueous buffer in the presence of bovine serum albumin to give the parent phenols, which is the acyclic analog of Kemp's elimination reaction of 5-nitrobenzisoxazole **28**. The process can be used as a spectrophotometric assay for formaldehyde under aqueous neutral conditions.

Introduction

Simple spectroscopic assays for the selective detection of small organic molecules are useful tools for high-throughput screening systems in chemistry and biology, such as assays for enzyme activities.[1] The analyte often displays a unique functional group that can be detected by relying on its specific chemical properties such as acidity,[2] metal chelating abilities,[3] or sensitivity to oxidizing agents.[4] This includes classical TLC-staining methods, such as visualizing amines with ninhydrin, fluorescamine, and 4-chloro-7-nitrobenzofurazane,[5] or phenols by diazonium coupling. Aldehydes and ketones are important functional groups that should be easily detectable selectively in biological media since the free carbonyl group is not present in proteins. Acetaldehyde can be detected by fluorescence in organic solvent upon reaction with 4-hydrazino-7-nitro-benzofurazane **4**,[6] or by an enzyme-coupled reaction with alcohol dehydrogenase in aqueous buffer.

Herein we report that oximes formed between aldehydes and the nitrophenol and umbelliferone derived oxyamines **1**–**3** undergo a chromo/fluorogenic fragmentation in aqueous buffer (Scheme 1). The reaction can be used as a simple assay for formaldehyde at neutral pH.



Scheme 1 Aldehyde detection *via* oxime fragmentation and tagging reagents for carbonyl compounds.

Results and discussion

In the course of developing high-throughput screening assays for enzymes, we were interested in finding a reagent to detect carbonyl products spectroscopically, in particular formaldehyde released upon hydrolysis of acyloxymethyl ethers by lipases in aqueous buffer.[7] 7-Hydrazino-4-nitro-benzofurazane **4** undergoes a fluorogenic hydrazone formation with acetaldehyde in organic solvent.[6] In our hands however, we did not detect any modulation of fluorescence in aqueous media. Other aromatic hydrazines such as dinitrophenyl hydrazine **5** and acridone **6**,[8] which can be used for tagging carbonyl groups, also did not produce any useful signal modulation in aqueous buffer in the presence of carbonyl compounds. We therefore set out to develop a specific fluorogenic or chromogenic reagent for the detection of carbonyl groups.

We decided to investigate oxyamines of nitrophenol or umbelliferones as carbonyl probes. Oxyamines generally react rapidly with carbonyl compounds to form stable oximes even at low concentration. The reaction is well known for the chemoselective ligations of various macromolecular fragments,[9] and can be used iteratively for oligomer synthesis.[10] In the case of nitrophenyl and umbelliferyl oximes, the strong acidity of nitrophenol and umbelliferone was expected to induce fragmentation of the oxime bond by β-elimination, leading to the liberation of the colored or fluorescent phenolate anion. Similar phenolate release processes induce a >20-fold increase in signal intensity and form the basis of many pH-indicators and fluorogenic and chromogenic enzyme substrates.

Phenyloxyamines are most efficiently prepared by direct *O*-amination of the corresponding phenols. Amination of nitrophenol, umbelliferone and 4-methylumbelliferone was realized by reacting the potassium phenolates with *O*-(mesitylenesulfonyl)-hydroxylamine ($MtsONH_2$) at 0 °C in DMF.[11] Although only modest yields were obtained, pure oxyamines were readily obtained by separation of the unreacted phenols by basic extraction. A series of oximes was prepared by reacting freshly prepared oxyamines with various aliphatic and aromatic aldehydes (Scheme 2). Oximes were formed exclusively as *E*-stereoisomers except with acetaldehyde, where both *E* and *Z* isomers were present.

† Electronic supplementary information (ESI) available: NMR spectra. See http://www.rsc.org/suppdata/ob/b4/b400314d/

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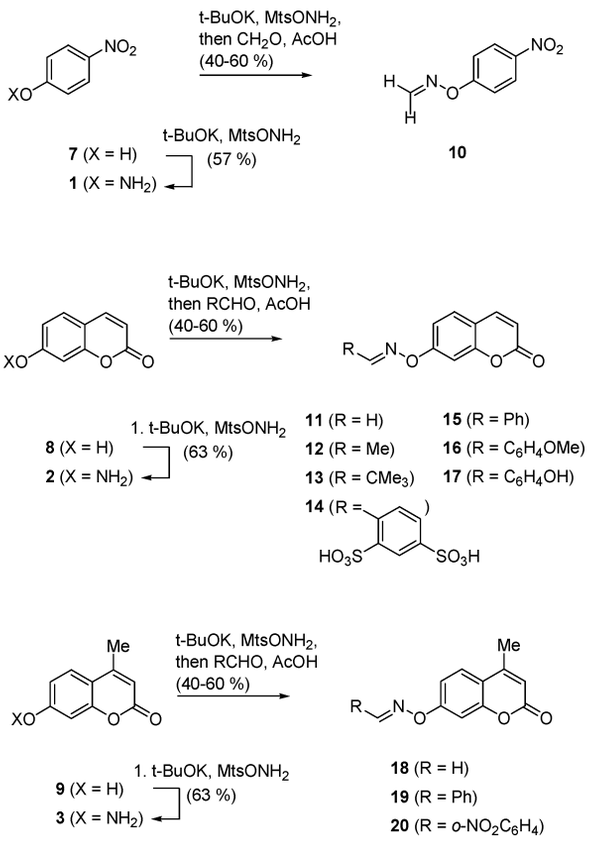


Scheme 2 Synthesis of chromo/fluorogenic oxyamines and oximes.

We next investigated the behaviour of oximes in aqueous buffer. All aldehyde oximes underwent a rapid decomposition at basic pH (>11) to the corresponding phenols and nitriles. The formation of umbelliferone and nitrophenol was confirmed by HPLC-analysis. The formation of nitrile was confirmed by HPLC and MS analysis in the case of benzaldehyde oximes **15** and **19** giving benzonitrile and with oxime **20** giving 2-nitrobenzonitrile. By contrast, the corresponding acetone oximes, which were readily formed from **1**–**3** and traces of acetone, were completely stable under basic conditions. At neutral pH all oximes were stable, however oximes of aliphatic aldehydes and of electron-poor aldehydes **14** and **20** underwent elimination in the presence of bovine serum albumin (BSA). The strongest reaction was observed with the formaldehyde oximes **10**, **11** and **18**, and oxime **14** derived from benzaldehyde-2,4-disulfonate. The fragmentation process can be interpreted as a β-elimination across the oxime double bond, which appears to be catalyzed by bases and by BSA.

The umbelliferyl oxime fragmentation reactions were accompanied by a strong increase of fluorescence. The nitrophenyloxyamine derivative **10** similarily underwent a chromogenic release of nitrophenol. These reactions might allow a direct spectrophotometric aldehyde assay if they could be coupled directly to the oxime bond formation reaction starting from the oxyamine as test reagent. We first tested the reaction using the stoichiometric reaction of oxyamine **2** with either formaldehyde, acetaldehyde, pivaldehyde or acetone at 100 μM each at neutral pH in the presence of BSA (0.2 mg mL^{-1}) as catalyst. While acetone and pivaldehyde gave no signal, the rate of phenol release was enhanced two-fold in the presence of acetaldehyde and ten-fold with formaldehyde. Nitrophenyloxyamine **1** gave similar results. A useful signal modulation as a function of formaldehyde concentration was observed when incubating the oxyamine at neutral pH in the presence of BSA (Fig. 1).

Several procedures were explored to improve the assay. Since oximes are rapidly formed in acidic medium, but decompose at basic pH, we attempted to generate a signal using the following

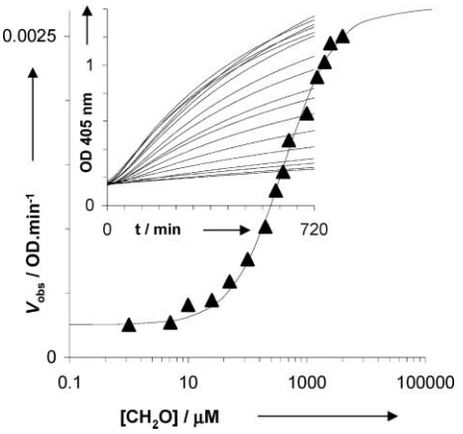
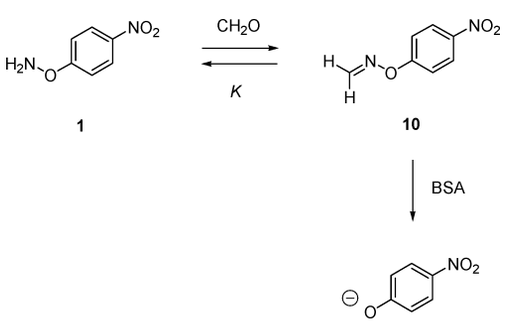


Fig. 1 Formaldehyde assay using nitrophenyloxyamine **1**. Conditions: formaldehyde (1 μM to 4 mM) in aq. 20 mM BisTris buffer pH 7.2, 100 μM **1**, 0.2 mg mL^{-1} BSA, 25 °C. Absorbance changes at λ = 405 nm were followed for each concentration assay (0.1 mL each) in half-area clear bottom polystyrene 96-well microtiter plates using a SpectraMAX 250 microtiterplate reader. The line was calculated from the double-reciproqual fitting in Fig. 2. Insert: OD time profiles. Plot: maximum rate of OD increase as a function of formaldehyde concentration.

sequence: 1) addition of nitrophenyloxyamine **1** (100 μM) to an aqueous solution of aldehyde (2 mM); 2) addition of acetic acid to induce oxime formation; 3) addition of a strong base (NaOH, Na_2CO_3 or ethanolamine) to trigger chromogenic decomposition of the oxime to nitrophenolate. Under these conditions decomposition was immediate in all samples (acetaldehyde, pivaldehyde, benzaldehyde, anisaldehyde) after addition of base. However, the unreacted oxyamine **1** also rapidly decomposed to nitrophenolate. The sequence was therefore modified by adding excess acetone after step 2) to convert unreacted oxyamine to a stable acetone oxime before addition of base. The procedure was successful for the titration of formaldehyde, and gave results similar to those obtained with the neutral BSA-catalyzed procedure described above. All other aldehydes failed to produce any signal upon addition of base. In the same manner, treatment with excess formaldehyde after step 2) induced decomposition of all test samples upon basification.

The observations above suggest that the nitrophenyloximes exist in equilibrium in aqueous solution, which shifts rapidly either to the stable acetone oxime in the presence of excess acetone, or to the base-labile formaldehyde oxime in the presence of formaldehyde (Scheme 3).



Scheme 3 Preequilibrium formation of oxime **10** and BSA-catalyzed decomposition.

The existence of a dynamic oxime-formation equilibrium is supported by the response curve of oxyamine **1** to formaldehyde (Fig. 2). Indeed the rate of nitrophenyl release follows the Michaelis–Menten equation ($1/V$ is linear *vs.* $1/[CH_2O]$), which can be interpreted in terms of a rapid formaldehyde oxime preequilibrium followed by a slower decomposition catalyzed by BSA. Oxime bond formation may or may not be catalyzed by BSA, in any event the observed equilibrium constant (K = 400 μM) corresponds to the formaldehyde concentration

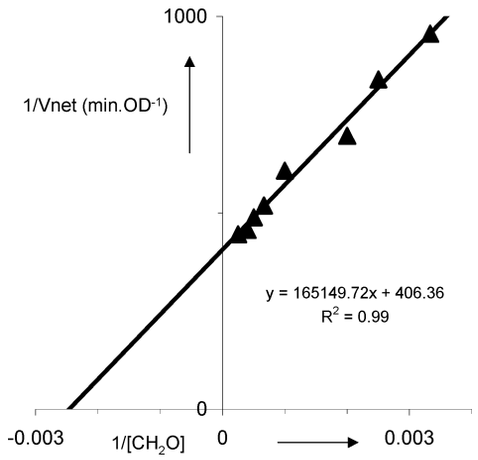


Fig. 2 Double reciproqual plot of the rate of nitrophenol release as $1/V_{net}$ as a function of formaldehyde concentration as 1/[CH_2O]. Conditions: formaldehyde (300 μM to 4 mM), aq. 20 mM BisTris buffer pH 7.2, 100 μM **1**, 0.2 mg mL^{-1} BSA, 25 °C.

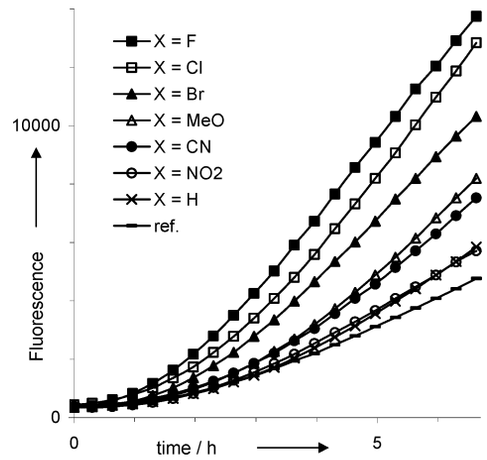
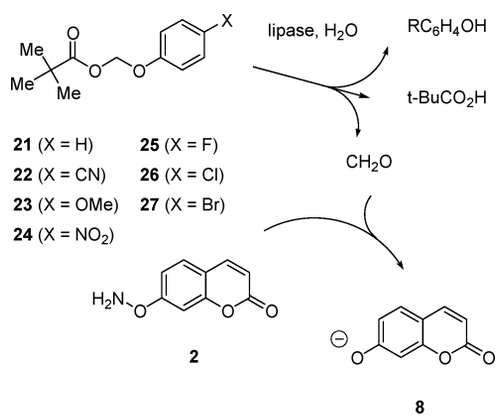


Fig. 3 Lipase assay for POM-ether hydrolysis using oxyamine **2** for formaldehyde detection. Conditions: 100 μM **2**, 100 μM POM-ether (**21**–**27**), 50 μg mL^{-1} *Pseudomonas sp. B* lipoprotein lipase (Fluka 62336), 0.5 mg mL^{-1} BSA, 20 mM aq. BisTris buffer pH 7.2, 5 % v/v DMF, 30 °C. Assays (0.1 mL) were followed in individual wells of round-bottom polypropylene 96-well-plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters λ_{ex} = 360 ± 20, λ_{em} = 460 ± 20 nm).

inducing 50% of signal modulation. This value is clearly above the total concentration of oxyamine used in the assay (100 μM), implying that oxime bond formation is not quantitative under the conditions of the assay. The value K = 400 μM can be interpreted in terms of an equilibrium constant of oxime bond formation, and compared with the known equilibrium constant for imine-bond formation between aliphatic amines and aldehydes in aqueous solution, which is in the range of K = 10 mM.[12] This implies that nitrophenyl oximes have stability constants similar to aldehyde imines and exist in a rapid equilibrium with their constituents. These results are consistent with other studies directed at dynamic combinatorial equilibria with hydrazone derivatives.[13]

We also attempted to use our assay to detect the formaldehyde released by lipase-catalyzed hydrolysis of pivaloyloxymethyl (POM) ethers **21**–**27** of various non-fluorescent phenols (Scheme 4) using the fluorogenic oxyamine **2** and BSA to catalyze the formaldehyde oxime decomposition. A time-dependent fluorescence increase was observed over a course of hours, a time-scale typical for the slow hydrolysis of such substrates with lipases (Fig. 3).

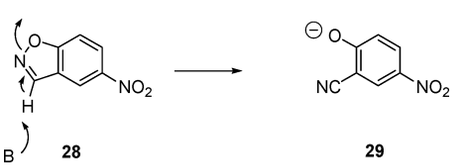


Scheme 4 Fluorescence detection of formaldehyde from lipase hydrolysis.

As above, the assay was limited by a significant spontaneous decomposition of oxyamine **2** in the absence of aldehyde, resulting in a significant background signal and a relatively low signal to background ratio.

Conclusion

A series of new fluorogenic and chromogenic oxyamine reagents was prepared by amination of nitrophenol and umbelliferones. The oxyamines formed oximes with carbonyl compounds in organic solvent. In water, the oximes undergo chromogenic or fluorogenic fragmentation under basic conditions. The process provides an assay for formaldehyde in water. The overall process is similar to that occuring in the one-pot conversion of aldehydes to nitriles using aminomethanesulfonic acid.[14] The acceleration effect of BSA observed at neutral pH for the fragmentation of oximes of aliphatic and electron-poor aromatic aldehydes must be highlighted. Indeed, BSA is well known to also catalyze the so-called Kemp's-elimination of 5-nitrobenzisoxazole **28** to form the yellow nitrophenolate **29** (Scheme 5), a reaction which has attracted enormous interest as a simple model for carbon deprotonation processes.[15] The fragmentation of our chromogenic and fluorogenic oximes by β-elimination represents the exact acyclic analog of Kemp's elimination. These oximes can be structurally varied at will by simply changing the aldehyde, and they may therefore prove useful as simple, structurally easily modulable spectroscopic probes for studying carbon-deprotonation processes in aqueous environment.



Scheme 5 Kemp's elimination is the cyclic analog of oxime fragmentation.

Experimental

All reactions were followed by TLC on Alugram SIL G/UV_{254} silica gel sheets (Macherey-Nagel) with detection by UV or with 0.5% phosphomolybdic acid solution in 95% EtOH. Silica gel 60 (Macherey-Nagel 230–400 mesh) was used for flash chromatography. Melting points were determined on a Kofler apparatus or with a Büchi 510 apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-300 spectrometer.

O-(4-Nitrophenyl) hydroxylamine 1

The following procedure is typical: potassium *tert*-butoxide (81 mg, 0.72 mmol) was added to a solution of nitrophenol **7** (100 mg, 0.72 mmol) in methanol (25 mL). After evaporation of the methanol under vacuum, the resulting yellow solid was dissolved in DMF (10 mL). *O*-Mesitylenesulfonylhydroxylamine

(154 mg, 0.72 mmol) was added and the solution stirred for 30 min at 0 °C. The mixture was then taken up with ethyl acetate (50 mL) and the organic layer washed with saturated solution of 1 M NaOH (2 × 100 mL) and water (2 × 100 mL). The organic layer was dried with anhydrous sodium sulfate and evaporated under vacuum to give **1** (72 mg, 57%) as a yellow solid. Mp: 122 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.10 (d, 2 H, J = 7.4 Hz), 7.18 (d, 2 H, J = 7.4 Hz), 5.97 (s, 2 H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 166.9, 139.6, 126.1, 115.7; ESI-HRMS: calcd. for $C_6H_7N_2O_3$ $[M + H]^+$: 155.0457; found: 155.0456.

7-(Aminooxy)-2*H*-chromen-2-one 2

The same procedure as above starting with umbelliferone **8** (100 mg, 0.62 mmol) gave **2** (69 mg, 63% yield) as a white solid. Mp 165–166 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.98 (d, 1 H, J = 9.4 Hz), 7.58 (d, 1 H, J =8.6 Hz), 7.25 (s, 2 H), 7.12 (d, 1 H, J = 2.3 Hz), 7.02 (dd, 1 H, J = 8.6, 2.3 Hz), 6.25 (d, 1 H, J = 9.4 Hz); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 165.4, 160.7, 155.6, 144.7, 129.6, 112.5, 111.2, 100.4; ESI-HRMS: calcd. for $C_9H_8NO_3$ $[M + H]^+$: 178.0504; found: 178.0504.

7-(Aminooxy)-4-methyl-2*H*-chromen-2-one 3

The same procedure as above starting with 4-methylumbelliferone **9** (100 mg, 0.57 mmol) gave **3** (63 mg, 58% yield) as a brown solid. Mp: 170 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.65 (br d, 1 H, J = 8.7 Hz), 7.25 (s, 2 H), 7.12 (d, 1 H, J = 2.2 Hz), 7.06 (dd, 1 H, J = 8.7, 2.2 Hz), 6.20 (br s, 1 H), 2.40 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 164.9, 160.2, 154.6, 153.4, 126.2, 112.9, 110.8, 110.5, 100.0, 18.1; EI-HRMS: calcd. for $C_{10}H_9NO_3$ $[M]^+$: 191.058243; found: 191.058170.

4-Methyleneaminooxynitrophenol 10

The following procedure is typical: potassium *tert*-butoxide (81 mg, 0.72 mmol) was added to a solution of 4-nitrophenol **7** (100 mg, 0.72 mmol) in methanol (25 mL). After evaporation of the methanol under vacuum, the resulting yellow solid was dissolved in DMF (10 mL). *O*-Mesitylenesulfonylhydroxylamine (154 mg, 0.72 mmol) was added and the solution stirred for 30 min at 0 °C. The mixture was then taken up with ethyl acetate (50 mL) and the organic layer washed with water (2 × 100 mL) and dried with anhydrous sodium sulfate. A few drops of acetic acid and an aqueous solution of formaldehyde (21 mg, 0.72 mmol) were added to the organic layer. After complete evaporation of ethyl acetate the resulting solid was purified by silica gel chromatography (3 : 2 hexane : ethyl acetate) to give **10** (43 mg, 36%) as a white solid. Mp: 74–75 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.23 (d, 2 H, J = 9.3 Hz), 7.45 (d, 1 H, J = 7.1 Hz), 7.25 (d, 2 H, J = 9.3 Hz), 6.92 (d, 1 H, J = 7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 163.6, 142.3, 131.6, 125.8, 114.2; EI-HRMS: calcd. for $C_7H_6N_2O_3$ $[M]^+$: 166.037842; found: 166.037980.

7-[(Methyleneamino)oxy]-2*H*-chromen-2-one 11

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and an aqueous solution of formaldehyde (18 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **11** (56 mg, 48%) as a white solid. Mp: 128-129 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.15 (d, 1 H, J = 9.3 Hz), 7.83 (d, 1 H, J = 9.1 Hz), 7.75 (d, 1 H, J = 5.8 Hz), 7.39 (d, 1 H, J = 5.8 Hz), 7.27–7.24 (m, 2 H), 6.48 (d, 1 H, J = 9.3 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 161.6, 160.4, 155.3, 145.3, 144.5, 130.2, 114.2, 113.8, 111.4, 101.6; EI-HRMS: calcd. for $C_{10}H_7NO_3$ $[M]^+$: 189.042593; found 189.042600.

Acetaldehyde *O*-(2-oxo-2*H*-chromen-7-yl) oxime 12

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and acetaldehyde (27 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **12** (75 mg, 60%) as a white solid. Mp: 96–97 °C; ^{1}H NMR (300 MHz, DMSO-d_6): for *E* isomer: δ = 8.04–8.00 (m, 1 H), 7.69 (d, 1 H, J = 9.3 Hz), 7.45 (q, 1 H, J = 5.5 Hz), 7.17–7.09 (m, 2 H), 6.35 (d, 1 H, J = 9.3 Hz), 2.05 (d, 3 H, J = 5.5 Hz); for *Z* isomer: 8.04–8.00 (m), 7.68 (d, J = 9.3 Hz), 7.17–7-09 (m), 6.33 (d, J = 9.3 Hz), 2.01 (d, J = 5.5 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) for *Z* and *E* isomers: δ = 161.5, 160.1, 155.0, 155.0, 154.4, 153.4, 144.2, 129.7, 129.6, 113.5, 113.3, 113.2, 113.0, 111.3, 110.9, 101.3, 100.9, 14.9, 12.5; EI-HRMS: calcd. for $C_{11}H_9NO_3$ $[M]^+$: 203.058243; found: 203.058750.

2,2-Dimethylpropanal *O*-(2-oxo-2*H*-chromen-7-yl) oxime 13

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and pivalaldehyde (53 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **13** (64 mg, 42%) as a white solid. Mp: 78 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.03 (d, 1 H, J = 9.4 Hz), 7.99 (s, 1 H), 7.69 (d, 1 H, J = 8.3 Hz), 7.14–7.09 (m, 3 H), 6.33 (d, 1 H, J = 9.4 Hz), 1.20 (s, 9 H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 164.0, 161.7, 160.1, 155.0, 144.2, 129.7, 113.4, 113.1, 111.0, 101.0, 34.2, 26.8; EI-HRMS: calcd. for $C_{14}H_{15}NO_3$ $[M]^+$: 245.105194; found: 245.105240.

4-((*E*)-{[(2-oxo-2*H*-chromen-7-yl) oxy]imino}methyl)benzene-1,3-disulfonic acid 14

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and benzaldehyde-2,4-disulfonic acid (191 mg, 0.62 mmol) followed by preparative RP-HPLC purification gave **14** (147 mg, 56%) as a brown solid. Mp: 77–78 °C. ^{1}H NMR (400 MHz, CD_3OD): δ = 9.41 (s, 1 H), 8.40 (dd, 1 H, J = 2.0, 0.5 Hz), 8.12 (d, 1 H, J = 8.0 Hz), 7.87 (ddd, 1 H, J = 8.0, 2.0, 0.5 Hz), 7.82 (d, 1 H, J = 9.3 Hz), 7.52 (d, 1 H, J = 8.6 Hz), 7.23 (d, 1 H, J = 2.3 Hz), 7.14 (dd, 1 H, J = 8.6, 2.3 Hz), 6.21 (d, 1 H, J = 9.3 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ = 166.3, 159.3, 155.8, 150.8, 148.4, 148.2, 133.1, 131.3, 130.9, 128.8, 118.0, 116.7, 115.4, 105.4; ESI-HRMS: calcd. for $C_{16}H_{12}NO_9S_2$ $[M + H]^+$: 425.9953; found: 425.9968.

Benzaldehyde *O*-(2-oxo-2*H*-chromen-7-yl) oxime 15

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and benzaldehyde (66 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **15** (73 mg, 45%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.49 (s, 1 H), 7.79–7–76 (m, 2 H), 7.70 (d, 1 H, J = 9.4 Hz), 7.53–7.45 (m, 4 H), 7.35 (d, 1 H, J = 2.3 Hz), 7.18 (d, 1 H, J = 8.5, 2.3 Hz), 6.32 (d, 1 H, J = 9.4 Hz,); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 161.5, 160.1, 155.1, 154.1, 150.4, 144.2, 131.3, 130.5, 129.8, 129.0, 128.0, 113.7, 113.3, 111.2, 101.4; EI-HRMS: calcd. for $C_{18}H_{11}NO_3$ $[M]^+$: 265.073893; found 265.073760.

4-Methoxybenzaldehyde *O*-(2-oxo-2*H*-chromen-7-yl) oxime 16

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and 4-methoxybenzaldehyde (84 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **16** (102 mg, 56%) as a white solid. Mp: 124 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.35 (s, 1 H), 7.61 (d, 1 H, J = 9.5 Hz), 7.37–7.27 (m, 2 H), 7.25–7.17 (m, 2 H), 7.06 (dd, 1 H, J = 8.6, 2.3 Hz), 6.94 (dd, 1 H, J = 8.6, 2.3 Hz), 6.25 (d, 1 H, J = 9.5 Hz), 3.81 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 162.6, 161.5, 160.4, 156.0, 153.4, 143.7, 132.3, 130.4, 129.1, 121.5, 118.1, 114.1, 114.0, 112.1, 111.9, 102.8, 55.8; ESI-HRMS: calcd. for $C_{17}H_{14}NO_4$ $[M + H]^+$: 296.0922; found: 296.0924.

4-Hydroxybenzaldehyde *O*-(2-oxo-2*H*-chromen-7-yl) oxime 17

Application of the procedure above starting from umbelliferone **8** (100 mg, 0.62 mmol) and 4-hydroxybenzaldehyde (75 mg, 0.62 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **17** (80 mg, 46%) as a white solid. Mp: 122 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.27 (br s, 1 H), 8.78 (s, 1 H), 8.17 (d, 1 H, J = 9.5 Hz), 7.85–7.81 (m, 3 H), 7.41 (d, 1 H, J = 2.3 Hz), 7.32 (dd, 1 H, J = 8.8, 2.3 Hz), 7.01 (d, 2 H, J = 8.8 Hz), 6.48 (d, 1 H, J = 9.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 162.2, 160.8, 160.5, 155.5, 154.2, 144.6, 130.2, 130.1, 121.5, 116.3, 113.8, 113.5, 111.5, 101.5; ESI-HRMS: calcd. for $C_{16}H_{12}NO_4$ $[M + H]^+$: 282.0766; found: 282.0762.

4-Methyl-7-[(methyleneamino)oxy]-2*H*-chromen-2-one 18

Application of the procedure above starting from 4-methylumbelliferone **9** (200 mg, 1.13 mmol) and an aqueous solution of formaldehyde (30 mg, 1.13 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **18** (115 mg, 50%) as a white solid. Mp: 98–99 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.75 (d, 1 H, J = 8.7 Hz), 7.63 (d, 1 H, J = 5.6 Hz), 7.27 (d, 1 H, J = 5.6 Hz), 7.17–7.11 (m, 2 H), 6.27 (q, 1 H, J = 1.2 Hz), 2.42 (d, 3 H, J = 1.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 161.2, 159.9, 154.3, 144.9, 126.8, 114.6, 112.8, 111.9, 110.7, 102.1, 101.2, 18.1; EI-HRMS: calcd. for $C_{11}H_9NO_3$ $[M]^+$: 203.058243; found: 203.058070.

Benzaldehyde *O*-(4-methyl-2-oxo-2*H*-chromen-7-yl) oxime 19

Application of the procedure above starting from 4-methylumbelliferone **9** (100 mg, 0.57 mmol) and benzaldehyde (60 mg, 0.57 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **19** (70 mg, 44%) as a white solid. Mp: 122 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.93 (s, 1 H), 7.98 (dd, 2 H, J = 8.0, 2.1 Hz), 7.90 (d, 1 H, J = 8.8 Hz), 7.68–7.63 (m, 3 H), 7.44–7.43 (m, 1 H), 7.37 (dd, 1 H, J = 8.8, 2.1 Hz), 6.41 (s, 1 H), 2.57 (s, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 161.8, 160.3, 154.8, 154.4, 153.6, 131.7, 130.9, 129.4, 128.3, 127.1, 114.9, 112.2, 111.2, 101.7, 18.5; EI-HRMS: calcd. for $C_{17}H_{13}NO_3$ $[M]^+$: 279.089543; found: 279.089290.

2-Nitrobenzaldehyde *O*-(4-methyl-2-oxo-2*H*-chromen-7-yl) oxime 20

Application of the procedure above starting from 4-methylumbelliferone **9** (200 mg, 1.13 mmol) and 2-nitrobenzaldehyde (170 mg, 1.13 mmol) followed by flash chromatography purification (3 : 2 hexane : ethyl acetate) gave **20** (115 mg, 31%) as a yellow solid. Mp: 153–154 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.09 (s, 1 H), 8.19 (dd, 1 H, J = 8.1, 1.5 Hz), 8.13 (d, 1 H, J = 7.7, 1.5 Hz), 7.79 (td, 1 H, J = 7.7, 1.5 Hz), 7.70 (td, 1 H, J = 8.1, 1.5 Hz), 7.59 (d, 1 H, J = 8.8 Hz), 7.35 (d, 1 H, J = 2.5 Hz), 7.17 (dd, 1 H, J = 8.8, 2.5 Hz), 6.22 (s, 1 H), 2.46 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 163.8, 162.7, 157.1, 156.0, 154.4, 150.7, 146.3, 136.9, 134.8, 132.6, 129.6, 127.8, 127.6, 114.8, 113.6, 104.3, 20.7; ESI-HRMS: calcd. for $C_{17}H_{13}N_2O_5$ $[M + H]^+$: 325.0824; found: 325.0824.

Kinetic measurements

All substrates were diluted from stock solutions in 50% DMF, and stored at +4 °C. Assays (0.1 ml) were followed in individual wells of round-bottom polypropylene 96-well-plates (Costar) using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems, filters λ_{ex} = 360 ± 20, λ_{em} = 460 ± 20 nm), or of polystyrene 96-well-plates (Costar) using a Spectramax 250 Microplate Spectrophotometer (Molecular Devices).

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