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New Indolyl Substrates for Chromogenic and Fluorogenic Detection of Esterase Activity in Solution

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Abstract—Three indolyl substrates for chromogenic detection of esterase activity have been synthesised. Hydrolysis of the non-coloured esters **1** catalysed by hog liver esterase, affords after oxidation the coloured water soluble indolinones **3** with absorption maxima at 478, 502 and 540 nm, respectively. The rate of hydrolysis was found to be slower compared to that of *p*-nitrophenyl acetate in all three cases. However, the chromophores obtained after hydrolysis and oxidation are advantageous in that they have their absorption maxima at longer wavelengths than *p*-nitrophenolate. Hydrolysis of **1a** was also monitored using fluorescence spectroscopy since the obtained intermediate **2a** is fluorescent.

Introduction

Chromogenic substrates are commonly used for colourimetric detection of enzyme activity. Upon enzymic action, these substrates release a free chromophore which differ significantly in absorption maximum from that of the substrate-bound chromophore. *p*-Nitrophenol is an example of a widely used chromophore in chromogenic substrates.[1,2] *p*-Nitrophenyl acetate (PNPA), used for detection of esterases, has an absorbance maximum at 270 nm while free *p*-nitrophenolate has an absorbance maximum at 400 nm. Although this is a sufficiently long wavelength for many purposes detection at longer wavelengths would be desirable in some systems to eliminate the problem of background absorption e.g. from cell material.

Another group of chromogenic substrates for hydrolytic enzymes is derived from indolyl chromophores, e.g. 5-bromo-4-chloro-3-indolyl butyrate (BCIB). These substrates are hydrolysed to a 3-hydroxyindole which readily oxidises in air to form an indigo dye that precipitates from the medium. This chromogenic system is used for detecting enzymatic activity in cells and tissues, on blots and in gels.[3–6]

Although there are numerous chromogenic substrates for detection of enzymes in solution known today, they mainly have *p*-nitrophenol or *p*-nitroaniline[7] as the leaving chromophores and thus detection is at relatively short wavelengths. Little work has been done to develop substrates with detection at long wavelengths with large shifts in absorption maxima upon enzymic action. Furthermore we wish to obtain a multiplex detection system where different enzymes can be detected simultaneously for rapid screening. This could be acquired using a system of non-coloured substrates with different enzyme specificity which release chromophores of various colours after enzyme catalysis. In order to achieve substrates that can detect enzyme activity in solution at longer wavelengths than *p*-nitrophenolate and as a first step towards a multiplex detection system we have synthesised new indolyl acetates **1** with a 4-pyridyl substitution at the 2-position. Analogous to that of BCIB these substrates generate after hydrolysis intermediate indoxyls **2** which are easily oxidised to the coloured indolinones **3**. However, in contrast to the indigo dye produced after hydrolysis of BCIB the released chromophores **3** from these substrates stay in solution. Hydrolysis of the non-coloured acetates **1** catalysed by esterase generate the intermediate indoxyls **2** which in the case of **2b** and **2c** are subsequently oxidised by air to the deeply red and purple dyes **3b** and **3c** with absorption maxima at 502 and 540 nm, respectively (Scheme 1). Air oxidation of the neutral indoxyl **2a** to **3a** is much slower but rapid oxidation can be accomplished with addition of an oxidising agent such as potassium ferricyanide.[8] However the intermediate indoxyl **2a** has an absorption maximum of 410 nm which is a red-shift of 130 nm from that of **1a**. Thus chromogenic detection is possible even without oxidation to the indolinone **3a**. The absorption maximum of the final oxidised product **3a** is at 478 nm. The relatively high extinction coefficients of the dyes **3a**, **3b** and **3c** (10600, 11300 and 13700 $M^{-1}\,cm^{-1}$, respectively)[9] make them useful as chromophores in chromogenic substrates.

Keywords: chromogenic; fluorogenic; esterase; indolyl; *p*-nitrophenyl acetate.

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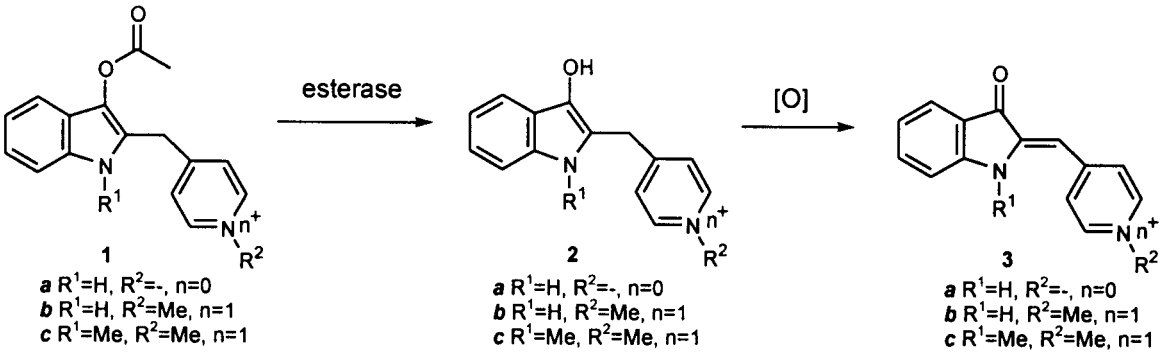


Scheme 1.

We also found that **2a** is fluorescent which opens the possibility of fluorogenic detection of esterases with substrate **1a**.

Results and Discussion

Synthesis

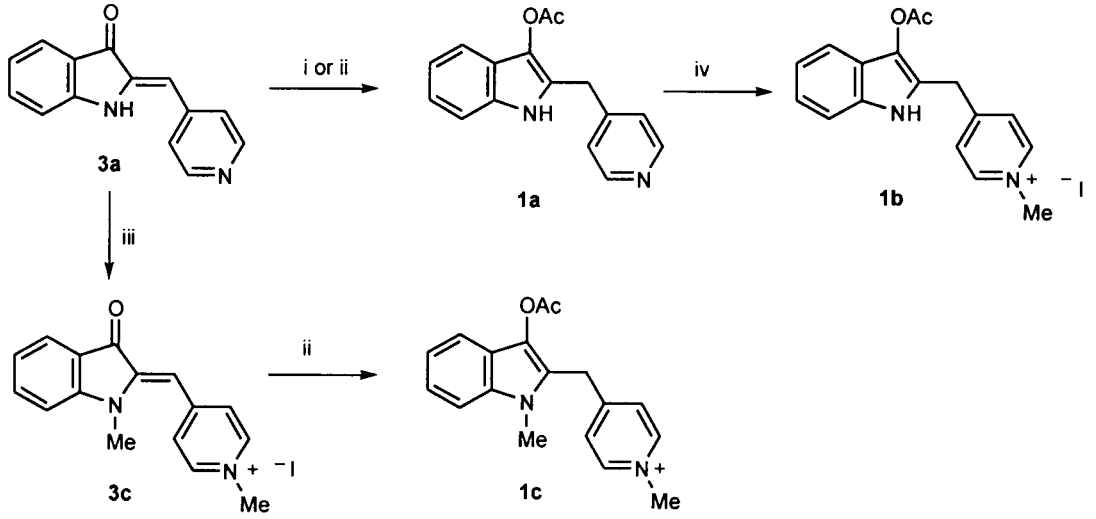
N-Acetylindoxyl was synthesised from anthranilic acid in three steps as previously described.[10,11] Condensation of *N*-acetylindoxyl with pyridine-4-carboxaldehyde in *N*,*N*-dimethyl formamide using triethylamine as base under a nitrogen atmosphere afforded 2-(pyridin-4-ylmethylene)-1,2-dihydro-indol-3-one **3a** in 83% yield. This yield is slightly higher than previously reported, where the condensation is performed under aqueous conditions.[9,12] Only one isomer was obtained.

Two ways to prepare the ester **1a** was developed (Scheme 2). *Method A:* reduction of **3a** with sodium borohydride in methanol together with cerium chloride heptahydrate under a nitrogen atmosphere, followed by acylation of the indoxyl compound with acetic anhydride and 4-dimethyl-amino-pyridine in dichloromethane produced **1a** in an overall 50% yield. Cerium chloride heptahydrate worked as an efficient catalyst and decreased the reaction time substantially. *Method B:* reduction of the indolinone **3a** by tin in a mixture of acetic anhydride and acetic acid (1:1, v/v) under a nitrogen atmosphere afforded **1a** in 90% yield.[13]

Methylation of **1a** with methyl iodide in dichloromethane at reflux for 4 h produced the monomethylated ester **1b** in 63% yield. Synthesis of the dimethylated ester **1c** directly from **1a** with iodomethane and potassium *tert*-butoxide as base was not feasible. Instead, the ester **1c** was prepared from **3a** by dimethylation to **3c** followed by esterification using method B. The esterification went in 84% yield and the methylation in *N*,*N*-dimethyl formamide using potassium *tert*-butoxide as base afforded the indolinone **3c** in 90%.[9]

UV–Vis experiments

Hydrolysis of the three substrates **1** catalysed by hog liver esterase was followed by UV–Vis spectroscopy. The change in absorption as a function of time during hydrolysis of the different substrates is shown in Fig. 1a–d. The new absorption bands afforded by hydrolysis of **1b** and **1c**, respectively, with absorption maxima at 502 and 540 nm are identical to those of the synthesised compounds **3b** and **3c**. No indoxyl intermediate **2b** and **2c** could be observed during hydrolysis which shows that oxidation of the intermediates is fast. In kinetic experiments, however, in



Scheme 2. *Reagents and conditions:* (i) 1—$NaBH_4$, $CeCl_3 \times 7H_2O$, MeOH, rt, 5 min, 2—Ac_2O, 4-DMAP, DCM, 45 min; (ii) Sn, AcOH/Ac_2O (1:1, v/v), 70°C, 2–2.5 h; (iii) MeI, $KOBu^t$, DMF, rt, 20 h; (iv) MeI, DCM, reflux 4 h.

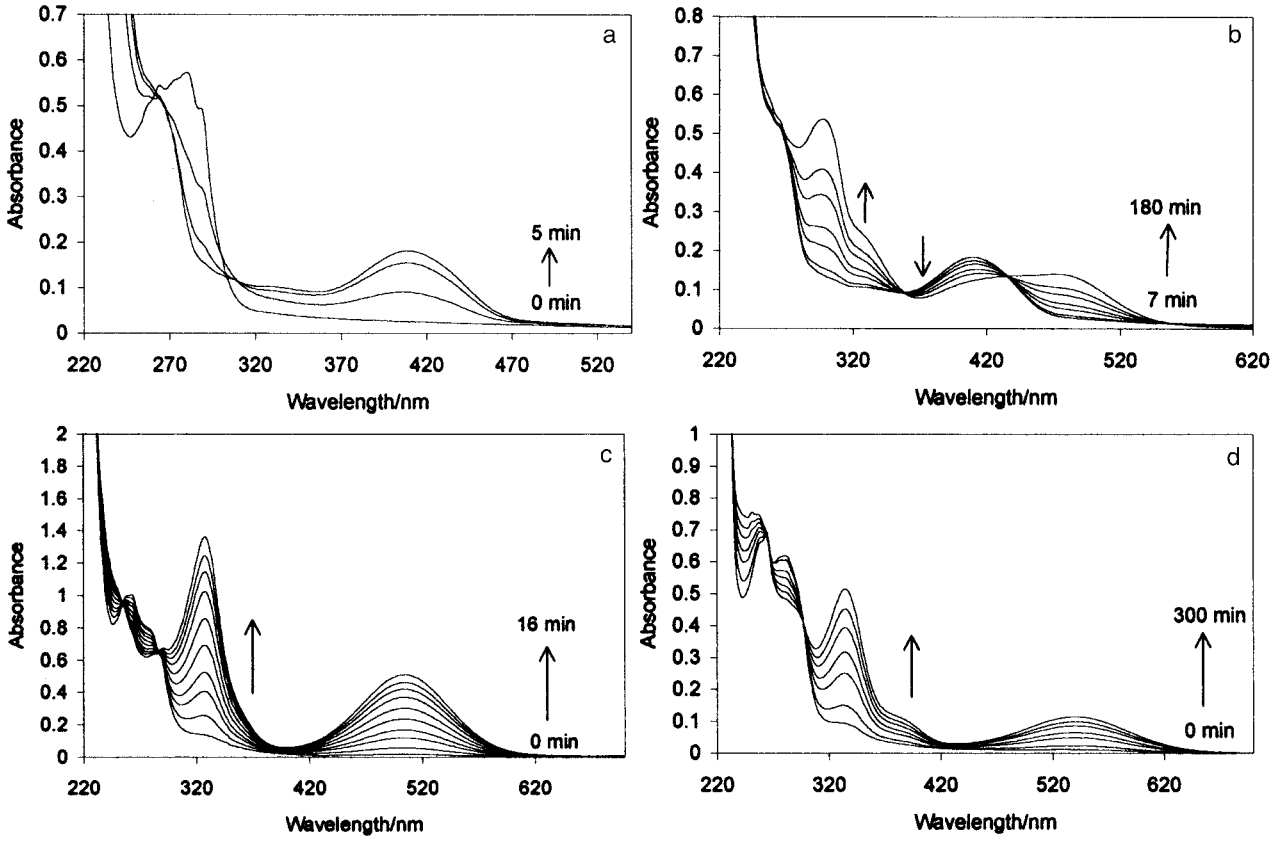


Figure 1. The change in absorption as a function of time when the substrates were incubated with esterase at 22°C: (a) and (b) **1a**, (c) **1b** and (d) **1c**. The enzymatic reactions were carried out in phosphate buffer (3.4 ml, 0.05 M K_2HPO_4, pH 8.0) with esterase (4 μl, 9.2 mg ml^{-1}, 1360 units ml^{-1}) (Unit definition: one unit will hydrolyse 1.0 μmol of ethyl butyrate per minute at pH 8.0 at 25°C) and: (a) and (b) **1a** (7.1×10^{-5} M); (c) **1b** (9.2×10^{-5} M); (d) **1c** (1.1×10^{-4} M).

which the absorbance of the final products **3b** and **3c** was followed over time, a typical curve for consecutive reactions was afforded. Thus there is a slight build-up of the intermediate indoxyls. But when using potassium ferricyanide as oxidant a good fit to pseudo-first-order kinetics was obtained.

During hydrolysis of the neutral ester **1a** an intermediate **2a** with absorption maximum at 408 nm was observed. The intensity of this absorption band slowly decreased and new bands consistent with the absorption spectrum of compound **3a** with absorption maxima at 478 and 298 nm were formed. Thus the intermediate **2a** is not as easily oxidised as intermediates **2b** and **2c**. In order to accomplish a more rapid oxidation, **1a** was hydrolysed in the presence of potassium ferricyanide. The absorption band of the intermediate **2a** at 408 nm was not observed during hydrolysis and only the absorption band corresponding to the oxidised product **3a** at 478 nm could be seen.

The faster air-oxidation of the intermediates **2b** and **2c** might be rationalised by the stabilising cyanine resonance structures afforded after oxidation to the dyes **3b** and **3c**. To investigate this, **2a** was protonated by adding concentrated hydrochloric acid after which air-oxidation occurred immediately to produce protonated **3a**, showing that oxidation is faster when there is a charge on the pyridine nitrogen since the product obtained is stabilised by resonance structures.

^{1}H NMR experiments

The hydrolysis of **1a** was also followed by ^{1}H NMR to elucidate the structure of the intermediate formed (data not shown). The peak at 2.34 ppm corresponding to the acetyl group decreased in intensity during hydrolysis and a new peak at 1.87 ppm from acetic acid was observed. The peak at 4.05 ppm corresponding to the bridging methylene group decreased in intensity with time at the expense of a new peak at 4.10 ppm. The peaks in the aromatic region decreased to form new similar signals at somewhat different chemical shifts. These NMR results are consistent with the formation of compound **2a**. A second species was observed in the spectra with two doublets at 3.48 and 3.80 ppm both with a coupling constant in the geminal region (J=13 Hz). These doublets may arise from a species where the bridging methylene protons are magnetically non-equivalent, because of restricted rotation around the bridging methylene bond. The amount of this species was, however, very low and fairly constant with time. Hydrolysis of the substrates **1b** and **1c** with esterase were also followed by ^{1}H NMR. The spectra achieved after complete hydrolysis were consistent with that of **3b** and **3c**.

Kinetic measurements

Estimations of the second order rate constants for the esterase catalysed hydrolysis of the different substrates and PNPA were obtained from the slopes of the linear

Table 1. Second-order rate constants for the different substrates

Substrate	λ_{max}/nm	λ_{max}/nm (product)	k_2[a]/M^{-1} s^{-1}
1a	281	410 (**2a**)	2.5×10^5
1b	263	502 (**3b**)	1.8×10^4 [b]
1c	264	540 (**3c**)	1.6×10^3 [b]
PNPA	310	400	5.0×10^6

[a] Second-order rate constant for the enzyme catalysis. Determined from the slope of linear plots of pseudo-first-order rate constants for hydrolysis of the substrates versus concentration of enzyme.
[b] Using potassium ferricyanide as oxidant.

plots of 3 pseudo-first-order rate constants versus the concentration of enzyme (Table 1). The pseudo-first-order constants for hydrolysis of the methylated esters **1b** and **1c** were determined, using potassium ferricyanide as oxidant, by following the absorbance at 502 and 540 nm, respectively with time. We should stress that since these are consecutive reactions pseudo-first-order kinetics is not apparent, but still, reasonable estimations of the pseudo-first-order constants could be obtained because the second oxidation step in presence of potassium ferricyanide is so fast. Since the intermediate indoxyl **2a** has an absorbance band at 408 nm, well separated from that of the starting material, the rate constant for substrate **1a** could be obtained without the use of an oxidant.

The slower hydrolysis of the cationic substrates **1b** and **1c** (Table 1) is probably due to a reduced affinity for the active site of the enzyme because of their high hydrophilicity. The second order rate constant for the hydrolysis of the dimethylated **1c** is substantially lower than for the monomethylated **1b**, considering the small difference in structure. This might be due to inhibition of the enzyme by the substrate induced by the methyl group on the indole moiety. However this has not been thoroughly investigated as yet.

Fluorescence measurements

The hydrolysis of **1a** can also be monitored using fluorescence spectroscopy since the intermediate indoxyl **2a** was shown to be fluorescent. The change in fluorescence as a function of time during hydrolysis of substrate **1a** is shown in Fig. 2. The sample was excited at 400 nm and the emission scan ranged from 440 to 540 nm. A peak was observed in the range of 460 to 490 nm, consistent with indoxyl fluorescence as reported by Gehauf et al.[14] This indicates that the fluorescence arises from the indoxyl moiety. The fluorescence intensity began to decrease after 5 min due to oxidation of **2a** to the non-fluorescent **3a**. However, at the initial stage of the reaction the rate of the oxidation is negligible compared to that of the hydrolysis reaction. Thus **1a** can be used in fluorometric detection of esterase activity. The detection limit was not determined because of the instability of the fluorescent product. Design of an analogous substrate without the tendency to oxidise in air is in process and will be reported in due time.

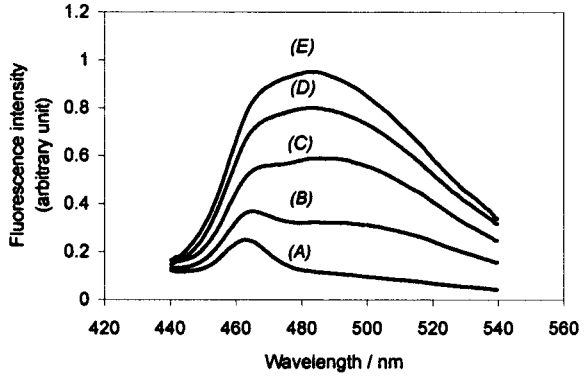


Figure 2. The change in the fluorescence spectrum as a function of time when substrate **1a** was incubated with esterase at 25°C. The enzymatic reaction was carried out with **1a** (1.4×10^{-5} mol dm^{-3}) and esterase (50 μl, 0.012 mg ml^{-1}, 1.7 units ml^{-1}) in phosphate buffer (Na_2HPO_4–NaH_2PO_4 0.067 M, pH 7.06). The sample was excited at 400 nm. (a) 0 min; (b) 0.5 min; (c) 1 min; (d) 3 min; (e) 5 min.

Conclusions

The newly synthesised indolyl acetates **1** can be used as chromogenic substrates for detection of esterase activity. The released chromophores have their absorbance maxima at longer wavelengths than *p*-nitrophenolate and in contrast to the indigo dyes produced after hydrolysis of known indolyl substrates such as BCIB they stay in solution. Although the acetates **1** are less reactive than PNPA towards esterases we believe that these types of chromogenic substrates have prospects in that analogous substrates can be designed which have high extinction coefficients, absorption at long wavelengths and perhaps even similar reactivity towards esterases as PNPA. It should be possible to change the absorption maximum of the free chromophore in these new types of chromogenic substrates by altering the aryl group to, e.g. a quinoline moiety instead of a pyridine, thus extending the conjugated system to afford an increase in absorption maximum. By having e.g. a phosphate ester instead of an acyl ester function the same chromophores could possibly be used in detection of phosphatases. Further studies are under way to explore the potential of these types of substrates in a multiplex detection system.

Experimental

Column and flash column chromatography was performed using aluminium oxide (activated, neutral, approx. 150 mesh) deactivated by addition of water to Brockman grade III. Melting points were determined on a Mettler FP82HT hot-stage microscope and are uncorrected. ^{1}H (400 MHz) and ^{13}C (100.6 MHz) NMR spectra were recorded at rt using a Varian UNITY-400 NMR spectrometer. Chemical shifts are in ppm, relative to solvent peaks which were calibrated as follows: DMSO (δ 2.50 for ^{1}H and δ_C 39.5 for ^{13}C NMR), CD_3OD (δ 3.31 for ^{1}H and δ_C 49.1 for ^{13}C NMR), D_2O (δ 4.80 for ^{1}H NMR) and $CDCl_3$ (δ_C 77.0 for ^{13}C NMR); *J* values are given in Hz. High resolution mass spectra were recorded using a VG ZabSpec instrument. IR spectra were run on a Perkin–Elmer 1600 FT-IR instrument. UV–Vis spectra were measured on a Varian Cary4 spectrophotometer. Fluorescence spectra were recorded using a SPEX τ2 spectrofluorometer.

N-Acetylindoxyl was synthesised from anthranilic acid in

three steps as previously described.[10,11] 1-Methyl-2-(N-methyl-4-pyridinium-4-ylmethylene)-1,2-dihydro-indol-3-one **3c** was prepared as previously reported.[9] Commercially available reagents were purchased from Aldrich and used without further purification. Hog liver esterase was purchased from Fluka as a suspension in 3.2 M ammonium sulfate solution (148 units/mg protein; 9.2 mg/ml).

2-(Pyridin-4-ylmethylene)-1,2-dihydro-indol-3-one 3a. *N*-Acetylindoxyl (0.50 g, 28.5 mmol), pyridine-4-carboxaldehyde (0.31 g, 28.5 mmol) and triethylamine (0.5 ml, 36 mmol) were stirred under a nitrogen atmosphere in *N*,*N*-dimethyl formamide (2.5 ml) at rt for 18 h. The solution was poured into water and the precipitate formed was collected by filtration, washed with water and air-dried to give **3a** as an orange-red solid (0.46 g, 83%). Mp 205–206°C (from methanol); UV–Vis: 298 (35500 M^{-1} cm^{-1}), 478 (10600 M^{-1} cm^{-1}) nm; IR (KBr): 3125 (NH), 1691 (C═O), 1614 (C═C) cm^{-1}; ^{1}H NMR (DMSO): δ 10.05 (1H, s, NH), 8.63 (2H, d, *J*=6.0 Hz, ArH), 7.65 (2H, d, *J*=6.0 Hz, ArH), 7.61 (1H, d, *J*=8.0 Hz, ArH), 7.57 (1H, t, *J*=8.0 Hz, ArH), 7.15 (1H, d, *J*=8.0 Hz, ArH), 6.96 (1H, t, *J*=8.0 Hz, ArH), 6.51 (1H, s, CH); ^{13}C NMR (DMSO): δ 186.7, 154.3, 150.2, 141.4, 137.1, 136.7, 124.5, 123.5, 120.5, 119.6, 112.6, 105.5; HR-FAB-MS *m*/*z* Found: 223.0816 $C_{14}H_{11}N_2O$ (MH^+): requires *M*, 223.0871).

These data were consistent with those reported for the previously prepared compound.[9]

2-(Pyridin-4-ylmethyl)-3-indolyl acetate 1a. *Method A:* 2-Pyridin-4-ylmethylene-1,2-dihydro-indol-3-one **3a** (0.10 g, 0.45 mmol) and cerium chloride hepta hydrate (0.17 g, 0.45 mmol) was dissolved in methanol (10 ml). Sodium borohydride (17 mg, 0.45 mmol) was added and a vigorous evolution of hydrogen gas followed. The mixture was stirred under a nitrogen atmosphere at rt for 5 min. The solvent was removed by distillation, without exposure of the mixture to air. The residue was dissolved in dichloromethane (15 ml). Acetic acid anhydride (0.05 ml, 0.53 mmol) and 4-dimethylamino pyridine (15 mg, 0.1 mmol) was added and the mixture was stirred under a nitrogen atmosphere at rt for 45 min. The mixture was washed with saturated aqueous sodium hydrogen carbonate (2×20 ml). The aqueous phase was extracted with dichloromethane (2×25 ml). The combined extracts were washed with water (2×25 ml) and brine (2×25 ml), dried over Na_2SO_4 and concentrated. The reddish residue was recrystallised from ethyl acetate to give **1a** (60 mg, 50%) as white crystals. Mp 181–182°C; UV–Vis: 281 (8100 M^{-1} cm^{-1}); IR (KBr): 3137 (NH), 1750 (C═O), 1208 (C–O) cm^{-1}; ^{1}H NMR (DMSO): δ 11.01 (1H, s, NH), 8.47 (2H, d, *J*=6.0 Hz, ArH), 7.28 (1H, d, *J*=7.8 Hz, ArH), 7.27 (1H, d, *J*=7.8 Hz, ArH), 7.23 (2H, d, *J*=6.0 Hz, ArH), 7.07 (1H, t, *J*=7.8 Hz, ArH), 6.97 (1H, t, *J*=7.8 Hz, ArH), 4.01 (2H, s, CH_2), 2.30 (3H, s, COMe); ^{13}C NMR ($CDCl_3$): δ 169.8, 148.8, 133.7, 127.7, 124.6, 124.1, 122.7, 121.2, 120.3, 117.4, 117.3, 111.6, 30.91, 20.82; HR-EI-MS *m*/*z* Found: 266.1055 $C_{16}H_{14}N_2O_2$ (M^+): requires *M*, 266.1039.

Method B: In an alternative method,[13] **3a** (30 mg, 0.135 mmol) was dissolved in concentrated acetic acid (3 ml). Acetic acid anhydride (3 ml) and tin powder (0.24 g, 2.0 mmol) was added and the mixture was stirred under a nitrogen atmosphere at 70°C for 2.5 h. Unreacted precipitates were filtered off and the filtrate was concentrated. The residue was dissolved in dichloromethane (20 ml) and washed with saturated aqueous sodium hydrogen carbonate (2×25 ml). The aqueous phase was extracted with dichloromethane (2×25 ml). The combined extracts were washed with water (2×25 ml) and brine (2×25 ml), dried over Na_2SO_4 and concentrated. The yellow residue was purified by column chromatography on neutral Al_2O_3 with methanol–dichloromethane (1:99) to give **1a** (32 mg, 90%). This compound showed identical physicochemical properties to those reported above.

2-(*N*-Methylpyridinium-4-ylmethyl)-3-indolyl acetate iodide 1b. 2-Pyridin-4-ylmethyl-3-indolyl acetate **1a** (33 mg, 0.12 mmol) and methyl iodide (21 μl, 0.33 mmol) were heated at reflux in dichloromethane (3 ml) under a nitrogen atmosphere for 4 h. After cooling a precipitate was collected by filtration and washed with ethyl acetate to afford **1b** as a white solid (32 mg, 63%). Mp 172–173°C (from ethyl acetate); UV: 263 (10900 M^{-1} cm^{-1}) nm; IR (KBr): 3344 (NH), 1735 (C═O), 1227 (C–O) cm^{-1}; ^{1}H NMR (CD_3OD): δ 8.75 (2H, d, *J*=6.6 Hz, ArH), 7.89 (2H, d, *J*=6.6 Hz, ArH), 7.34 (1H, d, *J*=8.0 Hz, ArH), 7.30 (1H, d, *J*=8.0 Hz, ArH), 7.14 (1H, t, *J*=8.0 Hz, ArH), 7.05 (1H, t, *J*=8.0 Hz, ArH), 4.38 (2H, s, CH_2), 4.33 (3H, s, NMe), 2.35 (3H, s, COMe); ^{13}C NMR (DMSO): δ 169.5, 157.9, 145.1, 133.1, 127.2, 125.6, 123.2, 121.8, 120.5, 119.3, 116.9, 111.6, 47.36, 30.03, 20.49; HR-FAB-MS *m*/*z* Found: 281.1291 $C_{17}H_{17}N_2O_2$ (M^+): requires *M*, 281.1290.

1-Methyl-2-(*N*-methylpyridinium-4-ylmethyl)-3-indolyl acetate iodide 1c. The indolinone **3c** (16 mg, 42 μmol) was dissolved in concentrated acetic acid (2 ml) and acetic acid anhydride (2 ml). Tin powder (76 mg, 0.64 mmol) was added and the mixture was stirred at 70°C under a nitrogen atmosphere for 2 h. Unreacted precipitates were filtered off and the filtrate was concentrated. The residue was purified by flash chromatography on neutral Al_2O_3 with NH_4Br (25% aq.)–methanol (1:9) to give **1c** and ammonium bromide, which was removed by trituration with dichloromethane. Evaporation of the solvent afforded **1c** as a white solid (15 mg, 84%). Mp 155–156°C; UV: 264 (8500 M^{-1} cm^{-1}), 280 (7700 M^{-1} cm^{-1}) nm; IR (KBr): 1750 (C═O), 1203, 1179 (C–O) cm^{-1}; ^{1}H NMR (DMSO): δ 8.86 (2H, d, *J*=6.4 Hz, ArH), 7.84 (2H, d, *J*=6.4 Hz, ArH), 7.48 (1H, d, *J*=8.0 Hz, ArH), 7.38 (1H, d, *J*=8.0 Hz, ArH), 7.20 (1H, t, *J*=8.0 Hz, ArH), 7.07 (1H, t, *J*=8.0 Hz, ArH), 4.51 (2H, s, CH_2), (3H, s, NMe), 3.56 (3H, s, NMe), 2.32 (3H, s, COMe); ^{13}C NMR (DMSO): δ 169.6, 157.6, 145.2, 134.2, 127.0, 126.9, 124.9, 122.0, 119.9, 119.5, 117.0, 110.0, 47.34, 29.91, 28.36, 20.47; HR-FAB-MS *m*/*z* Found: 295.1465 $C_{18}H_{19}N_2O_2$ (M^+): requires *M*, 295.1447.

Kinetic measurements

The kinetic experiments were carried out using a Varian Cary4 spectrophotometer by following the absorbance at 400 nm (*p*-nitrophenolate), 408 nm (**2a**), 502 nm (**3b**) or 540 nm (**3c**) as a function of time. The buffer solution used was 0.05 M potassium hydrogen carbonate with pH

8.0. In a typical kinetic experiment 50 μl of an esterase suspension (0.23 mg ml^{-1}, 34 units ml^{-1}) was added to 3.0 ml of a freshly prepared substrate solution in a 1 cm quartz cuvette. The substrate solutions were prepared by adding 10 μl of a substrate stock solution in acetonitrile or distilled water to the buffer. The concentrations of the substrates were determined from their absorption maxima and were in the region of 4–40 μM. An excess of potassium ferricyanide was added in experiments with substrates **1b** and **1c**. The pseudo-first-order rate constants were determined by fitting the experimental data to a single exponential function. The second order rate constants were determined from linear regression analysis of the experimentally measured pseudo-first-order rate constants as a function of three enzyme concentrations. The absorption spectrums after complete hydrolysis of **1b** and **1c** were consistent with the synthesised compounds **3b** and **3c**.

^{1}H NMR experiments

Phosphate buffered (0.05 M; pH 8) deuterium oxide (0.7 ml) was used as solvent and a suspension of hog liver esterase (4 μl, 9.2 mg ml^{-1}, 1360 units ml^{-1}) was added to the NMR-tube and spectra were recorded at appropriate time intervals. To facilitate dissolution of the less water soluble **1a**, it was initially dissolved in 0.1 ml deuterated dimethyl sulfoxide. The spectra of the different substrates after complete hydrolysis were consistent with the synthesised compounds **3**.[9]

Acknowledgements

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