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Synthesis and characterization of the aqueous solution chemistry of the food-derived carcinogen model *N*-acetoxy-*N*-(1-methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide and its *N*-pivaloyloxy analogue

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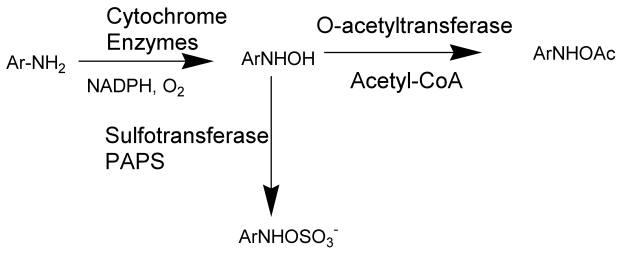
Abstract—We report the synthesis of *N*-acetoxy-*N*-(1-methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide, **7**, its *N*-pivaloyloxy analogue, **9**, and improved synthesis of indole-2-acetonitrile, **3** (70% in five steps from indole-2-carboxylic acid), the carcinogenic amine Trp-P-2, **4** (40% from **3**), and the nitro compound, **5** (40% from **4** by oxidation with H_2O_2 using $Mo(CO)_6$ catalyst). In aqueous solution at neutral pH, **7** primarily undergoes C–O bond cleavage to yield the hydroxamic acid, **8**, but under the same conditions the sterically hindered **9** decomposes predominately by N–O bond cleavage with a pH independent rate constant that is 7.5-fold smaller than that for **7**. In the pH range 0.5–7.0 three different processes for the decomposition of **9** were detected by kinetics. Only the process that dominates at neutral pH generates a nitrenium species that can be trapped by N_3^-.

1. Introduction

The heterocyclic amines derived from cooked protein containing food have been shown to be highly carcinogenic and mutagenic.[1] The heterocyclic amines are classified into two categories: the IQ type (IQ, MeIQx, MeIQ, etc.), that contains the imidazoquinoline or imidazoquinoxaline structure and the non-IQ type (Glu-P-1, Trp-P-1, Trp-P-2, AαC, etc.) that contain the 2-aminopyridine moiety. Non-IQ amines are formed by pyrolysis of proteins and amino acid mixtures.[2] These materials have also been isolated in parts per billion concentrations from broiled and fried meats, fish and protein-rich plant material and have been shown to be mutagenic to *Salmonella* in the presence of rat liver homogenates.[2] These heterocyclic amines are now considered to be probable human carcinogens.[3]

The food-derived heterocyclic amines are promutagens/procarcinogens that require oxidative metabolism for activation. The likely activation processes are shown in Scheme 1.[4] We have been studying the selectivity of the nitrenium ions derived from these heterocyclic esters.[5] The synthesis of the hydroxylamine or hydroxamic acid ester derivatives of these heterocyclic amines is essential for studying the selectivity of the nitrenium ion derived from these amines. There have been several reported syntheses of Trp-P-2.[6,7] Herein we report improvements in the yield of synthesis of Trp-P-2, **4**, and synthesis and characterization of its corresponding hydroxamic acid ester derivatives **7** and **9**. Surprisingly, at neutral pH, **7** does not, to any significant extent, undergo N–O bond cleavage to generate a nitrenium ion, the dominant neutral pH reaction of other ester derivatives of heterocyclic hydroxylamines and hydroxamic acids.[5] Instead, **7** undergoes predominant C–O bond cleavage to generate the hydroxamic acid, **8**. The more sterically hindered **9** does undergo N–O bond cleavage at neutral pH.



Scheme 1.

The synthesis of **4** was previously reported by Takeda et al.[7] starting from indole-2-acetonitrile, **3**, in a one-pot procedure using CH_3CN and $AlCl_3$ in 15% yield (Scheme 2). The indole-2-acetonitrile was synthesized from indole-2-carboxylic acid in five steps in 36% yield.[8]

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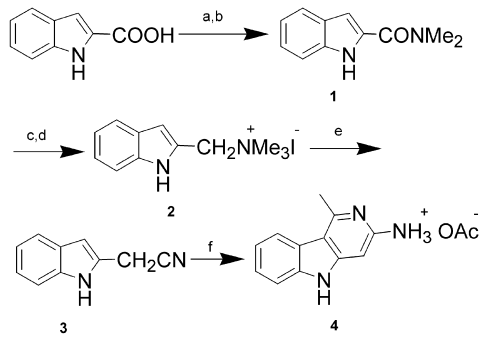


Scheme 2. (a) $SOCl_2$, C_6H_6, cat. DMF, 50°C, (b) $(CH_3)_2NH$, C_6H_6, RT, 95% (a+b), (c) LAH/THF, 50°C, 98%, (d) MeI/EtOAc, 50°C, 99%, (e) KCN/DMF, 76%, (f) CH_3CN, $AlCl_3$, 90°C, 18 h, MeOH/AcOH, 40%.

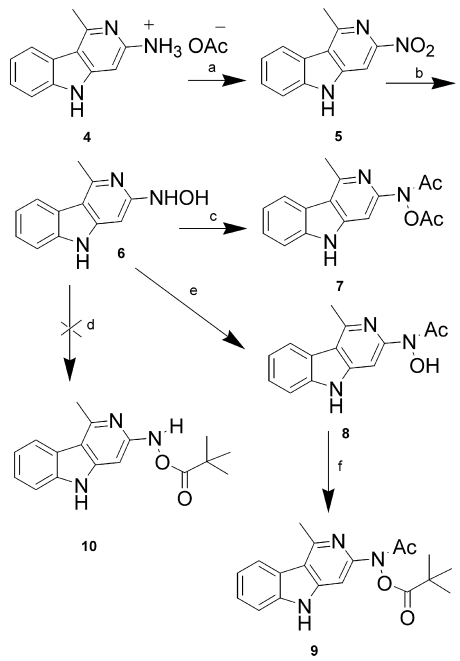
2. Results and discussion

We improved the yield of **3** by modifications of the original procedure.[8] By completely removing the benzene-thionyl chloride solution prior to treatment with dimethylamine gas in the first step, instead of reducing to 1/3 volume as reported, the yield of **1** was increased from 60 to 95% and the formation of the 3-chlororindole-2-carboxamide by-product was eliminated. During the transformation of the quaternary ammonium iodide **2** to the nitrile **3** by S_N2 displacement, the replacement of the reported solvent MeOH by DMF improved the yield. Prior to this modification, the methanol also acted as a nucleophile and gave the corresponding ether compound in addition to the desired nitrile **3** in equal amounts. The use of DMF resulted in a single product without need of column chromatography. The overall yield of **3** was 70% starting from indole-2-carboxylic acid compared to 36% previously reported.[8]

The yield of **4** from **3** was also improved by modification of the reported procedure.[7] After the reaction, the excess $AlCl_3$ was quenched with water, the pH of the solution was adjusted to 4–5 and the reaction mixture was extracted with Et_2O to remove the neutral reaction products. The water layer was then made basic with 2 M K_2CO_3 until pH>9. The product was isolated from the basic aqueous solution and purified as described in the literature.[7] The Et_2O layer gave unreacted **3** in ca. 50% yield. Longer reaction time (72 h) did not increase the yield of **4**. Based on the isolated yield of the recovered unreacted starting material **3**, the yield of **4** was increased from the reported 15 to 40% (Scheme 2). The overall yield of **4** starting from indole-2-carboxylic acid was 28%.

The synthesis of **7** requires the oxidation of **4** to the corresponding nitro compound, **5**. The reported synthesis of **5** from **4** requires the use of Na_2WO_4, $(CF_3CO)_2O$, and H_2O_2 with a yield of 12%.[9] Attempts were made to increase the yield of this procedure without any success. However, the reported oxidation of Glu-P-1 and Glu-P-2 to the corresponding nitro compound using $Mo(CO)_6$ gave 60–70% yield.[10] The oxidation of **4** using $Mo(CO)_6$ and H_2O_2 following the reported procedure for Glu-P-1 and Glu-P-2 gave **5** in 40% yield after 1 h (Scheme 3).

A selective and controlled reduction of the nitro compound with 5% Pd/C and hydrazine monohydrate gave the



Scheme 3. (a) $Mo(CO)_6$, CF_3COOH, $(CF_3CO)_2O$, 30% H_2O_2, 40%, (b) 5% Pd/C, $NH_2NH_2{\cdot}H_2O$, THF, −20°C, 1 h, 96%, (c) 2.2 equiv. Et_3N, 2.2 equiv. AcCl, THF, 0°C, 30 min, 98%, (d) Et_3N, $(CH_3)_3CCOCN$, THF, −20°C, 30 min, 37%, (f) 1.3 equiv. $(CH_3)_3CCOCl$, 1.3 equiv. Et_3N, THF, 0°C, 1 h, 91%.

corresponding hydroxylamine, **6**.[11] Although synthesis of **6** has been reported from the nitro compound it was characterized only by its mass spectrum.[9] We report more detailed characterization here. The attempted synthesis of the ultimate carcinogenic ester, **10**, with pivaloyl cyanide[12] was unsuccessful, as the ester decomposed spontaneously during workup. The acyl cyanide procedure does provide isolated *N*-arylhydroxylamine esters in cases in which these compounds are less reactive.[5a,b] The hydroxylamine, **6**, was treated with 2.2 equiv. of acetyl chloride and triethylamine at 0°C in THF for 30 min to give the hydroxamic acid ester, **7**, after workup. The ester, **7**, was 99% pure according to HPLC and NMR (Scheme 3). The pivalic acid ester, **9**, was made in modest yield by successive addition of acetyl chloride and pivaloyl chloride. The intermediate hydroxamic acid, **8**, was isolated and characterized.

Kinetics of the decomposition of **7** and **9** were examined by UV and HPLC methods at 20°C in 5 vol% CH_3CN–H_2O, μ=0.5 ($NaClO_4$) in NaH_2PO_4/Na_2HPO_4, AcOH/NaOAc, and $HCO_2H/NaHCO_2$ buffers (0.02–0.06 M) at pH>2.5 and in $HClO_4$ solutions at pH≤2.5. For both compounds the initial decomposition of the ester at neutral and acidic pH was followed by a much slower decomposition of an initial reaction product that complicated the determination of rate constants by the UV method. This subsequent reaction was more pronounced for **7**. This was not a problem for kinetics followed by the HPLC method because HPLC made it possible to directly monitor the concentration of the ester without interference from subsequent reactions. In spite of this complication, it was possible to determine reliable rate constants for the decomposition of **7** and **9** by UV methods either by fitting the absorbance vs time data to a consecutive first-order rate equation or by monitoring the

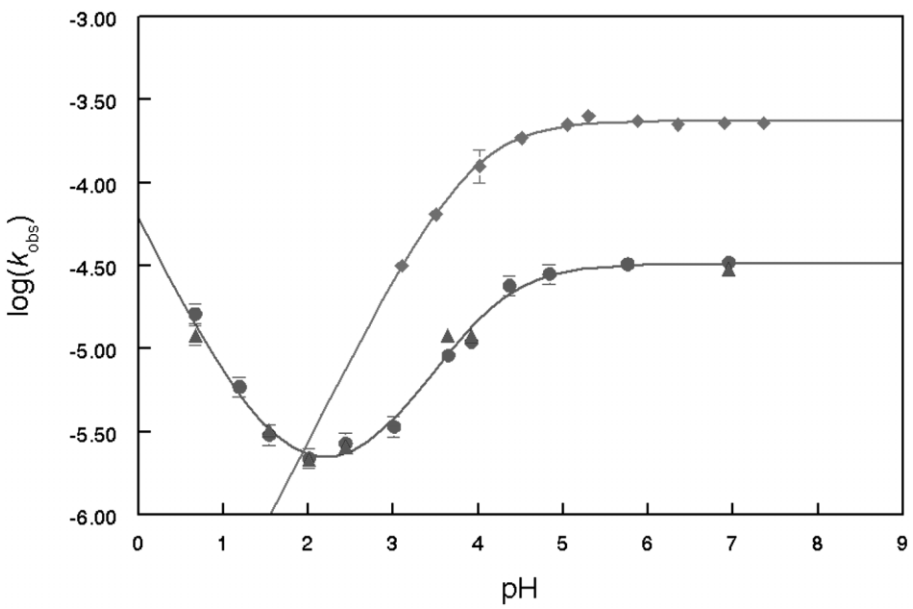


Figure 1. log k_{obs} vs pH for **7** and **9** at 20°C. **7**: ◆. **9**: ▲ (HPLC), ● (UV). Data were fit to Eq. (1) for **9** and to the last term of Eq. (1) for **7** by non-linear least-squares methods.

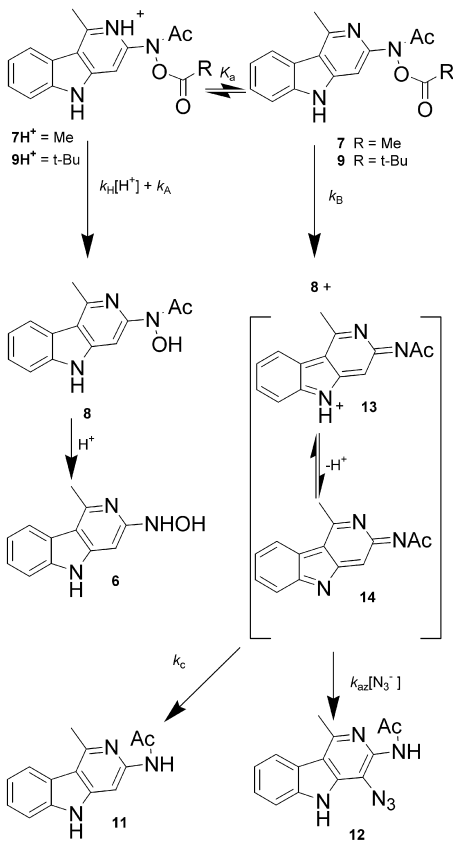
disappearance of the ester at an isosbestic point for the subsequent reaction. Rate constants for decomposition of **7** and **9**, k_{obs}, were buffer independent, but did exhibit pH-dependence. A plot of log k_{obs} vs pH is shown in Figure 1 for both compounds.

$$k_{obs} = (k_H(10^{-pH})^2 + k_A\ 10^{-pH} + k_B K_a)/(K_a + 10^{-pH}) \quad (1)$$

The pH dependence of the decomposition of **9** was fit quite well by Eq. (1). Only the last term ($k_B K_a/(K_a+10^{-pH})$) was necessary to fit the data taken over a smaller pH range for **7**. All of these terms have been observed for other esters of heterocyclic hydroxylamines and hydroxamic acids.[5] For **9**, the first term of Eq. (1) is consistent with acid-catalyzed decomposition of the conjugate acid **9H**$^+$, and the second term is consistent with spontaneous decomposition of **9H**$^+$ (Scheme 4).[5] The last term of Eq. (1), common to both **7** and **9**, is consistent with spontaneous decomposition of the neutral ester (Scheme 4).[5] Parameters obtained from the kinetic fits are provided in Table 1. The kinetically determined pK_as were equivalent, within experimental error, to those obtained by spectrophotometric titration of **7** and **9**.

The last term of Eq. (1) is consistent either with spontaneous N–O bond cleavage to generate a nitrenium ion, or with an uncatalyzed acyl-transfer reaction to H_2O (Scheme 4).[5] Examination of reaction products for the decomposition of **7** at pH 7.0 showed that the hydroxamic acid, **8**, accounted for ca. 90% of reaction products. Slow decomposition of this product accounts for the subsequent reaction observed by UV spectroscopy. Clearly, C–O bond cleavage dominates for this compound. This is somewhat surprising since all previously examined carboxylic acid esters of heterocyclic hydroxylamines or hydroxamic acids undergo predominant N–O bond cleavage at neutral pH.[5]

The more sterically hindered **9** decomposes 7.5-fold more slowly than **7** at neutral pH, and the yield of **8** is reduced to 40% for this compound. In its place, the amide, **11**, (Scheme 4) becomes the major product. In the presence of N_3^- the yield of **11**, but not **8**, decreases. The N_3^--adduct, **12**, is formed in place of **11** (Fig. 2). This occurs without a change in k_{obs} at [N_3^-] sufficiently high to completely trap out **11** (k_{obs}=(3.4±0.1)×10^{-5} s^{-1} measured by HPLC in the presence of 10 mM N_3^- in pH 6.95 0.02 M $H_2PO_4^-$/HPO_4^{2-} buffer compared to (3.1±0.1)×10^{-5} s^{-1} in the same



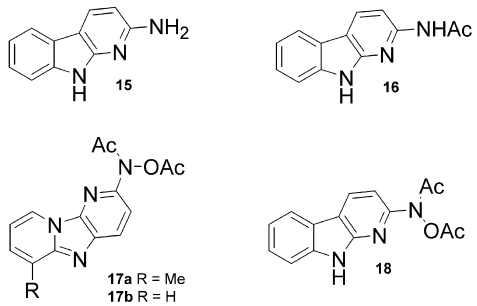
Scheme 4.

Table 1. Kinetic and titration parameters at 20°C

Ester	pK_a (kinetics/titration)	10^5k_H ($M^{-1}s^{-1}$)	10^6k_A (s^{-1})	10^5k_B (s^{-1})
7	3.92±0.04			24±1
7	4.15±0.06			
9	4.12±0.07	5.8±0.6	1.5±0.2	3.2±0.2
9	4.11±0.03			

buffer in the absence of N_3^-).[13] The mechanism of Scheme 4 is consistent with these observations.

Trapping of the nitrenium ion, **13**, and/or its conjugate base, **14**, by N_3^- can explain the decreasing yield of **11** in the presence of N_3^- provided that **11** is also generated from **13** and/or **14**. Previously we have shown that the conjugate acid–base pairs of several heterocyclic nitrenium ions, capable of deprotonation to form a quinoid-like conjugate base such as **14**, are subject to rapid reduction.[5b,d] The yield of **8** is not affected because the N_3^- trapping occurs in a step subsequent to the decomposition of **9** via the competing N–O and C–O bond cleavage processes. The trapping ratio, k_{az}/k_c, in pH 6.95 0.02–0.06 M $H_2PO_4^-/HPO_4^{2-}$ buffer is buffer concentration-independent with an average value of $(3.9\pm0.8)\times10^4$ M^{-1}. More extensive trapping studies at different pH will be required to determine if this is the trapping ratio for **13** or **14**, or an average value intermediate between the two. The results do show that at neutral pH the N-acetylated Trp-P-2 intermediates are more selectively trapped by N_3^- than are the corresponding intermediates from AαC, **15** ($k_{az}/k_c=1.2\times10^3$ M^{-1}), and *N*-acetylAαC, **16** ($k_{az}/k_c=4.5\times10^2$ M^{-1}).[5b,d] We have previously shown that the mutagenicity of the parent amines to *Salmonella typhimurium* TA 98 or TA 100 correlates positively with the selectivity of the corresponding nitrenium species for reaction with N_3^- at neutral pH.[14] Trp-P-2 is about 2 orders of magnitude more mutagenic than AαC in both of these *Salmonella* strains.[15] This is in accord with our observation that at neutral pH the *N*-acetylTrp-P-2 nitrenium species are about 2 orders of magnitude more selective for reaction with N_3^- than are the *N*-acetylAαC intermediates.



Small amounts of **12** can be detected by HPLC when **7** undergoes decomposition at neutral pH in the presence of N_3^-. This shows that N–O bond cleavage is a minor component of the decomposition of **7** (ca. 5%). Rate constants for nucleophilic attack on the carboxyl carbon of pivalic acid esters are typically 1 to 2 orders of magnitude smaller than those for acetic acid esters.[16] Taking into account that C–O bond cleavage represents ca. 40% of the decomposition rate of **9** and 95% of the decomposition rate of **7** at neutral pH, the replacement of Me by *tert*-Bu in the acyloxy component of the molecule slows the acyl-transfer by a factor of ca. 18-fold. This allows the N–O bond cleavage to become the dominant, although not exclusive, process in **9**.

It is possible that the spontaneous decomposition of **7** into **8** via the k_B pathway involves intramolecular nucleophilic attack of the pyridyl N in a 5-member ring transition state. An intramolecular general base catalysed attack of H_2O involving a 7-member ring appears less likely. If the intramolecular process does occur, the decreased rate of decomposition of **9** via the acyl-transfer path may be due to the steric bulk of the *tert*-Bu group hindering the participation of the pyridyl N in a cyclic transition state much as is the case in an intermolecular process.[16] Our current data cannot distinguish these mechanistic

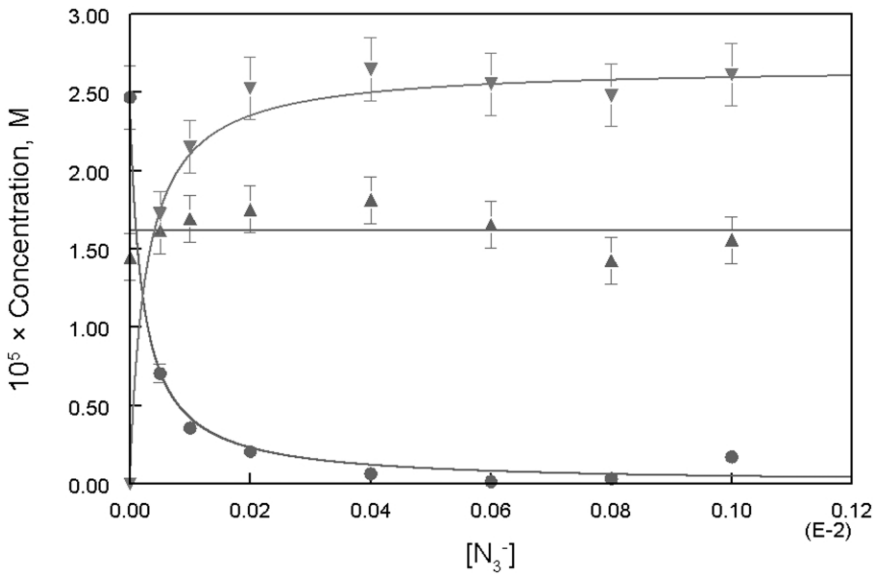


Figure 2. Yields of **8** (▲), **11** (●), and **12** (▼) as a function of [N_3^-] in 0.06 M pH 6.95 phosphate buffer. Data for **11** and **12** were fit to standard equations, described elsewhere,[5] that provide $k_{az}/k_c=(3.9\pm0.5)\times10^4$ M^{-1}. The line for **8** is the average value of its yield throughout the experiment.

possibilities, but the intramolecular nucleophilic process may explain why $\mathbf{7H}^+$ is less reactive than **7** (Fig. 1) even though the leaving group in $\mathbf{7H}^+$ is presumably better.

The reactions that **9** undergoes under acidic conditions were also examined. HPLC analysis shows that at both pH 2.0 and 0.7 the initial product of decomposition of **9** is **8**, and that **8** subsequently decomposes into **6**. Similar to other heterocyclic hydroxylamines we have investigated, **6** is not subject to rapid Bamberger rearrangement under moderately acidic conditions, so it is reasonably stable in the acidic pH range.[5c,d] The acid-catalysed decomposition of $\mathbf{9H}^+$ to generate **8** that is governed by $k_H[H^+]$ dominates at pH 0.7, accounting for ca. 90% of the observed rate of decomposition of **9** at this pH. This reaction is similar to that previously observed for the Glu-P derivatives **17a** and **17b**.[5c]

Spontaneous decomposition of the conjugate acid of the ester (k_A) has been observed previously for the *N*-acetyl-AαC derivative **18**, but in that case the hydroxylamine was generated directly from **18** with no intermediate hydroxamic acid.[5d] In the present study HPLC data show that the hydroxamic acid, **8**, is definitely the initial product of the decomposition of **9** at pH 2.0, a pH at which the k_A term is the dominant kinetic term, accounting for 70% of the overall reaction. Decomposition of the initially formed **8** into the hydroxylamine, **6**, occurs with the same rate constant observed for authentic **8** at this pH (6.6×10^{-7} s^{-1}). The first step of this process for **9** is apparently attack of H_2O on the ester carboxyl of $\mathbf{9H}^+$ to generate **8** via acyl-transfer.

It is now clear that the propensity for C–O vs N–O bond cleavage in ester derivatives of heterocyclic *N*-acetyl-*N*-arylhydroxamic acids at neutral pH is strongly dependent on structure. While **17a** and **17b** decompose exclusively by N–O bond cleavage,[5c] **18** exhibits ca. 15% C–O bond cleavage,[5d] and **7** decomposes with ca. 95% C–O bond cleavage under the same conditions. The more sterically hindered ester **9** still undergoes 40% C–O bond cleavage. This variation in reaction type is likely controlled by the relative stability of the respective nitrenium ions, the leaving group ability of the hydroxamates, and possibly the efficiency of intramolecular nucleophilic attack by the pyridyl N. Since both reactions exhibit the same kinetics at neutral pH it is necessary to examine product distributions to determine which reaction is occurring in individual cases.

3. Conclusion

We were able to increase the yield of the synthesis of indole-2-acetonitrile, **3**, from the previously reported 36 to 70%. The overall yields of the carcinogenic amine, **4**, and the nitro derivative, **5**, starting from indole-2-carboxylic acid, were also improved to 28 and 11%, respectively from the previously reported overall yields of 5 and <1%, respectively. The model carcinogen, **7**, undergoes predominant C–O bond cleavage at neutral pH to generate the hydroxamic acid, **8**. This reaction is suppressed in the more sterically hindered pivalic acid ester, **9**, so that N–O bond cleavage to generate nitrenium species competes with acyl-transfer. Under acidic conditions **9** decomposes to **8** via spontaneous decomposition of $\mathbf{9H}^+$ and by acid-catalyzed hydrolysis of $\mathbf{9H}^+$.

4. Experimental

4.1. General

All solvents were distilled before use. All reagents were used as received. *N*-(1*H*-indole-2-ylmethyl)-*N*,*N*-dimethylamine and *N*-(1*H*-indole-2-ylmethyl)-*N*,*N*,*N*-trimethylammonium iodide (**2**) were synthesized following the reported procedure.[8]

4.1.1. *N*,*N*-Dimethylindole-2-carboxamide (1). This compound was synthesized following the procedure published by Schindler[8] with a few modifications. A few drops of DMF were added to allow the indole-2-carboxylic acid to completely dissolve into benzene instead of heating the solution. The excess thionyl chloride was removed by evaporating the entire benzene solution instead of reducing it to 1/3 volume. Dimethylamine was added directly into the acyl halide solution by bubbling dimethylamine gas into the solution instead of adding the dimethylamine as a benzene solution. The product was obtained in 95% yield: mp 177–180°C (lit.[8] 180–182°C).

4.1.2. 2-(Cyanomethyl)indole (3). This compound was synthesized following the procedure published by Schindler[8] with one modification. DMF was chosen as the solvent instead of CH_3OH. The product was obtained in 76% yield: mp 93–95°C (lit.[8] 96–98°C).

4.1.3. 1-Methyl-5*H*-pyrido[4,5-*b*]indol-3-ylammonium acetate (4). This compound was synthesized following the published procedure by Takeda, et al.[7] with minor modifications. To a solution of **3** (1.5 g, 9.6 mmol) dissolved in 50 mL of CH_3CN was added $AlCl_3$ (15 g, 0.11 mol) portion-wise at 0°C over 30 min. After the addition, the mixture was refluxed for 18 h. Then water was added to quench the excess $AlCl_3$ and the pH of the solution was adjusted to 4–5. It was then extracted with Et_2O (3×25 mL). The Et_2O extracts were combined and dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give 0.75 g (50%) of **3**. The aqueous layer was made basic with 2 M K_2CO_3. To the aqueous solution 5–10% (w/v) of Rochelle's salt was added and the solution extracted with CH_2Cl_2 (5×100 mL) and EtOAc (5×100 mL). The combined organic extract was dried over anhydrous Na_2SO_4. The organic solution was evaporated under reduced pressure. The resulting crude product was dissolved in a small amount of MeOH, and a few millilitres of EtOAc and a few drops of AcOH were added. The crystalline precipitate was collected and washed with EtOAc to give 400 mg of pure **4**. Chromatography (silica gel, 5% MeOH/EtOAc) of the mother liquor yielded an additional 100 mg of **4**. The yield of the reaction is 40% (based on the recovered **3** from the Et_2O layer). ^1H NMR (300 MHz, DMSO-d_6) δ: 10.98 (1H, s), 7.82 (1H, d, J=7.6 Hz), 7.30 (1H, d, J=7.6 Hz), 7.22 (1H, t, J=7.9 Hz), 7.09 (1H, t, J=7.9 Hz), 6.21 (1H, s), 5.65 (2H, br), 2.67 (3H, s), 1.90 (3H, s).

4.1.4. 3-Nitro-1-methyl-5*H*-pyrido[4,5-*b*]indole (5). To a mixture of 4.4 mL of $(CF_3CO)_2O$ and 0.7 mL of CF_3COOH was added 1.35 mL (12 mmol) of 30% H_2O_2 in a dropwise fashion at 0°C. To the cold mixture was added 0.135 g, (0.51 mmol) of $Mo(CO)_6$ in small portions. A solution of Trp-P-2-acetate (**4**), 0.25 g (0.97 mmol) in 15 mL of CH_2Cl_2 and 0.7 mL of CF_3COOH, also cooled to 0°C, was added in a dropwise fashion over 20–30 min to the stirred oxidizing media. The reaction mixture was cooled in an ice bath throughout the reaction. Reaction progress was monitored by TLC with precoated silica gel plates (EtOAc). The reaction was complete within 1 h of the last addition of **4** based on TLC. The solution was made basic with ca. 20 mL of cold 2 M K_2CO_3, ca. 40 mL of brine was added, and the resulting mixture was extracted with CH_2Cl_2 (3×25 mL) and EtOAc (3×25 mL). The combined organic extracts were evaporated under reduced pressure without drying, and the crude product was subjected to column chromatography on silica gel (EtOAc) to give **5** that exhibits no impurities in its ^{1}H NMR spectrum, yield: 0.088 g (40%), ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.40 (1H, br), 8.28–8.26 (2H, m), 7.72 (d, 1H, J=8.2 Hz), 7.61 (t, 1H, J=7.1 Hz), 7.40 (t, 1H, J=7.9 Hz), 3.02 (3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 152.4 (C), 151. 5 (C), 144.3 (C), 141.6 (C), 127.9 (CH), 123.0 (CH), 121.2 (CH), 121.1 (C), 120.2 (C), 112.2 (CH), 99.8 (CH), 23.2 (CH_3); LC/MS (ESI, positive ion mode): $C_{12}H_{10}N_3O_2$ $(M+H)^+$ requires *m/e* 228.07, found 228.00.

4.1.5. 3-Hydroxyamino-1-methyl-5*H*-pyrido[4,5-*b*]indole (6). 21.4 mg (0.095 mmol) of **5** was dissolved in 15 mL of dry THF. To this solution was added 21 mg of 5% Pd/C. The solution was cooled to −20°C while stirring under N_2. To the solution was added 20 μL of hydrazine monohydrate. The reaction was monitored by HPLC (C_8 column, 254 nm, 6/4 MeOH/H_2O, flow rate=1 mL/min). When the peak area for **5** was below 10% of the starting concentration, the Pd/C was immediately filtered by vacuum filtration. The Pd/C was washed thoroughly with THF. The combined THF was evaporated under vacuum to dryness to give 19.3 mg of the hydroxylamine **6** (96% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.31 (s, 1H), 8.49 (br, 2H, exchangeable), 7.92 (d, 1H, J=7.7 Hz), 7.41 (d, 1H, J=7.7 Hz), 7.30 (t, 1H, J=7.2 Hz), 7.16 (t, 1H, J=7.7 Hz), 6.69 (s, 1H), 2.75 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 161.0 (C), 150.5 (C), 147.0 (C), 139.6 (C), 124.2 (CH), 122.3 (C), 120.8 (CH), 119.6 (CH), 111.9 (C), 110.5 (CH), 85.2 (CH), 23.0 (CH_3); LC/MS (ESI, positive ion mode): $C_{12}H_{12}N_3O$ $(M+H)^+$ requires *m/e* 214.24, found 214.10.

4.1.6. *N*-Acetoxy-*N*-(1-methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide (7). 19.3 mg, (0.091 mmol) of **6** was dissolved in 15 mL of dry THF and the resulting solution was stirred under N_2 at 0°C. 28 μL, (0.20 mmol) of triethylamine was added followed by the addition of 14 μL, (0.20 mmol) of acetyl chloride. The solution was stirred for 30 min, and then quenched with 10 mL of 5% aqueous $NaHCO_3$. The mixture was extracted thoroughly with EtOAc (3×15 mL). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, filtered and evaporated under vacuum to yield 0.026 g of product (98% yield). IR (ATR): 3285, 1790, 1685, 1610, 1380 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.85 (1H, br), 8.11 (d, 1H, J=7.8 Hz), 7.58 (d, 1H, J=7.3 Hz), 7.55 (1H, s), 7.47 (t, 1H, J=6.9 Hz), 7.29 (t, 1H, J=7.9 Hz), 2.88 (3H, s), 2.31 (3H, s), 2.17 (3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 169.0 (C), 151.1 (C), 148.6 (C), 146.1 (C), 145.3 (C), 140.4 (C), 126.3 (CH), 122.1 (CH), 120.9 (C), 120.4 (CH), 116.5 (C), 111.4 (CH), 97.4 (CH), 23.4 (CH_3), 22.4 (CH_3), 18.1 (CH_3); LC/MS (ESI, positive ion mode): $C_{14}H_{14}N_3O_2$ $(M+H-C_2H_2O)^+$ requires *m/e* 256.2, found 256.1; high-resolution MS (ES): $C_{16}H_{16}N_3O_3$ $(M+H)^+$ requires *m/e* 298.1191, found 298.1194.

4.1.7. *N*-Hydroxy-*N*-(1-methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide (8). 16 mg, (0.075 mmol) of **6** was dissolved in 5 mL of dry THF and the solution was stirred under N_2 at −20°C. 11.6 μL, (0.083 mmol) of triethylamine was added followed by the stepwise addition of 5.8 μL, (0.083 mmol) of acetyl chloride over a 5 min period. The solution was stirred for 30 min during which time the mixture was allowed to warm to 0°C. The reaction was then quenched with 15 mL of brine. The mixture was extracted thoroughly with EtOAc (3×15 mL). The combined organic extract was dried with anhydrous Na_2SO_4, filtered and evaporated under vacuum. The residue was taken up into a minimum volume of EtOAc, and subjected to column chromatography on silica gel (EtOAc) to yield 7 mg of **8** (37%). IR (KBr): 3425, 3280, 1659, 1602, 1581, 1378 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.74 (1H, br), 10.57 (1H, br), 8.10 (1H, d, J=7.9 Hz), 7.55–7.53 (1H, m) 7.55 (1H, s), 7.44 (1H, t, J=7.5 Hz), 7.27 (1H, t, J=7.2 Hz), 2.91 (3H, s), 2.23 (3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 169.0 (C), 150.7 (C), 148.6 (C), 145.3 (C), 140.3 (C), 126.0 (CH), 122.0 (CH), 121.1 (C), 120.2 (CH), 115.7 (C), 111.2 (CH), 97.4 (CH), 23.2 (CH_3), 22.7 (CH_3); high-resolution MS (ES): $C_{14}H_{14}N_3O_2$ $(M+H)^+$ requires *m/e* 256.1086, found 256.1080.

4.1.8. *N*-Pivaloxy-*N*-(1-methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide (9). 10 mg, (0.039 mmol) of **8** was dissolved in 8 mL of dry THF and the solution was stirred under N_2 at 0°C. 6.0 μL, (0.043 mmol) of triethylamine was added followed by the stepwise addition of 5.3 μL, (0.043 mmol) of pivaloyl chloride over a 5 min period. The reaction progress was monitored by HPLC (C_8 column, 254 nm, 75/25 MeOH/H_2O containing 50 mg/L deferoxamine mesylate, buffered with 0.05 M 1:1 KOAc/AcOH, flow rate of 1.0 mL/min). Additional triethylamine and pivaloyl chloride (ca. 20% of the original volumes) were added to bring the reaction to completion. The reaction was quenched by addition of 15 mL of brine followed by 1 mL of 5% aqueous $NaHCO_3$. The mixture was extracted thoroughly with EtOAc (3×15 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum to yield 12.1 mg of **9** (91%). IR (ATR): 3285, 2922, 1780, 1674, 1610, 1579, 1379 cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 11.79 (1H, br), 8.09 (1H, d, J=7.7 Hz), 7.68 (1H, s), 7.55 (1H, d, J=8.1 Hz), 7.45 (1H, t, J=7.0 Hz), 7.28 (1H, t, J=7.9 Hz), 2.86 (3H, s), 2.19 (3H, s), 1.36 (9H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.0 (C), 169.0 (C), 151.6 (C), 146.8 (C), 146.2 (C), 141.2 (C), 127.0 (CH), 122.8 (CH), 121.8 (C), 121.45 (CH), 116.8 (CH), 112.2 (C), 97.1 (CH), 38.1 (C), 27.9 (CH_3), 24.0 (CH_3), 23.4 (CH_3); high-resolution MS (ES): $C_{19}H_{22}N_3O_3$ $(M+H)^+$ requires *m/e* 340.1661, found 340.1661.

4.1.9. *N*-(1-Methyl-5*H*-pyrido[4,5-*b*]indol-3-yl)acetamide (11). This compound was made from **4** in the same manner that **8** was made from **6** except that 2 equiv. of triethylamine were utilized. IR (KBr): 3250, 1672, 1610, 1378 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 11.58 (1H, br), 10.42 (1H, br), 8.08 (1H, s), 8.02 (1H, d, J=7.8 Hz) 7.47 (1H, d, J=7.9 Hz), 7.38 (1H, t, J=7.4 Hz), 7.22 (1H, t, J=7.4 Hz), 2.84 (3H, s), 2.09 (3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 169.0 (C), 150.7 (C), 148.0 (C), 146.0 (C), 140.0 (C), 125.2 (CH), 121.6 (C), 121.4 (CH), 120.0 (CH), 114.1 (C), 111.0 (CH), 92.4 (CH), 24.0 (CH_3), 22.8 (CH_3); high-resolution MS (ES): $C_{14}H_{15}N_3O$ $(M+H)^+$ requires *m/e* 240.1137, found 240.1133.

4.1.10. *N*-(1-Methyl-4-azido-5*H*-pyrido[4,5,*b*]indol-3-yl)acetamide (12). The pivalic acid ester **9** (11 mg, 0.032 mmol) was dissolved in 2.5 mL of dry, distilled DMF. This solution was added in a stepwise fashion (0.5 mL at 6 h intervals) to 100 mL of a pH 7.0, 5 vol% CH_3CN-H_2O, μ=0.5 ($NaClO_4$), 0.04 M NaH_2PO_4/Na_2HPO_4 buffer containing 2.0 mM NaN_3 that was maintained at 20°C. The reaction mixture was extracted with EtOAc (4×20 mL) 48 h after the last addition of **9**. The combined EtOAc extracts were washed with ice-cold 20% NaOH (2×15 mL) and then with brine (1×15 mL). The EtOAc solution was then dried over anhydrous Na_2SO_4 and evaporated under vacuum to yield **12** contaminated with ca. 30% of a mixture of **8** and **11**. Since **12** decomposes upon attempted purification, spectral analysis was performed on the crude isolated sample. IR (ATR): 3248, 2127, 1664 cm^{-1}; ^1H NMR (300 MHz, CD_2Cl_2) δ: 9.13 (1H, s), 8.2 (1H, brs), 7.95 (1H, d, J=9.0 Hz), 7.47–7.42 (2H, m), 7.33–7.27 (1H, m), 2.68 (3H, s), 2.32 (3H, s); ^{13}C NMR (75 MHz, CD_2Cl_2) δ: 126.8 (CH), 122.3 (CH), 121.5 (CH), 111.8 (CH), 30.0 (CH_3), 27.4 (CH_3); LC-MS (ESI, positive ion mode): $C_{14}H_{13}N_4O$ $(M-N_2+H)^+$ requires *m/e* 253.1, found 253.2.

4.2. Kinetics and product studies

All kinetics were performed in 5 vol% CH_3CN-H_2O, μ=0.5 ($NaClO_4$) at 20°C. Buffers used to maintain pH were 0.02–0.06 M NaH_2PO_4/Na_2HPO_4, AcOH/NaOAc, and $HCO_2H/NaHCO_2$ at pH>2.5 and $HClO_4$ solutions at pH≤2.5. NaN_3 solutions were prepared at high concentration (0.45 M for NaN_3), μ=0.5, at pH 7.00 and diluted into the working range with the phosphate buffers of the same pH and ionic strength. Detailed procedures and solvent purifications can be found elsewhere.[5]

Stock solutions of **7** and **9** were made in DMF at concentrations of ca. 1.0×10^{-2} M. Injection of 15 μL of these stock solutions into 3.0 mL of solution generated reaction solutions of initial concentrations of ca. 5.0×10^{-5} M. Wavelengths monitored for kinetics studies were 245, 255, 265, and 320 nm at all pH for both **7** and **9**. Initial absorbance measurements for **7** for the spectrophotometric titration were made at 254 nm. Initial absorbance measurements for **9** for the spectrophotometric titration were made at 255 and 265 nm. Absorbance vs time data were fit to a standard first-order rate equation or consecutive first-order rate equation to provide k_{obs}. Peak area data for **9** obtained by HPLC methods described below were also fit to a standard first-order rate equation to obtain k_{obs}. More detailed procedures are described elsewhere.[5]

Reaction products were monitored by HPLC with UV detection at 254 nm. HPLC conditions were: C_8 reverse-phase analytical column, 75/25 or 50/50 MeOH/H_2O eluent, containing 50 mg/L deferoxamine mesylate, buffered with 0.05 M 1:1 KOAc/AcOH, flow rate of 1.0 mL/min.

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