Synthesis and reactivity of a Kemp's acid amide as a potential prototype anticancer prodrug

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Abstract: An imide of Kemp's acid was synthesised. Ultraviolet spectroscopy was used to monitor its ring-opening reaction in alkaline solution to an amide, which was found to be stable in its fully ionised state at the high pH. The reactions of the amide on reduction in pH to neutrality were then also studied by UV spectroscopy and showed a 2 phase reaction which does not involve any reformation of imide. The first phase is likely to be the hydrolysis of the amide via neighbouring group catalysis by a carboxylic acid group to form anhydride and amine, a reaction which has potential in anti-tumour prodrug application because it should be highly sensitive to pH and thereby faster at the higher pH of tumour tissue than in healthy tissue. Further study to analyse the detail of the reactions by NMR spectroscopy will now follow and accurate rate measurements of relevance to prodrug application will be undertaken.

Keywords: amide hydrolysis kinetics; imide; Kemp's acid; prodrug

Introduction

Tumour hypoxia is a phenomenon which occurs in solid tumour tissue, where the oxygen level and extracellular pH are both lower than in the surrounding healthy tissue. The lower pH is thought to be due to inefficient clearance of cellular waste and metabolic acids from the hypoxic region. This can lower the extracellular pH to below pH 6.3 (Denny, 2001) which is as much as 1 pH unit lower than normal body tissue which is actively regulated and mediated at pH 7.35-7.45.

Extracellular pH is one of the few known differences between healthy tissue and solid tumour tissue, and it provides potential for targeting cancer cells with prodrugs. A prodrug is a drug which is administered in an inactive form and is metabolised in the body to release a cytotoxin (cell- killing chemical) faster at the lower pH in tumours, with less effect on healthy tissue. Because of this there has been investigation into possible molecular systems that can exploit this small difference in pH. Some such molecular systems are amides with neighbouring carboxylic acid groups. These molecules have increased rates of hydrolysis due to intramolecular acid group catalysis (Figure 1) by the un-ionised carboxylic acid group on the amide functionality. The rate of hydrolysis in molecules such as Kemp's acid (Kemp & Petrakis, 1981) and its derivatives (Figure 3) is accelerated because of the enforced stereochemistry, which places all of the acid and amide functional groups in the axial position with the methyls preferring to stay equatorial to prevent the chair structure of the molecule flipping into the other chair conformation. The hydrolysis reaction is of interest because amides that undergo hydrolysis produce an anhydride and also release an amine group, as in Figure 1 (**c**). For reaction at pH 6 or 7 this requires more than one acid group as mono-carboxylic acids are nearly fully ionised by pH 6 and (**e**) is the dominant form so there are almost no non-ionised acid groups available for amide hydrolysis, so hydrolysis rates decrease severely. However, a molecule with two carboxylic acid groups in its structure with have a higher second p*K*a value meaning that there will still be some non-ionised acid group available for intramolecular catalysis at pH 6 -7. A non-ionised acid group increases the rate of hydrolysis, allowing the reaction from (**a**) to (**c**) to proceed at biological pH. If this intramolecular reaction is fast enough at tumour pH, there should also be a significant rate difference in cytotoxin release between pH 6.3 and pH 7.3 at which there will be much less of

the second acid group in non-ionised form, so that the effect of the released cytotoxin on healthy tissue would be reduced.

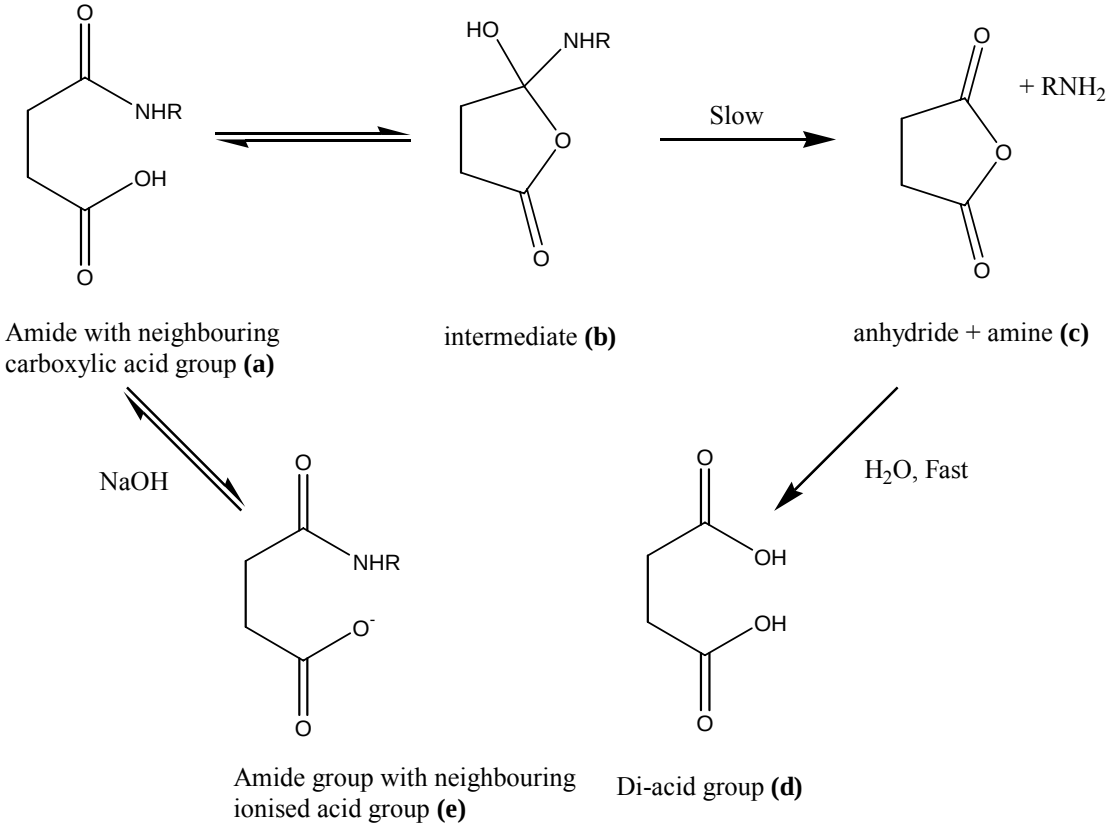

Figure 1: Scheme for hydrolysis reaction of an amide by a neighbouring carboxylic acid group

Molecules such as Kemp's acid and its derivatives (Figure 3) are therefore ideal for this type of study, due to the enforced proximity for rapid rate and also the presence of two carboxylic acid groups on the Kemp's amide molecule (**4**; Figure 3) for adequate reactivity at pH 6.3. Two previous studies that have been conducted on Kemp's acid amides and their rate constants were by Menger & Ladika (1987) and Curran, Borysenko, Abelleira & Messier (1994). Menger reported a $t_{1/2}$ of 8 minutes for the intramolecular catalysed cleavage of a tertiary amide under typical biological conditions (neutral pH and ambient temperature). Curran's work reported a rate constant for tertiary amide derivatives of Kemp's acid that was 1000 times higher than that for a corresponding secondary amide. Both of these studies were conducted with aliphatic amides, whereas aromatic amides are of interest for the purposes of this paper and further research, as there are good arylamine cytotoxins available and fewer good aliphatic cytotoxins (Menger et al. 1987 & Curran et al. 1994). A problem does stem from choosing aromatic amides over aliphatic ones, and that is the possible formation of imides from the arylamides instead of the preferred hydrolysis reaction. Studies by Billett et al. (2006) on cyclopentane-1, 2, 3, 4-tetracarboxylic acid systems with arylamide functionalities showed that the formation of imide was preferred at pH 5-7 over hydrolysis to form amide (Figure 2), which hindered any possible prodrug application (Menger et al. 1987, Curran et al. 1994 & Billett et al. 2006). Any studies concerning arylamides then needs to consider this possibility.

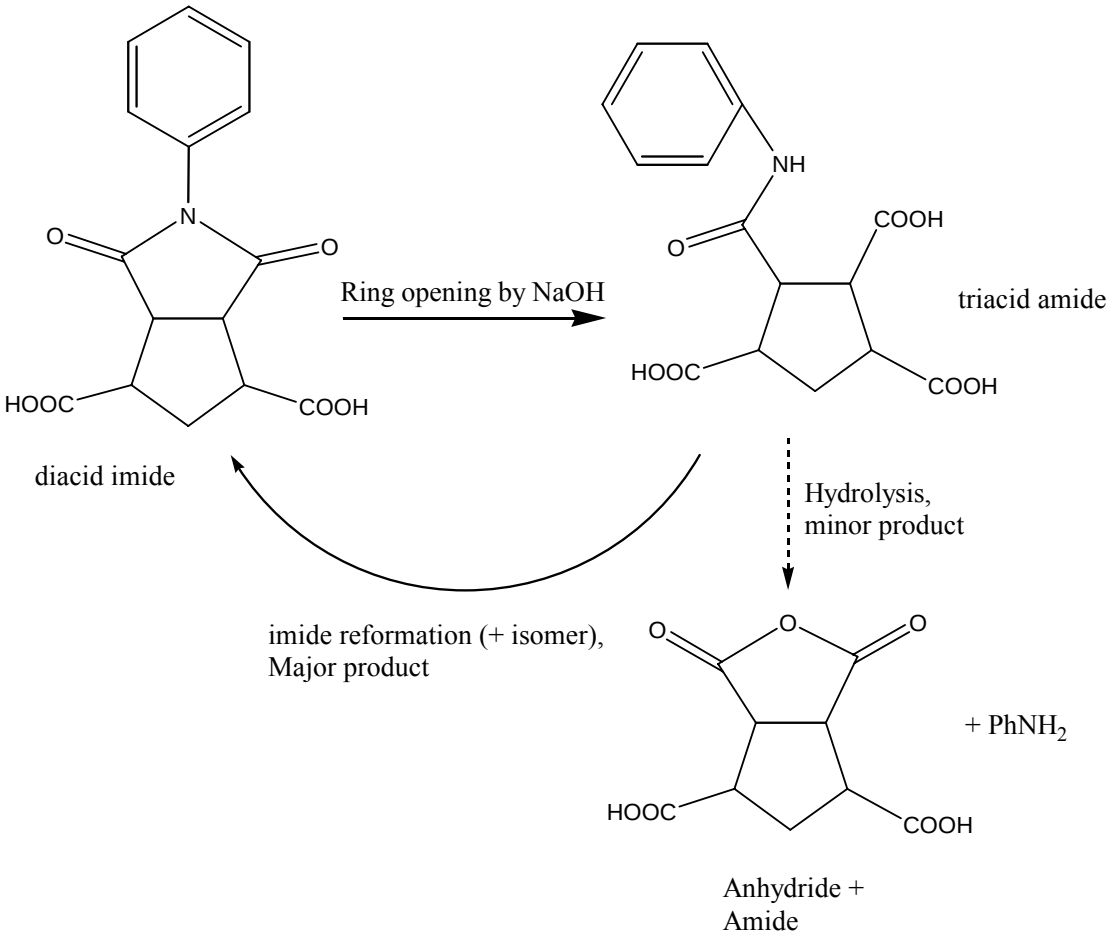

Figure 2: Imide reformation as the major product as opposed to hydrolysis as reported by Billett et al. (2006).

Current plan

The attachment of a cytotoxic arylamine to the molecule is far down the line of research, as current research is concerned with the synthesis and characterisation of a pure imide (**3;** Figure 3), which can then be opened (reaction 3) to form an amide. The imide ring opening can be studied by UV spectroscopy as there is a UV detectable aromatic group on the molecule which changes in UV absorbance with hydrolysis. Study of this reaction by UV is conducted in potassium hydroxide (KOH) to see how quickly the ring opening reaction is occurring, and also to see if there is any subsequent hydrolysis of amide occurring. These conditions if found to be suitable could be later extended to a synthetic scale for formation of a neutral amide by neutralisation though work by Menger et al. (1987) with aliphatic amides showed the diacid amide formed by hydrolysis was too reactive to isolate. At this exploratory stage however, pH of the sample is then lowered to pH 7 and 6 to see how fast the amide reacts and also to see if UV changes suggest the formation of any imide as well as any hydrolysis product. The main ultimate interest in this research is to measure the absolute rate at roughly pH 6 to see if the amine release is fast enough to have possible prodrug applications and then measure how much slower the rate is at pH 7.3 to see how selective cytotoxic activity would be for tumour tissue vs. healthy tissue.

EXPERIMENTAL METHODOLOGY

General procedures

NMR spectra were run at 300 or 400MHz (^{1}H) on Bruker Avance 300 FT-NMR and Bruker Avance DRX400 FT-NMR spectrometers. Infrared spectra were recorded as liquid solution spectra in KBr discs on a Perkin-Elmer spectrum 100 FT-IR spectrometer. UV-vis studies were conducted on a Kontron Uvikon 860 spectrophotometer.

Synthesis of 2

Oxalyl chloride (1.69 mL, 5 x excess) was added dropwise to a solution of Kemp's acid (0.5 g) in 25 mL of dichloromethane on an oil bath at 55^{o}C. This system was then refluxed for four hours at 55^{o}C and left to cool overnight. This produced a clear solution, from which the solvent was removed under vacuum, which yielded crude white crystals. These were then dissolved in dichloromethane (10 mL) and heated at 55^{o}C for two hours in an attempt to purify the crystals. Upon removal of solvent via vacuum, fine white crystals were obtained. IR (liquid solution in CH_2Cl_2): 1746 (anhydride C=O) 1770 (anhydride C=O), 1802 cm^{-1} (acid chloride C=O). ^{1}H NMR ($CDCl_3$): δ 2.74 (d, 2H, equat CH), 2.04 (d, 1H, equat CH), 1.39 (d, 1H, axial CH), 1.34 (s, 6H, Me-C-COO), 1.32 (s, 3H, Me-C-COCl), 1.25 (d, 2H, axial CH).

Synthesis of 3

The anhydride acid chloride was dissolved in distilled pyridine (5 mL), to which *p*-anisidine (1.2 mol equivalent, 0.298 g), and a catalytic amount of dimethylaminopyridine were added. The solution was heated at 90^{o}C for two days under nitrogen. It was then extracted into saturated sodium bicarbonate (140 mL), which was acidified with hydrochloric acid (70 mL, 2 M) down to pH 2, and extracted with dichloromethane (2 x 20 mL), the extract was washed with distilled water (10 mL) and then dried over $CaCl_2$. Excess solvent was removed under vacuum. This yielded light brown crystals. ^{1}H NMR ($CDCl_3$): δ 7.00 (d, 2H, CH-C-OMe), 6.88 (d, 2H, CH-C-N), 3.78 (s, 3H, OMe), 2.83 (d, 2H, equat CH), 2.16 (s, 6H, CH_3-C-CO-N), 2.13 (d, 1H, equat CH), 1.45 (d,1H, axial CH), 1.32 (s, 3H, CH_3-C-COOH), 1.26 (d, 2H, axial CH).

Results and discussion

Reaction 1

The anhydride acid chloride **2** was prepared from Kemp's acid **1** under reflux in dichloromethane solvent with excess oxalyl chloride as suggested by Rebek et al. (1985). This was used instead of $SOCl_2$, as oxalyl chloride is more reactive and leaves fewer residues. The reaction was conducted with a $CaCl_2$ drying tube and in glassware that had been dried overnight in an oven to remove as much water as possible, as any water present in the system may lead to the hydrolysis of the acid chloride product back to the acid. The reaction solution was placed under gentle reflux until the reaction solution turned clear. This occurs because **1** is insoluble in the dichloromethane solvent due to its acid groups, whereas the product **2** is soluble in the solvent. Removal of solvent and oxalyl chloride from the reaction solution under vacuum left crude white crystals. A purification was done on the crude crystals to try and obtain a better product. Using the same reflux system as for the original reaction, dichloromethane was added to dissolve the crystals, which were left to reflux for two hours and then left to stand overnight. Vacuum line removal of the excess solvent left fine white crystals.

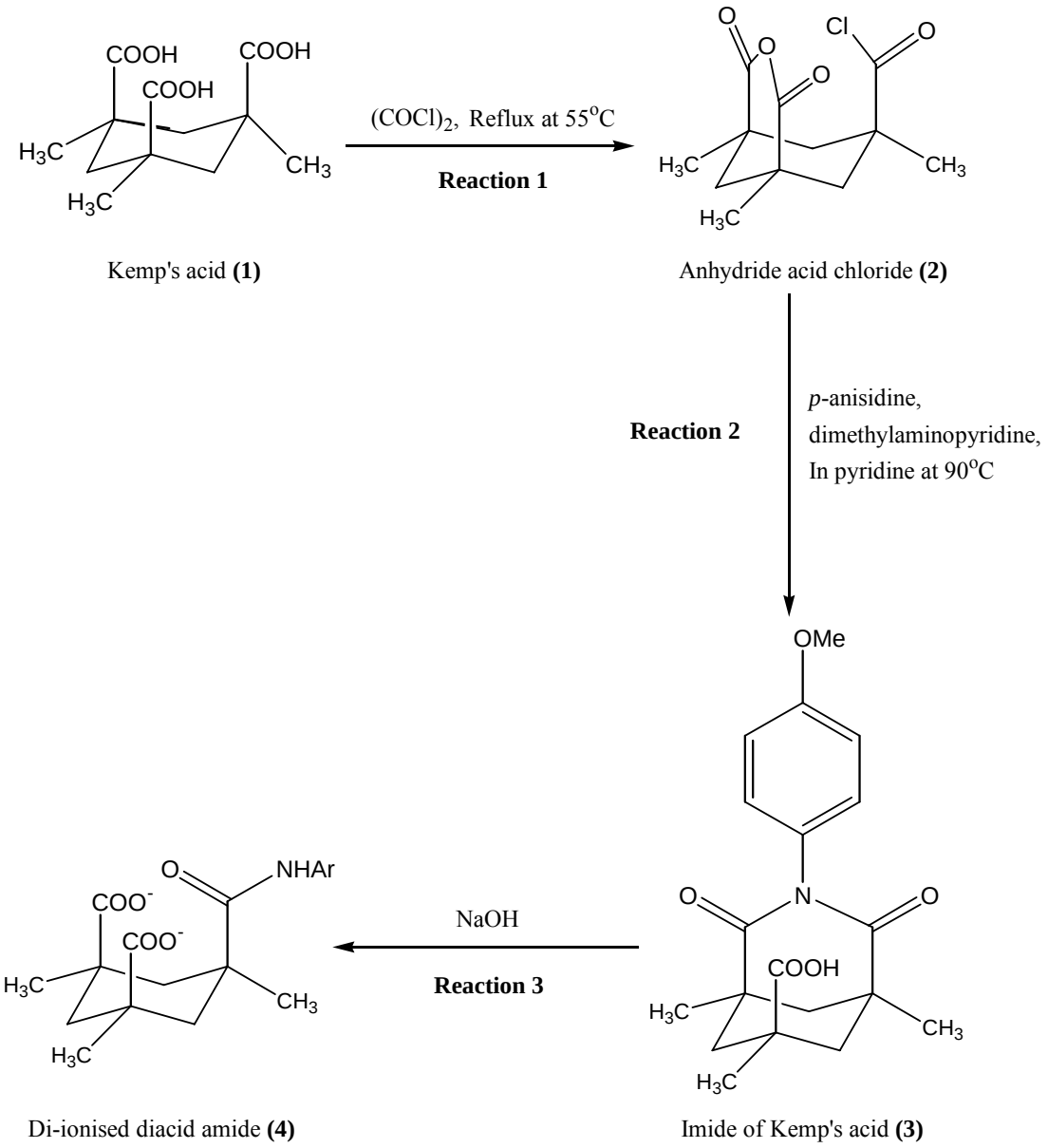

Figure 3: Synthesis of imide

Reaction 2

Pyridine (AR) was distilled over KOH and dried with molecular sieves to remove as much water as possible. The glassware to be used was dried overnight. The imide **3** was prepared by reacting **2** with 1.2 mol equivalent of *p*-anisidine and a catalytic amount of dimethylaminopyridine in pyridine solvent. This reaction solution was heated under nitrogen for two days. A 1mL sub-sample was taken, and was extracted by the method illustrated below and the sample then analysed by IR and ^{1}H NMR (in $CDCl_3$). The NMR of the sample showed that the imide product had been formed along with some impurities. After NMR, a white solid had formed on top of the $CDCl_3$ solution, the solid was removed and analysed IR and melting point and was found to be Kemp's acid, showing that in the reaction mix some of the original material had been reformed.

An extraction method based on the work of Rebek et al. (1985) was employed to separate the desired imide from the other reaction components. The main reaction solution was extracted via five steps (as summarised below):

Step 1: The main solution was extracted with sodium bicarbonate, which ionises the COOH group on the imide so that it dissolves in the aqueous medium.
$RCOOH + HCO_3^- \longrightarrow RCOO^- + H_2O + CO_2$
$RCOO^-$ is water miscible, but pyridine and dimethylaminopyridine may also dissolve; there was no insoluble material.

Step 2: The aqueous bicarbonate extract was acidified with 2M hydrochloric acid to pH 2. This neutralises the acid but ionises all the pyridine and aromatic amines (Figure 4).

Step 3: Two extractions were done with dry dichloromethane (20 mL) in a separating funnel. The desired acid imide product can be extracted into dichloromethane (as it is not water soluble) and leave the other ionised compounds in the water layer.

Step 4: The two organic fractions were combined and washed with distilled water (10mL) to remove any inorganic residues. The combined fractions were then dried with granular $CaCl_2$ and filtered.

Step 5: Removal of dichloromethane under vacuum yielded brown crystals.

The brown crystals were analysed by ^1H NMR ($CDCl_3$) and this showed that this product was much cleaner than the previous sub-sample, and contained only the desired imide product as assessed by comparison with the spectrum for a close analogue with a butyl group in place of methoxy on the aromatic ring, as previously reported by Rebek et al. (1985).

Reaction 3

The diacid amide **4** is formed from **3** in alkaline NaOH (0.04 M) which opens the imide ring but keeps the product in the unreactive di-ionised state. This type of opening reaction was done by Billett et al.(2006) on a similar aryl imide diacid in a cyclopentane 1, 2, 3, 4-tetracarboxylic acid system. Reactions of this type can be studied by UV-vis spectrometry to see the rate of the reaction and also to try and detect if there are any competing reactions, or any hydrolysis reaction of product diacid amide to form amine and Kemp's acid.

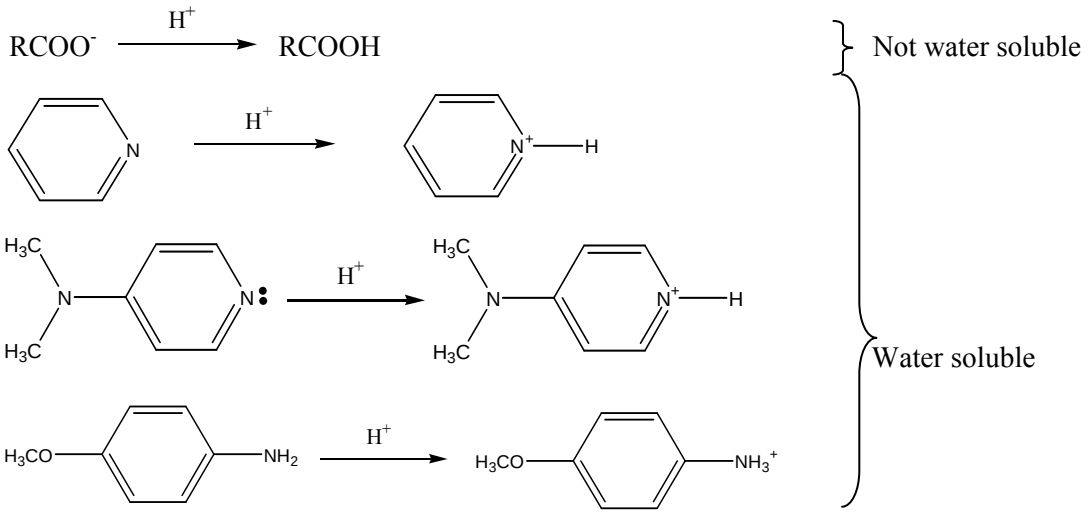

Figure 4. Ionisation of acid and aromatic amine groups in step 2 of extraction

UV STUDIES TO MONITOR THE TIME COURSE OF REACTIONS

These scanning studies were initiated with the ultimate goal of seeing whether **4** will hydrolyse to **1** and amine and/or reform **3**, as both products were seen in similar studies done by Billett et al.(2006). When it is known what reactions are occurring, further studies can be done to measure the rate constants of the reaction of amide **4** as a function of pH.

Initially the reaction of **3** was studied in 0.04 M NaOH at pH 12.4, and showed increased absorbance at 245 nm which is indicative of the imide being hydrolysed to **4**, but could also be **3** → amine (NH_2Ar) + **1**. The spectra were collected over forty, 50-minute intervals and the last two were collected after consecutive 250 minute periods (one at 250 min, other at 500 min). The reaction appeared from sequential spectra to be of a single reaction with a tight isosbestic point at 233 nm. This suggested that only reaction **3** → **4** was occurring and that **4** was stable in the conditions in its fully ionised state, which was confirmed by the following experiments.

The pH of the solution in the cell at completion of reaction was then lowered to 6.7 by adding KH_2PO_4 solution. The solution was monitored again by repetitive UV scanning to completion. There were two aims in this experiment (apart from an early interest in reaction rates).
(i) If the previous reaction had produced **4** and it was stable at high pH, its hydrolysis reaction (or some other reaction) at pH 6.7 was now to be expected by analogy with earlier studies of Kemp's amides, whereas if **4** had already hydrolysed on to amine and Kemp's acid at high pH (not shown in Figure 1) no further reaction could occur and there would be no spectral changes at the lower pH. In the event there were major spectral changes so this was good evidence that they are those of the reaction of the amide **4** and that it was the stable product of the alkaline ring-opening.
(ii) The spectral changes might show whether the amide was reacting to reform the imide and/or amine and acid by hydrolysis as expected from previous studies with Kemp's acid (Menger et al. (1987) & Curran et al. (1994)) in which the amides were always tertiary so were unable to form imides.
In the event, there was an initial reaction with a tight isosbestic point at about 233 nm (which was seemingly coincident with that observed at higher pH for the imide to amide reaction), but over time this isosbestic point was lost and a further decrease in absorbance indicated the likelihood of a second reaction.

As a result of the coincidence of the two isosbestic points, a likely explanation was initial rapid formation of imide followed by an ongoing reaction of imide responsible for the later UV changes. However, part of the reaction solution used in this study had been kept aside at the same temperature and at about the time of the halfway point of the initial reaction at pH 6.7 this sample was raised in pH to 12.4 (equivalent to the pH of the 0.04 M NaOH) the aim being to detect the reversal of imide formation back to amide, with increasing absorption as in the initial scans referred to above for 0.04 M NaOH. As it eventuated, no reaction was observed which negates the possibility that there was any imide now present, and suggests that if any imide is formed at pH 6.7 from the amide it must be minimal, or otherwise have reacted itself so quickly at this pH that it is not present at the mid-stage of the apparent initial amide reaction. This evidence suggests that the coincidence of the isosbestic points in the two reactions must indeed be a coincidence, i.e. that the actual product of the initial amide reaction, the amide, and the imide must all have very similar absorbance intensity (extinction coefficient) at 233 nm.

A similar test was done on the original reaction solution after reaction to completion at pH 6.7 in which its pH was raised to 12.4 and the solution then re-monitored, this also showed the absence of any reaction and therefore of any imide at completion. Later, a sample of imide was dissolved directly into pH 7 buffer solution and there were no absorbance changes over

several days; the imide is quite stable at this pH. This effect negates any possibility that the two-phase reaction of amide could be the formation of imide followed by a reaction of imide.

One possibility is that, as amide hydrolyses through the formation first of anhydride and amine followed by anhydride hydrolysis, the second "phase" as noted by UV spectroscopy in the pH 6.7 reaction could be anhydride hydrolysis with the isosbestic point of 233 nm applying to the preceding anhydride and amine formation. To roughly check this possibility we took an as yet insufficiently pure sample of anhydride and reacted it in pH 7 buffer solution, monitoring absorbance changes. The anhydride showed significant reduction in absorbance below 250 nm which could show up if the anhydride were formed during the reaction of amide but the rate of reaction appeared possibly to be too fast to be the second phase in the reaction of amide. However, it cannot be ruled out yet and a similar test with purified anhydride is the next experiment needed on the path to establish the nature of the two phases in the amide reaction.

Conclusion

The results obtained so far have not been clear-cut. UV studies on the reactions of the imide to amide and subsequent amide reactions have shown that imide reformation does not occur; this is promising as far as prodrug application is concerned as the hydrolysis reaction is the model for cytotoxin release. The two phase reaction observed at pH 6-7 may be due to the anhydride reacting slowly after amine release and this will be investigated with a pure sample of the anhydride. If confirmed this would not be an impediment to prodrug application as it could occur only after cytotoxin is released. Further work will involve synthesising more pure imide, characterising it and performing further UV studies on it to determine rates of reaction and the all important rate differential between pH 6.3 and 7.3. The question of the second phase of the amide reaction remains to be resolved, but it if is not anhydride reaction, probably the best approach will be to study all the reported reactions here by NMR (Nuclear Magnetic Resonance) spectroscopy in D_2O solution as NMR spectral characteristics will be much more diagnostic then those of UV spectroscopy. If results are positive, the aromatic amine group on the imide may be substituted for an aromatic cytotoxic amine with possible prodrug applications then to be assessed.

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Author Notes

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